TIME DEPENDENCE OF IMMUNORESPONSE OF DIFFERENT EPITOPES OF gp41 AND ITS RELATION TO THE STRUCTURE OF SHORT PEPTIDE FRAGMENTS OF gp41

Dalibor STYS[a,*], Jan NOVAK[b], Viktor KRCHNAK[c,**], Josef VAGNER[c], Petr STROP[a,**] and Milos BUDESINSKY[a]

[a] *Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, 166 10 Prague 6, The Czech Republic*
[b] *Institute of Hematology and Blood Transfusion, 101 03 Prague 10, The Czech Republic*
[c] *Institute of Sera and Vaccines, 101 03 Prague 10, The Czech Republic*

Received December 30, 1994
Accepted April 15, 1995

Twenty amino acid residues peptides derived from the N-terminal domain of glycoprotein gp41 exhibit four different types of reactivity with human sera in the course of seroconversion. The differences in the accessibility of the peptide by immune apparatus can result from different binding of the knobs formed by trimers of glycoprotein gp120 to the surface of the viral particle. The region covered with peptides of higher immunoresponse may be involved in binding of knobs to the viral surface. The loss of knobs during ageing is probably the source of differences in the time-dependence of immunoresponse of different epitopes. Peptide fragments covering amino acids 580–588 and 592–612 have been already studied in detail from the point of view of their reactivity with sera and structure. Here we describe new reactive region between the residues 612–629. The structure features of corresponding peptide in solution (studied by ^{1}H NMR spectroscopy) are different from those previously reported. In this particular case peptides corresponding to different regions of gp41 differ also in their structures in solution prior to the binding to the antibody.

Peptide fragments of the N-terminal domain of gp41 are known for their reactivity with human HIV-1 positive sera[1]. The gp41 protein is involved in holding the knobs formed by trimers of gp120 on the viral surface[2]. Virions are losing knobs during ageing and new, previously hidden parts of gp41 appear on the surface. The ability of human immune system to form antibodies against certain gp41 fragments in different stages of ageing may therefore be determined not only by the number of viral particles but also by their stage of development. The relation between the stage of development of the disease and the age of the viral particles is of general interest. This led us to test the

* Present address: Plant Cell Biology, University of Lund, S-22007 Lund, Sweden.
**Present address: Selectide Corporation, Tucson, AZ 85737, U.S.A.

immunoresponse of sera taken from patients in various stages of AIDS against the peptides mapping the N-terminal domain of the gp41.

It has been known for a long time that peptides which react with sera have a tendency to form well defined structure in solution[3]. The existence of peptides with different reactivities, which is a clear indication that different epitopes are present in their structures, enabled us to study the extent to which differences in immunoresponse correlate with the differences in the structure of peptides in solution.

RESULTS

The sequence of N-terminal part of HIV-1 (the LAV isolate) gp41 (spanning over 586 – 631 region) was divided into fourteen peptides, each with 20 amino acid residues, shifted by 2 residues (Table I). These peptides were subjected to testing with anti-HIV-1 Seroconversion Panel C. Four types of reactivity were observed (Fig. 1). Most of the peptides showed only slight growth of response during the seroconversion (type 1, peptides p-15-1, p-15-3 to p-15-6, p-15-9 and p-15-11 to p-15-14). This type of reactivity is represented in Fig. 1 by peptide p-15-3. Peptide p-15-7 showed high level

TABLE I
Sequences of peptides used for tests of immune reaction. The sequences of known epitopes are in bold, the peptides of high immunity reaction are underlined

Peptide	Position	Sequence
p-15-14	586 – 605	KQLQARIL**AVERYLKDQQLL**
p-15-13	588 – 607	LQARIL**AVERYLKDQQLLGI**
p-15-12	590 – 609	ARIL**AVERYLKDQQLLGIWG**
p-15-11	592 – 611	IL**AVERYLKDQQLLGIWGCS**
p-15-10	594 – 613	**AVERYLKDQQLLGIWGCSGK**
p-15-9	596 – 615	**ERYLKDQQLLGIWGCSGKLI**
p-15-8	598 – 617	**YLKDQQLLGIWGCSGKLIC**T
p-15-7	600 – 619	**KDQQLLGIWGCSGKLIC**TTA
p-15-6	602 – 621	**QQLLGIWGCSGKLIC**TTAVP
p-15-5	604 – 623	**LLGIWGCSGKLIC**TTAVPWN
p-15-4	606 – 625	**GIWGCSGKLIC**TTAVPWNAS
p-15-3	608 – 627	**WGCSGKLIC**TTAVPWNASWS
p-15-2	610 – 629	**CSGKLIC**TTAVPWNASWSNK
p-15-1	612 – 631	**GKLIC**TTAVPWNASWSNKSL

of reactivity in all samples of Panel C (type 2). Peptide p-15-8 showed a significant linear increase of reactivity during seroconversion (type 3) whereas the peptides p-15-2 and p-15-10 showed a non-linear course of reactivities (type 4).

The peptide p-15-10 contains at its N-terminus (amino acids 594 – 600) a region which forms a linear epitope (epitope 1) as has been revealed by careful studies using both infected sera and monoclonal antibodies[4,5]. The next region forming an epitope 2 found in those studies[4,5] corresponds to the C-terminus of peptide p-15-8. This observation leads us to the conclusion that the existence of the epitope at one of the termini of the peptide may be favourable for its reactivity because of its ability to form appropriate structure with minimal influence from other part of the sequence. The peptide p-15-2 contains only part of the epitope 2 at its N-terminus, and its reactivity is similar to that of peptide p-15-10 which contains epitope 1 at its N-terminus. A new epitope should be found at the C-terminus of p-15-2 (epitope 3).

Structures of peptides corresponding to epitopes 1 and 2 have been studied by NMR (refs[6,7]) and two different structures have been found. We have studied the structure of the peptide p-15-2 to learn the structure of epitope 3.

Complete sequence-specific assignment of the proton resonances has been obtained using DQF-COSY (ref.[4]), TOCSY (ref.[8]) and ROESY (ref.[9]) spectra measured at 5 and 30 °C. Chemical shifts of protons at 5 °C are given in Table II. To distinguish between "folded" and "random-coil" conformation of peptide in solution the following NMR criteria are commonly used: (i) chemical shift distribution, (ii) vicinal J(H,H) values, (iii) temperature changes of NMR parameters, (iv) interproton nuclear Overhauser enhancements (NOE). The observation of interproton NOE's provides most valuable structural information (for review see refs[10,11]). For each type of secondary structure an appropriate pattern of NOE's can be found[12]. In a small linear peptides the existence of one well defined tertiary structure in water solution is rare. It is common that solvation is energetically superior to the forces holding folded conformation in a peptide[10].

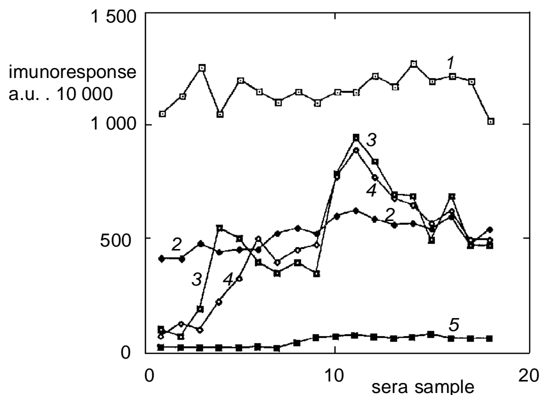

FIG. 1
Time course of intensity of immune reaction of peptides to infected sera. *1* p-15-7, *2* p-15-8, *3* p-15-10, *4* p-15-2, *5* p-15-3

In the NMR spectra of the peptide p-15-2 several indications of the existence of folded secondary structure have been found. Figure 2 shows differences between the observed and "random-coil" values of chemical shifts of αH protons. It has been shown[13,14], that the differences larger than +0.1 and/or –0.1 ppm can be taken as significant for "folded" form if they appear at least at four neighbouring residues with the same sign (positive differences indicating β-strand and negative ones α-helical segment). According to this rule we could expect at least helical features in the C-terminal region of peptide p-15-2 between N-14 and K-20 residues.

TABLE II
Proton chemical shifts in peptide p-15-2 at 5 °C

Residue	NH	αH	βH	Other protons
Cys-1	–	4.37	3.31, 3.18	–
Ser-2	8.8	4.6	3.83	–
Gly-3	8.71	4.03, 3.79	–	–
Lys-4	8.25	4.36	1.95	γH 1.35, δH 1.63, εH 2.88, εNH 7.56
Leu-5	8.03	4.26	1.72, 1.53	γH 1.57, δH 0.90, 0.85
Ile-6	8.07	4.11	1.83	γH 1.44, 1.14, 0.88, δH 0.83
Cys-7	8.63	4.78	3.26, 3.01	–
Thr-8	8.27	4.41	4.2	γH 1.15
Thr-9	8.29	4.3	4.14	γH 1.17
Ala-10	8.43	4.3	1.32	–
Val-11	8.29	4.33	1.93	γH 0.89
Pro-12	–	4.37	2.23, 1.82	γH 1.93, δH 3.83, 3.57
Trp-13	8.33	4.37	3.26, 3.11	H-2 7.19, H-4 7.54, H-5 7.08, H-6 7.22, H-7 7.43, NH 10.08
Asn-14	7.41	4.23	2.22, 1.96	γNH 7.37[a]
Ala-15	8.03	3.91	1.21	–
Ser-16	8.24	4.27	3.77	–
Trp-17	7.9	4.59	3.18	H-2 7.12, H-4 7.44, H-5 7.08, H-6 7.22, H-7 7.43, NH 10.04
Ser-18	7.91	4.26	3.69, 3.59	–
Asn-19	8.27	4.55	2.77, 2.70	γNH 7.62[a]
Lys-20	8.24	4.17	1.80, 1.68	γH 1.34, δH 1.56, εH 2.95, εNH 7.48

[a] The second γNH proton of Asn has not been determined due to the spectral overlap.

The fingerprint region of the ROESY spectrum indicating the sequential assignment is shown in Fig. 3. In the major part of the sequence, both the $d_{\alpha,N}(i, i)$ and $d_{N,N}(i, i + 1)$ connectivities have been found. The intensity of $d_{N,N}(i, i + 1)$ cross-peaks is not uniform and, in some cases, is relatively high, indicating that the peptide does not exist in the fully extended β-chain conformation. In the major part of sequence also $d_{\beta,N}(i, i)$ connectivities have been found, indicating the rigidity of the secondary structure. Schematic diagram summarizing the observed NOE contacts to backbone protons and their intensities is shown in Fig. 4.

Disulfide bridge between Cys-1 and Cys-7 at the N-terminus of peptide p-15-2 leads to the formation of a cyclic structure. Connectivities have been found between the NH of Ser-2 and βH of Trp-13 and Trp-17, one of the αH protons of Gly-3 and backbone NH proton of Trp-17, εNH of Lys-4 and αH and backbone NH of Lys-20. They clearly indicate that also the C-terminal linear region of the peptide is involved in a folded structure. The temperature coefficient of εNH of Lys-4 is lower than that of εNH Lys-20 which indicates that a hydrogen bond is formed between this proton and oxygen

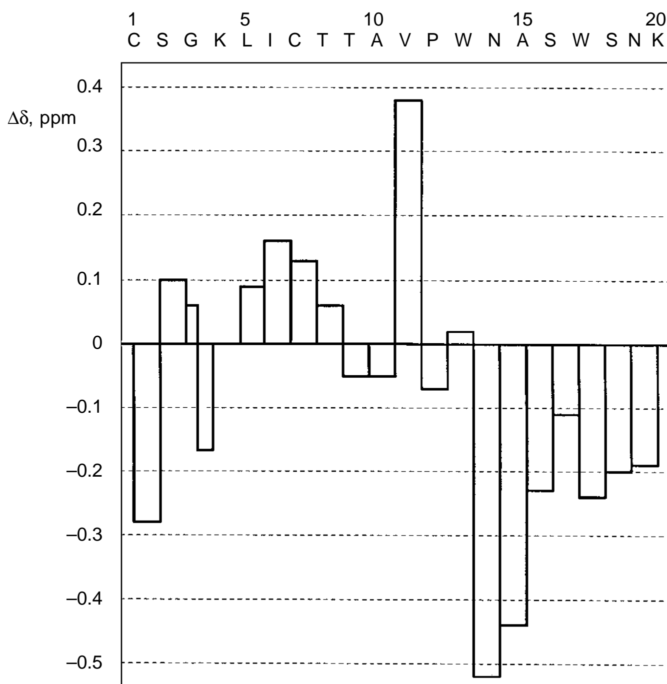

FIG. 2
Chemical shift differences between the observed and "random-coil" chemical shift values of αH protons in peptide p-15-2 (according to the refs[15,16])

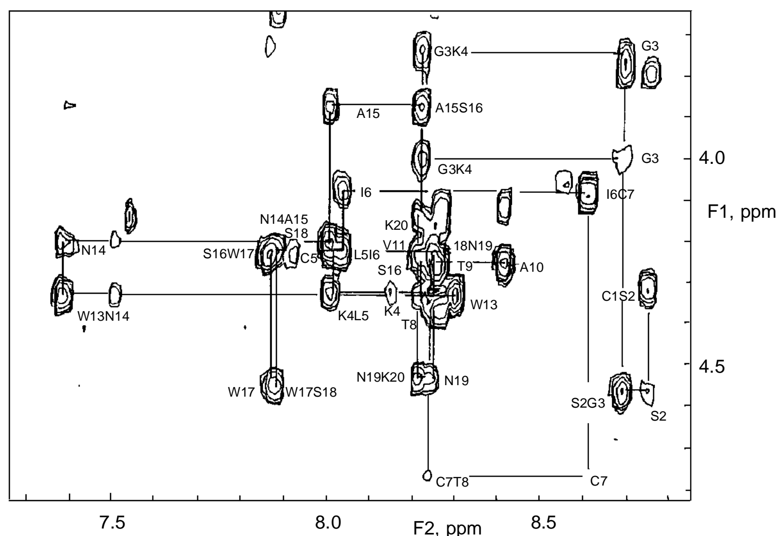

FIG. 3
Portion of the 500 MHz ROESY spectrum of p-15-2 at 5 °C showing the NH–αH region. The sequential $d_{\alpha,N}(i, i + 1)$ are indicated

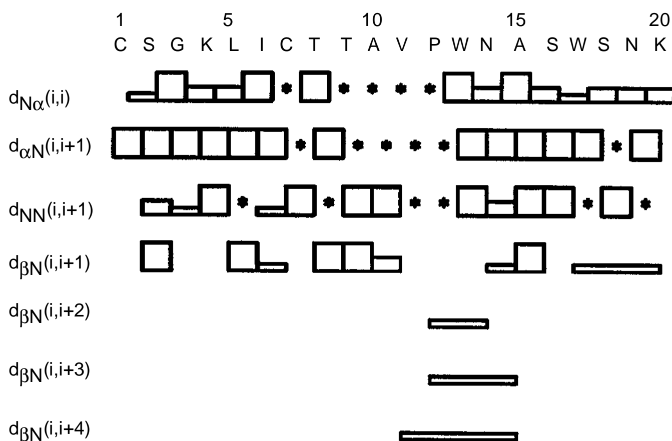

FIG. 4
Schematic diagram showing the intensities of the NOE connectivities to backbone protons in peptide p-15-2. The asterisk indicates that a crosspeak could not be unambigously assigned due to the resonance overlap

of C-terminal $CONH_2$ or the backbone carbonyl of Asn-19. Low temperature coefficients −3.2 and −3.6 . 10^{-3} ppm/K have been found for the backbone NH protons of Ser-2 and Leu-5, which indicates, that they probably form a hydrogen bond. Other nonsequential crosspeaks have been found between the residues Val-11 and Ala-15. Several crosspeaks have been found between the backbone NH protons and side-chain protons of non-neighbouring residues as well as the contact between H-4 of the indole ring of Trp-13 and βH of Ala-15. A turn-like extended structure seems to be present in this region. The existence of this turn is supported also by low *J*(NH,αH) values for

TABLE III
Temperature dependence of coupling constants and chemical shifts of NH protons

Residue	*J*(NH,αH)		Δδ/Δ*T*, ppm/ °C . 10^3	
	at 30 °C	at 5 °C	backbone NH	side-chain NH
Cys-1	–	–	–	
Ser-2	8.3	7.8	−3.2	
Gly-3	5.6, 5.7	6.0, 5.1	−6.4	
Lys-4	7.7	[a]	−8.8	εNH −2.8
Leu-5	6.7	[a]	−3.6	
Ile-6	7.3	7.4	−9.2	
Cys-7	8.3	7.8	−8.0	
Thr-8	[a]	[a]	−6.8	
Thr-9	[a]	[a]	−9.5	
Ala-10	6.6	5.1	−9.5	
Val-11	9.4	[a]	−11.2	
Pro-12	–	–	–	
Trp-13	7.4	5.1	−13.6	
Asn-14	[a]	[a]	+3.6	γNH −4.8
Ala-15	5.0	[a]	−5.6	
Ser-16	[a]	[a]	−6.0	
Trp-17	6.8	6.8	−3.2	
Ser-18	7.2	6.9	−2.8	
Asn-19	6.9	[a]	−4.4	
Lys-20	[a]	[a]	−6.0	εNH −3.2

[a] The value of parameter could not be determined due to peak overlap.

Ala-10 and Trp-13 (Table III) which are in the range typical for certain types of β-turn and which increase with temperature to the values typical for random structure. Non-sequential NOE contacts found in the structure of peptide p-15-2 are indicated in Fig. 5.

The extreme temperature coefficients were found for backbone NH protons of Asn-14 (+3.6 . 10^{-3} ppm/K) on one side and Val-11 and Trp-13 (−11.2 . 10^{-3} and −13.6 . 10^{-3} ppm/K, respectively) on the other side. This, together with significant temperature changes in the chemical shifts of many protons and loss of most of the long- and medium-range connectivities is an indication of a temperature-induced conformational change in peptide.

In the rest of the sequence only the long-range contact of εH proton of Lys-20 to H-2 proton of the indole ring of Trp-17 has been found. The stability of the structure is indicated by the existence of many $d_{N,\beta}(i, i + 1)$. Lower temperature coefficients have been found for the backbone NH protons of Trp-17, Ser-18 and Asn-19. These observations show that the peptide p-15-2 has a defined secondary as well as tertiary structure not only in the cyclic part of the molecule containing the residues 1 to 7 but also in the linear part of the peptide chain.

DISCUSSION

The study of reactivities of peptides of N-terminal domain of gp41 protein clearly shows differences in immunobehaviour of different epitops. The epitope represented by the peptide p-15-8 (epitope 2) shows linear immunoresponse during seroconversion, whereas epitopes represented by the peptides p-15-2 (epitope 3) and p-15-10 (epitope 1) show

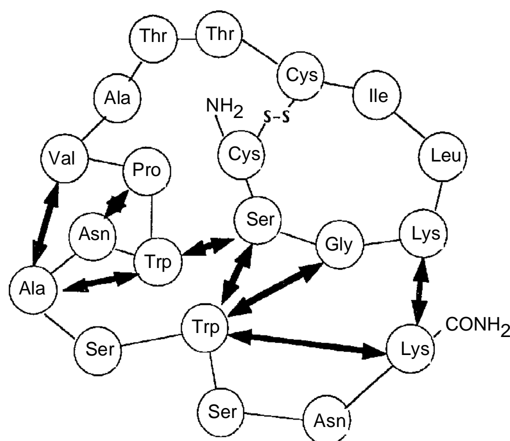

FIG. 5
Scheme of the non-sequential NOE contacts found in the structure of peptide p-15-2

non-linear immunoresponse. These differences reflect changes the total amount of viral particles as well as in the accessibility of certain epitopes to the immune system. This is consistent to the widely accepted theory that gp41 is involved in holding the knobs, formed by trimers of gp120, on the surface of the viral particle[2]. The loss of the knobs during ageing causes appearance of further parts of gp41 on its surface. In these newly-accessible parts of the protein sequence the epitopes represented by peptides p-15-2 and p-15-10 may be found although the non-linearity of immune response should be also considered.

The structures of peptides spanning the sequence 582–588 and 594–604 in the SIV virus, which correspond to the regions 594–600 and 606–616 in the LAV isolate of HIV-1, virus have been already determined. In the work of Dyson et al.[7] on the SIV fragments, the region 582–588 has a rigid extended structure and the region 594–604 forms a nascent helix. This is in contradiction to the previous observation of the same group[6] in which in the HIV-1 peptide, spanning the region 603–609 (no isolate referred) which corresponds to the region 609–616 in the LAV isolate where the extended structure has been found for the oxidized and random structure for the reduced form of the cystein. As it is hard to believe that the homologous regions in SIV and HIV-1 virus will adopt different structures, one should expect that the residues preceding the cystine ring are important for the nascent helix formation. In our work we have found highly similar structure of the cystine ring as has been found in the work of Oldstone et al.[10]. The minor differences can be probably caused by the interaction of this region to the C-terminal part of the peptide.

As the peptide p-15-2, which shows a pattern of reactivity similar to that of p-15-10, contains part of a known epitope (residues 1–7), the structure responsible for differences in the reaction with human sera should be found in the C-terminal region of the peptide. This region adopts a turn-like conformation, followed by a short, extended region. Such type of structure has not been found for any of the epitope regions previously studied. This suggests that for each of epitopes there exists corresponding peptide structure in solution.

EXPERIMENTAL

Preparation of Synthetic Peptides

Peptides derived from N-terminal domain of HIV-1 gp41 were prepared by continuous-flow solid-phase multiple peptide synthesis as described by Krchnak and Vagner[15]. HPLC purified peptides displayed correct amino acid composition and showed the expected molecular peak in FAB mass spectrum.

Sera

Anti-HIV-1 Seroconversion Panel C was obtained from the Boston Biomedica, Inc., MA, U.S.A. It represents a samples from serial bleeds collected from individual plasma donors during a 10 weeks

period of antigen-antibody seroconversion. Panel C was well characterized by B.B.I. using 10 anti-HIV-1 commercially available ELISA test kits, one anti-HIV-1 Western blot test and one HIV-1 antigen ELISA test.

ELISA

ELISA with fourteen peptides were carried out by a standard solid-phase method. The peptides were used in a free form. Microelisa plates (Novogen, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague) were coated with peptide (1 μg/well) in bicarbonate buffer (pH 9.6) at room temperature for 1 h. Free binding sites on the plates were saturated using 1% bovine serum albunime (BSA) in the same buffer. The BSA solution was removed after 1 h at room temperature, diluted sera (1 : 50) were added, and the mixture was incubated at room temperature for 1 h. After several washings, peroxidase-conjugated swine antibody to human IgG diluted 1 : 2 500 was added. The colour was developed by incubation with *o*-phenylenediamine. The composition of the buffers and details of the ELISA procedure have been described elsewhere[16].

Preparation of Sample for NMR Spectroscopy

Lyophilized peptide p-15-2 was dissolved in the mixture of 90% H_2O and 10% D_2O to give a final concentration of 8 mmol/l. The concentration of the peptide was controlled by amino acid analysis. The pH of the self-buffering solution was 3.95 which is in the range suitable for NMR measurements due to low exchange rate of NH protons.

NMR Structural Studies

All proton NMR spectra of peptide p-15-2 were recorded at 500 MHz frequency on the Varian UNITY-500 spectrometer. Dioxane was used as an internal standard and the chemical shifts recalculated to tetramethylsilane using relation δ(dioxane) = 3.75. The phase sensitive DQF COSY (ref.[4]), TOCSY (ref.[8]) (spinlock time 80 ms) and ROESY (ref.[9]) spectra (mixing time 200 ms) were acquired at 5 and 30 °C for 2 048 data points using 8 scans for each of 256 values of t_1 increment. Before Fourier transformation the spectra were zero-filled in t_1 to the final data matrix size 2 048 × 1 024 points. The temperature coefficients were calculated with data obtained from two 2D-TOCSY spectra spectra at 5 and 30 °C. The coupling constants J(NH,αH) were determined from 1D spectra acquired at 5 and 30 °C for 32k data points with 128 scans.

Authors thank to Prof. J. F. Allen for carefull reading of the manuscript. Work was supported by the grant No. 45568 of the Academy of Sciences of the Czech Republic and by grant No. 0782-5 of Czech Ministry of Health.

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