CONFORMATIONAL FEATURES OF A SYNTHETIC CYCLIC PEPTIDE CORRESPONDING TO THE COMPLETE V3 LOOP OF THE ELI HIV-1 STRAIN IN WATER

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The disulfide bridge closed cyclic peptide corresponding to the whole V3 loop of the envelope protein gp120 of the ELI HIV-1 strain was synthesized and examined by proton 2D NMR spectroscopy in water. Although the peptide is mainly conformationally flexible, a turn appears to be present at an N-terminal glycosylation site, while in the C-terminal half of the peptide the data point toward nascent helical structures. Similar conformational preferences in aqueous solution were observed in other V3 loop peptides, especially for the Ile28-Gly30 tripeptide part.

Key words: 2D NMR; ELI sequence V3 loop; Conformation in water; gp120; HIV-1.

The initial stage in human immunodeficiency virus type 1 (HIV-1) infection of human CD4+ T-cells and macrophages involves the viral envelope proteins gp120 and gp41 (refs[1,2]), which exist as a dimeric complex on the mantle of the free virus. In the first step of infection gp120 binds to the CD4 receptor on the host cell surface, thereby inducing structural changes in gp120 which disrupt the dimeric gp120-gp41 complex and expose new domains of gp120 (refs[3,4]). The next steps in infection are poorly understood but do involve the two envelope proteins, which act together to finally attain fusion of the host and viral membranes[5].

The part of gp120 which generates the largest response from the body's immune system is its third variable loop[6,7] (the V3 loop). This cysteine-bridged loop between residues 303 and 338 of gp120 (ref.[8]) is highly variable between different HIV-1 strains but still plays an important role in infection[9,10]. Antibodies directed towards this V3 loop do not inhibit gp120–CD4 binding, but can prevent subsequent infection[11], which is interesting with regard to the development of a vaccine. Attempts to generate V3 loop antibodies that react to a broad range of strains, however, have been impeded by

the variability of the V3 loop. Only antibodies directed toward the conservative central region of the V3 loop have been successful in neutralizing a broader selection of strains[12,13]. Since the heterogeneity of the V3 loop does not interfere with its function in viral entry, this suggests that a common conformational feature exists for all V3 loops of infectious strains. The heterogeneity remains important for determining HIV-1 tropism, however, as the major determinants of viral tropism are situated within this loop[14–16].

Previous structural studies on V3 loops have established that the conserved central region (GPGR) has a turn conformation[17–20] and that the C-terminus is inclined to form helices[21–24], which are amphipathic for most strains[24]. In contrast, the ELI V3 loop has a central region which is highly mutated compared to the previously examined strains (GLGQ instead of GPGR) (Fig. 1), and a C-terminal helix, if present, would not be amphipathic. A structural examination by NMR of this "exotic" ELI V3 loop could therefore assist in understanding which features are important for V3 loop functioning.

EXPERIMENTAL

Solid Phase Synthesis

The peptide was sythesized by a stepwise solid-phase method in an automated peptide synthesizer (model 430A, Applied Biosystems, Inc.), according to the *tert*-butoxycarbonyl–trifluoroacetic acid protocol, on a Cys-(4-MeBzl) phenylacetamidomethyl resin (Applied Biosystems). Trifunctional amino acids were protected as follows: Arg (*p*-toluenesulfonyl), Asp (*O*-cyclohexyl ester), Cys (4-methylbenzyl), His (benzyloxymethyl), Lys (2-chlorobenzyloxycarbonyl), Ser and Thr (benzyl ether), Tyr (2-bromobenzyloxycarbonyl). Amino acids were introduced using symmetric anhydride activation in dimethylformamide

V3 ELI

1 5 10 15 20 25 30

C **A** R P **Y Q** N T R **Q R T P** I G **L** G **Q S** L Y T T **R S R S** I I G Q A H C

V3 RF

1 5 10 15 20 25 30 35

C T R P N N N T R K S I **T K** G P G R **V I** Y **A** T G **Q** I I G D I R **K** A H C

V3 Consensus

1 5 10 15 20 25 30 35

C T R P N N N T R K S I H I G P G R A F Y T T G E I I G D I R Q A H C

FIG. 1

Diagram showing the V3 loop consensus sequence and the sequences of the V3 loop peptides from gp120 of the RF and ELI HIV-1 variants. The predicted β turn region is underlined. Boldfaced amino acids are substitutions of, or insertions in, the consensus sequence

(single coupling for the twenty first residues then double coupling), except for Asn, Arg and Gln, which were introduced using the dicyclohexylcarbodiimide–hydroxybenzotriazole activation protocol. At the end of the synthesis, the amino-terminal protecting Boc group was removed, and the peptide was acetylated using acetic anhydride. Final deprotection was done by hydrogen fluoride procedure, for 1 h at 0 °C. The cleaved deprotected peptide was precipitated with cold diethyl ether and then dissolved in 5% acetic acid and lyophilized. The crude peptide thus obtained was dissolved at the molar concentration of 60 μmol l^{-1} in a 0.1 M ammonium acetate buffer (pH 8.5) and stirred under air bubbling during 48 h.

The buffer was then concentrated under reduced pressure and lyophilized. The crude cyclized peptide was purified by reversed-phase HPLC on a 5 μm, 300 A Nucleosil C18 (Macherey Nagel) column (9 × 500 mm) with an acetonitrile–water–0.05% trifluoroacetic acid solvent system, using a 80 min gradient of 0–60% acetonitrile.

The purity of the final peptide was checked by analytical reversed-phase HPLC on a 5 μm, 100 A Vydac C18 column (4.6 × 250 mm) with an acetonitrile–0.05% aqueous trifluoroacetic acid solvent system, using a 30 min gradient of 0–60% acetonitrile (after 5 min isocratic elution at 0% acetonitrile). The compound eluted as a sharp peak at 26 min, and was more than 98% pure. The identity was determined by amino acid analysis after total acid hydrolysis and molecular mass determination, performed by plasma desorption mass spectrometry on a Bio Ion 20. For $C_{163}H_{266}N_{56}O_{50}S_2$ calculated: 3 875.4; found: 3 874.7.

NMR Spectroscopy

The NMR sample was a 4.0 mM solution of the peptide in a mixture of 90% H_2O and 10% D_2O at a pH of 2.6. The solution was not buffered. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate was added as an internal chemical shift reference.

All proton NMR spectra were acquired on a Bruker AM-500 spectrometer. Two-dimensional NMR experiments performed on the water solution ELI V3 peptide sample at 278.0 K were a magnitude COSY (ref.[25]), a DQF-COSY (ref.[26]), a TOCSY (spin-lock time τ_{SL} 55 ms)[27] and a NOESY (mixing time τ_m 400 ms)[28]. For the extraction of the temperature gradients of the amide NH proton chemical shifts ($\Delta\delta_{NH}/\Delta T$), additional ROESY spectra (τ_{SL} 200 ms)[29] were recorded at 278.4, 281.3 and 284.8 K. The H_2O peak was suppressed by selective irradiation during a 1.4 s relaxation delay[30]. The irradiation was continued during the mixing time in the NOESY spectrum. The spin-lock used in the TOCSY spectra, with a field strength of 7.25 kHz, was an MLEV-17 sequence preceded by a 1.5 ms trim pulse. In the ROESY spectra continuous irradiation with a field strength of 3.09 kHz was used for spin-locking. The spectra were acquired in the F2 dimension with 2 048 real data points and in F1 with 512 t1-increments. The Time Proportional Phase Incrementation method[31] (TPPI) was used for phase-sensitive acquisition. The data were processed on a Bruker X32 workstation using the UXNMR program. A squared cosine window function was used in the F2 dimension, a $\pi/3$ shifted sine function in the F1 dimension, except for the processing of the magnitude COSY, where a squared sine function was used in F2 and a sine function in F1. The Fourier-transformed spectra had 2 048 data points in both dimensions, giving a resolution of 3.4 Hz/pt. A polynomial baseline correction was performed in the F2 dimension. Spectral analysis was done manually on plotted spectra and on a Silicon Graphics Crimson workstation with the program PRONTO (ref.[32]).

RESULTS

Resonance Assignments

The proton resonances were assigned according to the established sequential strategy[33] using 2D COSY and TOCSY spectra to recognize amino acid spin systems and 2D ROESY and NOESY spectra to identify sequential connectivities. Table I shows the complete assignment of all protons with their chemical shift values as extracted from the 2D NOESY spectrum at 278 K in water. In Fig. 2 the sequential connectivities from Gly15 to Cys34 are displayed. The low chemical shift dispersion resulted in extensive overlap of the resonances, especially for the side chain protons of the arginine residues. The assignment was also hindered by weak signals probably belonging to *cis* proline isomers of the main peptide. The interresidual NOE contacts that were observed for the ELI peptide in water are summarized in Fig. 3.

Conformation in Water

All observations demonstrate that the ELI peptide exists in aqueous solution as a dynamic mixture of multiple conformations. The chemical shift values of the protons closely approach random coil values[34,35], and NOEs indicative of secondary structure (e.g. $d_{\alpha\alpha}(i, j)$ and $d_{\alpha N}(i, j)$ NOEs for β-sheet and $d_{\alpha N}(i, i + 3)$, $d_{\alpha\beta}(i, i + 3)$ and $d_{\alpha N}(i, i + 4)$ for α-helix) are not observed. Also, both strong sequential $d_{\alpha N}(i, i + 1)$ and $d_{NN}(i, i + 1)$ NOEs are present throughout the peptide, indicating that both the α and β regions of conformational space are being sampled.

A number of observations indicate that some nascent structure is present, however. The NOEs that indicate this structuring are all weak, and do certainly not belong to the major conformations adopted by the peptide. In the N-terminal part of the peptide, three $d_{\alpha N}(i, i + 2)$ NOEs (Cys1-Arg3, Tyr5-Asn7 and Asn7-Arg9) are present, accompanied by some backbone–side chain and side chain-side chain contacts. The temperature coefficients of the amide protons of Gln6, Thr8 and Arg9 (Table I) are also below average (lower than –7 ppb/K, while most values are around –8.5 ppb/K). This indicates that these amide protons are to some level protected from the solvent. Especially the value for the Gln6 amide proton (–4.5 ppb/K) is very low and could indicate hydrogen bonding. Structuring also appears to be present in the Ser19-Arg24 region. Two $d_{NN}(i, i + 2)$ NOEs are present in this region (Leu20-Thr22 and Thr22-Arg24), combined with several side chain–backbone and side chain–side chain contacts, and the amide proton temperature coefficients (Table I) have below average values for the Tyr21-Arg24 residues. Finally, two $d_{\alpha N}(i, i + 2)$ NOEs were observed in the C-terminal region (Ile28-Gly30 and Ile 29-Gln31).

The observed backbone–backbone contacts ($d_{\alpha N}(i, i + 2)$ and $d_{NN}(i, i + 2)$) indicate that nascent helical structures[36] are preferred by the peptide. Also, the sequential $d_{NN}(i, i + 1)$

TABLE I

^{1}H Resonance assignments and amide proton chemical shift temperature coefficients for the V3 ELI peptide in water at pH 2.6 and *T* 278 K

Residue	Chemical shift, ppm						
	NH	$(-\Delta\delta_{NH}/\Delta T)^a$	$C^\alpha H$	$C^\beta H$	$C^\gamma H$	$C^\delta H$	Others
Cys-1	8.57	(8.6)	4.66	3.18, 2.90			
Ala-2	8.72	(8.9)	4.34	1.38			
Arg-3	8.39	(8.6)	4.60	1.79	1.67	3.20	7.26 ($N^\varepsilon H$)
Pro-4			4.39	2.23, 1.79	1.97	3.76, 3.60	
Tyr-5	8.43	(9.8)	4.49	3.03, 2.99		7.11	6.82 ($C^\varepsilon H_2$)
Gln-6	8.26	(4.5)	4.27	2.01, 1.91	2.26		7.61, 6.99 ($N^\varepsilon H_2$)
Asn-7	8.56	(8.1)	4.71	2.88, 2.79			7.75, 7.06 ($N^\delta H_2$)
Thr-8	8.25	(6.9)	4.30	4.30	1.22		
Arg-9	8.47	(5.9)	4.31	1.87^b, 1.78^b	1.63^b	3.18^b	7.25 ($N^\varepsilon H$)
Gln-10	8.41	(9.1)	4.31	2.09, 1.98	2.36		7.65, 7.00 ($N^\varepsilon H_2$)
Arg-11	8.60	(8.6)	4.37	1.86^b, 1.76^b	1.63^b	3.19^b	7.26 ($N^\varepsilon H$)
Thr-12	8.35	(10.2)	4.59	4.15	1.26		
Pro-13			4.44	2.30, 1.88	2.06, 1.98	3.89, 3.70	
Ile-14	8.39	(10.6)	4.13	1.84	1.50, 1.21	0.87	0.92 ($C^\gamma H_3$)
Gly-15	8.58	(8.0)	3.95				
Leu-16	8.36	(10.3)	4.34	1.66, 1.62	1.64	0.93, 0.89	
Gly-17	8.70	(8.1)	3.94				
Gln-18	8.31	(8.6)	4.37	2.13, 1.99	2.34		7.68, 6.99 ($N^\varepsilon H_2$)
Ser-19	8.55	(8.1)	4.43	3.90, 3.85			
Leu-20	8.40	(8.8)	4.27	1.50^b, 1.39^b	1.50^b	0.88, 0.82	
Tyr-21	8.27	(6.7)	4.62	3.11, 2.94		7.10	6.81 ($C^\varepsilon H_2$)
Thr-22	8.12	(6.4)	4.37	4.25	1.20		
Thr-23	8.26	(5.6)	4.30	4.26	1.24		
Arg-24	8.46	(6.4)	4.34	1.87^b, 1.79^b	1.65^b	3.18^b	7.25 ($N^\varepsilon H$)
Ser-25	8.42	(9.8)	4.40	3.90, 3.85			
Arg-26	8.50	(7.5)	4.35	1.89^b, 1.78^b	1.65^b	3.19^b	7.26 ($N^\varepsilon H$)
Ser-27	8.39	(10.0)	4.45	3.84			
Ile-28	8.33	(9.5)	4.21	1.87	1.45, 1.17	0.85	0.89 ($C^\gamma H_3$)
Ile-29	8.34	(9.2)	4.13	1.85	1.51, 1.21	0.87	0.92 ($C^\gamma H_3$)
Gly-30	8.66	(10.5)	3.98, 3.93				

NOEs for the ELI V3 peptide are relatively strong compared to two previously studied V3 loop peptides[23,24]. No NOEs characteristic of a turn in the central region (GLGQ) could be discerned.

DISCUSSION

The ELI peptide, despite its exotic sequence, corresponds quite well with previously studied V3 loops[17,18,21–24] when the regions where structuring occurs are compared.

The region around the glycosylation site[8] of the V3 loop (Gln6, usually Asn6), is relatively structured. A turn, which could be important for exposing Gln6 for glycosylation, has been observed for other V3 loop peptides in this region[18,22–24], and also seems to be present in the ELI peptide: two $d_{\alpha N}(i, i + 2)$ NOEs (Tyr5-Asn7 and Asn7-Arg9) indicate backbone folding, and the temperature coefficient for the amide proton of Gln6 is very low, indicating hydrogen bonding.

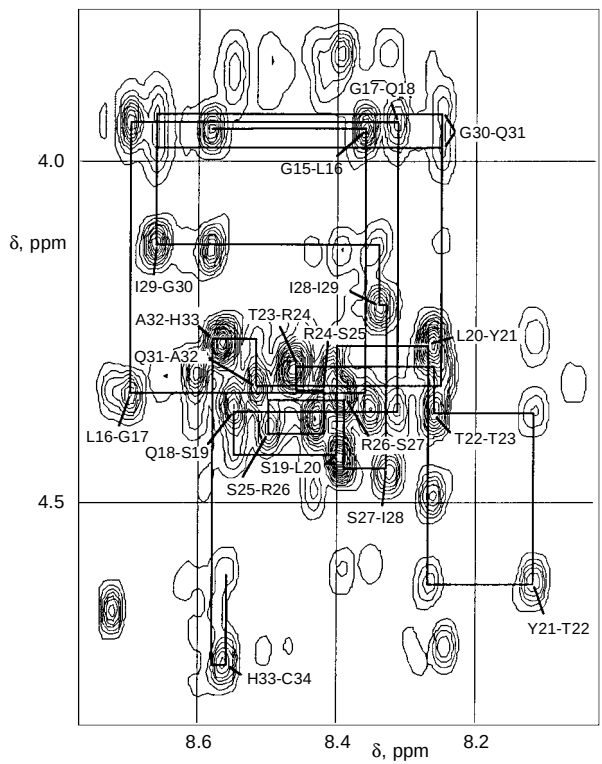

FIG. 2
Fingerprint region of the NOESY spectrum at 278 K of the V3 ELI peptide in water. The sequential walk from G15 to C34 is shown

While the central GPGR region has a turn conformation in all previously studied V3 loop peptides, no NOEs confirming a turn could be observed for the corresponding GLGQ part of the ELI peptide. However, its presence cannot be completely excluded since the Leu16-Gln18 $d_{\alpha N}(i, i + 2)$ NOE, which is a major indication for a turn, is obscured by overlap. If the turn is not present, this is probably due to the divergent sequence of the ELI peptide in this region (Fig. 1): no proline residue is present at position 16, and this residue is known to assist turn formation.

The region C-terminal to the turn is also relatively structured. The Ser19-Arg24 region is characterized by two $d_{NN}(i, i + 2)$ NOEs, a range of other NOE contacts, and lowered temperature coefficients for the amide protons from Tyr21-Arg24. Nearer the C-terminus two $d_{\alpha N}(i, i + 2)$ NOEs (Ile28-Gly30 and Ile29-Gln31) are observed.

The C-terminal region of V3 loops is known to prefer helical conformations, especially in TFE–water solutions[21–24]. Although we were not able to study the ELI V3 peptide in TFE–water, the presence of $d_{NN}(i, i + 2)$ and $d_{\alpha N}(i, i + 2)$ NOEs, and the relatively high intensity of the sequential $d_{NN}(i, i + 1)$ NOEs observed in the C-terminal region of this peptide in water indicate some preference for the α region of conformational space[36]. These NOEs are comparable to the NOEs observed for the RF V3 loop peptide in water[24], and this peptide did adopt helical conformations in TFE–water mixtures, indicating that the helical tendency of the ELI V3 peptide could be comparable.

A major similarity between the ELI, RF and Consensus V3 loop peptides is the presence of the $d_{\alpha N}(i, i + 2)$ NOEs in their Ile-Ile-Gly parts (in the Consensus V3 loop peptide, the $d_{\alpha N}(i, i + 2)$ NOE between the first Ile and the Gly can be present, but is obscured by overlap). This Ile-Ile-Gly part corresponds to positions 28–30 in ELI and 26–28 in RF and Consensus, and although these parts were not aligned in a sequence

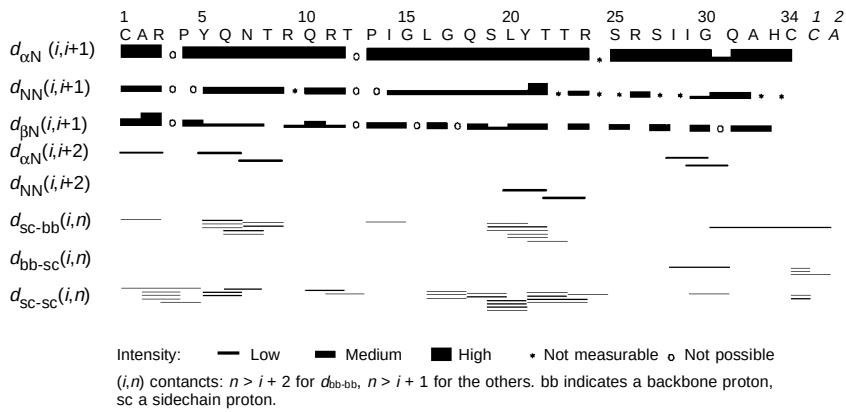

FIG. 3
Diagram showing the NOEs connections for the V3 ELI peptide in aqueous solution at pH 2.6 and *T* 278 K

comparison by LaRosa et al.[12], we suggest that they are equivalent, so that the Arg24-Ser27 part of ELI substitutes the Gly24-Glu25 part of Consensus, and that the Asp29-Arg31 part of Consensus is deleted in ELI (Fig. 1). This Ile-Ile-Gly tripeptide part is relatively conserved: of the 245 amino acid sequences examined by LaRosa et al., the first isoleucine is substituted with a non-conservative amino acid only 5 times, the glycine only 12 times. The central isoleucine is less conservative and is different in 35 cases. Also, this tripeptide fragment is situated behind two residues which are important in determining viral tropism, and is located in the expected helix area. These observations indicate that the Ile-Ile-Gly tripeptide fragment could be an important and common structural factor in V3 loops.

This study shows that the ELI V3 loop peptide has a preference for nascent helix in its C-terminal part. Actual helix formation could not be observed, and it is thus not clear if a C-terminal helix can be formed in this ELI peptide. It has been suggested that amphipathicity of a C-terminal V3 loop helix enables interaction with a biological membrane, and that this amphipathicity could thus be an important factor for HIV functioning. Most HIV strains can form amphipathic C-terminal helices in their V3 loop, but, in contrast, the ELI strain does not possess this characteristic, and this raises questions to the importance of amphipathic helices. However, HIV is known for its diversity, and it is still possible that a different mechanism (other than helix formation) plays a role in V3 loop functioning. In this regard, the ELI V3 loop could form another structure in contact with a biological membrane, and thus still be able to function, or a different mode of entry could exist for this strain. On the other hand, if this ELI V3 loop does form a C-terminal helix, the helix formation itself (regardless of amphipathicity) would be important. More research is necessary to determine the important structural factors of the V3 loops of these divergent strains.

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