Effects of single α -to- β residue replacements on recognition of an extended segment in a viral fusion protein

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ABSTRACT: Partial replacement of α -amino acid residues with β -amino acid residues has been established as a strategy for preserving target-engagement by helix-forming polypeptides while suppressing susceptibility to proteolysis. The impact of β -residue incorporation within polypeptides that adopt less regular conformations, however, has received less attention. The HRC domains of fusion glycoproteins from pathogenic paramyxoviruses contain a segment that must adopt an extended conformation in order to engage the HRN domain in the post-fusion state and drive merger of the viral envelope with a target cell membrane. Here we examine the impact of single α -to- β substitutions within this extended N-terminal segment of the engineered HRC peptide VIQKI. Stabilities of helix-bundles formed with a native viral HRN have been evaluated, the structures of five helix-bundles have been determined, and antiviral efficacies have been measured. Many sites within the extended segment show functional tolerance of α -to- β substitution. These results offer a basis for future development of protease-resistant inhibitors of paramyxovirus infection.

The disruption of interactions within or between proteins is an attractive basis for drug design. This strategy requires molecules that can engage large surfaces on target proteins, a goal that is generally difficult to achieve with small molecules (MW<1000).^{1–3} Clinically useful inhibitors of pathogenic protein-protein interactions are typically engineered proteins or large peptides. Antibodies and other large proteins are expensive to produce.⁴ Smaller polypeptides offer the advantage of chemical synthesis but suffer from rapid degradation by endogenous proteases.^{1–3} One strategy for retaining the benefits of medium-sized polypeptides while minimizing proteolytic susceptibility is to incorporate non-natural subunits into the peptidic backbone.^{5–12} The challenge inherent in this approach is to maintain target protein engagement despite backbone alteration. We have shown that the recognition properties of an α -helix can be retained after periodic replacement of α -amino acid residues with β residues.^{13–22} The resulting “ α/β -peptides” adopt an α -helix-like conformation but resist enzymatic degradation. However, there is no general strategy for implementing α -to- β modifications in the context of other polypeptide conformations.

Here we explore α -to- β backbone modification in the context of an extended polypeptide segment that is crucial in the paramyxovirus infection cycle. We focus on an eight-residue portion of the fusion (F) glycoprotein of human parainfluenza virus 3 (HPIV3). The F protein orchestrates fusion of the HPIV3 envelope and the outer membrane of a target cell. This process involves a complex set of structural transitions within an F trimer that ultimately leads to the formation of a six-helix bundle assembly between N-

terminal heptad repeat (HRN) and C-terminal heptad repeat (HRC) domains of F.^{23–25} In the post-fusion state, the N-terminal portion of the HRC domain (residues 449–456) adopts an extended conformation, while the majority of the HRC domain residues (457–484) form an α -helix (**Figure 1A**).²⁶

Figure 1. (A) HPIV3 F ectodomain in postfusion conformation (PDB 1ZTM), (B) VIQKI co-assembled with HPIV3-HRN (PDB 6NRO), (C) sequence of VIQKI, (D) structure of a β^3 -residue.

We have evaluated the structural and functional consequences of α -to- β replacement throughout the extended segment in the context of an engineered peptide that inhibits HPIV3 infection. The outcome of this fundamental study provides a foundation for future efforts to develop antiviral α/β -peptides that contain β

residues along their entire length. Agents of this type could be valuable not only for paramyxoviruses but also for coronaviruses, which have long extended segments in the HRC domains of their fusion proteins.^{27–29}

This study began with a previously reported peptide derived from the HRC domain of HPIV3, designated VIQKI (**Figure 1C**), which blocks infection by both HPIV3 and respiratory syncytial virus (RSV).^{30–32} These two viruses are leading causes of fatal lower respiratory infections in young children.^{33–35} A crystal structure of VIQKI co-assembled with HPIV3-HRN revealed a six-helix bundle assembly similar to the HRC/HRN assembly in the post-fusion conformation of the HPIV3 F protein (**Figure 1B**).³⁰ As expected, two distinct conformations were observed within the VIQKI peptide, an α -helix spanning residues corresponding to 457–484 of the F protein, and an extended conformation for residues corresponding to 449–456. Our studies focused on backbone modifications in the extended portion of VIQKI (**Figure 1**, dashed boxes).

A set of VIQKI analogues was synthesized in which each of the eight N-terminal residues was replaced individually with the homologous β^3 -amino acid residue (**Figure 1D**). Thus, each replacement preserved the side chain found in VIQKI but introduced an extra CH_2 unit into the backbone. The effect of each single α -to- β^3 substitution on assembly stability with HPIV3-HRN was examined using variable-temperature circular dichroism (VT-CD) spectroscopy. When combined with a 51-residue peptide corresponding to the HPIV3-HRN domain, each α / β -VIQKI variant formed a helical assembly, as detected by CD minima at 208 and 222 nm. Each assembly exhibited a cooperative thermal transition at 222 nm, which allowed us to determine apparent thermal denaturation ($T_{m,\text{app}}$) values, based on the mid-point of each transition (Table 1). For each α / β -VIQKI variant, a $\Delta T_{m,\text{app}}$ value was calculated based on comparison with $T_{m,\text{app}}$ for VIQKI itself [$\Delta T_{m,\text{app}} = T_{m,\text{app}}(\text{HPIV3-HRN+VIQKI } \beta^3\text{-variant}) - T_{m,\text{app}}(\text{HPIV3-HRN+VIQKI})$].

Table 1. Apparent thermal denaturation temperatures ($T_{m,\text{app}}$) of co-assemblies formed between HPIV3-HRN+VIQKI β^3 -variant pairs

Peptide	$T_{m,\text{app}}$ (°C) ^a	$\Delta T_{m,\text{app}}$ ^b
VIQKI	88.6	
VIQKI-V449(β^3 V)	89.0	+0.4
VIQKI-A450(β^3 A)	89.2	+0.6
VIQKI-L451(β^3 L)	88.7	+0.1
VIQKI-D452(β^3 D)	89.1	+0.5
VIQKI-P453(β^3 P)	86.1	-2.5
VIQKI-I454(β^3 I)	86.7	-1.9
VIQKI-D455(β^3 D)	81.5	-7.1
VIQKI-I456(β^3 I)	86.2	-2.4

^a 1:1 mixture of Inhibitor (50 μM) and HRN (50 μM) peptides.

^b $\Delta T_{m,\text{app}} = T_{m,\text{app}}(\text{HPIV3-HRN+VIQKI } \beta^3\text{-variant}) - T_{m,\text{app}}(\text{HPIV3-HRN+VIQKI})$

The VT-CD analysis revealed that several α / β -VIQKI variants, with substitutions at V449, A450, L451 or D452, formed helical assemblies with stabilities comparable to that of the prototype α -peptide VIQKI. Moderate decreases in assembly stability were

observed for α -to- β modification at P453, I454 or I456. The variant with substitution at D455 was highly destabilized.

Our previous crystal structure of VIQKI co-assembled with HPIV3-HRN revealed multiple inter- and intramolecular interactions among the first nine residues of VIQKI that may be important for inhibitor binding and antiviral efficacy.³⁰ The aliphatic side chains of L451, I454, and I456 of VIQKI make hydrophobic contacts with the HPIV3-HRN core trimer. The backbone carbonyl of D452 forms a hydrogen bond with the side chain of Y178 within HPIV3-HRN. The carboxylate side chain of D455 within VIQKI forms a hydrogen bond with the backbone amide N-H of S457. This $i, i+2$ interaction may serve as an N-terminal helix cap to initiate α -helical secondary structure at S457.

To gain insight into the structural impact of the α -to- β substitutions, we co-crystallized five VIQKI variants with the HPIV3-HRN peptide, including D452(β^3 D), P453(β^3 P), I454(β^3 I), D455(β^3 D), and I456(β^3 I). Each structure was solved by molecular replacement using a search model based on the HPIV3-HRN+VIQKI structure (PDB 6NRO).³⁰ Chains A and B from 6NRO were truncated to include residues 153–173 (chain A) and 460–480 (chain B). All hydrogen atoms and side chains were removed prior to molecular replacement. Crystallographic models were refined to 1.70 Å [HPIV3-HRN+VIQKI-D452(β^3 D), (PDB 6PZ6)]; 1.87 Å [HPIV3-HRN+VIQKI-P453(β^3 P), (PDB 6PRL)]; 1.49 Å [HPIV3-HRN+VIQKI-I454(β^3 I), (PDB 6VAS)]; 1.79 Å [HPIV3-HRN+VIQKI-D455(β^3 D), (PDB 6PYQ)]; and 2.17 Å [HPIV3-HRN+VIQKI-I456(β^3 I), (PDB 6V3V)].

Figure 2. X-ray crystal structures of VIQKI and β^3 -variants co-assembled with HPIV3-HRN. (A) HPIV3-HRN+VIQKI, PDB:6NRO; (B) HPIV3-HRN+VIQKI-D452(β^3 D), (PDB:6PZ6); (C) HPIV3-HRN+VIQKI-P453(β^3 P), (PDB:6PRL); (D) HPIV3-HRN+VIQKI-I454(β^3 I), (PDB:6VAS); (E) HPIV3-HRN+VIQKI-D455(β^3 D), (PDB:6PYQ); (F) HPIV3-HRN+VIQKI-I456(β^3 I), (PDB:6V3V). HPIV3-HRN (orange), VIQKI and β^3 -variants (green) with β^3 -residues highlighted in violet.

In each of the five new structures, the single β^3 residue substitution within the extended N-terminal segment did not affect the α -helical conformation of residues 457–484. However, the impact of substitution on the extended conformation differed among the five

variants (**Figure 2**). In three of the five co-crystal structures, those containing P453(β^3 P), I454(β^3 I), or D455(β^3 D) substitutions, the backbone modification resulted in a significant remodeling of the extended conformation in the N-terminal region relative to VIQKI.

For VIQKI-P453(β^3 P), contacts involving the I454 and I456 side chains and hydrophobic residues from the HPIV3-HRN trimer were retained, as was the helix capping interaction of D455. However, this substitution nucleated a hairpin turn that reoriented residues 449–452, with loss of contacts observed between these residues in VIQKI and HPIV3-HRN (**Figures 2C, 3B**). For VIQKI-I454(β^3 I), only residues 455–484 could be resolved. No significant electron density was observed for the β^3 I residue or residues 449–453.

In the co-assembly of VIQKI-D455(β^3 D) and HPIV3-HRN, residues 452–484 of the VIQKI variant were resolved. However, the transition from extended to α -helical conformation, which occurred at S457 in the HPIV3-HRN+VIQKI structure, was not observed for the D455(β^3 D) variant. Instead, VIQKI-D455(β^3 D) adopted a helical conformation from residue 453 to 484 (**Figures 2E, 3C**). We hypothesize that the additional backbone carbon atom between the carboxylate side chain of β^3 D455 and the backbone amide N-H of S457 in this variant, relative to VIQKI itself, interferes with the helix-capping interaction involving the side chain of D455 in VIQKI.

Figure 3. 2mFo-DFc weighted electron density maps for β^3 -residues in (A) HPIV3-HRN+VIQKI-D452(β^3 D), (B) HPIV3-HRN+VIQKI-P453(β^3 P), (C) HPIV3-HRN+VIQKI-D455(β^3 D), (D) HPIV3-HRN+VIQKI-I456(β^3 I). β^3 -residues are shown in violet.

In the crystal structures of VIQKI-D452(β^3 D) and VIQKI-I456(β^3 I) co-assembled with HPIV3-HRN, the extended conformation observed for VIQKI itself was retained, although in both variants the two terminal residues, V449 and A450, could not be resolved. In both new structures, key interactions between the inhibitor and the HPIV3-HRN core were preserved. These interactions include contacts of side chains from L451, I454, and I456 (or β^3 I456) with hydrophobic residues in the HRN trimer, as well as hydrogen bonds between the backbone carbonyl of D452 with the

side chain hydroxyl of HRN residue Y178. Although the helix capping interaction was present in both of these VIQKI variants, the distance between the D455 carboxylate and S457 backbone N was longer in the I456(β^3 I) variant relative to the D452(β^3 D) variant. This increased length may reflect the additional backbone carbon between the hydrogen bond donor and acceptor in the I456(β^3 I) variant.

The impact of each single α -to- β substitution on inhibitory activity toward HPIV3 infection was evaluated using plaque reduction assays. Hep2 cell monolayers were infected with engineered recombinant HPIV3 that expresses green fluorescent protein (GFP) in the presence of various concentrations of each VIQKI variant, and the extent of infection was determined by quantification of fluorescence. The results are shown in two formats. **Figure 4A** shows extent of inhibition of infection as a function of VIQKI variant concentration. **Figure 4C** shows the ratio of extent of inhibition for each variant relative to VIQKI itself at the experimental concentration closest to the IC_{50} of VIQKI (16 nM). The results for D455(β^3 D) show that a single α -to- β substitution in this 36-mer can cause a significant decrease in inhibitory activity. However, most of the α -to- β substitutions seem to be well tolerated.

Although VIQKI was derived from the HRC domain of HPIV3 F, this engineered peptide also inhibits RSV infection.³⁰ **Figures 4B,D** show that the effect of α -to- β substitutions on efficacy against RSV was much more pronounced than the effect on efficacy against HPIV3. Most VIQKI variants were significantly less effective relative to VIQKI itself. However, the variants containing L451(β^3 L) or D452(β^3 D) substitutions seemed to match VIQKI in efficacy against RSV infection. Differences between the effects of specific α -to- β replacements on HPIV3 inhibition vs. RSV inhibition presumably arise from the observation that the N-terminal segment of VIQKI adopts different conformations when co-assembled with HPIV3-HRN vs. RSV-HRN.³⁰ It is noteworthy that two backbone modifications, L451(β^3 L) and D452(β^3 D), are tolerated in both systems.

Figure 4. Anti-viral efficacy of VIQKI β^3 -variants against HPIV3 or RSV. Peptide activity against HPIV3 (left) or RSV (right) were determined by plaque reduction assay in infected Hep-2 cell monolayers. Data are shown as (A–B) percent inhibition of infection as a function of inhibitor concentration and (C–D) a ratio of the percent inhibition of each β^3 variant with the percent inhibition of VIQKI. Data are expressed as mean $\pm$ standard deviation ($n=3$ separate experiments).

Systematic α -to- β replacement has been explored for several peptide antigens that adopt extended conformations when bound

to an MHC.³⁶ The outcome of substitutions has varied by position within each antigen. A systematic α -to- β series was recently reported for a 10-residue phosphopeptide that binds in an extended conformation to 14-3-3 proteins.³⁷ β^3 substitution near either end caused modest declines in affinity (~ 10 -fold), but more severe losses in affinity were observed for substitutions closer to the center of the peptide. Based on these varied precedents, it was not obvious that α -to- β substitutions would be structurally and functionally tolerated within the extended portion of an HPIV3 inhibitor.

We have used a dual-inhibitory antiviral peptide with a well-characterized mode of target engagement to explore the effects of replacing α -amino acid residues by homologous β^3 residues within a segment that is extended in the bioactive conformation. This modification preserves the side chain but alters the backbone via introduction of a CH_2 unit. It could not have been predicted that some sites in the prototype inhibitor, VIQKI, would tolerate α -to- β substitution while retaining the ability to inhibit infection by two distinct viruses, which require distinct conformations of the segment containing the backbone modification. This work offers a step toward development of protease-resistant dual HPIV3/RSV inhibitors that might address life-threatening childhood infections for which there are no current drugs. In addition, given the presence of long extended segments in the post-fusion HRC/HRN assemblies formed by coronavirus fusion proteins^{27–29}, these results encourage application of the backbone modification strategy to inhibitor development directed toward these pathogens.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Additional figures, synthetic procedures, and characterization data for all synthesized peptides, as well as protocols for plaque reduction and thermal denaturation assays (PDF)

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