Kinetic Dependence to HIV-1 Entry Inhibition*

Received for publication, February 14, 2006, and in revised form, June 19, 2006 Published, JBC Papers in Press, June 27, 2006, DOI 10.1074/jbc.M601457200

H. Kirby Steger and Michael J. Root†

From the Department of Biochemistry and Molecular Biology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Infection by human immunodeficiency virus type 1 (HIV-1) involves the fusion of viral and cellular membranes mediated by formation of the gp41 trimer-of-hairpins. A designed protein, 5-Helix, targets the C-terminal region of the gp41 ectodomain, disrupting trimer-of-hairpins formation and blocking viral entry. Here we show that the nanomolar inhibitory potency of 5-Helix (IC_{50} \sim 6 \text{ nM}) is 4 orders of magnitude larger than its subpicomolar binding affinity (K_{D} \sim 0.6 \text{ pM}). This discrepancy results from the transient exposure of the 5-Helix binding site on gp41. As a consequence, inhibitory potency is determined by the association rate, not by binding affinity. For a series of 5–Helix variants with mutations in their gp41 binding sites, the IC_{50} and K_{D} values poorly correlate. By contrast, an inverse relationship between IC_{50} values and association rate constants (k_{on}) extends for over 2 orders of magnitude. The kinetic dependence to inhibition places temporal restrictions on an intermediate state of HIV-1 membrane fusion and suggests that access to the C-terminal region of the gp41 ectodomain is largely free from steric hindrance. Our results support the importance of association kinetics in the development of improved HIV-1 fusion inhibitors.

HIV-12 cellular invasion proceeds through fusion of viral and cellular membranes, a process mediated by the viral surface glycoprotein Env in response to binding cellular receptors (1, 2). Functional Env is composed of two noncovalently linked subunits, gp120 and gp41, arranged as a trimer of heterodimers on the virion surface. The interaction of gp120 with cellular CD4 and a chemokine receptor promotes structural rearrangements that ultimately lead gp41 to insert its N-terminal fusion peptide segment into the target cell membrane (Fig. 1A). Eventually, gp41 collapses into a stable, compact trimer-of-hairpins conformation essential for efficient membrane fusion (3).

The core of the gp41 trimer-of-hairpins is a bundle of six α-helices formed by two hydrophobic heptad repeat sequences (HR1 and HR2) within each of the three gp41 ectodomains (4–7). The N-terminal HR1 segments form a central, trimeric coiled coil. The C-terminal HR2 segments pack in an antiparallel manner into hydrophobic grooves on the exterior of the HR1 coiled coil. The antiparallel packing of HR1 and HR2 segments ensures that the fusion peptide and transmembrane region of gp41 are brought into close proximity (Fig. 1A). Since the fusion peptide is inserted within the target cell membrane and the transmembrane region is embedded within the viral membrane, formation of the gp41 trimer-of-hairpins promotes the juxtaposition of the two membranes (6). The similarity of the gp41 trimer-of-hairpins to the fusion-active conformations of glycoproteins from distantly related enveloped viruses suggests that this structure plays a critical role in promoting viral membrane fusion (see Ref. 3 and references therein).

Peptides derived from the HR2 region of the gp41 ectodomain can disrupt formation of the gp41 trimer-of-hairpins, thereby inhibiting HIV-1 entry (4, 8, 9). Denoted as C-peptides, they work in a dominant negative fashion by binding the gp41 HR1 regions exposed in the extended, prehairpin intermediate conformation (4, 10–13). Once bound, they prevent association of the HR1 and HR2 segments, thus blocking viral membrane fusion. That C-peptides bind an intermediate state in the fusion process is supported by the following observations: 1) C-peptides can precipitate gp41 only after Env binds cellular receptors (12, 13); 2) the extent of inhibition depends on C-peptide concentration present during membrane fusion and not on preincubation levels (12); 3) conditions that alter the rate of gp41-mediated membrane fusion influence the inhibitory potency of C-peptides (14–16); and 4) at intermediate concentrations that block fusion pore formation, C-peptides can lock gp41 in a state that still permits lipid mixing (17). Despite the transient exposure of their binding site, some C-peptides possess potent (low nanomolar) inhibitory activity in vitro. One C-peptide, T20 (enfuvirtide), also demonstrates antiviral activity in vivo and has been approved by the FDA for treatment of HIV-1 infected individuals (18, 19).

In a complementary manner, agents that specifically target the gp41 HR2 segment also block HIV-1 entry. N-peptides, derived from the gp41 HR1 region, can inhibit through this mechanism, but their tendency to aggregate in physiological solutions severely limits their potency (4, 20–22). However, engineered proteins that expose all or part of the HR1 coiled coil in soluble form display potent, broad-spectrum inhibitory activity (mid-picomolar to low nanomolar). In one design strategy, three HR1 and two HR2 segments that form the core of the gp41 trimer-of-hairpins have been linked into a single polypeptide, denoted 5-Helix (23). 5-Helix lacks a third HR2 segment,
Kinetic Dependence to HIV-1 Entry Inhibition

Inhibition of HIV-1 membrane fusion by 5-Helix. A, a working model of HIV-1 membrane fusion depicting the proposed conformational changes of gp41 (adapted from Ref. 23). In its native state, much of gp41 is covered by a canopy of gp120 monomers (green). The interaction of cellular receptors (CD4 and chemokine receptor, not shown) with gp120 stimulates gp41 to extend and insert its N-terminal fusion peptide (red) into the target cell membrane. Eventually, the HR1 (gray) and HR2 (blue) regions of gp41 interact, collapsing the glycoprotein into its trimer-of-hairpins state that juxtaposes the viral and target cell membranes. 5-Helix inhibits by targeting the gp41 HR2 region prior to trimer-of-hairpins formation. For clarity, the gp41 cytoplasmic domain has been omitted. B, inhibition of cell-cell fusion by 5-Helix (filled squares) and its variant 5-Helix N3-D4 (open squares). HIV-1 Env-expressing effecter cells were cocultured with receptor-bearing target cells in the presence of varying inhibitor concentrations. The resulting syncytia numbers were normalized to the amount observed in the absence of protein. The data points represent the mean ± S.E. of at least three independent experiments. C, effect of 5-Helix preincubation on viral infection. HIV-1 pseudotyped with HXB2 Env was incubated with or without 30 nM 5-Helix for 45 min at 37 °C. The viral mixture was then diluted 10-fold in the presence or absence of 5-Helix before being applied to receptor-bearing target cells. The Pre and Inf designations refer to 5-Helix concentrations during the preincubation and infection steps, respectively. The data, normalized to the observed infectivity of virus not exposed to 5-Helix, represent the mean ± range of mean for duplicate samples. The experiment has been repeated three times with similar results.

and this vacancy creates a single, high affinity C-peptide binding site. In a second design strategy, N-peptides of various lengths are linked to trimerizing scaffolds such as a soluble coiled coil (21, 24, 25) or a six-helix bundle (22). These chimeric inhibitors expose three C-peptide binding sites, and multiplicity of interaction likely plays a role in their inhibitory mechanism.

It is unclear at what point during fusion the gp41 HR2 segment becomes accessible to engineered inhibitors like 5-Helix. Some evidence suggests that these designed proteins might bind the native conformation of Env prior to receptor engagement. Unlike C-peptides, 5-Helix can precipitate Env from cellular and viral membranes in the absence of CD4 or coreceptor (26, 27). This interaction enables a recombinant 5-Helix toxin to kill Env expressing cells without the need for receptor activation (26). However, the extent of receptor-independent interaction is relatively small and may merely reflect 5-Helix binding to a misfolded, nonfusogenic form of Env. Moreover, the interaction is enhanced in the presence of receptors (26–28), suggesting that 5-Helix might bind to an intermediate state of fusion. Consistent with this interpretation, inhibition of the HR2 region is sensitive to gp41 mutations that disrupt trimer-of-hairpins formation and alter the kinetics of membrane fusion (16, 29).

Here, we take an alternate approach to investigate the temporal accessibility of the gp41 HR2 segment during membrane fusion. Using a series of 5-Helix variants with mutations within their C-peptide binding sites, we have probed the relationship between inhibitory potency, binding affinity, and association kinetics. We reasoned that the potency of an inhibitor capable of binding native Env should largely depend on its binding affinity. Contrary to this expectation, we find that binding affinity is a poor predictor of inhibitory potency. Rather, an inverse relationship is observed between IC50 (50% inhibitory concentration) and the second-order association rate constant (k on). This precise dependence is predicted for an inhibitor that targets a transient state in the fusion process. Our results place temporal and spatial restrictions on the exposure of the C-terminal region of the gp41 ectodomain.

**EXPERIMENTAL PROCEDURES**

**Construction, Purification, and Characterization of 5-Helix Variants**—Mutations of Ala and Asp were introduced alone or in combination into 5-Helix at residues Val549, Leu556, Gln563, and Val570 of the N40 segments (numbering according to the HXB2 Env sequence of the HR1 region). These residues fall in the e-position in a helical wheel diagram of the HR1 coiled coil (23) and make primarily hydrophobic contacts with the HR2 segment. For the Single Ala, Single Asp, Double Ala, and Triple Ala variants, substitutions were made in all three N40 segments simultaneously. For reasons of protein stability, substitutions were incorporated into the third N40 segment only for the Double Asp and Ala + Asp variants (see supplemental material for more details on variant construction, purification, and characterization).

All 5-Helix proteins were recombinantly expressed in *Escherichia coli* and purified under denaturing conditions using metal chelate chromatography. Protein refolding took place on nickel-nitrilotriacetic acid–agarose beads (Qiagen) in 4 M guanidine hydrochloride by slow cooling from 90 °C to room temperature. Monomers were separated from aggregates by gel filtration (Sephacryl S200 HR (Amersham Biosciences)) in 10 mM Tris/100 mM NaCl, pH 8.0). Protein was determined to be >95% pure by SDS-PAGE analysis. The concentrations of all 5-Helix variants were determined by absorbance at 280 nm in 6 M guanidine hydrochloride (30). Molar ellipticity ([θ]222) and melting temperature (T m) for each variant were determined by circular dichroism spectroscopy and are reported in Table 1.

**Preparation of Fluorescently Labeled C-peptide R-C37**—The R-C37 peptides utilized in the study contain HR2 residues 625–661 from either HXB2 or JRFL Env (numbering according to the HXB2 sequence). They were generated from cysteinated C-peptide Cys-C37-H2 obtained by proteolysis of the recombinantly expressed trimer-of-hairpins construct CGG-NC1.1 (see Ref. 23 and supplemental material for details). The peptide was fluorescently labeled using rhodamine-5-maleimide (Molecular Probes) and purified to homogeneity by reverse phase high performance liquid chromatography (Waters...
Kinetic Dependence to HIV-1 Entry Inhibition

5-Helix consists of three 40-residue N-peptides and two 38-residue C-peptides alternately connected (N-C-N-C-N) by five-amino acid Gly/Ser linkers. X-ray crystallographic studies confirm that the core of 5-Helix adopts the structure of the gp41 trimer-of-hairpins minus one HR2 helix (Fig. 2A). The hydrophobic groove created by this vacancy interacts strongly with C-peptides and enables 5-Helix to inhibit Env-mediated membrane fusion at low nanomolar concentrations (Fig. 1B). The reported IC50 values are independent of viral concentrations for infectivity studies or effector cell concentrations in cell-cell fusion assays (see supplemental Fig. S2). Thus, 5-Helix inhibitory potency reflects an intrinsic property of the Env interacting with target cells and is not limited by mass action due to the amount of Env on viral or cellular membranes.

Preincubating virus at high 5-Helix concentrations prior to diluting for infection does not enhance 5-Helix inhibitory activity (Fig. 1C). Thus, if 5-Helix interacts with Env prior to receptor engagement, there is sufficient time for the protein to re-equilibrate with its binding site during infection.

\[ \Delta f = \Delta f_{\text{min}} + \frac{\Delta f_{\text{max}} - \Delta f_{\text{min}}}{2[C37]_0} \left( \frac{([C37]_0 - [5H]_0 - K_D)}{1 + He^{-k_{on}t}} + \frac{P}{1 - He^{-k_{off}t}} \right) \]

where

\[ P = \sqrt{([C37]_0 - [5H]_0 - K_D)^2 + 4[C37]_0 K_D} \]

\[ \Delta f_{\text{min}} \text{ and } \Delta f_{\text{max}} \text{ were fluorescence differences from samples in which R-C37 was fully bound and fully unbound, respectively, and } [C37]_0 \text{ and } [5H]_0 \text{ refer to the total (bound + unbound) concentrations of R-C37 and 5-Helix variant. The } K_D \text{ values obtained using this method were within 30% of the values determined using either a fluorescence resonance energy transfer-based competition assay for tight binding variants (} K_D < 200 \text{ pM} \text{) or a direct R-C37 fluorescence assay for weaker binding variants (data not shown).} \]

For association kinetics measurements, solutions of R-C37 (1 nM) and 5-Helix variant were mixed together in a Kinexa 3000 flow line for a short amount of time before passage through the flow cell. The degree of binding was varied by adjusting either the incubation time (2.9 to 7.2 s, depending on the flow rate) or 5-Helix variant concentration. To determine the value of \( k_{\text{off}} \), a full titration of 5-Helix variant concentration was performed (see Fig. 4, A and B). The fluorescence differences (\( \Delta f \)) from duplicate samples were fit to a general bimolecular kinetics model.

\[ H = \frac{[C37]_0 + [5H]_0 + K_D - P}{[C37]_0 + [5H]_0 + K_D + P} \]

The values \( P, \Delta f_{\text{min}}, \Delta f_{\text{max}}, [C37]_0, \) and \([5H]_0 \) have the same meaning as in Equation 1. Here, \( t \) is the incubation time, and \( K_D \) is the equilibrium dissociation constant determined previously. For wild type 5-Helix and the Q563D variant, the \( k_{\text{on}} \) values were confirmed using a fluorescence-based association assay (see supplemental Fig. S1). Moreover, the wild type 5-Helix/R-C37 dissociation rate constant (\( k_{\text{off}} \)) determined by fluorescence competition agrees (within 50%) with the product \( K_D k_{\text{on}} \) as expected for a biomolecular interaction (data not shown).

RESULTS

Inhibitory Activity of 5-Helix and Its Variants—5-Helix consists of three 40-residue N-peptides and two 38-residue C-peptides alternately connected (N-C-N-C-N) by five-amino acid Gly/Ser linkers (23). X-ray crystallographic studies confirm that the core of 5-Helix adopts the structure of the gp41 trimer-of-hairpins minus one HR2 helix (Fig. 2A).3 The hydrophobic groove created by this vacancy interacts strongly with C-peptides and enables 5-Helix to inhibit Env-mediated membrane fusion at low nanomolar concentrations (Fig. 1B). The reported IC50 values are independent of viral concentrations for infectivity studies or effector cell concentrations in cell-cell fusion assays (see supplemental Fig. S2). Thus, 5-Helix inhibitory potency reflects an intrinsic property of the Env interacting with target cells and is not limited by mass action due to the amount of Env on viral or cellular membranes.

Preincubating virus at high 5-Helix concentrations prior to diluting for infection does not enhance 5-Helix inhibitory activity (Fig. 1C). Thus, if 5-Helix interacts with Env prior to receptor engagement, there is sufficient time for the protein to re-equilibrate with its binding site during infection.

3 K. Champagne and M. J. Root, manuscript in preparation.
Kinetic Dependence to HIV-1 Entry Inhibition

FIGURE 2. Effect of C-peptide binding site mutations on the inhibitory activity of 5-Helix. A, lateral (top) and axial (bottom) views of a ribbon diagram derived from the crystal structure of 5-Helix.N3-D4.3 N40 segments (gray) are connected to C36 segments (blue) by five amino acid Gly/Ser linkers (red). In this 5-Helix variant, the third N40 segment has been modified by the substitution of Asp for Val549, Leu556, Glu563, and Val570 (indicated in magenta space-filling format). These four residues point into the C-peptide binding interface. B, titration of cell-cell fusion by 5-Helix and the indicated 5-Helix variants. Data points represent the mean ± S.E. of three to seven independent experiments and have been fit to Langmuir functions (solid lines) of the following form: \( f_{\text{synctia}} = \frac{1}{1 + ([5H]/K_{50})} \).

5-Helix can equilibrate with native Env, then inhibitory potency should correlate with intrinsic binding affinity. Conversely, if inhibition occurs through an intermediate state with a lifetime insufficient to reach equilibrium, then potency will be influenced by association kinetics.

To distinguish between these possibilities, we have constructed a series of 5-Helix variants with mutations in one or more of four residues that form predominantly hydrophobic contacts with the HR2 segment (Fig. 2A). When all four are mutated to Asp, the resulting protein, 5-Helix.N3-D4, displays no inhibitory activity despite being stably folded in the appropriate structure (Figs. 1B and 2A). Here, we have mutated the residues individually or in combination to Ala or Asp. The IC\(_{50}\) values of the 5-Helix variants range from 54 nM (0.85 x IC\(_{50}\), wild type) to 970 nM (150 x IC\(_{50}\), wild type) in a cell-based fusion assay (Fig. 2B, Table 1). In general, variants with Asp substitutions tend to have lower potencies than variants with only Ala substitutions, consistent with the hydrophobic nature of the interaction between 5-Helix and the HR2 segment. Variants with one Ala substitution (Single Ala) have IC\(_{50}\) values indistinguishable from that of wild type 5-Helix. Remarkably, variants with even three Ala substitutions (Triple Ala) are still effective inhibitors, with IC\(_{50}\) values within a factor of six of the wild type 5-Helix IC\(_{50}\).

Relationship of Binding Affinity to Inhibitory Potency—Solution-phase binding affinities were determined using a Kinexa 3000 instrument. Equilibrated mixtures of 5-Helix and a rhodamine-labeled C-peptide (R-C37) were passed through a fluorescence flow cell containing a small amount of 5-Helix-conjugated beads. A portion of the free (unbound) R-C37 was specifically retained in the bead pack, resulting in an increase in fluorescence signal directly proportional to the free concentration of R-C37 in solution. In the experiments depicted in Fig. 3A, a solution of 30 pm R-C37 generates a fluorescence signal that increases linearly with the amount of solution passed through the flow cell. Addition of 19 pm 5-Helix to this solution reduces the fluorescence signal by 60%, indicating that only 40% of R-C37 remains unbound in solution. From full titrations of 5-Helix at several R-C37 concentrations, we have determined the \( K_D \) to be 0.65 pm for the wild type protein (Fig. 3B).

The binding affinities of the mutant variants span more than 5 orders of magnitude. While weakly inhibiting variants tend to have relatively low binding affinities, the IC\(_{50}\) and \( K_D \) values show little correlation in general (Table 1). For instance, the \( K_D \) values for the four Single Ala variants range from 0.79 to 63 pm, but this 80-fold variance is not reflected in their IC\(_{50}\) values (Figs. 2B and 3C). Likewise, binding is disrupted more than 4 orders of magnitude in the Triple Ala variants, yet their IC\(_{50}\) values are less than 6-fold higher than the wild type IC\(_{50}\). However, an Ala-Asp variant (V549D/L556A) that binds with similar affinity as the Triple Ala variants inhibits with 150-fold lower potency than wild type 5-Helix. The lack of overall correlation between IC\(_{50}\) and \( K_D \) values is inconsistent with an equilibrium thermodynamic interpretation of inhibition (Fig. 3D).

Influence of Association Kinetics on 5-Helix Inhibition—Based on our results, we postulate that the inhibitory target of 5-Helix is exposed during a short-lived intermediate state. The simplest model that depicts this inhibition mechanism is summarized by Scheme 1. Here, N, I, and F symbolize the native, intermediate, and the fusogenic conformations of gp41, respectively. The rate constants \( k_{on} \) and \( k_{off} \) describe the kinetics of 5-Helix (5H) binding to I, where the ratio \( k_{off}/k_{on} = K_D \). The constants \( k_f \) and \( k_r \) refer respectively to the unidirectional rates out of I and out of I-5H (the inhibited intermediate state). The irreversible transition from I-5H to a hypothetical dead end state (D) is required to prevent the entire reaction from sinking into F, even in the presence of 5-Helix. The results of this study do not describe the nature of this dead end conformation.

Using this scheme, we can understand the 10\(^{4}\)-fold discrepancy between IC\(_{50}\) and \( K_D \) for wild type 5-Helix. Productive inhibition by 5-Helix requires that its binding rate (\( k_{on}[5H] \)) be on the order of or greater than \( k_r \). Although 5-Helix at low picomolar concentrations is capable of binding more than 50% of Env, the reaction takes several hours to reach equilibrium (even if association occurs at the diffusion limit). Since the intermediate state of gp41 exists for much shorter durations (17, 28), 5-Helix cannot significantly bind and fusion proceeds unabated. When the concentration is raised into the nanomolar range, 5-Helix binds Env with much faster kinetics, and inhibition is observed.

The kinetic restriction to inhibition predicts that more slowly associating 5-Helix variants will require higher concentrations in order to inhibit. Thus, we expect IC\(_{50}\) values to vary inversely with \( k_{on} \). To test this prediction, we have measured the solution-phase association of 5-Helix and R-C37 by contin-
uous flow method using the Kinexa 3000 (Fig. 4A). The association rate constant for wild type 5-Helix is 2.6 × 10^7 M^{-1} s^{-1}, very close to the diffusion-limited association rate constant for bimolecular interactions (Fig. 4B). The relatively potent variants that contain only Ala substitutions have association rate constants within a factor of five of the wild type value (Table 1, Fig. 4C). By contrast, the association rate constants of the least potent inhibitors (Ala-Asp and Double Asp variants) are up to 150-fold lower than the wild type value. In general, an inverse relationship between the IC_{50} and k_{on} extends for over 2 orders of magnitude (Fig. 4C), consistent with the predictions of the inhibition mechanism.

**Estimating the Lifetime of the 5-Helix-sensitive Intermediate State**—The exact expression for IC_{50} derived from the inhibition Scheme 1 is as follows (see supplemental material for derivation).

\[ \text{IC}_{50} = \frac{k_f}{k_{on}} \left( 1 + \frac{k_{off}}{k_f} \right) \]  

(Eq. 5)

For the vast majority of variants, the IC_{50} and k_{on}^{-1} are nearly

| Variant Class | Mutant | 3x or N3 | IC_{50} ± SEM (nM) | K_{D} ± SEM (nM) | k_{on} ± SEM (x 10^7 M^{-1} s^{-1}) | [θ]_{222} ± SEM (deg cm^2 dmol^{-1}) | T_{m} (°C) |
|---------------|--------|---------|-------------------|-----------------|-------------------------------|-------------------------------|----------|
| WT | WT | 3x | 6.3 ± 1.9 | 0.00065 ± 0.00002 | 2.6 ± 0.3 | -28500 ± 330 | >95 |
| Single Ala | V549A | 3x | 7.9 ± 0.04 | 0.024 ± 0.007 | 2.5 ± 0.4 | -29900 ± 640 | >95 |
| | L556A | 3x | 5.4 ± 0.33 | 0.063 ± 0.007 | 2.6 ± 0.3 | -28100 ± 440 | >95 |
| | Q563A | 3x | 5.5 ± 0.98 | 0.0008 ± 0.0002 | 2.5 ± 0.4 | -28400 ± 750 | >95 |
| | V570A | 3x | 9.0 ± 2.0 | 0.023 ± 0.008 | 1.4 ± 0.2 | -27000 ± 580 | >95 |
| Single Asp | V549D | 3x | 47 ± 9.8 | 27 ± 9.7 | 0.55 ± 0.1 | -25200 ± 800 | 80 |
| | L556D | 3x | 23 ± 2.4 | 9.5 ± 1.8 | 0.73 ± 0.3 | -26700 ± 1500 | 70 |
| | Q563D | 3x | 25 ± 5.3 | 0.047 ± 0.005 | 1.0 ± 0.1 | -26600 ± 510 | >95 |
| | V570D | 3x | 76 ± 15 | 2.3 ± 0.39 | 0.35 ± 0.07 | -25900 ± 430 | 91 |
| Double Ala | V549A/L556A | 3x | 17 ± 1.5 | 11 ± 0.47 | 1.3 ± 0.4 | -28500 ± 180 | 81 |
| | V549A/Q563A | 3x | 12 ± 0.81 | 0.094 ± 0.008 | 2.8 ± 0.7 | -28000 ± 510 | 93 |
| | V549A/V570A | 3x | 17 ± 0.66 | 3.9 ± 0.29 | 1.2 ± 0.2 | -29200 ± 1500 | 88 |
| | L556A/Q563A | 3x | 8.7 ± 1.1 | 0.17 ± 0.009 | 2.7 ± 0.8 | -26100 ± 20 | 93 |
| | L556A/V570A | 3x | 22 ± 2.1 | 8.3 ± 0.95 | 1.0 ± 0.2 | -28200 ± 670 | 89 |
| | Q563A/V570A | 3x | 10 ± 0.69 | 0.047 ± 0.003 | 2.3 ± 0.7 | -28300 ± 310 | >95 |
| Ala+Asp | V549D/L556A | N3 | 900 ± 370 | 40 ± 7.3 | 0.028 ± 0.005 | -27300 ± 180 | >95 |
| | *L556A/Q563A | N3 | 71 ± 23 | 14 ± 4.4 | 1.1 ± 0.2 | -27700 ± 1600 | >95 |
| | L556A/V570A | N3 | 800 ± 190 | 55 ± 6.3 | 0.033 ± 0.002 | -26900 ± 1100 | >95 |
| | V549A/L556D | N3 | 970 ± 180 | 79 ± 18 | 0.014 ± 0.002 | -27100 ± 700 | >95 |
| | *L556D/Q563A | N3 | 150 ± 80 | 23 ± 4.3 | 0.79 ± 0.05 | -27900 ± 1200 | >95 |
| | L556D/V570A | N3 | 530 ± 210 | 33 ± 17 | 0.029 ± 0.005 | -25500 ± 410 | >95 |
| Double Asp | V549D/Q563D | N3 | 170 ± 48 | 33 ± 8.2 | 0.21 ± 0.04 | -27500 ± 1700 | >95 |
| | L556D/Q563D | N3 | 450 ± 100 | 250 ± 59 | 0.043 ± 0.001 | -28000 ± 1600 | >95 |
| | Q563D/V570D | N3 | 260 ± 14 | 8.0 ± 1.7 | 0.13 ± 0.01 | -27500 ± 1700 | >95 |
| Triple Ala | V549A/L556A/Q563A | 3x | 36 ± 5.6 | 48 ± 13 | ND | -24800 ± 1000 | 80 |
| | L556A/Q563A/V570A | 3x | 28 ± 2.3 | 38 ± 9.2 | ND | -27800 ± 790 | 90 |
| | V549A/Q563A/V570A | 3x | 26 ± 3.5 | 13 ± 4.2 | 0.60 ± 0.09 | -27800 ± 660 | 87 |
| D4 | N3-D4 | N3 | >30,000 | >20,000 | ND | -28100 ± 1500 | >95 |

**TABLE 1**

Structural, inhibitory and binding properties of 5-Helix and its variants

Disruption of the C-peptide binding site on 5-Helix was achieved by incorporating the indicated substitutions into either all three N40 segments (3x) or only the third N40 segment (N3, see supplemental material for details). For each variant, the helical fold and stability was confirmed by measurements of molar ellipticities at 222 nm ([θ]_{222}) and T_{m} using circular dichroism spectroscopy. The reported inhibitory potency (IC_{50}) for each variant was determined in the cell-cell fusion assay employing HIV-1HXB2 Env effector cells and CD4- and CXCR4-expressing targeting cells. The solution-phase KD and k_{on} were obtained using the Kinexa 3000 instrument by measuring the unbound C-peptide concentration in mixtures of R-C37 (EnvHXB2-derived) and 5-Helix variant. For the two Ala+Asp variants indicated with an asterisk, the IC_{50} and k_{on} values deviate from the linear correlation shown in Fig. 4C. Their k_{off} values, calculated as k_{off}/K_{D}, are the largest among the entire set of 5-Helix variants and essentially determine the magnitude of k_{off} in Equation 5 of the text. NA and ND signify “not applicable” and “not determined,” respectively.

*SEPTEMBER 1, 2006 • VOLUME 281 • NUMBER 35 • JOURNAL OF BIOLOGICAL CHEMISTRY*
Kinetic Dependence to HIV-1 Entry Inhibition

**FIGURE 3.** Binding affinities of 5-Helix proteins and their relationship to inhibitory potencies. A, the fluorescence response as equilibrated solutions of 5-Helix and rhodamine-labeled C-peptide (R-C37) were passed through the Kinexa 3000 flow cell containing 5-Helix-conjugated beads. Each 5-ml sample contained 30 pm C-C37 and the indicated concentration of 5-Helix and was incubated for 48 h at 25 °C before analysis. Beads were washed in a Tris-buffered saline solution before the start (S arrow) and after the end (E arrow) of sample flow. The difference in average fluorescence signal during these wash periods was proportional to the amount of unbound R-C37 in solution. The dotted line labeled “Buf” represents the instrument response to a solution lacking R-C37. B, full 5-Helix titration of R-C37 at equilibrium. Solutions of serially diluted 5-Helix and either 30 pm (squares) or 3 pm (circles) C-C37 were analyzed in duplicate. The fraction of unbound C-C37 was determined by analyzing the fluorescence data relative to the signals from samples in which C-C37 is fully bound and fully unbound. The data from both titrations were globally fit to an equilibrium bimolecular binding model (Equation 1) to obtain a KD value for each concentration of 5-Helix and the single Ala variants. The data points represent the mean ± range of mean of duplicate measurements and have been fit to Equation 1. The gap between titrations and the differences in their slopes indicate that these five proteins have widely varying KD values even though they inhibit with nearly identical potencies (see Table 1). D, the relationship between IC50 and KD values for 5-Helix and the six classes of variants. The data and error bars (both x and y) represent the mean ± S.E. of at least three independent experiments for both inhibitory potency and binding affinity. The dotted line shows the expected correlation predicted by the intermediate state inhibition model (Scheme 1) if all variants shared the same koff values of 2.5 × 10^7 M^-1 s^-1. This line is a solution to Equation 5 with koff replaced by koff/K0, and the rate constants K0 and koff set to 0.2 and 0.1 s^-1, respectively. Note that the log10 axes are not on the same scale. WT, wild type.

A similar linear dependence between IC50 and koff^-1 is observed for 5-Helix inhibition of HIV-1 pseudotyped with either HXB2 or JRFL Env (Fig. 5). Variations in the slopes of these relationships reflect differences in koff values that partially depend on target cell properties. For HXB2 virus, a steeper relationship is found for target cells that express higher levels of coreceptor CXCR4, indicating that HXB2 Env transits more rapidly through the 5-Helix-sensitive intermediate state when fusing to these cells. When infectivities of JRFL and HXB2 virus are measured on the same cell, different koff values are observed, although the reason (e.g. strain specific sequence differences or variations in coreceptor density) cannot be ascertained in these experiments. Nevertheless, the lower 5-Helix sensitivity of JRFL virus is a consequence of increased kinetic restriction and not due to reduced binding affinity.

**DISCUSSION**

In this study, we demonstrate four essential features of 5-Helix inhibition of HIV-1 membrane fusion. First, the degree of inhibition depends on 5-Helix concentration present during membrane fusion and not on preincubation levels. Second, 5-Helix binds with subpicomolar affinity but inhibits with nanomolar potency, a 10,000-fold discrepancy. Third, there is poor correlation between inhibitory potency and interaction affinity for a series of 5-Helix variants with disrupted C-peptide binding. Fourth, the IC50 values for these variants are inversely proportional to their association rate constants. The relationships between inhibitory potency, equilibrium binding affinity, and association kinetics lead us to conclude that 5-Helix targets a short lived intermediate state of gp41 during the membrane fusion process.

How can our proposed inhibitory mechanism (i.e. the transient exposure of the 5-Helix inhibitory target) be reconciled with the reported receptor-independent interaction of 5-Helix with Env (26, 27)? Recent evidence suggests that Env may exist in several forms on the surface of viruses and cells, with only a fraction being competent for membrane fusion (31, 32). This conformational diversity has been proposed to account for the inability of certain nonneutralizing antibodies to block the antiviral activity of a neutralizing antibody, despite being able to compete with the latter for binding to HIV-1. The presence of “misfolded,” nonfusogenic forms of Env in viral and cellular membranes might serve as effective immune decoys to forestall development of a potent neutralizing humoral immune response (33). We speculate that binding to one or more of these nonfusogenic conformations accounts for the receptor-independent interaction of 5-Helix with Env.

In our model, the lifetime of the 5-Helix-sensitive intermediate state of gp41 is related to the slope of the linear dependence between IC50 and koff^-1. The strain-specific variability in slopes observed for JRFL and HXB2 Env may reflect, in part, differences in their primary structure that alter transition to or stability of the trimmer-of-hairpins conformation. However, factors on the target cell, such as coreceptor density, also exert an

---

*Due to the temperature difference between measurements of association rate constants (25 °C) and inhibitory potencies (37 °C), the slope of the IC50 versus koff^-1 relationship (Fig. 4C) actually underestimates the true value of koff. Fluorescence binding experiments indicate that koff values increase by a small factor (2.8 ± 0.1) as temperature is raised from 25 to 37 °C (see supplemental Fig. S1). The main effect of this temperature difference would be to increase (by this same factor) the slope of the IC50 versus koff^-1 relationship, implying a shorter average lifetime for the 5-Helix-sensitive intermediate state.*
Kinetic Dependence of Inhibitory Potency on Association Rate

FIGURE 4. The correlation of 5-Helix association kinetics with inhibitory potencies. A, fluorescence response to R-C37 (1 nM) and 5-Helix (0–100 nM) mixed for 2.9 s in the Kinexa 3000 flow line before passage over 5-Helix-coated beads. Beads were washed in a Tris-buffered saline solution before the start (E arrow) and after the end (E arrow) of sample flow. The fraction of R-C37 remaining unbound in solution is proportional to the increase in fluorescence signal recorded after the final wash. B, for the experiment in A, the fraction of unbound R-C37 is plotted against 5-Helix concentration. The data represent the mean ± S.E. of four independent measurements and have been fit to a general bimolecular kinetics model (Equation 3) yielding a \( k_{on} \) value of 2.2 \( \times 10^7 \text{ M}^{-1} \text{s}^{-1} \). C, the inverse dependence of inhibitory potency on association rate for 5-Helix and the six classes of variants. In this double-log graph, the mean IC\(_{50}\) values obtained in the cell-cell fusion assay are plotted against the reciprocals of the mean \( k_{on} \) values. The error bars (both x and y) represent the S.E. of at least three independent measurements of both values. The starred points indicate the two Ala\( ^{+}\)Asp variants that have the largest calculated \( k_{on} \) values (\( k_{on}/K_{C} > 0.16 \text{ s}^{-1} \)). With the exception of these two points, the data have been fit to a linear regression with a y intercept of zero and a slope of 0.21 s\(^{-1}\). The symbol definitions are the same as those in Fig. 3D.

FIGURE 5. Kinetic dependence to 5-Helix inhibition of viral infectivity. HIV-1 pseudotyped with JRFL (blue) or HXB2 (black) Env was used to infect HOS-CD4-CCR5 cells. The HXB2 virus (red) was also used to infect HOS-CD4-Fusin cells, which express CXCR4 to a greater extent (Ref. 41 and H. K. Steger and M. J. Root, unpublished results). IC\(_{50}\) values for 5-Helix (squares) and two single Asp variants, Q563D (circles) and V570D (triangles), are plotted against the reciprocal \( k_{on} \) values determined using R-C37 peptides of the appropriate HXB2 or JRFL Env sequence. The data points represent the mean ± S.E. of at least three independent experiments and are fit to linear regressions with y intercepts of zero. The slopes (\( k_{on} \) values) are 0.58 s\(^{-1}\) for JRFL on HOS-CD4-CCR5 cells, 0.13 s\(^{-1}\) for HXB2 on HOS-CD4-CCR5 cells, and 0.32 s\(^{-1}\) for HXB2 on HOS-CD4-Fusin (CXCR4) cells.

Effect on \( k_{on} \) values. Increased coreceptor levels have been correlated with enhanced speed of Env fusion and decreased potency of C-peptide inhibitors (14). A similar effect is reported here for inhibition by 5-Helix. We are currently exploring how coreceptor binding to gp120 is coupled to the exposure of the gp41 HR2 region.

In addition to \( k_{on} \), the dependence of IC\(_{50}\) on \( k_{on} \) provides information regarding the spatial exposure of the gp41 HR2 region. Steric factors that significantly restrict 5-Helix accessibility to the inhibitory target will necessarily impose a maximum possible association rate constant (\( k_{max} \)) for 5-Helix binding to gp41. The minimum IC\(_{50}\) in this situation will be equal to \( k_{on}/k_{max} \) and the y intercepts of the IC\(_{50}\) versus \( k_{on} \) plots will take on this value (see supplemental material for details). The actual y intercepts of these plots closely approach zero, signifying that \( k_{max} \) is large and implying that 5-Helix encounters little steric hindrance in binding gp41. Instead, the minimum IC\(_{50}\) value appears to be determined by the same factors that limit the unhindered association of 5-Helix and C37 (e.g. diffusion through bulk solution). Unobstructed access to the gp41 HR2 region is unexpected for intermediate states of fusion that, in addition to the remainder of gp41 and the two membranes, may include gp120, CD4, a chemokine receptor, and other surface proteins on the virus (or effector cell) and target cell (34). However, minimal steric hindrance for 5-Helix inhibition is consistent with the neutralization properties of two large monoclonal antibodies, 2F5 and 4E10, that bind epitopes at the C terminus of the gp41 HR2 region (35–37).

When analyzed using a simple model of intermediate state inhibition (Scheme 1 and Equation 5), our data suggest that the HR2 region is accessible to 5-Helix for only a few seconds during the fusion process. The accuracy of this lifetime estimate depends on how well the interaction between 5-Helix and C37 in solution recapitulates 5-Helix binding to gp41 in the viral
Kinetic Dependence to HIV-1 Entry Inhibition

membrane. For reasons elaborated above, steric hindrance is unlikely to play a major role in limiting the rate of 5-Helix association with gp41. Other factors that might influence the kinetic parameters of gp41 binding include: 1) effects of the phospholipid bilayer on the local concentration of 5-Helix and 2) chemical properties of the HR2 segment not found in the C37 peptide (e.g. glycosylation at Asn\(^637\)). These factors could either enhance or reduce the rate of 5-Helix association with gp41, leading, respectively, to an over- or underestimation of the lifetime of the 5-Helix-sensitive intermediate state. The qualitative agreement of the data with the proposed model, however, suggests that if these factors come into play, they influence all 5-Helix variants to the same extent.

The transition from an activated Env species to trimer-of-hairpins formation has been estimated to take as long as 10–20 min (17, 28). These estimates were obtained from cell-cell fusion experiments by following the time course of HR1 coiled coil exposure, C-peptide inhibitory sensitivity, and fusion pore formation. The much shorter lifetime estimates suggested by our data specifically reflect the average duration of the HR2-exposed intermediate state to which 5-Helix can bind. This 5-Helix-sensitive state may last for only a small fraction of the time between Env activation and trimer-of-hairpins formation. Although modeled as a concerted reaction in Fig. 1A, this transition appears to be a multistep process that can be arrested at different stages under conditions suboptimal for membrane merger (15, 16, 38). Recent evidence suggests that the HR1 and HR2 regions are not exposed symmetrically as these intermediate states progress toward trimer-of-hairpins formation (16). Similar asymmetry in HR1 and HR2 exposure has been reported for paramyxovirus membrane fusion (39). The binding affinity of 5-Helix and the kinetic restriction of its inhibitory mechanism imply that Env arrested transiently in a state that suitably exposes the HR2 region should be substantially more sensitive to this inhibitor. Efforts are under way to identify the appropriate conditions that produce such an arrested state. Furthermore, the analytical methods developed here are quite general and might be utilized to report on the temporal and spatial exposure of the gp41 HR1 segment during the C-peptide sensitive intermediate state.

Our results support the significance of association kinetics as an important parameter in the development of improved fusion inhibitors targeting transient gp41 states. Antiviral potency might be enhanced through efforts that increase local inhibitor concentration near the viral membrane, possibly by designing multivalent agents or cross-linking inhibitors with surface binding motifs. Indeed, increased local inhibitor concentration likely accounts for the picomolar antiviral activity of the chimera multivalent N-peptide inhibitor CCIZN17 (24). This covalently linked trimeric peptide has three binding sites that each interact with a small portion of the gp41 HR2 region (40). Using one or two of its three binding sites, this inhibitor might interact with the virion surface through misfolded Env glycoproteins or previously triggered gp41 molecules. The remaining binding site(s) on CCIZN17 would thus be prepositioned to interact with an HR2 region of an adjacent Env immediately following its activation by cellular receptors. In this manner, CCIZN17 might partially overcome the kinetic restriction faced by 5-Helix and inhibit with much higher potency.

Acknowledgments—We thank S. Paul, R. M. Jones, M. Crothamel, J. Busillo, and G. Colceriu for their assistance during the course of these experiments; M. Kay and R. Doms for helpful discussions; and J. Benovic, C. Scott, J. Williams, and members of the Root laboratory for helpful discussions and critical manuscript evaluations.

REFERENCES

1. Eckert, D. M., and Kim, P. S. (2001) Annu. Rev. Biochem. 70, 777–810
2. Pierson, T. C., and Doms, R. W. (2003) Curr. Top. Microbiol. Immunol. 281, 1–27
3. Root, M. J., and Steger, H. K. (2004) Curr. Pharm. Des. 10, 1805–1825
4. Lu, M., Blacklow, S. C., and Kim, P. S. (1995) Nat. Struct. Biol. 2, 1075–1082
5. Chan, D. C., Fass, D., Berger, J. M., and Kim, P. S. (1997) Cell 89, 263–273
6. Weissenhorn, W., Dessen, A., Harrison, S. C., Skehel, J. J., and Wiley, D. C. (1997) Nature 387, 426–430
7. Tan, K., Liu, J., Wang, J., Shen, S., and Lu, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12303–12308
8. Wild, C. T., Shugars, D. C., Greenwell, T. K., McDanai, C. B., and Matthews, T. J. (2004) Proc. Natl. Acad. Sci. U. S. A. 91, 9770–9774
9. Jiang, S., Lin, K., Strick, N., and Neurath, A. R. (1993) Nature 365, 113
10. Chan, D. C., Chukotkin, C. T., and Kim, P. S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15613–15617
11. Rimsky, L. T., Shugars, D. C., and Matthews, T. J. (1998) J. Virol. 72, 986–993
12. Furuta, R. A., Wild, C. T., Weng, Y., and Weiss, C. D. (1998) Nat. Struct. Biol. 5, 276–279
13. Kilgore, N. R., Salzwedel, K., Reddick, M., Allaway, G. P., and Wild, C. T. (2003) J. Virol. 77, 7669–7672
14. Reeves, J. D., Gallo, S. A., Ahmad, N., Miamidian, J. L., Harvey, P. E., Sharron, M., Pohlmann, S., Sfakianos, J. N., Derdeyn, C. A., Blumenthal, R., Hunter, E., and Doms, R. W. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16249–16254
15. Melikyan, G. B., Markosyan, R. M., Hemmati, H., Delmedico, M. K., Lambert, D. M., and Cohen, F. S. (2000) J. Cell Biol. 151, 413–423
16. Abrahamyan, L. G., Mkrtchyan, S. R., Binley, J., Lu, M., Melikyan, G. B., and Cohen, S. F. (2005) J. Virol. 79, 106–115
17. Munoz-Barroso, I., Durell, S., Sakaguchi, K., Appella, E., and Blumenthal, R. (1998) J. Cell Biol. 140, 315–323
18. Lalezari, J. P., Henry, K., O’Hearn, M., Montaner, J. S., Pilleri, P. J., Trotter, B., Walsley, S., Cohen, C., Kuritzkes, D. R., Eron, J. J., Jr., Chung, J., DeMasi, R., Donatucci, L., Drobnis, C., DeLehantry, J., and Salgo, M. (2003) N. Engl. J. Med. 348, 2175–2185
19. Lazzarin, A., Cloet, B., Cooper, D., Rein, J., Arasteh, K., Nelson, M., Katlama, C., Stellbrink, H. J., Delfraissy, J. F., Lange, J., Huson, L., DeMasi, R., Wat, C., DeLehantry, J., Drobnis, C., and Salgo, M. (2003) N. Engl. J. Med. 348, 2186–2195
20. Wild, C., Oas, T., McDanai, C., Bolognesi, D., and Matthews, T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10537–10541
21. Eckert, D. M., and Kim, P. S. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 11187–11192
22. Louis, J. M., and Kim, P. S. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 29485–29494
23. Root, M. J., Kay, M. S., and Kim, P. S. (2001) Science 291, 884–888
24. Bianchi, E., Finotto, M., Ingallinella, P., Hrin, R., Carella, A. V., Hou, X. S., Schleif, W. A., Miller, M. D., Geleziunas, R., and Pessi, A. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 12993–12998
25. Louis, J. M., Neszfehvat, I., Chang, L., Clore, G. M., and Bewley, C. A. (2003) J. Biol. Chem. 278, 20278–20285
26. Root, M. J., and Hamer, D. H. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 5016–5021
27. Koshiba, T., and Chan, D. C. (2003) J. Biol. Chem. 278, 7573–7579
28. Dimitrov, A. S., Louis, J. M., Bewley, C. A., Clore, G. M., and Blumenthal, R. (2005) Biochemistry 44, 12471–12479
29. Follis, K. E., Larson, S. J., Lu, M., and Nunberg, J. H. (2002) J. Virol. 76, 7356–7362
30. Edelhoch, H. (1967) Biochemistry 6, 1948–1954
31. Herrera, C., Spenlehauer, C., Fung, M. S., Burton, D. R., Beddows, S., and Moore, J. P. (2003) J. Virol. 77, 1084–1091
32. Poignard, P., Mouland, M., Golez, E., Vivona, V., Franti, M., Venturini, S., Wang, M., Parren, P. W., and Burton, D. R. (2003) J. Virol. 77, 353–365
33. Moore, P. L., Crooks, E. T., Porter, L., Zhu, P., Cayanan, C. S., Grise, H., Corcoran, P., Zwick, M. B., Franti, M., Morris, L., Roux, K. H., Burton, D. R., and Binley, J. M. (2006) J. Virol. 80, 2515–2528
34. Hamburger, A. E., Kim, S., Welch, B. D., and Kay, M. S. (2005) J. Biol. Chem. 280, 12567–12572
35. Muster, T., Steindl, F., Purschke, M., Trkola, A., Klima, A., Himmler, G., Ruker, F., and Katinger, H. (1993) J. Virol. 67, 6642–6647
36. Zwick, M. B., Labrijn, A. F., Wang, M., Spenlehauer, C., Saphire, E. O., Binley, J. M., Moore, J. P., Stiegler, G., Katinger, H., Burton, D. R., and Parren, P. W. (2001) J. Virol. 75, 10892–10905
37. Ofek, G., Tang, M., Sambor, A., Katinger, H., Mascola, J. R., Wyatt, R., and Kwong, P. D. (2004) J. Virol. 78, 10724–10737
38. Markosyan, R. M., Cohen, F. S., and Melikyan, G. B. (2003) Mol. Biol. Cell 14, 926–938
39. Russell, C. J., Jardetzky, T. S., and Lamb, R. A. (2001) EMBO J. 20, 4024–4034
40. Sia, S. K., Carr, P. A., Cochran, A. G., Malashkevich, V. N., and Kim, P. S. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 14664–14669
41. Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhard, M., Di Marzio, P., Marmon, S., Sutton, R. E., Hill, C. M., Davis, C. B., Peiper, S. C., Schall, T. J., Littman, D. R., and Landau, N. R. (1996) Nature 381, 661–666