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Biophysical Chemistry 103 (2003) 51–65

Biophysical Chemistry

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Conformational study of linear and cyclic peptides corresponding to the 276–284 epitope region of HSV gD-1

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Received 24 February 2002; received in revised form 28 June 2002; accepted 3 July 2002

Abstract

The results of conformational analysis of linear and cyclic peptides from the ^{276}SALLEDPVG^{284} sequence of glycoprotein D of Herpes simplex virus are presented. The epitope peptides were synthesized by SPPS and on resin cyclization was applied for preparation of cyclic compounds. Circular dichroism spectroscopy, Fourier-transform infrared spectroscopy and nuclear magnetic resonance (NMR) were used to determine of the solution structure of both linear and cyclic peptides. The results indicated that the cyclopeptides containing the core of the epitope (DPVG) as a part of the cycle have more stable β-turn structure than the linear peptides or the cyclic analogues, where the core motif is not a part of the cycle. NMR study of H–SALLc(EDPVGK)–NH_2 confirm presence of a type I β-turn structure which includes the DPVG epitope core.

Keywords: Cyclic epitope peptides; On resin peptide cyclization; Conformation of cyclic peptides; HSV gD-1

1. Introduction

Herpes simplex virus (HSV) with two closely related serotypes, HSV-1 and HSV-2, is one of the most common infectious agents in humans. The glycoprotein D (gD) of HSV type 1 or 2 is a major envelope protein which is also expressed in the membrane of infected cells. It plays an important role in the initial stages of viral infection and induces high titres of virus neutralizing immune response, mediated partially by inhibition of viral fusion [1]. It is, therefore, a logical target for construction of subunit vaccines against HSV infection [2]. It has been shown, that peptides from the N-terminal region of HSV-1 gD (gD-1) can induce both B and T cell responses [3] and resulting antibodies proved to be able to neutralize HSV-1 in vitro [4–6]. We have demonstrated earlier, that the immunization with peptide 1–23 attached to branched chain polypeptide carriers resulted in antibodies with virus neutralizing activity and caused 50% survival of mice infected with lethal dose of HSV-1 [7]. Another epitope of HSV

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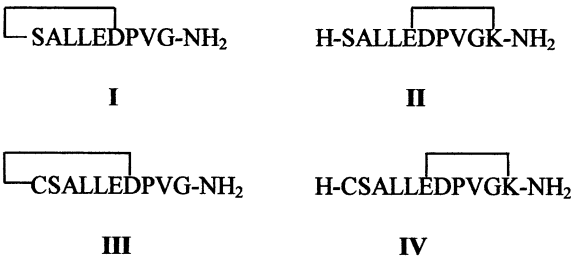
PII: S0301-4622(02)00232-6

gD has been identified by monoclonal antibodies and localized to the 268–287 region [8,9]. Truncated and overlapping peptides corresponding to this sequence were synthesized in our laboratory [10] and some of them were connected to branched chain polypeptide poly[Lys(DL-Ala$_m$)], where $m \sim 3$ (AK) [11]. The ^{276}SALLEDPVG^{284} epitope peptide coupled to AK polymer was immunogenic and induced protection of preimmunized mice (BALB/c and CBA) against lethal dose of HSV [12]. Immunological characterization of truncated peptides of this region suggested the ^{281}DPVG^{284} tetramer sequence as the epitope core [13].

To study the role of secondary structure on immunoreactivity, we have prepared various cyclic peptides corresponding to the SALLEDPVG sequence. First the DPVG core epitope as a guest sequence was inserted into the α-conotoxin GI as a host oligopeptide with well characterized 3-dimensional structure [14]. Replacement of 9–12 (RHYS) sequence in α-conotoxin GI by DPVG tetramer did not result significant changes in the solution conformation of the molecule. The characteristics of the primary and of the memory IgM and IgG type antibody responses showed that the bicyclic HSV-α-[Tyr^1]-conotoxin chimera is capable to induce strong antibody responses in C57/B1/6 mice. Data obtained with the C57/B1/6 serum as well as with IgG type monoclonal antibodies indicate that the antibodies recognise the DPVG motif presented in the bicyclic HSV-α-[Tyr^1]-conotoxin. Only some reactivity was also found with the monocyclic and not with the linear form of the chimera. These data suggest that the DPVG-specific antibody responses are highly dependent on the conformation of the chimera [15].

In another experiment two cyclic versions of ^{276}SALLEDPVG^{284} nonapeptide were prepared containing either a head-to-side chain lactam ring, where the epitope core motif is situated essentially outside of the cycle (*exo*-form) (**I**) or a side chain to side chain lactam ring between γ-carboxyl group of Glu and ε-amino group of a Lys residue attached to the C-terminal of the sequence [16]. In the latter case the DPVG motif is a part of the 6-residue lactam ring (*endo*-form) (**II**). Analogues containing cysteine at the N-terminal were also synthesized for further conjugation of cyclic peptides to polymeric carriers (**III** and **IV**) (Scheme 1).



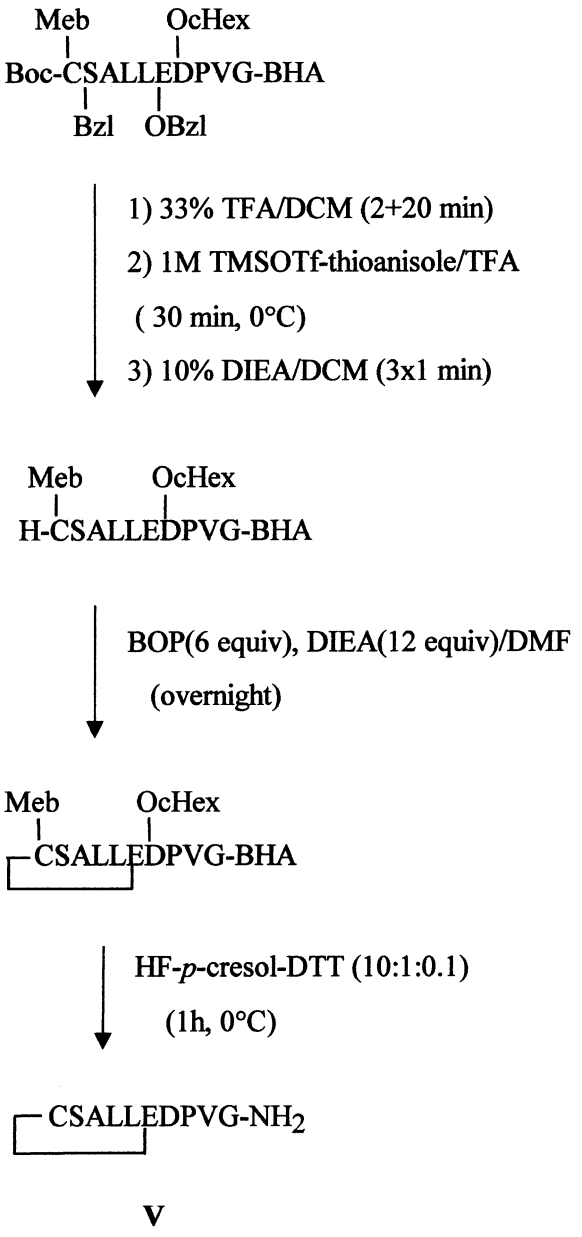
Scheme 1.

In this paper, we describe our findings on the comparative conformational analysis of linear and cyclic peptides (**I**–**IV**) carried out by circular dichroism (CD) spectroscopy, Fourier-transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR) spectroscopy. In addition, here we report on the synthesis of c(CSALLE)DPVG–NH_2 (**V**, Scheme 2) as a new variant, that contain free carboxyl group on the side chain of Asp, and the DPVG sequence localized fully outside of the cycle. A new linear peptide (SALLEDPVGK) was also synthesized to study the effect of ionic interaction between side chains containing amino and carboxyl groups. Results summarised in this paper might contribute to the design of epitope peptides of gD 276–284 region with desired conformation for optimal immunorecognition.

2. Materials and methods

2.1. Materials

Protected amino acids were obtained from Reanal (Budapest, Hungary), Bachem (Bubendorf, Switzerland) and Novabiochem (Laufelfingen, Switzerland). 4-Methylbenzhydrylamine (MBHA) and benzhydrylamine (BHA) resins were from Novabiochem and Bachem, respectively. Benzotriazol-1-yloxy-*tris*-(dimethylamino) phosphonium



Scheme 2.

hexafluorophosphate (BOP) (Novabiochem), *N*,*N*′-dicyclohexyl carbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) (Fluka, Buchs, Switzerland) were used as coupling reagents. Hydrogen fluoride (HF), trimethylsilyl trifluoromethanesulfonate (TMSOTf), trifluoroacetic acid (TFA), trifluoroethanol (TFE) as well as scavengers (dithiothreitol (DTT), anisole, thioanisole, *m*- and *p*-cresol) and diisopropylethylamine (DIEA) were Fluka products. Solvents for synthesis and high performance liquid chromatography (HPLC) were purchased from Reanal.

2.2. *Synthesis of c(CSALLE)DPVG–NH$_2$* (*V*) *cyclopeptide*

The cyclic peptide was synthesized on 0.5 g BHA-resin (0.23 mmol/g) by Boc (*terc*-butyloxycarbonyl) chemistry. Side chain of glutamic acid was protected with benzyl (Bzl) group, while ω-carboxyl group of aspartic acid was blocked as cyclohexyl (cHex) ester. The SH-group of Cys contained 4-methylbenzyl (Meb) protecting group and benzyl protection was used for Ser. Boc protection was removed with 33% TFA/DCM (2+20 min) followed by washing with DCM (5× 0.5 min), neutralization with 10% DIEA/DCM (3×1 min) and DCM washing again (4×0.5 min). The amino acid derivatives and coupling reagents (DCC and HOBt) dissolved in DCM–DMF 4:1 (V/V) were used in 3 M excess for the resin capacity. The coupling reaction was continued for 60 min at RT. Then, the resin was washed with DMF (2× 0.5 min) and DCM (3× 0.5 min). The efficiency of the coupling was checked by ninhydrine [17] or bromophenol blue [18] tests.

N-terminal Boc group was removed from the protected linear peptide prior to 'hard acid' deprotection [19]. Benzyl groups from Glu and Ser were cleaved by 1 M TMSOTf-thioanisole in TFA (10 ml total) in the presence of 0.2 ml *m*-cresol in 30 min at 0 °C. After washing the resin with DCM, neutralization was carried out with 10% DIEA in DCM (3×1 min). Cyclization was performed with six equivalents of BOP and 12 equiv of DIEA in DMF for 16 h. HF cleavage procedure (90 min, 0 °C) using 5% *p*-cresol as scavenger and 20 equiv DTT as reduction agent was applied for removal either remaining protecting groups or cyclic peptide from the resin. The crude peptide was purified by reverse phase-HPLC (RP-HPLC).

Yield:	12.8 mg (11.3%);
FAB-MS $[M+H^+]$:	984.4 (calc. 984.5);
Amino acid analysis:	Asp 1.04 (1), Ser 0.94 (1), Glu 1.08 (1), Pro 1.05 (1), Gly 0.97 (1), Ala 1.01 (1), Val 0.95 (1), Leu 1.95 (2).
Retention time:	30.4 min (determined by HPLC as described below)

2.3. Synthesis of H–SALLEDPVGK–NH_2

The linear peptide was built up on 0.25 g MBHA resin (1.1 mmol/g capacity) with the standard Boc protocol (as described). The side chain of lysine was protected with 2-chlorobenzyloxycarbonyl protecting group, while benzyl protection was used for Ser, Glu and Asp. The peptide was removed from the resin with 10 ml HF containing 0.5 g *p*-cresol. The crude peptide was purified by RP-HPLC.

Yield:	214.4 mg (76%);
FAB-MS $[M+H^+]$:	1026.4 (calc. 1026.6);
Amino acid analysis:	Asp 1.02 (1), Ser 0.91 (1), Glu 1.09 (1), Pro 1.09 (1), Gly 0.99(1), Ala 1.03 (1), Val 0.97 (1), Leu 1.98 (2), Lys 0.98 (1).
Retention time:	26.4 min (determined by HPLC as described below)

2.4. Reverse phase high performance liquid chromatography

Analytical RP-HPLC was performed on a Waters (Nihon Waters Ltd, Tokyo, Japan) HPLC system using a Phenomenex Jupiter C_{18} column (250×4.6 mm I.D.) with 5 μm silica (300 Å pore size) (Torrance, CA) as a stationary phase. Linear gradient elution (0 min 0% B; 5 min 0% B; 50 min 90% B) with eluent A (0.1% TFA in water) and eluent B (0.1% TFA in acetonitrile–water (80:20, V/V)) was used at a flow rate of 1 ml/min at ambient temperature. Peaks were detected at λ=214, 254 and 280 nm. The samples were dissolved in eluent A or in the 2:1 (V/V) mixture of eluent A and B. The crude products were purified on a semipreparative Phenomenex Jupiter C_{18} column (250×10 mm I.D.) with 10 μm silica (300 Å pore size) (Torrance). Flow rate was 4 ml/min. The same eluents with a linear gradient from 15% B to 65% B in 50 min were applied.

2.5. Amino acid analysis

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

2.6. Fast atom bombardment mass spectrometry

Fast atom bombardment mass spectra (FAB-MS) were obtained on a VG-ZA-2SEQ tandem mass spectrometer (Fisons, UK) equipped with a Cs^+ ion gun (30 keV). The peptide samples were dissolved in DMSO and mixed with glycerol matrix.

2.7. Circular dichroism

CD spectra were recorded on a Jobin Yvon Mark VI dichrograph (Longjumeau, France) at room temperature in quartz cells of 0.02-mm path length. The spectra were averages of three scans between 185 and 280 nm. NMR grade TFE and double distilled water were used as solvents. The concentration of the samples was 0.5–1 mg/ml. CD band intensities are expressed in molar ellipticity ($[\Theta]_{MR}$ in deg cm^2/dmol).

2.8. Fourier-transform infrared spectroscopy

Infrared spectra at a resolution of 2 cm^{-1} were obtained in TFE (Aldrich, NMR grade) solution with a Bruker IFS-55 (Karlsruhe, Germany) FTIR spectrometer using a 0.02 cm cell with CaF_2 windows. The sample concentration was 1.9–2.1 mg/ml. The contribution of the δ_{HOH} band appearing at 1633 cm^{-1} in TFE due to traces of water was removed on the basis of the $\nu_{as\ HOH}$ band ($\sim$3688 cm^{-1} for water in TFE) by subtracting spectra of water in TFE. The amide I region of the spectra was decomposed into component bands by the Levenberg–Marquardt nonlinear curve-fitting method using weighted sums of Lorentzian and Gaussian functions. The choice of the starting

parameters was assisted by Fourier self-deconvolution (FSD). The subtraction, curve-fitting and FSD routines were part of the instrument's software package (OPUS, version 2.2).

2.9. Nuclear magnetic resonance spectroscopy

Samples for NMR experiments were prepared by dissolving 2 mg of lyophilised peptide in 0.5 ml of H_2O/D_2O (9:1, V/V). pH was measured with a glass micro electrode, uncorrected for isotope effects, and adjusted to 5.0 by addition of DCl or NaOD. The temperature of the NMR probe was calibrated using a methanol sample. Sodium [3-trimethylsilyl 2,2,3,3-$^2H_4$] propionate (TSP) was used as an internal reference. NMR spectra were recorded on a Bruker AMX-600 (Karlsruhe, Germany) spectrometer. Phase-sensitive 2D correlated spectroscopy [20], total correlated spectroscopy (TOCSY) [21], nuclear Overhauser enhancement spectroscopy (NOESY) [22,23] spectra were recorded by standard techniques using presaturation of the water signal and the time-proportional phase incrementation mode [24]. NOESY mixing times were 200 ms. TOCSY spectra were recorded using MLEV17 with *z* filter spin–lock sequence [21] and an 80 ms mixing time. The $^1H-^{13}C$ heteronuclear single quantum coherence (HSQC) spectra [25] at natural ^{13}C abundance were recorded in 4-mM peptide samples in D_2O. Acquisition data matrices were defined by 2018×512 points in t_2 and t_1, respectively. Data were processed using the standard XWIN-NMR Bruker program on a Silicon Graphics computer. The 2D data matrix was multiplied by a square-sine-bell window function with the corresponding shift optimised for every spectrum and zero-filled to a $2\ k\times1\ k$ complex matrix prior to Fourier transformation. Baseline correction was applied in both dimensions. The 0 ppm ^{13}C δ was obtained indirectly by multiplying the spectrometer frequency that corresponds to 0 ppm in the ^1H spectrum, assigned to internal TSP reference, by 0.25144954 [26,27].

^1H-NMR spectra were assigned by standard 2D sequence-specific methods [28,29]. Then, the ^{13}C resonances were straightforwardly assigned on the basis of the cross-correlations observed in the HSQC spectra between the proton and the carbon to which it is bonded. The ^1H and ^{13}C δ-values of linear peptide and cyclic peptide **II** are presented in Tables 1–3 and have been deposited at the PESCADOR database (http://ucmb.ulb.ac.be/Pescador/).

Structures for the cyclic peptide **II** were calculated using the program DYANA [30] and an annealing strategy. The intensities of the non-sequential NOEs observed for peptide **II** were evaluated qualitatively and translated into upper limit distant constraints; strong (3.0 Å), medium (3.5 Å), and weak (4.5 Å). Pseudo atom corrections were added where necessary. Φ angles were constrained to the range $-180°$ to $0°$ except for Asp and Gly. Secondary structure in the resulting structures was determined by using the PROMOTIF v 2.0 program [31].

3. Results and discussion

3.1. Synthesis of cyclic peptides

The ^{276}SALLEDPVG^{284} immunodominant region of HSV gD-1 was selected for preparation of corresponding conformational restricted cyclic epitope peptides. Two cyclic epitope peptides (c(SALLED)PVG–NH_2 (**I**) and H-SALLc(EDPVGK)–NH_2 (**II**)) and their elongated versions with cysteine at the N-terminus (c(CSALLED)PVG–NH_2 (**III**) and H–CSALLc(EDPVGK)–NH_2 (**IV**)) were synthesized by solid phase methodology [12]. Using synthetic strategy based on differentially acid-labile benzyl/cyclohexyl protection, we have prepared an additional new cyclic peptide c(CSALLE)DPVG–NH_2 (**V**).

The linear precursor sequence of peptide **V** (Scheme 2) was built up on BHA resin by Boc chemistry. The side chain of Glu residue was protected as benzyl ester, removable by TMSOTf-thioanisole/TFA cleavage, while Asp was protected as cyclohexyl ester, stable under this deprotection condition [10]. Prior to on resin cyclization, the N-terminal Boc group was removed with 33% TFA/DCM followed by cleavage of benzyl groups (Ser, Glu) with 1 M TMSOTf-thioanisole/TFA at 0 °C for 30 min.

Table 1
Characteristic amide I frequencies and relative intensities (%) in the curve-fitted FTIR spectra of the linear and cyclic epitope peptides from 276 to 284 region of HSV gD-1

Peptide	Non-acceptor amide >C=Os		H-bond acceptor amide >C=Os			
	Free and distorted amides	Solvent exposed	Strongly solvated/ weakly H-bonded	β-turn	γ-turn	Bifurcated
H–SALLEDPVG–NH_2	1674 (49)[a] 1697 (5)		1656 (6)	1644 (21)[b]	1628 (5)	1601 (5)
H–SALLQDPVG–NH_2	1673 (56)[c]	1666 (12)		1644 (20)	1616 (3) 1628 (4)	1604 (5)
H–SALLENPVG–NH_2	1675 (69)[c]		1657 (5)	1642 (20)	1620 (5)	
H–SALLEDPVGK–NH_2	1674 (61)[c] 1695 (3)		1655 (6)	1642 (18)	1617 (4) 1629 (5)	1602 (3)
H–CSALLc(EDPVGK)–NH_2	1674 (48)[c] 1694 (5)		1654 (18)	1636 (23)	1615 (4)	1603 (2)
H–SALLc(EDPVGK)–NH_2	1673 (48)[c] 1691 (3)	1658 (8)	1648 (10)	1636 (22)	1614 (7)	1602 (2)
c(CSALLED)PVG–NH_2	1675 (29)[c] 1686 (6)	1663 (9)		1645 (42)[b]	1620 (5)	1607 (3)
c(SALLED)PVG–NH_2	1674 (20) 1686 (12)	1664 (6)	1653 (21)	1640 (12)	1615 (5) 1628 (10)	1601 (3)
c(CSALLE)DPVG–NH_2	1674 (27) 1689 (10)	1662 (11)		1646 (35)[b] 1631 (11)	1615 (5)	

[a] Relative intensity which means the percentage of a component band in the total integrated area of the amide I region (1600–1700 cm^{-1}). Bands of $\leqslant$2% relative intensity are not shown.
[b] These values may result from distorted β-turn structures with relatively weak H-bonds.
[c] Contains the contribution of ν_{as} COO^- of TFA^-.

Under this cleavage condition the peptide–BHA resin bond as well as the side chain of Cys(Meb) and Asp(OcHex) remain intact (Scheme 2). Cyclization on the resin using 6 equiv of BOP reagent in the presence of DIEA (12 equiv) was completed overnight and followed by HF cleavage. HPLC analysis of the crude product showed two main peaks of similar intensities (Fig. 1). The first eluting component was the cyclopeptide **V** (c(CSALLE)DPVG–NH_2). The other component was identified as the cyclic dimer **Va** resulting from double lactam formation between two different chains (Scheme 3).

The yield of peptide **V** (11% overall after purification) was intermediate between that of 7-residue lactam c(CSALLED)PVG–NH_2 (20%) and the similar 6-residue lactam c(SALLED)PVG–NH_2 (6–7%) [16]. The HPLC chromatogram (Fig. 1) and mass analysis of the peaks also indicated the absence of compounds with other ring size. This demonstrates the selectivity of cyclohexyl and benzyl protection under TMSOTf-thioanisole/TFA cleavage condition.

3.2. CD and FTIR analysis

Secondary structure of four linear and five cyclic analogues (Table 1) from HSV gD-1 276–284 sequence were studied by CD and FTIR spectroscopy. CD measurements were carried out in water, TFE and water-TFE 1:1 (V/V) mixture. The peptides in general showed U, C or C+U type CD spectrum characteristic for mixtures of conformers with considerable turn populations (for

Table 2
^{1}H and ^{13}C chemical shifts of the cyclic peptide **II** in aqueous solution at pH 5.5 and 5 °C (ppm, from TSP)

Residue	NH	$^{13}C_{\alpha}$	$C_{\alpha}H$	$^{13}C_{\beta}$	$C_{\beta}H$	Others	
S1		57.0	4.15	62.8	4.02, 3.98		
A2	8.82	52.2	4.37	19.0	1.39		
L3	8.45	55.0	4.31	42.2	1.62, 1.56	$^{13}C_{\gamma}$ 26.9, $C_{\gamma}H$ 1.63,	$^{13}C_{\delta}$ 24.6, 23.5, $C_{\delta}H_3$ 0.95, 0.90
L4	8.43	54.5	4.38	42.2	1.63, 1.58	$^{13}C_{\gamma}$ 26.9, $C_{\gamma}H$ 1.61	$^{13}C_{\delta}$ 24.6, 23.4, $C_{\delta}H_3$ 0.95, 0.88
	8.39	*4.35*					
E5	8.40	55.0	4.27	30.0	1.96, 1.96	$^{13}C_{\gamma}$ 33.7, $C_{\gamma}H$ 2.22, 2.18	
	8.37	*55.3*	*4.21*	*29.6*	*1.99, 1.99*	*34.4 2.38, 2.23*	
D6	8.82	51.7	4.86	41.3	2.85, 2.69		
	8.28	*51.2*	*4.74*	*43.2*	*2.68, 2.48*		
P7		64.6	4.34	32.2	2.36, 2.04	$^{13}C_{\gamma}$ 27.2, $C_{\gamma}H$ 2.07, 2.07	$^{13}C_{\delta}$ 51.2, $C_{\delta}H$ 4.04, 4.04
		63.2	5.04	34.4	2.42, 2.20	25.0 1.99, 1.83	50.1 3.60, 3.51
V8	8.21	64.0	3.96	31.5	2.17	$^{13}C_{\gamma}$ 21.5, 20.7, $C_{\gamma}H_3$ 1.01, 0.93	
	8.63	*64.3*	*3.87*	*32.1*	*2.09*	*21.3, 20.9 1.08, 1.01*	
G9	8.11	45.4	4.02, 3.95				
	8.62	*45.2*	*4.02, 3.83*				
K10	7.72	55.7	4.32	33.1	1.89, 1.79	$^{13}C_{\gamma}$ 25.3, $C_{\gamma}H$ 1.44, 1.31,	$^{13}C_{\delta}$ 30.0, $C_{\delta}H$ 1.56, 1.44
	7.99	*55.9*	*4.21*	*32.5*	*1.81, 1.81*	*24.6 1.36, 1.36*	*30.0 1.54, 1.48*
						$^{13}C_{\varepsilon}$ 41.7, $C_{\varepsilon}H$ 3.18, 3.07	
						41.4 3.41, 3.04	
						$N_{\zeta}H$ 8.07	
						8.07	
$CONH_2$	7.38, 7.30						
	7.76, 7.21						

The chemical shifts corresponding to the *cis* species are given in italics.

selected CD spectra see Figs. 2–4). This is in agreement with the FTIR spectra (Table 1), where significant amide I component bands approximately 1640 cm^{-1} (the so-called β-turn acceptor bands) [32] could be detected even in the case of linear peptides (H–SALLEDPVG–NH_2, H–SALLQDPVG–NH_2, H–SALLENPVG–NH_2, H–SALLEDPVGK–NH_2).

The CD spectra of linear H–SALLEDPVG–NH_2, H–SALLQDPVG–NH_2, H–SALLENPVG–NH_2 are almost identical in TFE, water or 1/1 its mixture (only the spectra of H–SALLENPVG–NH_2 is shown), however, some differences were detected in case of H–SALLEDPVGK–NH_2 (Fig. 2). These changes in the CD pattern may be explained by the interaction of ε-amino group of lysine residue with one of the side chain carboxyl (Glu or Asp) groups. The CD spectra of all linear peptides in water reflect the predominance of open (unordered) spectra. Strong negative $\pi-\pi^*$ band under 200 nm with small shoulder at approximately 220 nm ($n-\pi^*$) can be observed in these spectra. The presence of TFE in the solvent mixture results in shift of $\pi-\pi^*$ band toward the higher wavelength and increase of the $n-\pi^*/\pi-\pi^*$ ratio. The class C CD spectra found in TFE maybe indicative for the presence of folded (I or III type of β-turn) conformations. This tendency was more pronounced in case of H–SALLEDPVGK–NH_2 (Fig. 2). Under these conditions the formation of β-turn might be due to the DPVG sequence.

When this sequence is comprised in a cycle formed at the C-terminus, such as in cyclopeptides **II** or **IV** the β-turn is further stabilized. This is reflected in the lower frequency of the β-turn acceptor band (1636 cm^{-1}) suggesting a stronger C_{10} intramolecular H-bond and thus a higher conformational stability. Cyclopeptides **II** and **IV** also showed the smaller change in the CD spectrum when water was replaced by TFE (Fig. 3). This small change of the $n-\pi^*/\pi-\pi^*$ ratio in both

Table 3
^{1}H and ^{13}C chemical shifts of the linear peptide H-SALLQDPVG-NH_2 in aqueous solution at pH 5.5 and 5 °C (ppm, from TSP)

Residue	NH	$^{13}C_\alpha$	$C_\alpha H$	$^{13}C_\beta$	$C_\beta H$	Others	
Sl		57.0	4.15	62.8	4.02, 3.99		
A2	8.83	52.3	4.38	19.1	1.40		
L3	8.45	55.0	4.32	42.1	1.63, 1.58	$^{13}C_\gamma$ 26.9, $C_\gamma H$ 1.62	$^{13}C_\delta$ 24.6, 23,5, $C_\delta H_3$ 0.95, 0.89
L4	8.42	54.8	4.37	42.1	1.64, 1.58	$^{13}C_\gamma$ 26.9, $C_\gamma H$ 1.62	$^{13}C_\delta$ 24.6, 23,4, $C_\delta H_3$ 0.95, 0.89
Q5	8.48 *8.40*	55.2 *55.3*	4.33 *4.28*	29.6 *29.6*	2.07, 1.96 *2.06, 1.94*	$^{13}C_\gamma$ 33.6, $C_\gamma H$ 2.34, 2.34 *33.6 2.35,2.35* $N_\delta H$ 7.51, 7.23 *7.43, 7.27*	
D6	8.58 *8.22*	52.4 *51.4*	4.84 *4.75*	40.1 *42.3*	2.75, 2.54 *2.71, 2.49*		
P7		63.1 *62.7*	4.47 *4.95*	32.0 *34.4*	2.30, 1.94 *2.38, 2.12*	$^{13}C_\gamma$ 27.2, $C_\gamma H$ 2.05, 2.05 *24.8 1.96, 1.84*	$^{13}C_\delta$50.6, $C_\delta H$ 3.86, 3.77 *50.1 3.59, 3.49*
V8	8.48 *8.77*	62.9 *63.8*	4.05 *3.92*	32.3 *32.0*	2.10 *2.07*	$^{13}C_\gamma$ 20.7, 20.9, $C_\gamma H_3$ 1.00, 0.96 *21.2, 20.5 1.08, 0.97*	
G9	8.64 *8.73*	44.7 *44.6*	3.93, 3.90 *3.96, 3.85*				
$CONH_2$	7.51, 7.23 *7.43, 7.27*						

The chemical shifts corresponding to the *cis* species are given in italics.

cyclopeptides as compared to the respective linear versions indicates the increased rigidity, but without a significant loss of flexibility of these structures.

In the case of epitope peptides containing cycle on the N-terminus (**I**, **III** and **V**) with two consecutive Leu residues, the steric hindrance of their bulky side chains reduces the stability of β-turns within the cycle. In the CD spectrum of **III** measured in water some amount of folded conformations can be detected (Fig. 4a). In TFE (Fig. 4b) and TFE/water 1:1 (V/V) (data not shown) the CD spectra suggest the predominance of folded conformers, probably two types of β-I (or β-III) turns, but in water the flexibility of the molecule is still preserved (Fig. 4a). It is very likely that the unusual spectral feature is due to a mixture of relatively distorted β-turn structures. This is supported by the high intensity of the 1645 cm^{-1} band in the FTIR spectrum of **III** and also for **V**, characteristic for weakly H-bonded, distorted β-turn structures. The presence of an additional β-turn band at 1631 cm^{-1} in the FTIR spectrum of **V** may result from a second β-turn formed within the DPVG sequence, situated in this case entirely in the linear part of the molecule.

The low-frequency amide I component bands between 1628–1614 cm^{-1} and near 1600 cm^{-1} in the FTIR spectra of the investigated peptides might be indicative for other types of H-bonded folded steric arrangements, such as γ-turns and bifurcated structures are also present to some extent [33].

The low frequency values of FTIR suggest the presence of type I β-turn structure in epitope peptides. However, the CD spectra show not well defined turn structures. To confirm the type I β-turn structure the NMR study was also necessary.

3.3. NMR conformational investigation

The conformational properties of cyclic peptide **II** where the core epitope (DPVG) is part of the cycle and linear peptide H–SALLQDPVG–NH_2 in aqueous solution were investigated by NMR to

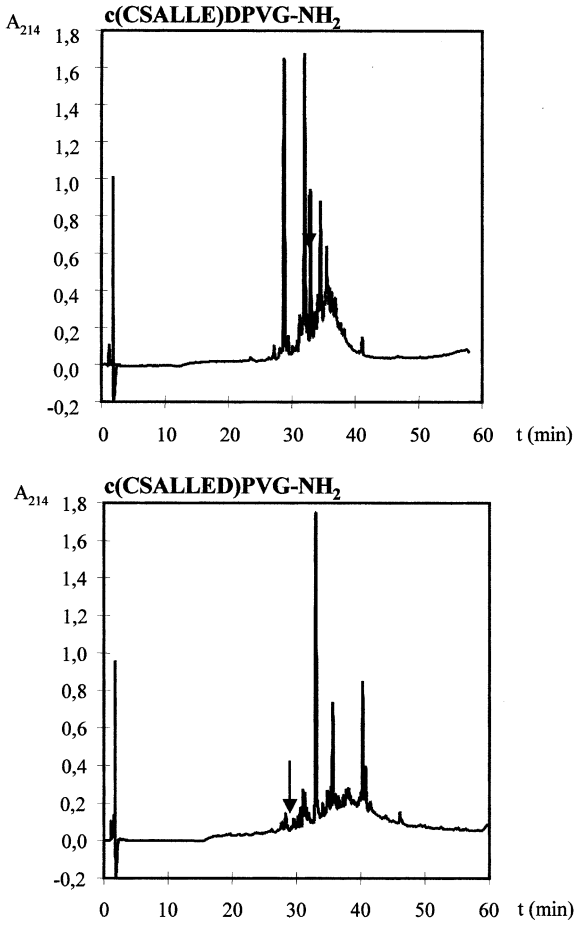
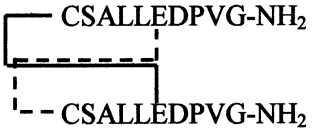


Fig. 1. HPLC profile of crude cyclopeptides c(CSALLE)DPVG–NH_2 and c(CSALLED)PVG–NH_2.

obtain further insights of structures, in particular to interpret differences in conformational behaviour between linear and cyclic peptide. In this case the restricted mobility of DPVG may be beneficial for NMR studies. The linear peptide contain Gln in position 5 can partly mimic the amide bond present in the cyclopeptide, between the side chains of glutamic acid and of Lys residues. The spectra of both cyclic peptide **II** and linear H–SALLQDPVG–NH_2 display a subset of minor signals corresponding to a species identified as the *cis* Pro conformer. The assignation was based on the fact that the ^{13}C δ difference between the Pro C_β and C_γ carbons in each peptide is larger for the minor (9.6 and 9.4 ppm in linear and cyclic peptide, respectively) than for the major species (4.8 and 5.0 ppm, respectively) [34]. The percentage of *cis* species is slightly higher in cyclic peptide **II** (19%) than in the linear peptide (12%; ~10% *cis* is commonly observed in any Pro-containing peptide). Structural characterization of the major *trans* species for both peptides was done on the basis of NMR parameters such as $C_\alpha H$ conformational shifts ($\Delta\delta_{C\alpha H} = \delta_{C\alpha H(observed)} - \delta_{C\alpha H(random\ coil)}$, ppm), $^{13}C_\alpha$ conformational shifts ($\Delta\delta_{C\alpha} = \delta_{C\alpha(observed)} - \delta_{C\alpha(random\ coil)}$, ppm), $^{13}C_\beta$ conformational shifts ($\Delta\delta_{C\beta} = \delta_{C\beta(observed)} - \delta_{C\beta(random\ coil)}$, ppm), temperature coefficients of NH amide protons and NOEs, (Fig. 5). The $C_\alpha H$, $^{13}C_\alpha$ and $^{13}C_\beta$ conformational shifts can be considered significant, and therefore indicative of a non-random conformation, if $|\Delta\delta_{C\alpha H}| > 0.05$ ppm, $|\Delta\delta_{C\alpha}| > 0.5$ ppm and $|\Delta\delta_{C\beta}| > 0.5$ ppm. The N-terminal and the Pro-preceding residues were excluded due to charge end effects, and to the large sequence effect of Pro residues [35], respectively. With these criteria, the large $C_\alpha H$, $^{13}C_\alpha$ and $^{13}C_\beta$ conformational shifts shown by cyclic peptide **II** at the 6–8 region indicate that the peptide adopts a defined conformation within that segment. In contrast, the $C_\alpha H$, $^{13}C_\alpha$ and $^{13}C_\beta$ conformational shift profiles for the linear peptide, where only Asp 6 (the Pro-preceding residue) and Val 8 are significant, suggest a mainly random coil state for this peptide. NH temperature coefficient data are in agreement with these conclusions. Thus, coefficients measured for all NH protons of the linear peptide and those of residues 2–6 of the cyclic peptide **II** are within the range observed for random coil peptides [36]. In contrast, those of residues 8–10, in particular residues 9 and 10, of peptide **II** are small in absolute value and very different from those of the corresponding 8–9



Va

Scheme 3.

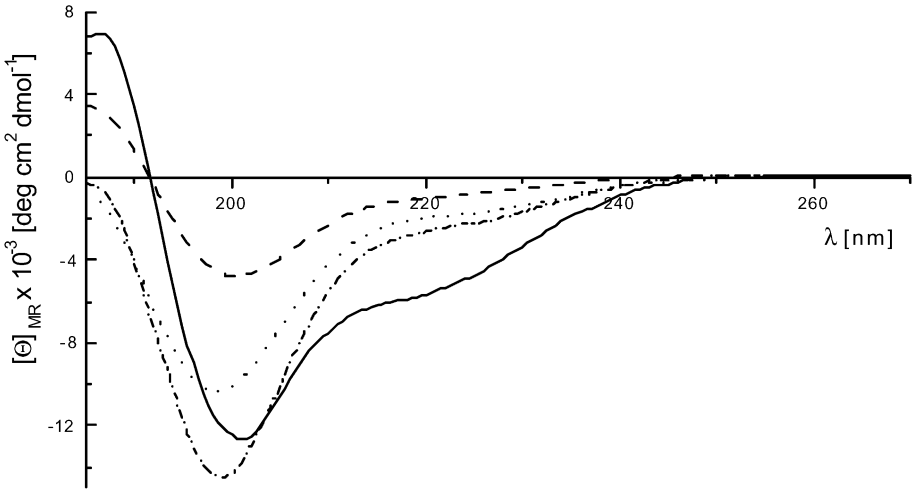


Fig. 2. CD spectra of linear peptides; H–SALLENPVG–NH_2 in water (-•-) and in TFE (—) and H–SALLEDPVGK–NH_2 in water (……) and in TFE (- -).

residues in the linear peptide, suggesting that they are protected from solvent and might be involved in intramolecular hydrogen bonds. The strongest evidence about the existence of a defined structure comes from the presence of non-sequential NOEs in the C-terminal region of the cyclic peptide **II**, again suggesting non-random conformation within region 6–10 (Fig. 5 and Table 4). The two $d_{\alpha N(i,i+2)}$ NOEs suggest that the non-random conformation might be a turn structure. In contrast, the absence of non-sequential NOEs in the linear peptide is indicative of a mainly random coil peptide.

Since the preferred structure adopted by peptide **II**, probably coexists in equilibrium with random-coil conformations, NOE data cannot be interpret-

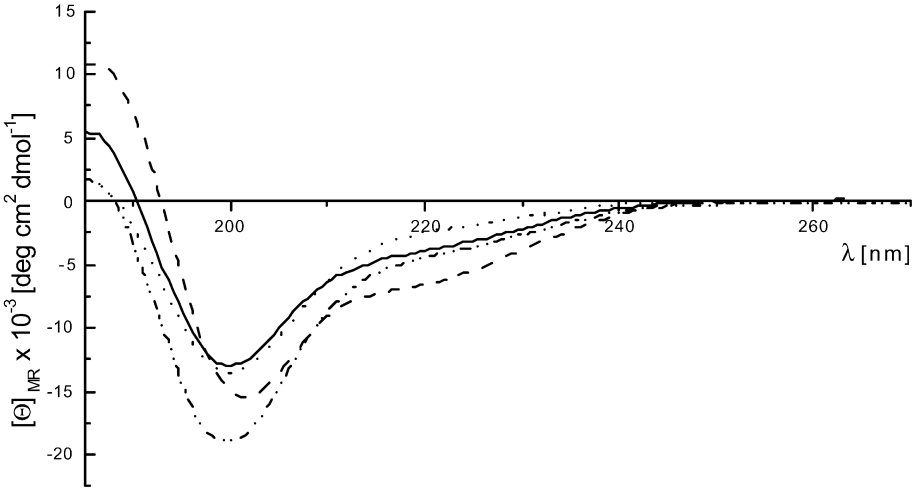


Fig. 3. CD spectra of cyclic peptides with DPVG core epitope in *exo*-position; H–SALLc(EDPVGK)–NH_2 in water (……) and in TFE (—) and H–CSALLc(EDPVGK)–NH_2 in water (-••-) and in TFE (- -).

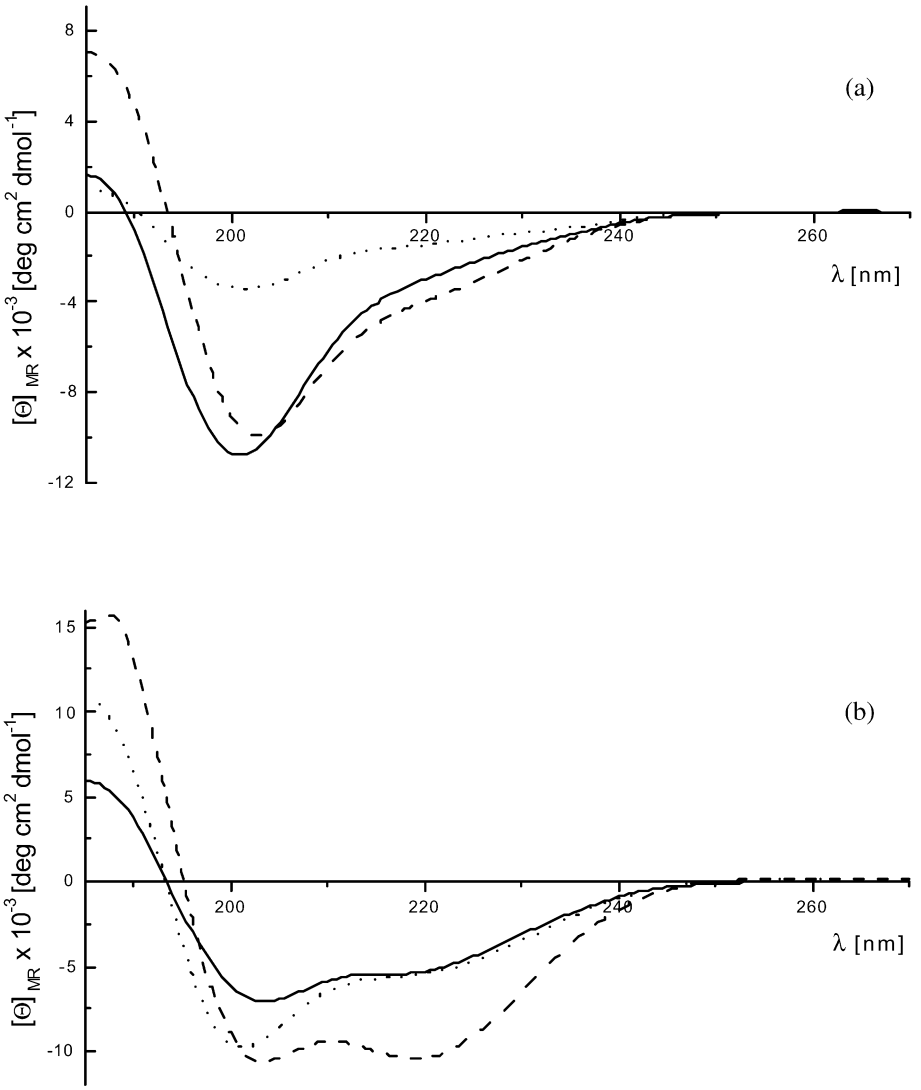


Fig. 4. CD spectra of cyclic peptides with DPVG core epitope in *endo*-position; c(SALLED)PVG-NH_2 (—), c(CSALLED)PVG-NH_2 (- -) and c(CSALLE)DPVG-NH_2 (……) in water (a) or TFE (b).

ed in terms of a unique structure. Nevertheless, we found it useful to perform a structure calculation to visualise the defined structure adopted by the cyclic peptide **II**. Since sequential NOEs are best excluded from structure calculation because random conformations contribute to their intensity, our structure calculation used as constraints only the 9 observed non-sequential NOEs (Fig. 5; Table 4). Despite the small number of distance constraints used, the resulting structures are well defined when only the region 5–10 is considered (Fig. 6). RMSDs for the 20 best calculated structures for peptide **II** are 2.5 ± 0.8 Å for the backbone atoms and 3.4 ± 0.9 Å for all heavy atoms, but they are reduced to 0.5 ± 0.2 Å and 1.1 ± 0.3 Å, respectively, when only residues 5–10 are considered. According to secondary structure analysis performed by using the PROMOTIF program

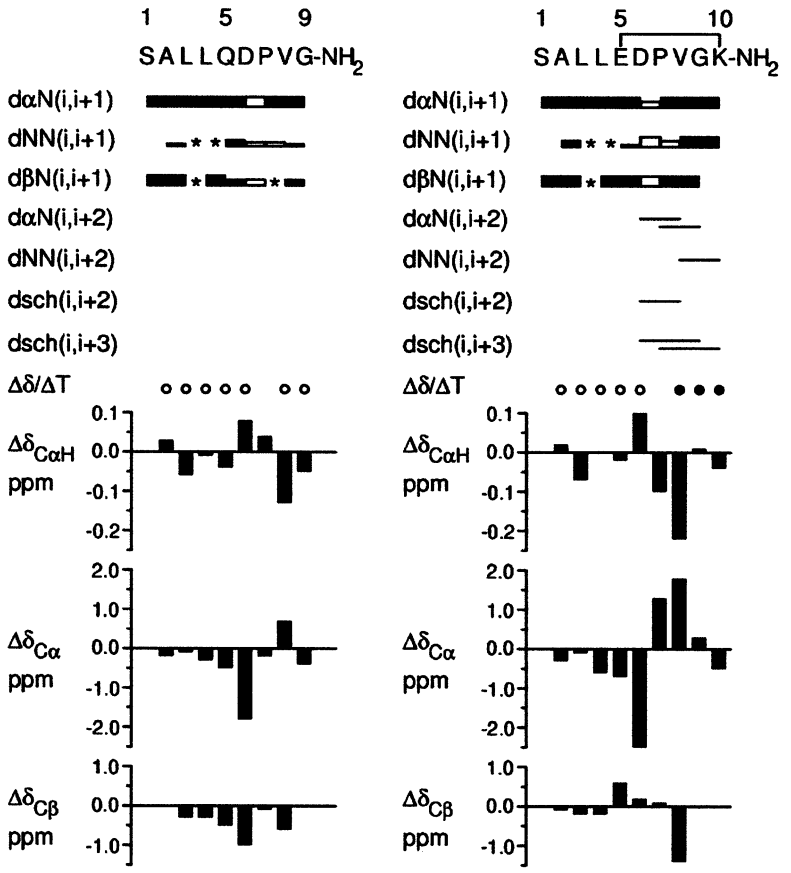


Fig. 5. Summary of NOE connectivities, temperature coefficients of the NH amide protons ($\Delta\delta/\Delta T$), $C_\alpha H$ conformational shifts ($\Delta\delta_{C\alpha H} = \delta_{C\alpha H(observed)} - \delta_{C\alpha H(random\ coil)}$, ppm, where $\delta_{C\alpha H(random\ coil)}$ values were taken from Bundi and Wüthrich [43], $^{13}C_\alpha$ conformational shifts ($\Delta\delta_{C\beta} = \delta_{C\beta(observed)} - \delta_{C\beta(random\ coil)}$, ppm, where $\delta_{C\alpha(random\ coil)}$ values were taken from Wishart et al. [35]), and $^{13}C_\beta$ conformational shifts ($\Delta\delta_{C\beta} = \delta_{C\beta(observed)} - \delta_{C\beta(random\ coil)}$, ppm, where $\delta_{C\beta(random\ coil)}$ values were taken from Wishart et al. [35]) as a function of sequence for linear peptide H–SALLQDPVG–NH_2 and for cyclic peptide II at pH 5.0 and 5 °C in aqueous solution. The thickness of the bars reflects the intensity of the sequential NOEs, i.e. weak, medium, and strong. The open bars indicate that $C_\delta H$ protons of Pro residue are involved in the corresponding NOE. An * indicates unobserved connectivity due to signal overlapping or closeness to diagonal; dsch indicates NOEs involving side chain protons. Close circles correspond to $|\Delta\delta/\Delta T| < 5\times10^{-3}$ ppm/K and open circles to $|\Delta\delta/\Delta T| > 6\times10^{-3}$ ppm/K. For Gly, the $\Delta\delta_{C\alpha H}$ value shown corresponds to the averaged of the two $C_\alpha H$ protons.

[31], residues DPVG form a type I β-turn, and residues PVDG another β-turn, classified as a type IV in 16 of the 20 calculated structures and as a type I in the other 4. A hydrogen bond between the carboxylic oxygen of Asp 6 and the NH proton of Gly 9 is present in 18 of the 20 calculated structures. This hydrogen bond is characteristic of the type I β-turn formed by residues DPVG [37]. The negative $\Delta\delta_{C\alpha H}$-values and the positive $\Delta\delta_{C\alpha}$-values of P7 and V8 residues in the cyclic peptide **II** as well as the negative $\Delta\delta_{C\beta}$-value of V8 residue are also consistent with the DPVG segment forming a type I β-turn (Fig. 5). These are the expected signs by considering the characteristic ϕ and ψ angles of these residues in positions i and $i+1$ of a type I β-turn and the relationship of the conformational shifts with the ϕ and ψ angles [27,38–42].

Table 4
Non sequential NOEs observed for the cyclic peptide H–SALLc(EDPVGK)–NH_2

Residue *i*	Residue *j*	NOE intensity
C_αH D6	NH V8	Weak
C_βH D6	NH V8	Strong
$C_{\beta'}$H D6	NH V8	Weak
C_βH D6	NH G9	Strong
C_αH P7	NH G9	Medium
C_αH P7	N_ζH K10	Medium
C_αH V8	CO–NH_2	Medium
C_αH V8	CO–NH_2	Weak
NH V8	NH K10	Weak

In brief, NMR data show significant differences in conformational behaviour between cyclic peptide **II** and its linear counterpart in aqueous solution. The cyclic peptide **II** adopts turn conformations spanning residues 6–10, while the linear peptide is in a mainly random coil state.

4. Conclusion

The binding of a peptide to antiprotein antibodies can be facilitated by the presence of a native like conformation of the peptide during formation of the peptide–antibody complex. The use of longer peptides does not necessarily lead to a higher level of binding since these compounds may adopt a conformation different from that of present in the native protein. Shorter peptides might fold more easily into the proper orientation required for binding to the antibody. A variety of approaches have been used to increase conformational similarity between peptide and intact protein. Since epitope sequences are frequently localized in turn or loop region of protein, cyclization of the peptide has often been used for this purpose, although it appears that information on the 3D structure of epitope is required to achieve the best results. The detailed 3D structure of HSV gD has

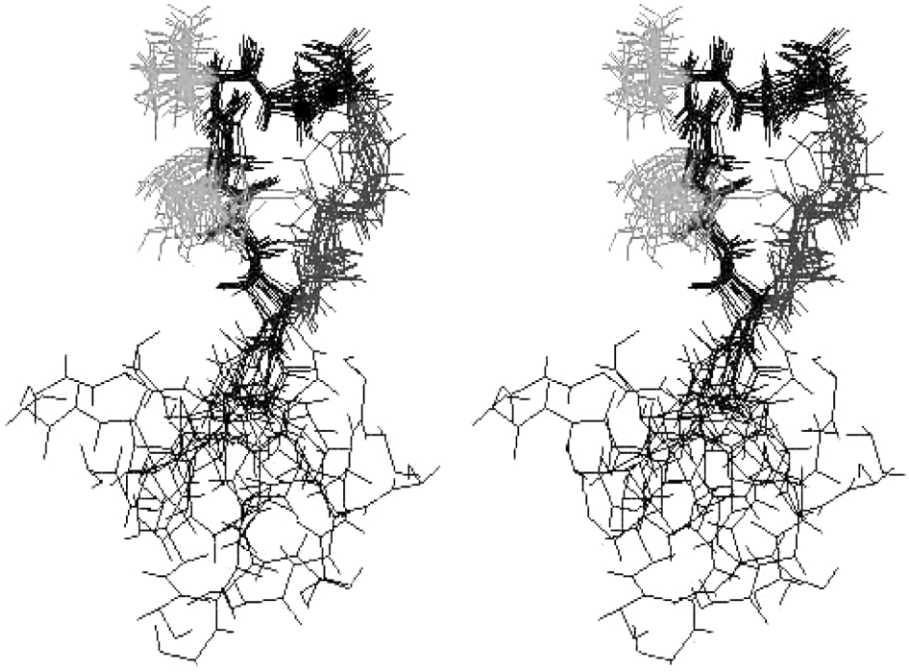


Fig. 6. Stereoscopic view of the best 20 structures calculated for cyclic peptide **II** superimposed over residues 5–10, showing the backbone atoms of the entire peptide chain in black, side chain atoms of residues 5 and 10 in grey and side chain atoms of residues 6–9 in light grey.

not known yet. Only combinations of different prediction methods offered some data for the secondary structure. These data suggest the presence of turn region localized to ^{281}DPVG^{284} tetrapeptide sequence, which is a proved core epitope of an immunodominant region of HSV gD-1.

In this study we have prepared linear and cyclic peptides to study the solution structure of ^{276}SALLEDPVG^{284} epitope sequence related compounds. CD and FTIR spectroscopy demonstrated β-turn structure even in linear peptides. CD, FTIR and NMR (H−SALLc(EDPVGK)−NH_2) measurements of cyclopeptides confirm the presence and increased stability of β-turn. The comparison of cyclopeptides (with DPVG in *endo* or *exo* position) suggested that the tendency to adopt this structure was dependent also on the location of the DPVG sequence.

The immunological properties of these peptides together with new analogues designed on the basis of this study are in progress.

Acknowledgments

This study was supported by grants from the Hungarian Research Fund (OTKA) No. T025834, T034866, T037719, from the Hungarian Ministry of Education FKFP No. 0101/1997, the Hungarian-Spanish Intergovernmental Program (E-13/1997) and COST Chemistry Action D13/0007/00. Work in Madrid was supported by the Spanish DGICYT Project No. PB98-0677 and European Project No. CEE B104-97-2086.

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