Mapping the Anatomy of the Immunodominant Domain of the Human Immunodeficiency Virus gp41 Transmembrane Protein: Peptide Conformation Analysis Using Monoclonal Antibodies and Proton Nuclear Magnetic Resonance Spectroscopy†

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Thirty-six monoclonal antibodies from mice and three from rats were raised against a peptide corresponding to the immunodominant domain of the transmembrane gp41 protein of human immunodeficiency virus (HIV) type 1 (LGLWGCSGKLIC; amino acid residues 598 to 609). Of these, three monoclonal antibodies from the mice and one from a rat also reacted with the corresponding peptide derived from the HIV type 2 transmembrane gp41 protein (amino acid residues 593 to 603; NSWGCAFRQVC). Immunochemical studies using a variety of synthetic peptides indicated that the cross-reactivity was due to antibody binding to CSGKLIC of HIV type 1 or CAFRQVC of HIV type 2. Single amino acid substitutions for a cysteine at either the amino or the carboxy end of the peptide interrupted antibody binding, indicating that the site recognized was the Cys-XXXXX-Cys loop. Similar results were obtained when the 11-mer HIV type 2 gp41 peptide (amino acids 593 to 603) was inoculated into mice to raise monoclonal antibodies. In this instance, of 30 monoclonal antibodies developed, 4 reacted with both HIV type 1 and HIV type 2 peptides. The conformation of a seven-residue peptide, CSGKLIC, corresponding to residues 603 to 609 of the gp41 immunodominant epitope of HIV-1 was investigated by proton nuclear magnetic resonance spectroscopy. The immunologically active form of CSGKLIC contains an intramolecular disulfide bond and maintains a preference for a folded conformation, apparently including a type I reverse turn about the residues SGKL. No such preference is observed for the reduced form of the peptide, which contains two thiol groups. The presence of the disulfide bond is thus integral to the formation of the structure of the loop in solution. In agreement with this finding, elimination of the possibility of loop formation by substitution of S for C at the amino or carboxy termini of the 7-mer is accompanied by the failure of antibody binding to this peptide.

Immunodominant linear epitopes occur in structural proteins of human immunodeficiency virus type 1 (HIV-1) and HIV-2 near the N termini of the respective transmembrane gp41 proteins (12, 14, 20, 26, 29). Synthetic peptides derived from these conserved domains show high specificity and sensitivity in detecting antibodies in sera from patients infected either with HIV-1 or HIV-2. Thus, these reagents have allowed the development of screening assays that discriminate between antibody responses to HIV-1 and HIV-2 (17a). These reactive regions are immunodominant in that >99% of HIV-infected individuals tested made antibody responses to them (12, 20). With HIV-1, the minimal epitope recognized was seven amino acids flanked at the N and C ends by cysteines (13).

Extensive testing with these peptides revealed that 61 (8%) of 722 patients reacted to both HIV-1 and HIV-2 (17a). Similarly, other studies demonstrated that 3 to 14% of HIV-positive West Africans possessed antibodies to both HIV-1 and HIV-2 in their sera (4, 11, 27). These observations suggested three possible causes, none of which need exclude the others. First, a single individual might be infected with both HIV-1 and HIV-2 (10, 22). As evidence to

support this contention, Rayfield and associates (22) demonstrated specific sequences for HIV-1 and HIV-2, using the polymerase chain reaction protocol to amplify DNA in peripheral blood mononuclear cells of a 28-year-old male, whereas Evans et al. (10) succeeded in isolating both viruses from peripheral blood mononuclear cells of a 37-year-old female. However, neither these investigators nor others have confirmed dual infection in the vast majority of individuals carrying antibodies to both HIV-1 and HIV-2. Therefore, dual infection might be an uncommon event. A second possibility is the formation of a single recombinant virus, an event observed previously with many but not all persistent murine retrovirus infections (1, 9, 15). As yet, there is no evidence for a novel recombinant AIDS virus. The third possibility is immunologic cross-reactivity between an amino acid linear determinant(s) or conformations shared between HIV-1 and HIV-2. This report addresses the last issue.

Such cross-reactivity was studied by generating monoclonal antibodies against the peptide sequences of the HIV-1 (LGLWGCSGKLIC) or HIV-2 (NSWGCAFRQVC) gp41 immunodominant domain. Experimentation with these monoclonal antibodies and a variety of synthetic peptides yielded evidence that immunologic cross-reactivity between HIV-1 and HIV-2 is dependent on the conformation of their Cys-XXXXX-Cys loops. Thus, the use of the two Cys-Cys 7-mers of HIV-1 and HIV-2 gp41 immunodominant domains

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should be of value in identifying instances of such cross-reactivity.

In addition, the conformation of the loop-containing heptapeptide CSGKLIC in solution was studied by proton nuclear magnetic resonance (NMR) spectroscopy. Local conformations of the immunogenic peptide in solution were defined by using one- and two-dimensional NMR studies for both the reduced (monomeric peptide containing two cysteine thiols) and oxidized (also monomeric, containing an intramolecular disulfide bond) forms of CSGKLIC. The chemical shift of the amide protons, the temperature dependence of the amide proton chemical shift, and the pattern of nuclear Overhauser effect (NOE) connectivities for each form were found to be markedly dissimilar. The results indicate that a preference for a folded conformation exists in the oxidized form of the peptide, but not in the reduced form.

MATERIALS AND METHODS

Peptide synthesis, generation of antibodies, and their quantitation. Peptides ranging in length from 6 to 24 amino acids were synthesized by the solid-phase method of Merrifield (19) either with an automated peptide synthesizer (430A; Applied Biosystems, Foster City, Calif.) or manually by the tea bag-resin method of Houghten (16). Peptide resins were cleaved by hydrogen fluoride, extracted, and analyzed for purity by high-performance liquid chromatography (HPLC), using a reverse-phase C₁₈ column.

Peptides used for immunization consisted of CGGLGLW GSCGKLIC, LGLWGSCGKLIC, CGGKYLDQDQARLN SWGCAFRQVC, or NSWGCAFRQVC. Each peptide alone or coupled to keyhole limpet hemocyanin carrier protein (5, 18) was inoculated into groups of four BALB/WEHI mice. After preimmunization sera were obtained, mice were injected at day 0 with 75 µg of peptide in complete Freund adjuvant given subcutaneously and intraperitoneally. Every 2 to 3 weeks thereafter, each mouse received 75 µg of peptide in phosphate-buffered saline with alum by the intraperitoneal route. Individual mice were bled 7 days after inoculation and their sera were tested by enzyme-linked immunosorbent assay (ELISA) for the presence of antibodies to the inoculated peptide. Upon documentation of specific antibodies, mice were inoculated at 3, 2, and 1 day prior to fusion of their spleen cells with 75 µg of peptide in phosphate-buffered saline administered intravenously. LOU/MN rats were inoculated with selected peptides by a similar protocol. Fusion of mouse or rat splenic lymphocytes to plasmacytoma cells followed the basic procedure of Kohler and Milstein (17) as modified in our reports (3, 23). Mouse splenocytes were fused to nonsecretor 6531 mouse myeloma cells, whereas rat splenocytes were fused to a nonsecretor Y3 AG1.23 rat myeloma line. After selection in hypoxanthine-aminopterin-thymidine medium, clones were obtained by limiting dilution and recloned.

Culture fluids and ascites induced by inoculation of selected hybridomas were screened for antibodies by an ELISA, as described before (12, 14). Each assay was run at least three times.

Inhibition of antibody binding was performed by mixing antibodies in serum, tissue culture, or ascites fluids with various concentrations of peptide, incubating for 30 min, adding the mixture to standard peptide-coated wells, and performing the standard ELISA.

NMR sample preparation. To prepare the reduced CSGKLIC sample, 4.2 mg of the lyophilized peptide was dissolved

in 90% ¹H₂O–10% ²H₂O and the pH was adjusted to 3.80 with 0.1 M NaOH and HCl solutions. The final volume was adjusted to 0.6 ml, and an internal standard of 5 µl of 0.06 M dioxan in ²H₂O was added. The final concentration for this sample was 8 mM. Some impurities were detected by NMR spectroscopy, principally a contamination with a very small amount of oxidized peptide. No further purification of the reduced sample was attempted.

The reduced peptide was oxidized in a dilute solution of 0.1 M ammonium bicarbonate, pH 7.9, by stirring overnight in air at room temperature. The solution was lyophilized to remove ammonium bicarbonate and was further purified by HPLC. Samples were prepared as described below to a final pH of 4.02 at two different concentrations, 6.9 and 11 mM. The NMR spectra acquired for the two solutions were identical, indicating that the peptide was monomeric under the conditions of the experiments.

¹H-NMR spectroscopy. All NMR spectra were recorded at 300 MHz on a Bruker MSL300 spectrophotometer. Dioxan was used as an internal standard; all spectra were referenced to (trimethylsilyl)propanesulfonic acid (TSS) at 0 ppm.

The temperature coefficients of the amide proton chemical shifts were calculated with data from one-dimensional spectra recorded at temperatures between 278 and 310 K.

Complete sequence-specific assignments of the proton resonances of the two forms of the peptide were obtained by using phase-sensitive two-dimensional double quantum filtered COSY (2QF COSY [21]) and rotating-frame NOESY (ROESY [2]) spectra. For the oxidized heptapeptide, two-dimensional spectra were acquired at different temperatures to confirm assignments, because of resonance overlap. Mixing times in the ROESY experiments were generally 100 to 150 ms.

RESULTS

Generation of monoclonal antibodies to HIV-1 and -2 gp41 peptides. Upon immunization with synthetic peptides corresponding to the immunodominant domains of the transmembrane gp41 protein of HIV-1 or HIV-2, mice and rats produced specific antibodies used as a source of monoclonal antibodies. Of these 39 monoclonal antibodies to the HIV-1 12-mer peptide (residues 598 to 609), 16 (41%) also reacted with the heptameric minimal epitope flanked by cysteines (residues 603 to 609; CSGKLIC) (Table 1). Most importantly, four of these monoclonal antibodies also reacted with the minimal heptameric HIV-2 peptide similarly flanked by cysteines (amino acids 597 to 603; CAFRQVC). Similarly, when peptides that encompassed the HIV-2 gp41 immunodominant epitope were used to immunize mice, four resulting monoclonal antibodies specifically reacted with the minimal seven-amino-acid peptide epitopes of both HIV-1 (CSGKLIC) and HIV-2 (CAFRQVC).

Three monoclonal antibodies generated after immunization with peptide sequences corresponding to HIV-1 and three resulting after immunization with HIV-2 peptides were selected for further study. All six monoclonal antibodies demonstrated highly reactive titers, even at endpoint dilutions, when tested against the HIV-1 or HIV-2 peptides (Table 2). However, none of the six monoclonal antibodies reacted with a peptide when the N-terminal cysteine was removed, disallowing the formation of an intramolecular disulfide bond (Table 2). These results were repeated in three other experiments. The immunoglobulin subset shown in Table 2 was typed by using isotype-specific murine and rat monoclonal antibodies.

TABLE 1. Generation of monoclonal antibodies to the peptides corresponding to the gp41 immunodominant domain of either HIV-1 or HIV-2^a

Immunogen	Host	Monoclonal antibodies					
		Total no.	HIV-1 amino acids:		HIV-2 amino acids:		HIV-1 HIV-2
			598-609	603-609	593-603	597-603	
HIV-1 gp41 peptide	Mouse	282	36	15	5	3	3
598 603 609	Rat	86	3	1	1	1	1
LGLWGCSGKLIC							
or							
595 598 609							
CGGEQLLGLWGCSGKLIC							
HIV-2 gp41 peptide	Mouse	610	14	4	30	4	4
593 597 603							
NSWGCAFRQVC							
or							
583 593 603							
CGGKYLDQDQARLNSWGCAFRQVC							

^a BALB mice or LOU/MN rats received a series of peptide injections (see Materials and Methods). Spleen cells from animals with anti-peptide antibodies in their sera were fused to mouse 6351 or rat Y3 AG1.23 myeloma cells. After cloning and recloning twice, supernatant fluids from each clone were tested for ability to bind to HIV-1 amino acids 598 to 609 (LGLWGCSKLIC) or 603 to 609 (CSKLIC) or HIV-2 amino acids 593 to 603 (NSWGCAFRQVC) or 597 to 603 (CAFRQVC).

The last experiments in this series judged the relative binding affinities of anti-HIV-1 peptide monoclonal antibody gp41-6 and anti-HIV-2 peptide monoclonal antibody 115.8 to the heptamer peptides of HIV-1 (CSGKLIC) or HIV-2 (CAFRQVC). Concentrations of peptide antigens varying from 10 to 0.01 μ g bound better to the heptamer contained in the immunizing peptide than to the nonimmunizing peptide (Table 3). The binding observed at this wide range of antigen dosages indicated reasonable efficiency and affinity of the antibodies under study.

Cross-reactive binding of monoclonal antibody to the peptides comprising the gp41 immunodominant domain of HIV-1 or HIV-2 is dependent on the integrity of the disulfide bond. Preliminary studies indicated the importance of the disulfide-bonded loop for monoclonal antibody binding to both HIV-1 and HIV-2 peptides (Table 2). To confirm and extend this observation, a series of peptides was synthesized in which either of the two cysteines was replaced by a serine. Only when the disulfide bond was present did significant binding

of either monoclonal antibody (gp41-6 or 115.8) to either of the HIV-1 or HIV-2 12-mer peptides occur (Table 4). Various concentrations of the heptamer peptide (residues 603 to 609; CSGKLIC) were used to inhibit binding of the original immunizing HIV-1 12-mer peptide (residues 598 to 609) to monoclonal antibody gp41-6. At a 20- μ g concentration, the binding of peptide amino acids 603 to 609 was inhibited >90%, with 50% inhibition at a concentration of 8 μ g. Peptide SGKLIC or CSGKLIS showed negligible inhibition at a 50- μ g concentration. Further, peptide CSGKLIC inhibited the binding of monoclonal antibody to the HIV-2 gp41 11-mer peptide.

Conformation of reduced and oxidized CSGKLIC peptide deduced from ¹H-NMR spectroscopy. One- and two-dimensional NMR techniques have been used to estimate the conformations present in solution of the heptamer CSGKLIC in the oxidized (intramolecular disulfide) and reduced (dithiol) forms. (Note that in the structural studies the residues in the heptamer are numbered 1 through 7.) Repre-

TABLE 2. Cross-reaction of monoclonal antibodies with peptides containing the Cys-Cys loop of the immunodominant epitopes of HIV-1 and HIV-2

Monoclonal antibody ^a to:	Immunoglobulin subtype	Endpoint dilution of ascitic fluid ^b					
		HIV-1 gp41 peptide			HIV-2 gp41 peptide		
		LGLWGCSGKLIC	CSGKLIC	SGKLIC	NSWGCAFRQVC	CAFRQVC	SAFRQVC
HIV-1							
41-6	IgG2b	520,000	270,000	30	4,000	500	30
4	IgG2b	28,480	25,000	10	1,280	420	30
75	IgG	2,560	2,560	10	2,540	640	20
HIV-2							
68.1	IgM	64,000	30,000	54	180,000	125,000	10
68.11	IgM	31,000	100,000	73	32,000	155,000	30
115.8	IgM	130,000	520	58	175,000	64,000	30
Normal mouse		14	17	10	10	35	10

^a Mouse monoclonal antibodies are HIV-1 41-6 and 4 and HIV-2 68.1, 68.11, and 115.8; 75 is a rat monoclonal antibody.

^b ELISA was read at 492 nm, using a concentration of 1 μ g of peptide per well.

TABLE 3. Binding of monoclonal antibodies (MoAb) to various concentrations of heptamer loop peptides of the immunodominant gp41 epitope of HIV^a

Concn of peptide (μg)	Endpoint dilution			
	MoAb of HIV-1 binding to:		MoAb of HIV-2 binding to:	
	HIV-1 peptide CSGKLIC	HIV-2 peptide CAFRQVC	HIV-1 peptide CSGKLIC	HIV-2 peptide CAFRQVC
10	4,700	1,100	1,400	92,000
1	300,000	150,000	6,000	240,000
0.2	1,200,000	400,000	680	2,700,000
0.1	190,000	80,000	110	660,000
0.2	14,000	13	43	320
0.01	20,000	21	40	620

^a Monoclonal antibody to HIV-1 was gp41-6 and that to HIV-2 was 115.8.

sentative regions of the 2QF COSY spectra at 278 K of the two forms of the peptide are shown in Fig. 1. At this temperature, the amide proton resonances of Lys-4 and Leu-5 are coincident in the oxidized heptamer. The resonance assignments, obtained through the use of 2QF COSY and ROESY (rotating-frame two-dimensional NOE) experiments, are given for both forms of the peptide in Table 5, together with the temperature coefficients of the amide proton chemical shifts and the ³J_{HNα} coupling constants.

ROESY spectra were also used to probe for local solution conformations. ROESY experiments were performed at a variety of temperatures for the oxidized heptamer to allow resolution of connectivities to overlapped amide proton resonances. For the reduced heptamer, only one ROESY spectrum was acquired, at 278 K, since all of the amide proton resonances are resolved at this temperature. Figures 2 and 3 show portions of the ROESY spectra acquired at 278 K of the reduced and the oxidized heptamers, respectively. In addition, Fig. 3 shows portions of the spectrum of the oxidized form acquired at 298 K to demonstrate the presence of the d_{NN(i,i+1)} NOE connectivity between Lys-4 and Leu-5, which is not observable at 278 K due to overlap of the amide proton resonances at this temperature. A summary of the NOE connectivities observed for the reduced and ox-

FIG. 1. Fingerprint region of 2QF COSY spectra of reduced (a) and oxidized (b) heptamer. NH-C^αH cross peaks are marked with the corresponding residue name. Numbers 2 through 7 correspond to residues 604 to 609 of the gp41 peptide representing the immunodominant epitope of HIV-1. The N-terminal residue of the peptide (Cys-1; residue 603) does not have an amide proton resonance.

dized heptamers is shown in Fig. 4, which includes information from ROESY spectra at several temperatures for the oxidized heptamer.

DISCUSSION

We show here that monoclonal antibodies generated in both mice and rats cross-react with the gp41 peptides corresponding to the immunodominant domains of HIV-1 and HIV-2 (12). This report extends our previous publications documenting the utility of designer peptides for analysis of HIV-1 and HIV-2 infection and the importance of the Cys-Cys loop in antibody recognition of the immunodominant domain of gp41 (12, 13, 25). Further, the NMR studies provide data on the conformational structure of this immunodominant gp41 domain of HIV-1.

Approximately 10% of the 39 monoclonal antibodies generated after immunization of mice and rats with the gp41 immunodominant domain peptide of HIV-1 were found to react with the heptamer minimal HIV-1 peptide and to cross-react with the HIV-2 peptide. Similarly, when the HIV-2 gp41 immunodominant peptide was used as an immunogen, 13% (4 of 30) of such monoclonal antibodies cross-reacted with the HIV-1 peptide. Cross-reactivity was clearly due to the conformation displayed by the seven-residue loop

TABLE 4. Cross-reaction between HIV-1 and HIV-2 shown by binding of monoclonal antibodies to the peptide analogs of the immunodominant gp41 epitope^a

Peptides	Endpoint dilution of monoclonal antibody to:	
	HIV-1: CSGKLIC ^b	HIV-2: CAFRQVC ^c
HIV-1		
CSGKLIC	126,000	1,400
SGKLIC	Nil	150
CSGKLIS	Nil	Nil
LGLWGSSGKLIC	1,000	140
HIV-2		
CAFRQVC	680	34,000
SAFRQVC	Nil	Nil
CAFRQVS	Nil	Nil
NSWGSAPFRQVC	Nil	400

^a Normal mouse sera or ascites fluid was not positive against these peptides in a dilution >1/30. Values of 1/30 or less are recorded as Nil.

^b Monoclonal antibody used was gp41-6.

^c Monoclonal antibody used was 115.8.

TABLE 5. Resonance assignments, temperature coefficients, and $^3J_{\text{HN}\alpha}$ coupling constants for oxidized and reduced heptamer

Residue no.	Chemical shift (ppm) (278 K, pH 3.8)						$-\Delta\delta/\Delta T$, 10 ³ (ppm/K) ^a	³ J _{HNα} (Hz)
	NH	C ^α H	C ^β H	C ^γ H	C ^δ H	C ^ε H		
Reduced heptamer (dithiol)								
Cys-1		4.30	3.15 3.08					
Ser-2	9.03	4.53	3.92				6.7	6.30
Gly-3	8.67	3.98					8.3	NM ^b
Lys-4	8.33	4.30	1.71 1.78	1.4	1.6 1.7	3.0	7.5	6.69
Leu-5	8.49	4.37	1.62	1.67	0.94 0.88		8.3	6.63
Ile-6	8.41	4.21	1.91	1.50 1.22	0.86	0.93 (C ^γ H ₃)	10.1	8.04
Cys-7	8.23	4.41	2.92				8.0	7.59
Oxidized heptamer (disulfide)								
Cys-1		4.47	3.18 3.38					
Ser-2	9.01	4.67	3.86 3.88				7.4	6.75
Gly-3	8.83	3.86 4.08					7.6	NM
Lys-4	8.19	4.48	1.79 1.83	1.42 1.22	1.73	3.04	8.8	7.80
Leu-5	8.19	4.31	1.72 1.76	1.66	0.96 0.92		3.4	6.60
Ile-6	7.96	4.26	1.88	1.22 1.51	0.89	0.94 (C ^γ H ₃)	9.5	8.61
Cys-7	8.42	4.61	3.28 3.09				9.1	7.47

^a Temperature coefficient of amide proton chemical shift = gradient of linear plot of chemical shift (δ) versus temperature (T). Since δ decreases with increasing T , the gradient has a negative sign.

^b NM, Not measured.

containing two cysteine residues at the carboxy ends of these peptides, because disruption of the disulfide bond resulted in the loss of antibody recognition. This was observed with substitution of a serine for a cysteine at either the amino or the carboxy end of the loop. Further, the 7-mer peptide, CSGKLIC, at a concentration of 20 μ g, blocked antibody binding to either the 12-mer HIV-1 or the 11-mer HIV-2 peptides. Immunochemical assays with dinitrophenol, which is commonly used to reduce the disulfide bond, were technically unsatisfactory due to solubility difficulties and were not used for peptide-antibody binding studies. Further, the HIV gp41 transmembrane peptides used here to generate monoclonal antibodies were earlier shown to induce antibodies upon immunization of rabbits (14) and to react with antibodies generated by humans during HIV infection (12), indicating their immunogenicity as well as recognition by the immune system during natural infection.

There are two major implications of these observations. First, we predict that the heptamer peptides, CSGKLIC for HIV-1 or CAFRQVC for HIV-2, would be valuable reagents in determining whether patients with dually positive sera are so because of immunologic cross-reactivity. Although dual infection with both viruses can occur (10, 22), the extremely few incidents reported indicate either difficulties with their isolation or identification by polymerase chain reaction or that dual infection is rare. Indeed, in preliminary studies, serum samples from 10 patients from the Ivory Coast (kindly supplied by K. DeCock and J. McCormick, Centers for Disease Control, Atlanta, Ga.) contained antibodies reactive to the HIV-1 12-mer peptide and the HIV-2 11-mer peptide. These 10 serum samples also reacted with the heptamer loop

peptides from HIV-1 and HIV-2. However, none of them reacted with peptide SGKLIC. Unfortunately, too little dually reactive serum was available to perform CSGKLIC, SGKLIC, and CSGKLIS peptide absorption studies. Hence, documentation of both types of HIV in some individuals may well be attributed to cross-reactive antigenic determinants. Further, the use of CSGKLIC, SGKLIC, CSGKLIS, CAFRQVC, and CAFRQVS reagents should be of value in resolution of this issue.

The second point of interest is that the immunochemical system described here can be manipulated by physical or chemical means for the study of molecular conformations and antibody-peptide interactions. NMR spectroscopy can be used for the elucidation of peptide conformational preferences in solution (6–8). The conformational constraint induced by the presence of an intramolecular disulfide bond in the heptamer peptide CSGKLIC considerably reduces the conformational space available to the peptide, allowing us to deduce the major conformational species present, using direct experimental information. Structural information obtained from the NMR spectra comes from several sources. First, the behavior of each of the protons of the molecule can in general be monitored independently of the others. This allows site-specific information to be obtained. Second, different NMR experiments give different information about these sites; for example, the temperature dependence of amide proton chemical shifts can give information on hydrogen bonding, and the NOE can be used to deduce interproton distances. These and other NMR data can be used to deduce peptide conformational preferences in solution.

The gradient of the linear plot of temperature versus the

FIG. 2. Portions of a 300-MHz ROESY spectrum ($\tau = 150$ ms) of the reduced heptamer at 278 K, pH 3.80. (a) NH- α region. Sequential NOE cross peaks between the C α H of each residue and the NH of the subsequent residue are marked, as well as the corresponding peaks for the intraresidue interaction (Fig. 1A). (b) NH-NH region. Diagonal corresponds to a one-dimensional NMR spectrum. Positions of sequential $d_{NN}(i,i+1)$ NOE cross peaks (not observed in this spectrum) are indicated.

chemical shift of the amide proton is termed the temperature coefficient. Typical values for this quantity range from 7×10^{-3} to 10×10^{-3} ppm/ $^{\circ}$ C for amide protons fully exposed, and hydrogen bonded, to solvent water (24). A lower numerical value indicates protection of the amide proton from solvent, perhaps by hydrogen bonding to another group from the peptide. The values of the temperature coefficients (Table 5) for the reduced heptamer are uniformly high, indicating that there is no protection of the amide protons from solvent (24). Similarly, for the oxidized form of the heptamer, all of the amide protons except that of Leu-5 are also apparently completely solvent exposed. The temperature coefficient of Leu-5 is lowered to a value of 3.4×10^{-3} ppm/K, which indicates some protection from exchange with solvent. Traditionally, this has been interpreted as an indication of an intramolecular hydrogen bond (8, 24). A possible hydrogen bond acceptor for the amide proton of Leu-5 in this sequence could be the carbonyl group of Ser-2.

The proximity of two protons can be deduced from NOE

FIG. 3. Portions of 300-MHz ROESY spectra ($\tau = 100$ ms) of oxidized heptamer at 278 K. (a) NH- α region. (b) NH-NH diagonal region. (Inset, c) Region of a 300-MHz ROESY spectrum at 298 K ($\tau = 150$ ms).

spectra. (The NOE depends on the inverse sixth power of the distance between the protons.) A number of NOEs are routinely observed in unfolded peptides, including that between the amide proton of a given residue and the C α H proton of the residue immediately preceding it [the $d_{\alpha N}(i,i+1)$ NOE]. When conformational preferences for other than extended-chain structures are present (6–8), other NOEs may be observed instead of or in addition to the $d_{\alpha N}(i,i+1)$ NOE. The pattern of NOE connectivities for the reduced form (Fig. 4, top) shows very strong $d_{\alpha N}(i,i+1)$ interactions. No $d_{NN}(i,i+1)$ NOE connectivities (i.e., between the amide protons of adjacent residues) are observed for this form of the peptide. This grouping of connectivities indicates extended-chain conformations in solution and excludes the possibility of significant populations of turn or helical conformations (7). By contrast (Fig. 4, bottom), the $d_{\alpha N}(i,i+1)$ NOE connectivities for the oxidized heptamer are weaker and there are now substantial $d_{NN}(i,i+1)$ NOE

FIG. 4. Schematic diagram summarizing the NOE connectivities observed in the NMR spectra of the reduced and oxidized heptamer peptides.

connectivities present in the ROESY spectra. We note that, at the higher temperature used to elucidate the Lys-4–Leu-5 interactions, all of the NOE cross peaks are weaker than at 278 K, probably due to higher exchange rates of the amide protons with solvent and a general higher degree of conformational freedom, to be expected at the higher temperature. In particular, the presence of the $d_{NN}(i,i+1)$ cross peak between Lys-4 and Leu-5 at 298 K is in itself a strong indicator of a significant population of a local folded conformation. In view of the conformational restriction imposed by the presence of the disulfide bond presumed to be present between Cys-1 and Cys-7, the indications of turn conformations in the oxidized heptamer are not surprising. The exact nature of these conformations can in some measure be inferred from the NMR data reported herein. The observation of relatively strong NOE connectivities between the amide protons of Gly-3 and Lys-4 and between Lys-4 and Leu-5, coupled with the very weak $d_{\alpha N}(i,i+1)$ NOE between Gly-3 and Lys-4, is strongly suggestive of the presence of a type I reverse turn. [A type II turn would show a strong $d_{\alpha N}(i,i+1)$ NOE between Gly-3 and Lys-4 and have a weak or absent $d_{NN}(i,i+1)$ between them.] The low temperature coefficient of the Leu-5 amide proton is consistent with the presence of conformers containing a hydrogen bond from the carbonyl group of Ser-2 to the Leu-5 amide proton, to be expected in a reverse turn from Ser-2 to Leu-5.

Conclusive identification of turn conformations must include other evidence such as the $^3J_{HN\alpha}$ coupling constant and the observation of a $d_{\alpha N}(i,i+2)$ NOE (30). The $^3J_{HN\alpha}$ coupling constants (Table 5) do not in general show much

variation, consistent with previous observations (7, 8) which indicate that these are the NMR parameters least sensitive to small populations of folded forms. In the present case, the observed coupling constants would not be inconsistent with the presence of an appreciable population of a type I turn conformation in the oxidized heptamer. For a type I turn, coupling constants of 4 Hz at position 2 (Gly-3) and 9 Hz at position 3 (Lys-4) are expected (28). By contrast, a type II turn is expected to have a small coupling constant at position 3 also (28). A comparison of the coupling constants (Table 5) shows that there is no significant difference between the constants for each residue for the two forms of the heptamer, with the exception of Lys-4, with which there is a significant increase in the oxidized CSGKLIC. This is consistent with the presence of a significant proportion of type I reverse turn conformations. The $^3J_{HN\alpha}$ coupling constants of the glycine residue are neither easy to calculate nor simple to interpret in the context of a reverse turn conformation, so these have not been considered in the present discussion. One of the diagnostic NOE connectivities for a reverse turn, the $d_{\alpha N}(i,i+2)$ NOE between Gly-3 and Leu-5, is not seen. At 278 K the NOE is obscured, due to resonance overlap of the Lys-4 and Leu-5 amides; it is also not observed at 298 K, where the amide proton resonances of Lys-4 and Leu-5 are resolved. As noted previously, the cross peaks in the ROESY spectrum at the higher temperature are in general much weaker; indeed, the Gly-3–Lys-4 amide-amide NOE, which is present at 278 K, is also absent from the 298 K spectrum, an indication of both the poor quality of the data available at the higher temperature and the strength of the Lys-4–Leu-5 amide-amide NOE observed here. The absence of the $d_{\alpha N}(i,i+2)$ NOE at the higher temperature may not therefore be surprising.

In conclusion, NMR studies have defined a conformation in solution for the peptide corresponding to the minimal immunodominant epitope of HIV-1, CSGKLIC. Observations with the oxidized heptamer indicate that it contains an intramolecular disulfide bond and an appreciable population of type I reverse turn conformations about the residues Ser-Gly-Lys-Leu of the sequence. Antibodies generated in experimental animals by immunization and in humans through infection apparently bind to this conformation. Reduction of the disulfide bond linking the flanking cysteines disrupts the turn conformation; replacement of cysteine residues disallows the disulfide and aborts the reaction between the antibody and the peptide antigen. Custom-designed (selected) peptides synthesized from these immunodominant domains of HIV-1 and -2 are of value for discriminating between HIV-1 and HIV-2 infections as well as documenting HIV serologic cross-reactivity.

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REFERENCES

1. Bosselman, R. A., F. Van Straaten, C. Van Beveren, I. M. Verma, and M. Vogt. 1982. Analysis of the *env* gene of a molecularly cloned and biologically active Moloney mink cell focus-forming proviral DNA. *J. Virol.* **44**:19–31.
2. Bothner-By, A. A., R. L. Stephens, J. Lee, C. D. Warren, and

- R. W. Jeanloz. 1984. Structure determination of a tetrasaccharide: transient nuclear Overhauser effects in the rotating frame. *J. Am. Chem. Soc.* **106**:811–813.
3. Buchmeier, M. J., H. A. Lewicki, O. Tomori, and M. B. A. Oldstone. 1981. Monoclonal antibodies to lymphocytic choriomeningitis and Pichinde viruses: generation, characterization, and cross-reactivity with other arenaviruses. *Virology* **113**:73–85.
4. Cot, M. C., M. Poulain, J. F. Delagneau, M. Peeters, and F. Brun-Vezinet. 1988. Dual HIV-1 and HIV-2 infection in West Africa supported by synthetic peptide analysis. *AIDS Res. Hum. Retroviruses* **4**:239–241.
5. Dyrberg, T., and M. B. A. Oldstone. 1986. Peptides as antigens: importance of orientation. *J. Exp. Med.* **164**:1344–1349.
6. Dyson, H. J., K. J. Cross, R. A. Houghten, I. A. Wilson, P. E. Wright, and R. A. Lerner. 1985. The immunodominant site of a synthetic immunogen has a conformational preference in water for a type-II reverse turn. *Nature (London)* **318**:480–483.
7. Dyson, H. J., M. Rance, R. A. Houghten, R. A. Lerner, and P. E. Wright. 1988. Folding of immunogenic peptide fragments of proteins in water solution. II. The nascent helix. *J. Mol. Biol.* **201**:201–217.
8. Dyson, H. J., M. Rance, R. A. Houghten, P. E. Wright, and R. A. Lerner. 1988. Folding of immunogenic peptide fragments of proteins in water solution. I. Sequence requirements for the formation of a reverse turn. *J. Mol. Biol.* **201**:161–200.
9. Elder, J. H., J. W. Gautsch, F. C. Jensen, R. A. Lerner, J. W. Hartley, and W. P. Rowe. 1977. Biochemical evidence that MCF murine leukemia viruses are enveloped (env) gene recombinants. *Proc. Natl. Acad. Sci. USA* **74**:4676–4680.
10. Evans, L. A., J. Moreau, K. Odehouri, D. Seto, G. Thomson-Honniebier, H. Legg, A. Barboza, C. Cheng-Mayer, and J. A. Levy. Simultaneous isolation of HIV-1 and HIV-2 from an AIDS patient. *Lancet* **ii**:1389–1391.
11. Foucault, C., O. Lopez, G. Jourdan, J. J. Fournel, P. Perret, and J. C. Gluckman. 1987. Double HIV-1 and HIV-2 seropositivity among blood donors. *Lancet* **ii**:165–166. (Letter.)
12. Gnann, J. W., J. B. McCormick, S. Mitchell, J. A. Nelson, and M. B. A. Oldstone. 1987. Synthetic peptide immunoassay distinguishes HIV type 1 and HIV type 2 infections. *Science* **237**:1346.
13. Gnann, J. W., J. A. Nelson, and M. B. A. Oldstone. 1987. Fine mapping of an immunodominant domain in the transmembrane glycoprotein of human immunodeficiency virus. *J. Virol.* **61**:2639–2641.
14. Gnann, J. W., P. L. Schwimmbeck, J. A. Nelson, A. B. Truax, and M. B. A. Oldstone. 1987. Diagnosis of AIDS using a 12 amino acid peptide representing an immunodominant epitope of human immunodeficiency virus. *J. Infect. Dis.* **156**:261.
15. Hartley, J. W., N. K. Wolford, L. J. Old, and W. P. Rowe. 1977. A new class of murine leukemia virus associated with development of spontaneous lymphomas. *Proc. Natl. Acad. Sci. USA* **74**:789–792.
16. Houghten, R. A. 1985. General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids. *Proc. Natl. Acad. Sci. USA* **82**:5131.
17. Kohler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of pre-defined specificity. *Nature (London)* **256**:495–497.
- 17a. Lambert, P., and A. Lemduci (World Health Organization). Unpublished data.
18. Liu, F.-T., M. Zinnecker, T. Hamaoka, and D. H. Katz. 1979. New procedures for preparation and isolation of conjugates of proteins and a synthetic copolymer of D-amino acids and immunochemical characterization of such conjugates. *Biochemistry* **18**:690–697.
19. Merrifield, R. B. 1969. Solid phase peptide synthesis. *Adv. Enzymol.* **32**:221–296.
20. Norrby, E., G. Biberfeld, F. Chiodi, A. von Gegerfeld, A. Nacler, E. Parks, and R. A. Lerner. 1987. Discrimination between antibodies and related retroviruses using site-directed serology. *Nature (London)* **329**:248.
21. Rance, M., O. W. Sorensen, G. Bodenhausen, G. Wagner, R. R. Ernst, and K. Wuthrich. 1983. Improved spectral resolution in Cosy ¹H NMR spectra of proteins via double quantum filtering. *Biochem. Biophys. Res. Commun.* **117**:479–485.
22. Rayfield, M., K. DeCock, W. Heyward, L. Goldstein, J. Krebs, S. Kwok, S. Lee, J. McCormick, J. M. Moreau, K. Odehouri, G. Schochetman, J. Sninsky, and C.-Y. Ou. 1988. Mixed human immunodeficiency virus (HIV) infection in an individual: demonstration of both HIV type 1 and type 2 proviral sequences by using polymerase chain reaction. *J. Infect. Dis.* **158**:1170–1176.
23. Rice, G. P. A., R. D. Schrier, and M. B. A. Oldstone. 1984. Cytomegalovirus infects human lymphocytes and monocytes: virus expression is restricted to immediate-early gene products. *Proc. Natl. Acad. Sci. USA* **81**:6134–6138.
24. Rose, G. D., L. M. Gierasch, and J. A. Smith. 1985. Turns in peptides and proteins. *Adv. Prot. Chem.* **37**:1–106.
25. Schrier, R. D., J. W. Gnann, A. J. Langlois, K. Shriver, J. A. Nelson, and M. B. A. Oldstone. 1988. B- and T-lymphocyte responses to an immunodominant epitope of human immunodeficiency virus. *J. Virol.* **62**:2531–2536.
26. Smith, R. S., R. B. Naso, J. Rosen, A. Whalley, Y.-L. Hom, K. Hoey, C. J. Kennedy, J. A. McCutchan, S. A. Spector, and D. D. Richman. 1987. Antibody to a synthetic oligopeptide in subjects at risk for human immunodeficiency virus infection. *J. Clin. Microbiol.* **25**:1498–1504.
27. Tedder, R. S., T. O'Connor, A. Hughes, H. N'jie, T. Corrah, and H. Whittle. 1988. Envelope cross-reactivity in Western blot for HIV-1 and HIV-2 may not indicate dual infection. *Lancet* **ii**:927–930.
28. Wagner, G., D. Neuhaus, E. Worgotter, M. Vasak, J. H. R. Kagi, and K. Wuthrich. 1986. Nuclear magnetic resonance identification of "half-turn" and ₃₁₀-helix secondary structure in rabbit liver metallothionein-2. *J. Mol. Biol.* **187**:131–135.
29. Wang, J. J. G., S. Steel, R. Wisniewski, and C. Y. Wang. 1986. Detection of antibodies to human T lymphotropic virus type III by using a synthetic peptide of 21 amino acid residues corresponding to a highly antigenic segment of gp41 envelope protein. *Proc. Natl. Acad. Sci. USA* **83**:6159.
30. Wright, P. E., H. J. Dyson, and R. A. Lerner. 1988. Conformation of peptide fragments of proteins in aqueous solution: implications for initiation of protein folding. *Biochemistry* **27**:7167–7175.