Extension of the Polarity-Dependent "Switch Phenomenon" of the gp120 Binding Domain as a Target for Antiviral Chemotherapy

Andreas Graf v. Stosch, Volker Kinzel, and Jennifer Reed*

Department of Pathochemistry, German Cancer Research Center, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany

Received August 28, 1995; Revised Manuscript Received November 6, 1995[⊗]

ABSTRACT: A 15-residue fragment within the major continuous domain of gp120 from HIV-1 that can bind independently to the CD4 receptor has been shown to have the property of behaving as a solvent polarity-dependent conformational switch. The switch behavior (cooperative transition from β-sheet to helical conformation as a function of solvent polarity), which is conserved among strains with the widest sequence variability possible, appears to be a prerequisite for the CD4-binding ability. A number of switch inhibitors have been identified that destroy the conformational switch in the 15-residue fragment and concurrently its ability to bind to CD4-expressing cells. It can now be shown that the switch behavior and its inhibition by substances with certain shared structural characteristics are not restricted to the 15-residue subfragment, but are reflected by the behavior of the entire 44-residue binding domain. Further, substances active as switch inhibitors have an immediate effect on the conformation of the 44-residue fragment in aqueous buffer whereas inactive substances do not. The predictive value of this as a screening method is demonstrated in testing a number of new potential switch inhibitory compounds.

The invasion of human immunodeficiency virus type 1 (HIV-1) into host cells is mediated by the attachment of the viral surface glycoprotein gp120 to its cellular receptor CD4 (Dalgleish et al., 1984; Klatzman et al., 1984; McDougal et al., 1986). The binding of gp120 to CD4 comprises the first contact between the virus and the cell initiating all later stages of the viral replication cycle. Inhibition of this protein/protein interaction by exogenous substances is an interesting target for anti-HIV strategies for two reasons. On the one hand, blocking of the gp120/CD4 contact would prevent any further step in the viral life cycle, especially the integration of the viral DNA into the human CD4 cell genome. On the other hand, it is of pharmaceutical advantage that drug delivery is restricted to body fluids and that factors promoting drug accumulation in the cytoplasm need not be taken into account for such an antiviral chemotherapy.

Lasky and co-workers (Lasky et al., 1987, 1988) identified a 44-amino acid region of gp120 extending from residue 397 to 440 that is directly concerned with binding to CD4 (Cordonnier et al., 1989). This laboratory has mapped a 15-amino acid sequence (HIV-1-Bi) within the Lasky binding region that (i) is 50% conserved within all HIV-1 strains, (ii) has a high secondary structure-forming potential, and (iii) retains the CD4-binding ability (Reed & Kinzel, 1991, 1993). Secondary structure analysis of such peptides derived from strains with the widest range of sequence variability in the HIV-1-Bi region has shown that they do in fact share a common structural component: on moving from a polar to an apolar environment, they undergo a sharp, concerted transition from β-sheet → helical structure known as the "switch phenomenon". The ability to exhibit this behavior directly corresponds with the ability of the peptides to bind to CD4-expressing cells. Classes of exogenous substances with the ability to bind to HIV-1-Bi peptides and to destroy the "switch behavior" [so that the secondary structure elements rise (helix) or fall (β-hairpin) linearly with increasing apolarity] have been identified and shown to be effective inhibitors of the peptide/CD4 interaction (Reed & Kinzel, 1994).

Production of a switch inhibitor sufficiently powerful to be used therapeutically requires that a large number of compounds be screened and that the number requiring testing be narrowed as far as possible by understanding the basis of their activity. For the latter purpose, a detailed knowledge of the structure of the switch domain with and without inhibitor would be extremely useful. Although the 15-residue HIV-1-Bi region from strain LAV (LAV15) is not suitable for ^{1}H NMR analysis in aqueous buffer (Graf v. Stosch et al., 1995), the 44-amino acid gp120 fragment originally identified as an independent CD4-binding moiety (Lasky et al., 1987, 1988) will remain in solution at concentrations high enough to allow structural studies with ^{1}H NMR. Accordingly, we undertook a structural study of the 44-residue Lasky fragment using ^{1}H NMR coupled with studies on the effect of the various switch inhibitor compounds on the larger peptide. Although the NMR investigations did not permit sufficient resolution to determine the precise nature of the interaction between inhibitor and the CD4-binding domain, the accompanying CD studies showed that, in contrast to their effect on the LAV 15-mer peptide, the presence of effective switch inhibitors caused an immediate change in the preferred conformation of the LAV 44-mer in aqueous solution. As this single-measurement technique would greatly facilitate screening for active compounds, it was tested on a number of new derivatives and found to have reliable predictive value. By using this method, a switch inhibitor has been found with an ID_{50} less than one-fifth of those previously measured.

* Correspondence should be addressed to this author at the Department of Pathochemistry, German Cancer Research Center, D-69120 Heidelberg, FRG; telephone, 49-6221-423256; fax, 49-6221-423259.

[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1995.

0006-2960/96/0435-411$12.00/0

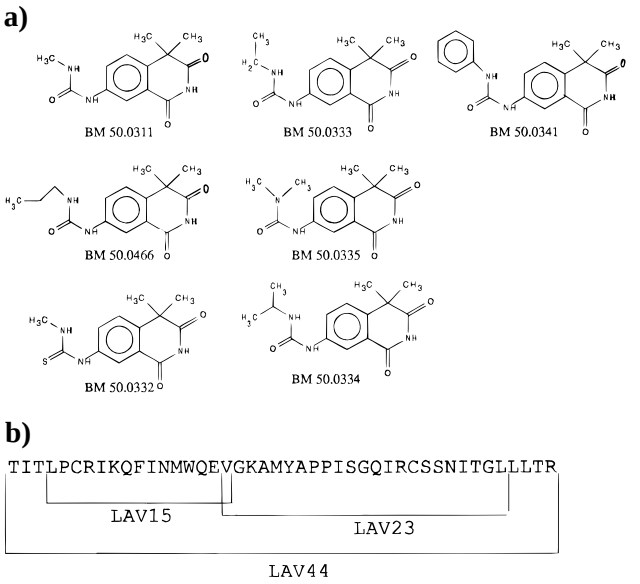

FIGURE 1: (a) Structural formulae for BM 50.0311 and all of its derivatives tested for their ability to change the LAV44 peptide secondary structure. (b) Sequence of LAV44 and derivative peptides used in this study.

MATERIALS AND METHODS

Peptides. Peptides corresponding to the entire 44-residue CD4-binding fragment of gp120 from HIV-1 strain LAV (T I T L P C R I K Q F I N M W Q E V G K A M Y A P P I S G-QIRCSSNITGLLLTR) were obtained from Bachem Biochemica GmbH, Heidelberg (LAV44).

The 15-residue switch domain (LAV15: LPCRIKQFIN-MWQEV) and a 23-residue peptide (LAV23: VGKA-MYAPPISGQIRCSSNITGL), both truncated versions of LAV44, have been synthesized by employing the Fmoc strategy (Merrifield, 1963; Carpino & Han, 1972) in a fully automated synthesizer (ABI 433). Peptide chain assembly was performed by using in situ activation of amino acid building blocks by 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU).

Inhibitors. CPF dipeptides [*N*-carbomethoxycarbonylprolylphenylalanyl benzyl ester, LL form (CPF1), and *N*-carboethoxycarbonylprolylphenylalanyl benzyl ester, DD form (CPF2)] (Figure 4) and BM 50.0311, as well as its derivatives (BM 50.0332, BM 50.0334, BM 50.0335, BM 50.0466, BM 50.0333, and BM 50.0341) (Figure 1), were the generous gift of Boehringer Mannheim. The BM compounds have no intrinsic dichroism. CPF dipeptides were dissolved as a concentrated solution (2 mg/125 μL) in TFE, and BM 50.0311 and its derivatives (1 mg/50 μL) were dissolved in 2 N NaOH, after which they could be brought into aqueous solution.

Circular Dichroism. Far-UV (190−240 nm) circular dichroism spectra were obtained using a Jasco J-500 or Jasco J-710 automatic recording spectropolarimeter. Calibration was carried out using 0.05% β-androsterone in spectral grade dioxane as a standard. Slit width was automatically adjusted to maintain a spectral resolution of 1.0 nm throughout the wavelength range.

Samples were measured in a 1 mm dichroically neutral quartz cuvette at a sensitivity of 2.0 mdeg/cm, a time constant of 2.0 s, and a scan speed of 5.0 nm/min. Spectra presented are a 4- or 2-fold signal average with identically measured and signal-averaged baseline subtraction, converted from millidegrees to mean residue ellipticity [θ_{mr}] for use in curve fitting and secondary structure analysis.

Curves were fitted as the linear sum of the CD spectra of model peptides in α-helix, β-sheet, extended coil, and reverse turn forms using data points between 190 and 235 nm (Yang et al., 1986). Model spectra for the first three structure types were an average of model peptide curves from the literature (Yang et al., 1986; Holzwarth & Doty, 1965; Quadrifolgio & Urry, 1968; Greenfield & Fasman, 1969; Adler et al., 1973; Brahms & Brahms, 1980) weighted according to the frequency of the respective side chains in proteins in general. Reverse turn model spectra were taken from Crisma et al. (1984).

Peptides were dissolved in the appropriate solvent system at a concentration of ≈100 μg/mL. Due to differences in the intrinsic dichroism of the inhibitor compounds used, the strategy of baseline correction was applied (Reed & Kinzel, 1994).

NMR Spectroscopy. Peptide samples were dissolved in 0.5 mL of 90% H_2O/10% D_2O (pH 5.5) to a final concentration of 1.0 mM. 3-(Trimethylsilyl)propionate (TSP) was added as the chemical shift reference. All NMR experiments were recorded on a Bruker AMX-600 spectrometer in the phase-sensitive mode using the time-proportional phase incrementation method (Marion & Wüthrich, 1983). Water suppression was carried out by selective presaturation, placing the carrier on the solvent resonances. Conventional pulse sequences were used for homonuclear correlation spectroscopy (COSY) (Aue et al., 1976) and nuclear Overhauser enhancement spectroscopy (NOESY) (Kumar et al., 1980). The mixing times in the NOESY experiment were set to 100 and 200 ms to exclude artificial peaks by spin diffusion. Total correlation spectroscopy data (TOCSY) were acquired by using the standard MLEV 17 spin−lock sequence and an 80 ms mixing time (Bax & Davies, 1985). The spectra were recorded at 278 and 293 K. The size of the acquisition data matrix was 2048 × 512 words in f_2 and f_1. Shifted square sine-bell window functions with the corresponding shift optimized for every spectrum were used for resolution enhancement, and baseline correction was applied in both directions.

The CoMFA (comparative molecular field analysis) procedure is an iterative multistep 3D QSAR method. The 3D structures of substances with inhibitory potential are generated and superimposed. A 3D grid is overlaid and field values are calculated for every grid box. Experimental data (e.g., binding constants) are linked to these physical results, and a partial least-squares analysis is performed. The CoMFA model is iteratively refined and is finally used to predict the effectiveness of drugs with related structural properties (Cramer et al., 1988; Kubinyi, 1993).

RESULTS

LAV15. Previous work has shown that the far-UV CD spectra of the LAV15 peptide (15-amino acid subfragment of the 44-residue CD4-binding domain synthesized according to strain LAV) exhibit an abrupt signal change as a function of the trifluoroethanol (TFE) concentration (Reed & Kinzel, 1991, 1993). A sharp conversion from CD curves typical of β-sheet to those indicating helical structure is observed at around 60% TFE (v/v). The addition of additional amounts of TFE leading to even more apolar solutions does

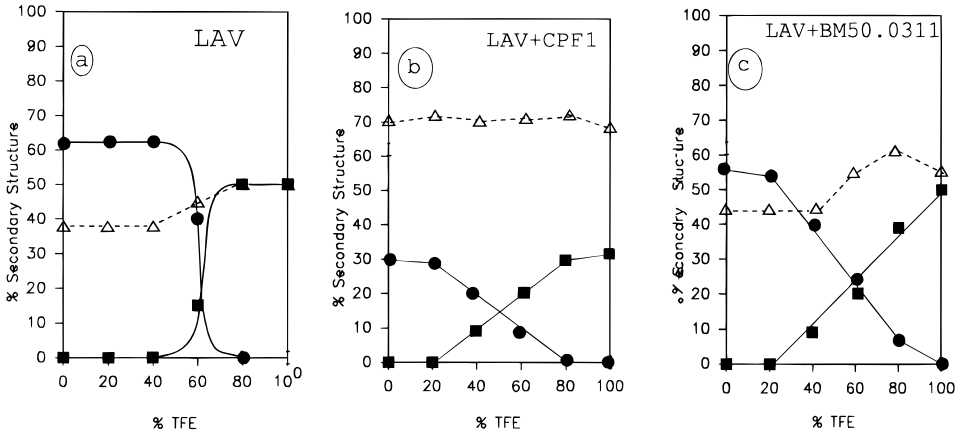

FIGURE 2: Secondary structure content of (a) LAV15 peptide, (b) LAV15 peptide and substance CPF1, and (c) LAV15 peptide and substance BM 50.0311 as a function of the TFE concentration (v/v). The secondary structure content is calculated from far-UV CD spectra measured at room temperature with concentrations of ~100 μg/mL peptide as standard (a) and the addition of 160 μg/mL CPF1 or (b) 10 μg/mL (350 mM) BM 50.0311 (c). The lines represent (■) α-helix, (●) β-sheet, and (△) random coil and the remainder of different turn types.

not result in any additional gain in helicity. A plot of the secondary structure content as a function of the TFE concentration is shown in Figure 2a. This behavior appears closely linked to the ability of the peptides to bind to CD4-expressing cells.

Several classes of switch inhibitors have been identified that have been previously shown to destroy this phenomenon (Reed & Kinzel 1994). Figure 2b shows the effect on LAV peptide behavior of one inhibitory derivative [*N*-carbomethoxycarbonylprolylphenylalanyl benzyl ester, LL (CPF1)] that was originally reported by Finberg and co-workers (1990) to be effective in preventing HIV infection of H9 tumor cells and human T-cells. Both the L,L and D,D stereoisomers (data not shown for the latter) are effective in eliminating the cooperativity of the β-sheet to helix transition. Linear increase in helical secondary structure and linear decrease in β-structure are also observed upon the addition of the inhibitor BM 50.0311 (Figure 2c).

As was shown in previous work by gp120/CD4 or LAV15/CD4 binding assays, these substances also block ligand/CD4 binding in a dose-dependent manner (Reed & Kinzel, 1994). Conversely, CPF dipeptide or BM 50.0311 derivatives with subtle modifications have proved to be ineffective as both binding and switch inhibitors.

LAV44. To verify the switch behavior and its inhibition as a physiologically relevant phenomenon, the original 44-amino acid Lasky binding domain was synthesized according to the sequence of HIV-1 strain LAV (LAV44). It has been shown that CD experiments with LAV44 confirm the former results with its subfragment LAV15. As the polarity of the medium is decreased, a sharp transition from $\beta \rightarrow$ helical structure is observed around 60% TFE, although it is masked to some extent by 29 additional residues. The portion of β-secondary structure at higher polarity ($\leq$50% TFE) is calculated to be $\approx$25%, while the remainder constitutes mainly random coil conformation. A comparison with LAV15 allows the conclusion that (i) the transition point at 60% TFE is conserved and (ii) the number of residues involved in the switch is the same in both cases (9 or 10 residues). The presence of three additional residues at the N-terminus and 26 additional residues at the C-terminus do not affect the solvent polarity-dependent cooperative refolding (Reed & Kinzel, 1993).

The LAV44 peptide is soluble at concentration ranges sufficiently high for 2D NMR measurements. 2D COSY, TOCSY, and NOESY spectra have been recorded in aqueous solution to identify regions with secondary structure elements. All TOCSY spectral peaks have been assigned and reflect the full ensemble of spin systems anticipated. The sequential assignment has been achieved by standard through-bond and through-space connectivity information. As expected from CD experiments, most of the 44 residues are in the random coil conformation. The ^{1}H chemical shifts approach their random coil (rc) values (Wüthrich, 1986), leading to clusters of intraresidue and sequential NH/CαH cross peaks within a very narrow range in the fingerprint region (Figure 3). All $\alpha H_i/NH_{(i+1)}$ NOEs, if not obscured by overlapping, were observed and their intensities compared relative to $\alpha H_i/NH_i$ cross peaks. The observation of strong sequential NOEs in comparison with intraresidue NOEs indicates the tendency toward extended chain structures (Dyson et al., 1988a,b). Medium range NOEs suggesting secondary structure elements or long range NOEs could not be detected. It must be emphasized that the existence of medium/long range NOEs within the fingerprint region cannot be excluded. Strong sequential or intraresidue NOEs possibly mask additional weaker medium/long range peaks.

Deviations from rc chemical shift values ($C_\alpha H - C_{\alpha,rc}H$) are usually interpreted as indications of regular secondary structure (Bruix et al., 1990). The overall average from Ile2 to Thr43 is calculated to be -0.035 ppm (not considering the three prominently positive values), suggesting no regular structure. On the other hand, the same calculation leads to a value of -0.09 ppm between Cys6 and Val18, which is significantly higher than the overall average. Deviations from rc values are obviously clustered along the N-terminal part, i.e., the LAV15 region. Three prominently positive difference values for Leu4 (+0.28), Ala24 (+0.22), and Pro25 (+0.16) are explained by the tendency of subsequent proline residues to shift their N-terminal neighbors by ca. +0.2 ppm. NH temperature coefficients—normally considered to be sensitive probes for detecting protected NH protons—did not reveal deviations from the expected values. Only the Gln16 peak remained unshifted, which might be due to the shielding effects of the Trp15 aromatic side chain.

Apart from the analogous switch mechanism in the TFE titration series, the behavior of LAV15 and LAV44 also

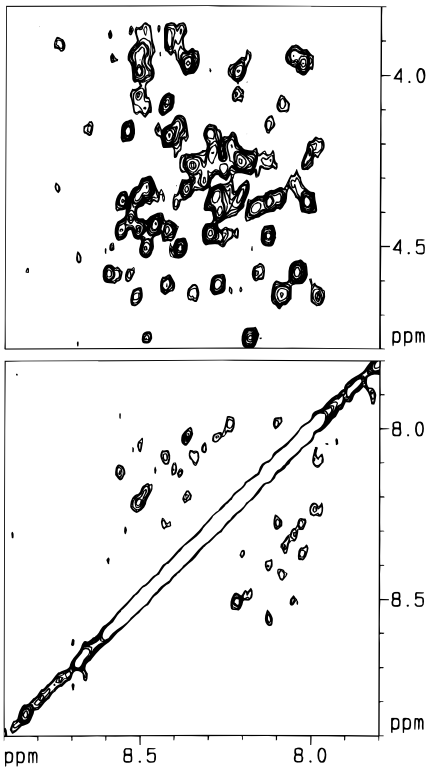

FIGURE 3: Selected regions of the 2D NOESY spectrum of the LAV44 peptide [1.0 mM in 90% H_2O/10% D_2O (pH 5.5), 20 °C, mixing time = 100 ms].

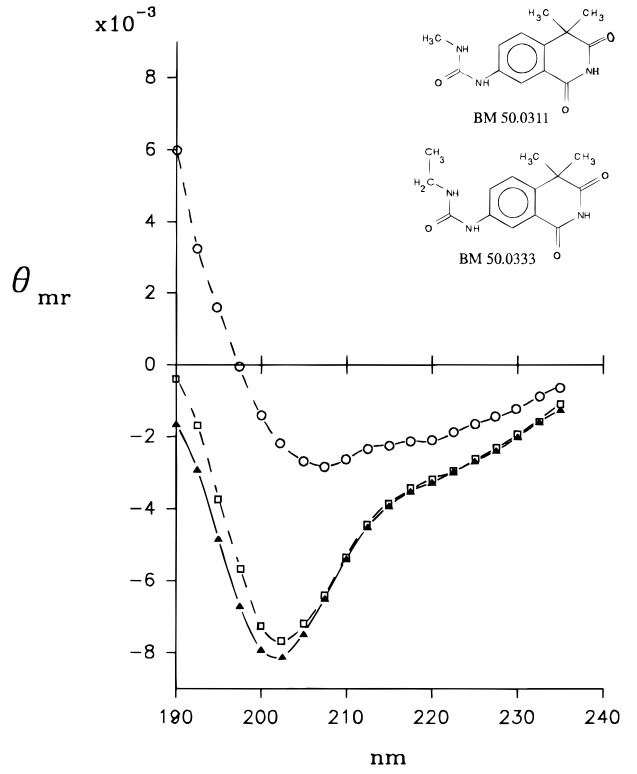

FIGURE 4: Far-UV CD spectra of the (▲) LAV44 peptide, (□) LAV44 and substance BM 50.0333, and (○) LAV44 and substance BM 50.0311. The spectra were measured in 10 mM Tris buffer (pH 7.5) at room temperature, with concentrations of ~100 μg/mL peptide as standard (▲) and the addition of 10 μg/mL BM 50.0333 (□) or 10 μg/mL BM 50.0311 (○).

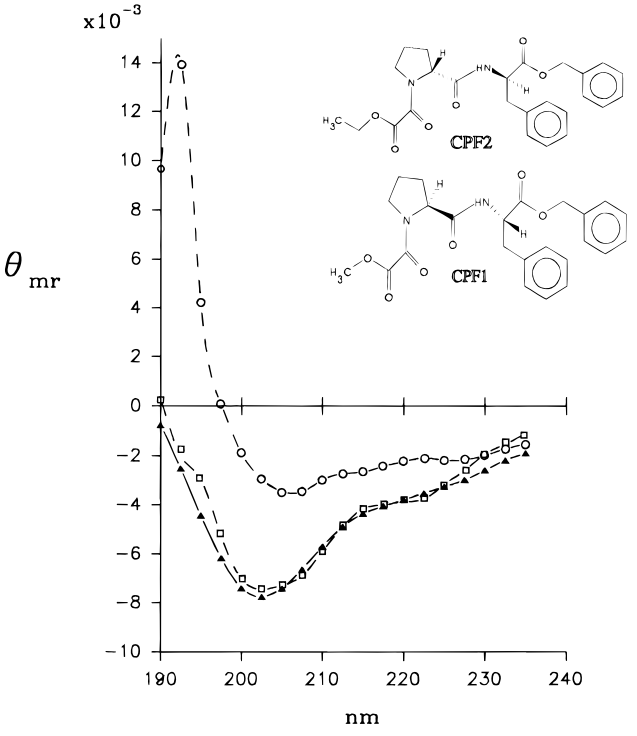

FIGURE 5: Far-UV CD spectra of the (▲) LAV44 peptide, (□) LAV44 and substance CPF2, and (○) LAV44 and substance CPF1. Spectra were taken under the same conditions as for Figure 4, with ~100 μg/mL LAV44 peptide solutions and the addition of 160 μg/mL CPF1 or CPF2, respectively.

concurs in their structural response upon the addition of the inhibitory substances (BM 50.0311, CPF1) and their ineffective derivatives. As shown in Figure 4, a convincing example of congruent behavior has been found. While the addition of BM 50.0311 to aqueous LAV44 peptide solution has a marked impact on the LAV44 secondary structure CD signal, the ethyl derivative (BM 50.0333) exhibits no effect on the LAV44 peptide CD curve, which nearly coincides with the original CD curve in the absence of any organic substance. These results agree with the former observation that BM 50.0311 with its methyl moiety alters the aqueous/TFE titration behavior of the HIV-1-Bi region, while the ethyl derivative is incapable of influencing this structural equilibrium (Reed & Kinzel, 1994). The CPF dipeptide derivatives exhibit the same pattern of behavior. Only the CPF1 inhibitor with a methoxy linkage, which destroys the cooperative transition, induces a structural change in the LAV44 peptide (Figure 5). In contrast, its ethoxy analog (CPF2), which is without effect on the LAV15 titration series, also fails to alter the LAV44 CD signal. Therefore, the inhibitory effect on the structural behavior coincides in a positive as well as a negative sense for LAV15 and LAV44.

The secondary structure content of the LAV44 peptide upon the addition of BM 50.0311 was calculated to be 15% helical and ≈85% rc and turn conformation. Therefore, the inhibitor induces a helical stretch and abolishes the β-like structure that accounts for 25% of the LAV44 peptide secondary structure in the absence of the inhibitor. Similar secondary structure content estimates, in particular a total loss of β-sheet conformation, were obtained for mixtures of LAV44 and the CPF1 dipeptide. As the response of the LAV44 peptide to active inhibitors could be seen in a single CD scan, it offered a considerable advantage as a screening method over TFE titration of the LAV 15-mer, which required a minimum of six spectra and was difficult to quantify for the determination of ID_{50}'s. Therefore, five additional derivatives of BM 50.0311 were tested for their

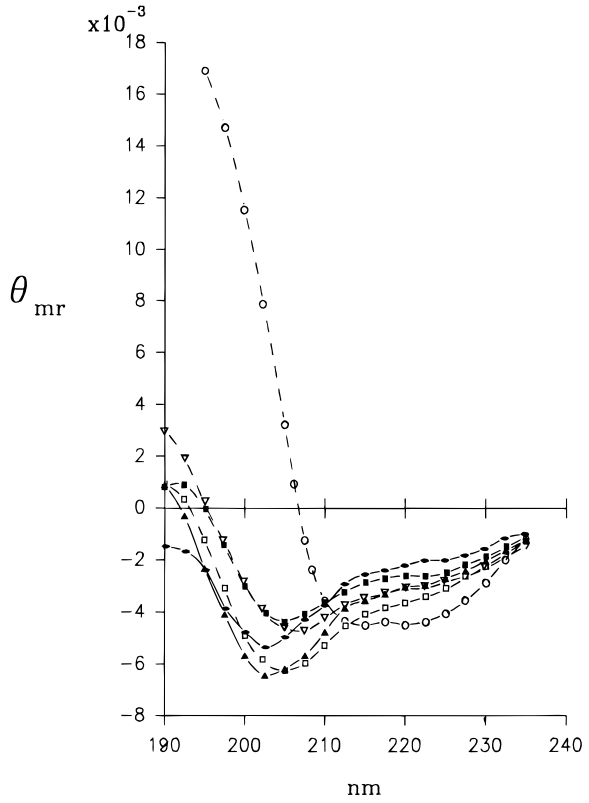

FIGURE 6: Far-UV CD spectra of LAV44 (▲) and five BM 50.0311 derivations are shown: (□) BM 50.0332, (●) BM 50.0334, (▽) BM 50.0335, (■) BM 50.0466, and (○) BM 50.0341. Spectra were taken under the same conditions as explained for Figure 4.

ability to change the LAV44 peptide secondary structure. All of these derivatives share the main structural characteristics of their progenitor, apart from subtle modifications along the tail moiety (Figure 1). Three substances have altered head groups, either destroying the H-bonding capacity of the terminal amide group (BM 50.0335) or substituting the methyl moiety by other alkyl groups (BM 50.0466 and BM 50.0334). These derivatives proved to have no effect on the LAV44 peptide structure (Figure 6). The results are not surprising since the most conservative modification possible at this particular site (methyl → ethyl) already destroys the inhibitory potential. A different kind of modification has been introduced in the fourth BM 50.0311 variant (BM 50.0332). The ureate linkage between the six-membered ring and the methyl group was replaced by a thioureate linkage. Interestingly, this minor modification also results in the total loss of structure changing activity (Figure 6).

In contrast to all derivatives mentioned so far, BM 50.0341, with a phenyl ring as head group, had a significant impact on the LAV44 secondary structure (Figure 6). Since this was the first independent prediction of inhibitory activity from the LAV44 peptide system, the substance BM 50.0341 was tested in the standard TFE titration system with the LAV 15-mer. The assay was complicated by the fact that BM 50.0341 apparantly promotes aggregation and precipitation of the LAV15 peptide in polar solutions. CD spectra taken at 10% intervals from 100% to 40% TFE, however, showed a linear conversion of β-sheet to helix as the polarity was reduced, with no evidence of an abrupt $\beta \rightarrow$ helix conversion at 60% TFE (data not shown). The induction of a conformational change in the LAV44 peptide in aqueous buffer

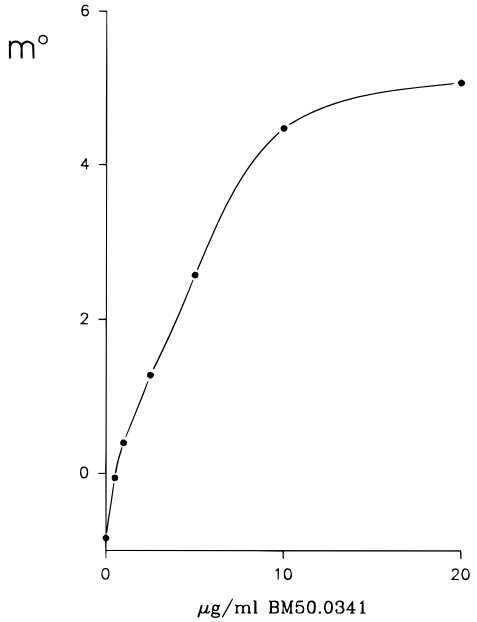

FIGURE 7: Dose dependence of the conformational change in the LAV 44-mer induced by BM 50.0341. The LAV 44-mer (100 μg/mL in 10 mM Tris, pH 7.5) was scanned from 190 to 240 nm in the presence of 0, 0.5, 1.0, 2.5, 5, 10, and 20 mg/mL BM 50.0341, and the rotational strength at 195 nm was plotted as a function of inhibitor concentration (10 μg/mL BM 50.0341, 30 μM).

therefore is a reliable indicator of switch inhibitory activity and may be used as a screening method.

CD spectra were repeatedly run for the LAV44 peptide in the presence of 1.5, 3, 7.5, 15, 30, and 60 μM BM 50.0341 to establish an ID_{50} for switch inhibition. Although a similar pattern was seen in all experiments (Figure 7 gives a representative example), a precise quantitative estimate of the ID_{50} was uncertain due to the sparingly soluble nature of the derivative, which introduced an element of variability at higher concentrations. A conservative estimate (i.e., assuming total solubilization of the derivative) gives an ID_{50} of $\sim$30 μM. (The concentration of the LAV 44-mer is 20 μM.) This is a roughly 5-fold improvement over the performance of the lead substance, BM 50.0311.

While such complementary results are, in principle, sufficient to postulate one common rationale behind the mode of action of the inhibitors in these two different assay systems, the possibility that the refolded area of LAV44 upon inhibitor addition is located at some point more distal to the N-terminal LAV15 sequence could not be excluded. Therefore, a 23-residue peptide (LAV23) corresponding to the C-terminal LAV44 sequence (Val414−Leu436) was synthesized and used as reference. Addition of either of the two active inhibitory substances, BM 50.0311 and the CPF1 dipeptide, had no effect on the CD signal of the C-terminal segment (data not shown). CD curves of mixtures of LAV23 and BM 50.0311 or CPF dipeptide do not indicate any structural change. These experiments confirm that both inhibitors recognize a common binding place on the LAV15 sequence (N-terminal part of LAV44) and that the induction of refolding within the LAV44 peptide and the loss of cooperativity in LAV15 are two different manifestations of the same physical phenomenon.

The finding that active switch inhibitors induce a conformational change in the LAV44 peptide simplifies the task of screening for such substances considerably. A single far-

UV scan of the peptide in the presence of the drug can be carried out in 20 min and requires only 300 μL of a 20 μM solution of the peptide and at most 200 μM drug. (Substances showing no activity below this level may be considered inactive for practical purposes.) Potential limitations exist in that the drug must be sufficiently soluble in aqueous solution, must not absorb strongly in the 190−240 nm region, and must show either no intrinsic dichroism (as is the case for the BM 50.0311 derivatives) or a dichroism unmodified by binding (which can then be subtracted as a buffer/drug baseline). Substances showing up positive in the single-scan test with the LAV44 peptide can then be subjected to the TFE titration test with the LAV15 peptide. If positive in both tests, a third screening to establish their ability to inhibit binding of the LAV15 peptide to CD4-expressing cells will be necessary.

DISCUSSION

Previous work has established the fact that the switch mechanism is a prerequisite for the CD4-binding ability of the HIV-1 surface glycoprotein gp120 (Reed & Kinzel, 1991, 1993, 1994). It has been shown that the cooperative refolding of the CD4-binding domain is a conserved feature of all strain sequences tested so far, in spite of the high overall sequence variability of gp120 (Hahn et al., 1985; Wong-Staal et al., 1985), and it has already been proposed that the polarity-dependent cooperative restructuring from $\beta \rightarrow$ helical conformation reflects a necessary and indispensable process for docking on the relatively apolar membrane-bound receptor. The switch at the CD4-binding domain is supposed to initiate further steps of refolding at other gp120 regions, which allows the transmembrane gp41 protein to act as a fusion domain.

The importance of the switch behavior is underlined not only by its conservation throughout strains with the widest range of sequence variability but also through the physiological effects of its inhibition. Evidence has been gathered that inhibitors from different substance classes with certain shared structural characteristics eliminate the cooperative transition and CD4-binding ability in parallel, as shown by gp120/CD4 and LAV15/CD4 binding assays. The entire 44-residue CD4-binding domain, as originally identified by Lasky et al. (1987, 1988), has now been subjected to NMR as well as CD spectroscopy to complement the information gained from the LAV15 switch domain (Reed & Kinzel, 1991, 1993, 1994). CD experiments have shown that the switch behavior is reproduced by the LAV44 peptide. The number of residues involved in the transition and the transition point at 60% TFE (v/v) are conserved, strongly supporting the idea that the same mechanism is effective in both LAV44 and its subfragment LAV15.

NMR studies with the aim of elucidating the LAV15 peptide structure in aqueous solution have been prevented by solubility problems at NMR concentration ranges, an observation not uncommon for peptides with β-hairpin structure (Graf v. Stosch et al., 1995; Barrow et al., 1991; Mutter & Hersperger, 1990). In contrast, the LAV44 peptide is soluble in aqueous solution at concentration ranges adequate for 2D NMR analysis. The NMR data, in contrast to the CD data, give no indication of regular secondary structure. Neither medium nor long range NOEs could be detected, and most ^{1}HCα chemical shifts approach their random coil values, although deviations are found in the N-terminal part of the sequence. However, it must be emphasized that weak, long range NOEs for β-sheet formation may be obscured by the overcrowded fingerprint region. In addition, a number of cases have been reported where NMR methods proved to be inadequate to detect small amounts of secondary structure originally indicated by CD experiments (Mendz, 1992). The data are consistent with the LAV44 peptide structure in fact being dominated by the random coil conformation, but with a remainder of rather flexible β-secondary structure ($\approx$25% calculated from CD curves) still existing, although not clearly delineated by NMR methods. It has been speculated that the switch domain embedded in the larger protein matrix of LAV44 has a less rigid β-conformation than in the LAV15 subfragment itself. This assumption is confirmed by CD experiments with mixtures of LAV44 and switch inhibitors or their ineffective derivatives, respectively. Addition of BM 50.0311 or CPF1 to aqueous LAV44 peptide solutions induces a dramatic structural change between the liganded and unliganded forms. Yet although both substances are effective in the LAV15 switch inhibition assay, they do not alter the initial LAV15 peptide structure in aqueous solution as observed by CD measurement. Only upon the addition of TFE is their effect on the structure detectable by a linear instead of a cooperative $\beta \rightarrow$ helical transition.

To verify that both phenomena—inhibition of the LAV15 peptide switch and the LAV44 structural change—originate from the same mechanistic mode of action, a peptide was synthesized (LAV23) corresponding to the C-terminal LAV44 peptide sequence excluding the switch domain. Both switch inhibitors failed to induce a structural change in this region, confirming that they bind to the switch domain with high specificity and evoke (i) a linear structure conversion in the LAV15 aqueous/TFE titration series and (ii) a structural change in the LAV44 peptide in aqueous solution.

The interrelation between these phenomena is documented not only by the inhibitory effects of CPF1 and BM 50.0311 in a positive sense but also by the ineffectiveness of their derivatives in both assays in a negative sense. Subtle modifications of BM 50.0311 at the alkyl head group render the analogs ineffective. As shown previously (Reed & Kinzel, 1994), the methyl $\rightarrow$ ethyl derivative proved to have no inhibitory potential in the LAV15 peptide/CD4 binding assay and to be without effect in eliminating the switch. Correspondingly, this substance fails to induce a structural change in LAV44. An analogous result has been found for CPF2, namely, that it is inefficient in (i) the LAV15/CD4 binding test, (ii) the LAV15 switch, and (iii) the LAV44 assay.

We have thus, first, identified a common trait of gp120 from different HIV-1 strains for the attachment to its CD4 receptor and, second, established a three-column method for screening substances for the potential to inhibit the viral docking process. The chemical characteristics of such substances have already been described extensively. Experiments with five additional derivatives of BM 50.0311 support previous assumptions (Reed & Kinzel, 1994). A planar tail structure seems to be absolutely necessary. Elongation of the methyl moiety by other alkyl groups results in a loss of activity. Nevertheless, the distribution of charges (possibly for the formation of H-bonds) has to be conserved, as seen for BM 50.0335 and BM 50.0332. The replacement of the

ureate linkage destroys the inhibitory potential just as a terminal tertiary amine (BM 50.0335) does. On the other hand, replacement of the methyl moiety by a phenyl group, which is expected to maintain the planar tail structure, not only retains activity but unexpectedly improves it, although the accompanying loss of solubility indicates that further derivatization will be necessary to produce optimal results. Two different approaches of QSAR (quantitative structure−activity relationship) are in progress to refine the structural requirements necessary for effective gp120/CD4 inhibition. First, testing of an array of derivatives has started in order to use these data for a CoMFA analysis (Cramer et al., 1988; Kubinyi, 1993). Second, NMR studies of the LAV44 peptide with an effective inhibitor are being carried out to elucidate the precise inhibitor binding place. Information about the stereochemical orientation on the target molecule will allow the rational design of substances with even higher specificity and affinity for therapeutic use.

ACKNOWLEDGMENT

The authors thank Mrs. Angelika Lampe for her excellent processing of the manuscript and Dr. A. Mertens of Boehringer Mannheim for his generosity in furnishing many of the compounds tested.

SUPPORTING INFORMATION AVAILABLE

One table is available showing chemical shift data of the peptide LAV44 in aqueous solution (pH 5.5, 293 K) (1 page). Ordering information is given on any current masthead page.

REFERENCES

Adler, A. J., Greenfield, N. J., & Fasman, G. D. (1973) *Methods Enzymol. 27*, 675−735.

Aue, W. P., Bartholdy, E., & Ernst, R. R. (1976) *J. Chem. Phys. 64*, 2229−2246.

Barrow, C. J., Colin, J., Zagorski, M. G., & Michael, G. (1991) *Science 251*, 179−183.

Bax, A., & Davies, D. (1985) *J. Magn. Reson. 65*, 355−360.

Brahms, S., & Brahms, J. (1980) *J. Mol. Biol. 138*, 149−178.

Bruix, M., Perello, M., Herranz, J., Rico, M., & Nieto, J. (1990) *Biochem. Biophys. Res. Commun. 167*, 1009−1014.

Carpino, L. A., & Han, G. Y. (1972) *J. Org. Chem. 37*, 3404−3409.

Cordonnier, A., Montagnier, L., & Emerman, H. (1989) *Nature 340*, 571−574.

Cramer, R. D., III, Patterson, D. E., & Bunce, J. D. (1988) *J. Am. Chem. Soc. 110*, 5959−5967.

Crisma, M., Fasman, G. D., Balaram, H., & Balaram, P. (1984) *Int. J. Pept. Protein Res. 23*, 411−419.

Dalgleish, A. G., Beverley, P. C. L., Clapham, P. R., Crawford, D. H., Greaves, M. F., & Weiss, R. A. (1984) *Nature 312*, 763−767.

Dyson, H. J., Rance, M., Houghton, R. A., Lerner, R. A., & Wright, P. E. (1988a) *J. Mol. Biol. 210*, 161−200.

Dyson, H. J., Rance, M., Houghton, R. A., Lerner, R. A., & Wright, P. E. (1988b) *J. Mol. Biol. 210*, 200−217.

Finberg, R. W., Diamond, D. C., Mitchell, D. B., Rosenstein, Y., Soman, G., Norman, T. C., Schreiber, S. L., & Burakoff, S. J. (1990) *Science 249*, 287−291.

Graf v. Stosch, A., Jiménez, M., Kinzel, V., & Reed, J. (1995) *Proteins 23*, 196−203.

Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108−4116.

Hahn, B. H., Gonda, M. A., Shaw, G. M., Popovic, M., Hoxie, J., Gallo, R. C., & Wong-Staal, F. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4813−4817.

Holzwarth, G., & Doty, P. (1965) *J. Am. Chem. Soc. 87*, 218−228.

Klatzman, D., Champagne, E., Chamaret, S., Gruest, J., Guetard, D., Hercend, T., Gluckman, J.-C., & Montagnier, L. (1984) Nature 312, 767−768.

Kubinyi, H., Ed. (1993) 3D QSAR in Drug Design. in *Theory, Methods and Applications*, ESCOM Science Publishers, Leiden.

Kumar, A., Ernst, R. R., & Wüthrich, K. (1980) *Biochem. Biophys. Res. Commun. 95*, 1−6.

Lasky, L. A., Nakamura, G., Smith, D. H., Fennie, C., Shimasaki, C., Patzer, D., Berman, P., Gregory, T., & Capon, D. J. (1987) *Cell 50*, 975−985.

Lasky, L. A., Nakamura, G., Smith, D. H., Fennie, C., Patzer, E., Shimasaki, C., Berman, P., Gregory, T., & Capon, D. J. (1988) in *Human Retroviruses, Cancer and AIDS, Approaches to Prevention and Therapy*, pp 257−267, Alan R. Liss, Inc., New York.

Marion, D., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 113*, 967−974.

McDougal, J. S., Kennedy, M. S., Sligh, J. M., Cort, S. P., Mawle, A., & Nicholson, J. K. A. (1986) *Science 231*, 382−385.

Mendz, G. L. (1992) in *Structure and Molecular Interactions of Myelin Basic Proteins and Its Antigenic Peptides* (Martinson, R. E., Ed.) CRC Press, Boca Raton, FL.

Merrifield, R. B. (1963) *J. Am. Chem. Soc. 85*, 2149−2154.

Modrow, S., Hahn, B. H., Shaw, G. M., Gallo, R. C., Wong-Staal, F., & Wolf, H. (1987) *J. Virol. 61*, 570−578.

Mutter, M., & Hersperger, R. (1990) *Angw. Chem., Int. Ed. Engl. 29*, 185−187.

Quadrifoglio, F., & Urry, D. W. (1968) *J. Am. Chem. Soc. 90*, 2755−2765.

Reed, J., & Kinzel, V. (1991) *Biochemistry 30*, 4521−4528.

Reed, J., & Kinzel, V. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6761−6765.

Reed, J., & Kinzel, V. (1994) *Biochemistry 33*, 10993−10998.

Wong-Staal, F., Shaw, G. M., Hahn, B. H., Salahuddin, S. Z., Popovic, M., Markham, P. D., Redfield, R., & Gallo, R. C. (1985) *Science 229*, 759−762.

Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, John Wiley & Sons, New York.

Yang, J. T., Wu, C.-S. C., & Martinez, H. M. (1986) *Methods Enzymol. 130*, 208−269.

BI952045W