Structure and Polymorphism of HIV-1 Third Variable Loops*

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The third variable (V3) loop of HIV-1 surface glycoprotein, gp120, has been the target of neutralizing antibodies. However, sequence variation inside the V3 loop diminishes its effectiveness as a potential vaccine against HIV-1. The elusive nature of the V3 loop structure prompted us to carry out a systematic study on different isolates in an attempt to identify a common structural motif in the V3 loop regardless of the amino acid sequence variability. We have previously determined the structural features of two V3 loops: V3 Thai and V3 MN. In this paper, we present the structure of two other variants: V3 Haiti and V3 RF. Our results show that similar secondary structures are observed in all the four V3 loops: a GPG(R/K/Q) crest in the center of the neutralizing domain, two extended regions flanking the central crest, and a helical region in the C-terminal domain. For the Haitian V3 loop, we also show how the conserved structural features are masked through a conformational switch encoded in the amino acid sequences on the C-terminal side of the GPGK crest.

A neutralizing determinant (1–8) located inside the V3 loop of the envelope glycoprotein, gp120, has been the target for protective immunity against the human immunodeficiency virus, type 1 (HIV-1). However, the amino acid sequence variation within the V3 loop has eluded the effectiveness of V3-based vaccine design (4, 5). Antibodies against the V3 loop generally exhibit type-specific neutralization profiles (6, 7), although a subset of anti-V3 antibodies specific for the less variable elements of the V3 loop show a broader range of neutralizing activity (7, 8). To better understand the effect of sequence variation on the structure and antigenicity of the HIV-V3 loop, we developed a method combining molecular modeling (9, 28) and two-dimensional NMR (10, 11) to analyze the global structure of the entire cyclic V3 loop and the local structure at the neutralizing determinant. We attempted to answer two specific questions: (i) Are there conserved secondary structural elements inside the V3 loop in spite of sequence variation? and (ii) Can the sequence variation inside the V3 loop mask this conserved secondary structure? Recently, we have shown (12) that in spite of the observed sequence variation, a conserved secondary structure is located inside the V3 loop. This structure consists of a solvent-accessible protruding motif (or a knob) spanning 8–10 residues with a central GPG(Q/R/K) type II turn at the crest of the knob (10–12). In this article, we demonstrate how amino acid sequence variation flanking the GPG crest can camouflage an otherwise conformationally pure epitope. For this purpose, we performed two-dimensional NMR and molecular modeling studies on two cyclic V3 loops from the Haitian and RF isolates (13):

V3-Haiti  
1  CCTRPNMTKPI 36 net charge +3
2  PMIIGYATG

V3-RF  
1  CCTRPNMTKPI 36 net charge +5
2  PMIIGYATG

(Cysteines at position 2 and 36 are S–S bridged; the first cysteine that is underlined in the sequence has a protective group on S; site-specific differences in sequence are marked in bold).

MATERIALS AND METHODS

Synthesis and Purification—The cyclic Haitian V3 loop was synthesized and purified by Dr. Anita Hong (Anaspec, CA). The cyclic RF V3 loop was synthesized and purified by Dr. G. M. Anantharamaiah, University of Alabama at Birmingham. Both Drs. Hong and Anantharamaiah provided their services under a contract with the AIDS Division of the National Institutes of Health.

NMR Spectroscopy—All NMR experiments on the RF V3 loop were carried out on the 600 MHz Bruker spectrometer at the University of Alabama at Birmingham, whereas the data on the Haitian V3 loop were collected on a 500 MHz Bruker AMX spectrometer at Chemistry, Science, and Technology-4, Los Alamos National Laboratory. NMR spectra were collected at 10°C with 3 mM peptide concentration (pH 5.5) for the Haitian V3 loop and at 5 mM peptide concentration (pH 5.5) for the RF V3 loop. All two-dimensional data were acquired in the phase-sensitive mode with the presaturation of the HDO signal during the relaxation delay. DQF-COSY data (14) were collected with the following acquisition parameters: t2 = 4096, t1 = 1024, relaxation delay = 1.5 s, number of transients = 32 for the Haitian V3 loop and number of transients = 48 for the RF V3 loop. Total correlated spectroscopy data (14) were collected with the following parameters: t2 = 2048, t1 = 1024, relaxation delay = 1.5 s, number of transients = 3248, isotropic mixing = 70 ms. NOESY (14) data were collected with similar acquisition parameters and with 75 and 250 ms of mixing time (τm) for the Haitian V3 loop and 200 and 400 ms for the RF V3 loop. The sequential assignments2 were performed by combining the total correlated spectroscopy, DQF-COSY, and NOESY data, processed on a SGI Indigo instrument using the Felix software (BioSym Inc.).

Structure Derivation—Two-dimensional NMR data of the Haitian V3 loop in water and in TFE/water mixture and for the RF V3 loop in water were analyzed with the aid of full-matrix NOEYS simulations, associated R-factor test, and energy calculations (10, 11). This produced a set of distance constraints (9–12, 28). The energy term (EDIST defining the
distance constraints) is added to the force field as in the work of Scheraga and co-workers (15). Monte Carlo simulated annealing (10, 11, 16) is performed to obtain a set of structures in agreement with NMR data. The maximum step size of the torsion angles was set at 15°, which produced an acceptance ratio of 0.20–0.30 for the 50,000 step MC cycle at each temperature. Full-matrix NOESY calculations were repeated for the final 50 low energy structures of the V3 loop. We define distances from the NOE data in terms of the upper and lower limits (range is above 0.5 Å). However, in our sampled structures the violations of these distances are ~0.22 Å. This implies that the uncertainty in distance estimate is always above 0.7 Å.

RESULTS

NMR Experiment on the Haitian V3 Loop—Fig. 1A shows the NOESY fingerprint HN-Hα region of the Haitian V3 loop in water at 10°C for 250 ms of mixing. Fig. 1B shows the DQF-COSY fingerprint HN-Hα region of the Haitian V3 loop in water at 10°C; the ranges of φ values are estimated from J_{HN-HN} coupling data. Note the presence of intra-residue HN-Hα and inter-residue HN(i+1)-Hα(i) cross-peaks. Proline residues (Pro5, Pro14 and Pro17) show Hα(i)-Hα(i+3) cross-peaks. Note that residues Cys2, Cys3, Thr3, and Arg4 do not show any cross-peaks. Fig. 1C shows the NOESY HN-HN region of the Haitian V3 loop in water at 10°C for 300 ms of mixing. Although a set of sequential HN-HN cross-peaks are observed in the C-terminal stretch (residues 28–33) of the V3 loop, no medium range NOEs like Hα(i)-Hβ(i+3) or HN(i)-Hα(i+3) cross-peaks are seen as a corroboratory evidence of a helical stretch. Fig. 3 summarizes the NOE data of the Haitian V3 loop in aqueous environment from the analyses of the data at 100 and 300 ms of mixing. Note that only a few Hα(i)-HN(i+1) cross-peaks are observed for the Haitian V3 loop in the aqueous environment. Seven nonsequential NOESY cross-peaks are observed (see panel of Fig. 3B).

Fig. 2A shows the NOESY fingerprint HN-Hα region of the Haitian V3 loop in a water/TFE (7:3) mixture at 10°C for 250 ms of mixing. Although the cross-peaks of the Haitian V3 loop are broader in the mixed solvent than in the aqueous environment, we are able to assign residues 5–36. Fig. 2B shows the NOESY HN-HN region of the Haitian V3 loop in the mixed solvent at 10°C for 250 ms of mixing. Due to the broadness of the peaks it is not possible to decipher the interaction of two HN protons that are close to the diagonal. However, quite a number of distinct HN-HN cross-peaks are observed in this cross-section. Fig. 3 summarizes the NOE data of the Haitian V3 loop in the mixed solvent from the analyses of the data at 75 and 250 ms of mixing. The NOE data of the Haitian V3 loop in the mixed solvent is distinct from that in water in the following respects. (i) Medium range Hα(i)-Hα(i+3) cross-peaks are observed for the residue pairs (27, 30), (31, 28), (32, 29), and (33, 30) which are indicative of a helical core spanning residues 27–33. (ii) Although there is a decrease in the absolute intensities of sequential HN-Hα and HN-HN cross-peaks, there is an enhancement in the relative HN-HN/HN-Hα cross-peak intensities for residues 26–34, which is again indicative of a helical structure in this segment. (iii) A few sequential Hα(i)-HN cross-peaks are observed in this stretch that are either weak or absent in the aqueous solvent. (iv) Finally, the Hα protons of

Fig. 1. NOESY (mixing time = 250 ms) and DQF-COSY cross-sections of the cyclic Haitian V3 loop in water (peptide concentration = 3.0 mM, pH 5.5, temperature = 10°C). A, the fingerprint HN-Hα region. B, DQF-COSY HN-Hα cross-section. C, the HN-HN region. For NOESY experiments, the acquisition parameters were as follows: t2 = 2048 data points, t1 = 1024 data points, relaxation delay = 1.5 s, number of transients = 32. The same acquisition parameters were used for the DQF-COSY experiment except for t2, which was increased to 4096 data points. Sequence specific assignments (14) were obtained starting from Phe21 (only Phe in the sequence) and moving backward and forward along the connectivity route until completion of the assignments. Note the resonance doubling of the residue Gly23, indicating a conformational equilibrium between the two forms. However, no additional NOEs to discriminate between the two conformations were observed.
observed in mixed populations; Gly^{18} shows a H("G18)-HN(F21) cross-peak (Fig. 3). The chemical shift of other residues in the second population are indistinguishable from the first. Although this conformational variant is also present in the aqueous solvent, the absence of the H("G18)-HN(F21) cross-peak suggests that such an interaction perhaps is not stabilized in a polar environment.

NMR Experiment on the RF V3 Loop—We have chosen the RF V3 loop to examine the effect of sequence variation on the overall folding of the V3 loop and the local structure at the GPG crest. The RF V3 loop is different from the Haitian V3 loop at eight positions and is more positively charged. Fig. 4A shows the NOESY fingerprint HN-H" region of the RF V3 loop in water at 10 °C for 400 ms of mixing. Note the presence of intra-residue HN-H" and inter-residue HN(i+1)-H"(i) cross-peaks except for the residues Cys^5, Cys^6, Thr^7, Arg^8, Pro^9, and Asn^10, possibly due to inherent flexibility in the N-terminal region. Fig. 4B shows the NOESY HN-HN region of the RF V3 loop in water at 10 °C for 300 ms of mixing. Although a set of sequential HN-HN cross-peaks are observed in the C-terminal stretch (residues 29–34) of the V3 loop, no medium range NOEs indicative of helical fragments are seen. Fig. 5 summarizes the NOE data of the RF V3 loop in the aqueous environment from the analyses of the data at 200 and 400 ms of mixing. Only intra-residue and sequential NOEs are observed. The J_{HN-HN} coupling constants from the DQF-COSY spectrum (Fig. 4C) provide the ψ values, which are converted into HN-H" intra-residue distances.\(^2\) The lack of solubility of the highly polar RF V3 loop prevented us from carrying out NMR experiments in the mixed water/TFE (7:3) solvent.

Structures of the Haitian V3 Loop—We have previously reported structural studies on the Thailand and MN V3 loops (10, 11). The NMR data for those two sequences revealed that the structure of the V3 loop contained a few reasonably well defined secondary structural elements, i.e. a GPGRI(Q) turn and a nascent C-terminal helix. However, the V3 loops are considerably flexible within the constraints of these secondary structural elements and the Cys^2-Cys^6 disulfide bridge. Therefore, we developed and applied a method to explore the extent of conformational flexibility of the V3 loop that is consistent with the NMR data (10, 11). Briefly, we employ the following steps. (i) We analyze the NMR data to assign secondary structural states, i.e. ranges of ϕ and ψ values, to the residues of the V3 loop. The NMR data for the aqueous form include the NOEs in Fig. 3 and the DQF-COSY data (Fig. 1C); the J_{HN-HN} coupling constants from the DQF-COSY spectrum provide the ψ values, which are converted into HN-H" intra-residue distances.\(^2\) Line broadening in the mixed solvent prevented us from obtaining a high quality DQF-COSY spectrum in the water/TFE (7:3) mixture. (ii) We obtain a set of starting (energy minimized) structures of the V3 loop subject to the ϕ and ψ values and disulfide bridge constraints. (iii) We then use these starting structures for Monte Carlo simulated annealing and energy minimizations for sampling the conformational space. (iv) We finally select a set of low energy structures (50 for the Haitian V3 loop) and analyze the conformational parameters to examine the nature of the flexibility.

Fig. 6 shows the ribbon models of the Haitian V3 loop in water (left) and water/TFE (7:3) mixture (right). The following color coding was used in these ribbon diagrams: gray for the N-terminal protruding loop at position T3-R10, green for the N-terminal extended β-strand flanking the GPG crest, magenta for the central β-turn at position G16-P17-G18-K19, yellow for the C-terminal extended β-strand flanking the GPG crest, and blue for the C-terminal segment D26-H35, which can form an α-helix. In water the C-terminal segment consists of
shown. However, in the representations only average positions for the side chains are and they do not sample a single rotamer conformation. In these representations only average positions for the side chains are shown. However, in the α-helical region for the mixed solvent structure, the side chains are organized in a cylindrical array as experimentally observed by the presence of a network of d\(^{13}\)([i,i+3]) and d\(^{15}\) sequential connectivities (Fig. 3B). Nonetheless, in both the structures (Fig. 6) the neutralizing epitope containing the central GPGK sequence forms a protruding loop even though the local structure and presentation of the loop in the two states are noticeably different. The aqueous structure of the Haitian V3 loop in Fig. 6 is the average of 50 sampled conformations that exhibit rms deviations below 1.5 Å with respect to the backbone atoms. Out of 50 sampled structures of the Haitian V3 loop in the TFE/water mixture, a small subset of six structures shows a large (>2.6 Å) rms deviation of the backbone atoms from the rest of the structures. The remaining 44 structures are within 1.6 Å rms deviations of the backbone atoms. The average structure in Fig. 6 is taken over these 44 structures.

Fig. 7 shows two conformations representing the aqueous environment (left) and the mixed solvent forms (right). The central region of the Haitian V3 loop containing the neutralizing determinant residues Ile\(^{13}\)-Pro\(^{14}\)-Met\(^{15}\)-Gly\(^{16}\)-Pro\(^{17}\)-Gly\(^{18}\)-Lys\(^{19}\)-Ala\(^{20}\)-Phe\(^{21}\)-Tyr\(^{22}\) is shown. The following color coding was used in these skeleton models: red for the central Gly\(^{16}\), Pro\(^{17}\)-Gly\(^{18}\)-Lys\(^{19}\) crest, green for the N-terminal Ile\(^{13}\)-Pro\(^{14}\), Met\(^{15}\) residues in extended conformation, and yellow for the C-terminal Ala\(^{20}\)-Phe\(^{21}\)-Tyr\(^{22}\) residues, which show a solvent-induced effect. In the water structure the C-terminal fragment is in an extended conformation (open state). In the mixed solvent, two types of chain folding are observed: one folded form is similar to that of the MN-V3 loop (11), whereas in the other, the GPG crest forms the typical type II β-turn followed by a type III β-turn involving residues Gly\(^{18}\)-Arg\(^{19}\)-Ala\(^{20}\)-Phe\(^{21}\), as evidenced by the presence of a medium range NOE between H\(^{\alpha}\)-G18 and H\(^{\alpha}\)-F21 (closed state). Such an S-shaped conformation has been previously reported for a peptide containing the V3 neutralizing determinant complexed to an antibody (18), and it will be referred as “arched” conformation for the rest of the paper. Our NMR data (Figs. 1A and 3) clearly indicated that these two states are simultaneously present in mixed solvent, whereas only the open state exists in aqueous solutions. We have not shown the open state structure of the Haitian V3 loop in the mixed solvent because it closely resembles the already published structure of the MN-V3 loop (11).

Structure of the RF V3 Loop—Fig. 8 shows the ribbon diagram for the structure of the RF V3 loop in water. Here, again the conformational analysis is done for 50 sampled low energy structures. All the sampled structures showed rms deviations of 0.22 ± 0.02 Å with respect to 79 independent distance constraints. The same color coding described in Fig. 6 was used here. The average structural features of the V3-RF in water resemble those observed for the Haitian and the MN V3 loops (11); however, the absence of any nonsequential NOE suggests
that the RF V3 loop structure is considerably more flexible than the Haiti-V3 and MN-V3 loops. Out of 50 sampled structures, a small subset of eight structures shows a large (>2.7 Å) rms deviations of the backbone atoms from the rest of the structures. The remaining 42 structures are within 1.7 Å rms deviations of the backbone atoms. The average structure in Fig. 8 is taken over these 42 structures.

**DISCUSSION**

Previous NMR studies on the Thailand and MN V3 loops (10, 11) and the current work on the Haiti and RF V3 loops (Figs. 6–8) can be summarized as follows: (i) A GPG-turn at the crest of the V3 loop is present in all the four sequences. (ii) Stretches of β-strand adjacent to the GPG-turn on the N- and C-terminal sides are common to all the four sequences. (iii) The residues in the C-terminal segment form a few turns in water and a helix in the less polar mixed solvent. (iv) In spite of the constraints of secondary structures (ii)–(iii) and the disulfide bridge, the V3 loop exhibits conformational flexibility as evidenced by the absence of long range NOESY interactions commonly observed in well folded globular proteins (14).

However, a "protruding knob" formed by the central GPG turn and the β-strands on either side emerges as the secondary structural feature conserved among diverse V3 loop sequences. The single crystal structure of the HIV-1 neutralizing antibody (monoclonal antibody 50.1) complexed to 16-residue-long linear MN-V3 fragment shows the hint of such a protruding knob, although the segment on the C-terminal side of the GPGR type II turn remains disordered (18). The crystallographic observation suggests that the protruding knob of the V3 loop that includes the neutralizing epitope might well be specifically recognized by the antibody. However, the conserved protruding knob of the V3 loop need not always be presented in its conformationally pure form because HIV may find a way to mask this conserved secondary structural element. In this work we report one such mechanism of masking as revealed by the closed state in Fig. 7. In this form of the Haitian V3 loop, the NMR data indicate an arching of the residues on the C-terminal side of the GPGR turn. This is a departure from the protruding knob motif that contains the central GPG turn and two β-strands on either side. Such an arched conformation of the neutralizing epitope has also been observed in an antibody (monoclonal antibody 59.1) complexed with a linear V3 fragment (18).

When combined with the single crystal data (17, 18), our NMR data (Refs. 10 and 11 and this work) indicate that the closed or arch conformation of the neutralizing epitope of the V3 loop is possible and can be recognized by the antibody. In addition, our data also indicate that an equilibrium between the closed and open state (Fig. 7) is possible for the same V3 loop sequence. The arching around Ala20-Phe21 tends to mask Lys19 and Ala20 (Fig. 7). The closed form of the V3 loop may camouflage some essential elements of the neutralizing epitope from the immune system. For instance, this masking will interfere with the binding of antibodies (8, 19) that recognize the PGRAF epitope.

Most importantly such a local masking of Ala20 and Phe21 should affect the proteolysis of the (Arg/Gln/Lys)19-Ala20 peptide bond by thrombin and trypsin (20, 21); the second enzyme

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Fig. 4. NOESY (mixing time = 400 ms) and DQF-COSY cross-sections of the cyclic RF V3 loop in water (peptide concentration = 5.0 mM, pH 5.5). A, the fingerprint HN-Hα region. B, the HN-HN region. C, DQF-COSY cross-sections of the cyclic RF V3 loop. For NOESY experiments, the acquisition parameters were as follows: $t_2 = 2048$ data points, $t_1 = 1024$ data points, relaxation delay = 1.5 s, number of transients = 32. Same acquisition parameters were used for the DQF-COSY experiment except for $t_2$, which was increased to 4 K. Sequence-specific assignments were obtained starting from Val20 (only Val in the sequence) and moving backward and forward along the connectivity route until completion of the assignments. Assignments in the fragment Cys1-Asn7 were not possible, presumably due to the intrinsic flexibility in the region Asn6-Asn7-Asn8.
FIG. 6. The ribbon diagrams describe representative folding patterns for the structures of the Haitian V3 loop in water (left) and in mixed water/TFE solvent (right). The following color coding was used in these ribbon diagrams: gray for the N-terminal protruding loop, green for the N-terminal extended β-strand flanking the GPG crest, magenta for the central β-turn at the GPG crest, yellow for the C-terminal extended β-strand flanking the GPG crest, and blue for the C-terminal segment, which can form an α-helix. In each case, the average is done over 50 sampled low energy structures. Ribbon models in the two cases correspond to the average structure. All the sampled structures of the Haitian V3 loop in water showed rms deviations of 0.24 ± 0.02 Å with respect to 95 independent distance constraints. All the sampled structures of the Haitian V3 loop in mixed water/TFE solvent showed rms deviations of 0.27 ± 0.02 Å with respect to 123 independent distance constraints. The structures of the Haitian V3 loop in water show a greater degree of flexibility than those in the mixed water/TFE solvent; this is due to the formation of the C-terminal helix in the mixed solvent.

FIG. 7. Two conformations representing the aqueous environment (left) and mixed solvent forms (right). The central region of the Haitian V3 loop containing the neutralizing determinant residues Ile13-Pro14-Met15-Gly16-Pro17-Gly18-Pro19-Ala20-Phe21-Tyr22 are shown. The following color coding was used in these skeleton models: green for the N-terminal Ile13-Pro14-Met15 residues in extended conformation, red for the central Gly16-Pro17-Gly18-Lys19 crest, and yellow for the C-terminal Ala20-Phe21-Tyr22 residues, which show solvent induced arching effect. Solvent-accessible areas were calculated using the Molecular Surface Package due to Connolly (27) with a probe radius of 1.5 Å. In the fragment Lys19-Phe22, the aqueous structure has a lower surface accessibility than the structure in the mixed TFE/water solvent.
lies on the T-cell surface. When gp120 is used as a substrate these two enzymes show exceptional specificity for cleavage of the (Arg/Gln/Lys)\(^{19}\)-Ala\(^{20}\) peptide bond inside the V3 loop. Most striking is the observation that the V3 loops in water (11) resemble those observed for the Haitian and the MN V3 loops in water (11).

Fig. 8. The ribbon diagrams describe representative folding pattern of the RF V3 loop in water. The same color coding described in the legend to Fig. 6 was used here. The structural features of the V3-RF in water resemble those observed for the Haitian and the MN V3 loops in water (11).

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