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The HIV-1 p66 homodimeric RT exhibits different conformations in the binding-competent and -incompetent NNRTI site

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Abstract

Non-nucleoside inhibitors of HIV-1 reverse transcriptase (RT), NNRTIs, which bind to the p66/p51 heterodimeric RT, also interact with the p66/p66 homodimer, whose structure is unknown. ^{19}F NMR of a single 4-trifluoromethylphenylalanine (tfmF) residue, incorporated into the NNRTI binding pocket of the p66/p66 homodimer at position 181, was used to investigate NNRTI binding. In the NNRTI-bound homodimer complex, two different ^{19}F signals are observed, with the resonance frequencies matching those of the NNRTI-bound p66/p51 heterodimer spectra, in which the individual p66- or p51-subunits were labeled with tfmF at positions 181. These data suggest that the NNRTI-bound p66/p66 homodimer conformation, particularly around residue 181, is very similar to that in the p66/p51 heterodimer, explaining why NNRTI binding to p66/p66 enhances dimer formation.

Keywords

HIV-1 reverse transcriptase; NNRTI; NMR; fluorine; single-site labeling

Introduction

HIV-1 reverse transcriptase (RT) is an essential enzyme in the HIV-1 lifecycle and has been a major drug target in the treatment of HIV-1 infection. RT is initially generated from the Gag-Pol polyprotein as a 66 kDa polypeptide by HIV-1 protease^{1–4}, which subsequently cleaves the C-terminal RNase H (RNH) domain from p66 to form the mature p66/p51 heterodimeric RT enzyme (Fig. 1A)^{5–10}. At present, two classes of RT inhibitors are in clinical use, nucleoside RT inhibitors (NRTIs), which bind to the enzyme's catalytic site and non-NRTIs (NNRTIs), which bind to an allosteric site. For both classes of inhibitors, structural studies have revealed that they only interact with the p66 subunit^{11–17}. In the crystal structure of apoRT, the NNRTI binding site on p66 is adjacent to β -strands $\beta 4$, $\beta 7$ and $\beta 8$, with Tyr residues Y181 and Y183 pointing approximately in the same direction and

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Supplementary information

Fig. S1, 1D ^{19}F NMR spectra of p66_{tfmF}/p51, p66/p51_{tfmF}, p66_{tfmF}, and p51_{tfmF} in the absence and presence of EFV; Fig. S2, 1D ^{19}F NMR spectra of (A) p66_{tfmF} and (B) p51_{tfmF} in the absence and presence of EFV; Table S1, ^{19}F Resonance frequencies, linewidths, and intensity for p66_{tfmF}.

Y188 in another (Fig. 1B) ¹⁸. In contrast, in the non-NNRTI-binding “inactive” p51 site, all three Tyr residue side chains point in the direction of Y183 (Fig. 1B) ¹⁸. Intriguingly, this “inactive” constellation is very similar to the conformation adopted by these three residues in the structure of the p66 subunit of the RT heterodimer complexed with the NNRTI Nevirapine (NVP) (Fig. 1C) ¹⁸ and, possibly, NNRTIs in general.

Although NNRTIs have been used as antiviral agents for more than 20 years, their molecular mechanism of action is complex and not completely understood. For some NNRTIs, such as Efavirenz (EFV), viral maturation is affected ^{19–21} and RT dimer formation is enhanced ^{22, 23}. Detailed biophysical studies using wild-type RT and dimer-dissociation mutants revealed that EFV interacts with a 1:1 stoichiometry with all possible dimers, p66/p66, p66/p51, and p51/p51, and promotes dimerization, although it also weakly binds to p66 and p51 monomers ^{24, 25}. Since p66/p66 and p51/p51 are homodimers, the question arises why only one NNRTI, but not two NNRTI molecules, bind to these dimers, implying that the two potential binding sites in the homodimers are not equivalent. The EFV binding site in monomeric p66 or p51 has been proposed to be identical to the NNRTI-binding site in the p66 subunit of wild-type RT ²⁵. Given that the p66/p66 homodimer is an intermediate RT species in viral maturation and NNRTIs may target this species, information about the interaction between NNRTIs and p66/p66 is vital to help understand this important step in the viral life cycle. In particular, structural data are necessary to explain the available biochemical and biological data.

Here, we examined binding of NNRTIs, i.e., EFV, NVP, and Rilpivirine (RPV), to the p66/p66 dimer and other RT dimers. To this end, we used p66 and p51 polypeptides, which contained 4-trifluoromethylphenylalanine (tfmF) incorporated at position 181, and recorded ¹⁹F solution NMR spectra. The trifluoromethyl group has particular advantages for use in ¹⁹F NMR. It possesses high sensitivity, compared to singly fluorinated amino acids such as F-tyrosines, given that three fluorine atoms are present. In addition, CF₃- groups, like a methyl groups, exhibit narrow lines because of their fast three-site jumps. This makes CF₃ and CH₃ ideally suited probes for large proteins ^{26–29}. We previously used the tfmF group and ¹⁹F solution NMR to investigate NNRTI binding to the p66/p51 RT heterodimer and showed that the highly plastic NNRTI binding site on p66 rigidifies upon drug binding and in drug-resistance RT mutants ³⁰. For the current study we prepared different dimers, containing fluorine labels on both chains or on only one of the two chains (p66_{tfmF}/p66_{tfmF}); p66/p51_{tfmF}; p66_{tfmF}/p51). ¹⁹F spectra of the p66/p66 homodimer revealed that the tfmF residue at position 181 senses two distinct chemical environments. Upon NNRTI binding, two different resonances are observed, similar in frequencies to the resonances in the equivalent RT/NNRTI complexes, suggesting that the local structural and electronic environment of the active and inactive NNRTI binding sites are already available in the p66/p66 homodimeric RT precursor. Overall, our data clearly identifies the interaction between NNRTIs and p66/p66, and demonstrates that only one site on the homodimer is competent to bind the inhibitors.

Materials and Methods

Preparation of tfmF-labeled p51, p66, and p66/p51

Specific introduction of trifluoromethyl-phenylalanine (tfmF) at position 181 in RT proteins were performed as previously described^{30, 31}. In brief, we used codon-optimized p66 and p51 constructs, containing C38V and C280S mutations, bearing an amber codon instead of the Y181 codon, and Strep- and hexa-histidine tags at N- and C-termini, respectively. The tfmF labeled proteins were produced in *E. coli* BL21 ai cells (Invitrogen), co-transformed with p51 or p66 constructs and the pDule2 RS vector, encoding the orthogonal amber tRNA/tRNA synthetase pair³². Two different RT polypeptides, harboring the tfmF amino acid at position 181 were prepared: p66_{tfmF} and p51_{tfmF}. Non-fluorinated protein was produced in *E. coli* BL21 (DE3) gold cells (Agilent Technologies, Santa Clara, CA). For the preparation of fluorinated/non-fluorinated heterodimer samples, equivalent amounts of cell pellets, containing the expressed p66 and p51 proteins, were mixed, lysed using a microfluidizer, and purified as described previously³⁰. As a final purification step, gel-filtration on a HiLoad 26/60 Superdex 200 column (GE Healthcare) was employed for all proteins, and the purified proteins were stored in 50% v/v glycerol at -80°C until further use.

Inhibitors

Efavirenz (EFV), and Nevirapine (NVP) were kindly provided by Dr. Sluis-Cremer. Rilpivirine (RPV) was purchased from Selleckchem (Houston, TX). All NNRTIs were stored in DMSO at concentrations of 10 mM.

NMR experiments

Protein samples were buffer exchanged into 25 mM sodium phosphate, 100 mM NaCl, 10% v/v D₂O, pH 6.8 in an Amicon Ultra concentrator (EMD Millipore, Billerica, MA) to a final volume of 350 µL. All ¹⁹F NMR spectra were recorded at 564.65 MHz on a 600 MHz Bruker AVANCE spectrometer, equipped with a CP TXO F/C-H-D triple-resonance z-axis gradient cryoprobe (Bruker Biospin, Billerica, MA). All data were processed with Topspin 3.1 (Bruker) and analyzed with MestReNova (Escondido, CA) as described previously³⁰. ¹⁹F 1D NMR spectra of p66_{tfmF} were recorded with and without NVP, EFV, and RPV at a protein concentration of 30 µM and 1:0, 1:0.5, 1:1 and 1:4 p66_{tfmF}:NNRTI ratios. ¹⁹F 1D NMR spectra of p66/p51_{tfmF} were recorded in the absence and presence of EFV at 1:1 molar ratio. For each spectrum ~16,000 scans were recorded and averaged.

Titration data analysis

For the RPV and NVP additions, the decrease in intensity of the inhibitor-free protein signals and the increase in the inhibitor-bound protein signals were used to determine the NNRTI dissociation constants, K_D , using Matlab software (Mathworks). Relative intensities, normalized with respect to the inhibitor-free signals, were plotted. For the EFV titration, relative intensity changes were compared to those calculated based on literature data, in which both monomer, [M], and dimer, [D], interactions with EFV, [I], were taken as: $K_{D1} = [D][I]/[DI] = 0.25 \mu\text{M}$; $K_{D2} = [MI][M]/[DI] = 0.4 \mu\text{M}$; $K_{D3} [M][I]/[MI] = 2.5 \mu\text{M}$; $K_{D4} = [M]^2/[D] = 4.2 \mu\text{M}$ ²⁴.

Results

Inhibitor-free spectra of apo p66_{tfmF}/p66_{tfmF}, p66_{tfmF}/p51, and p66/p51_{tfmF}

The p66 protein exists in a concentration-dependent monomer/dimer equilibrium in solution, with a reported dimerization constant of $\sim 4 \mu\text{M}$ ²³. We ascertained by multi-angle light scattering that p66/p51 RT (>90%) and the p66 ($\sim 80\%$) proteins are predominantly dimeric. The 1D ¹⁹F NMR spectra of the three different proteins, p66_{tfmF}/p66_{tfmF}, p66/p51_{tfmF} and p66_{tfmF}/p51 exhibit several resonances. The spectrum of p66_{tfmF}/p66_{tfmF} contains two resonances at -60.9 ppm and -61.9 ppm, with the lower field resonance possessing slightly larger intensity (Fig. 2, Table S1). Comparison of the spectrum from the homodimeric protein with that of heterodimeric RT, containing the ¹⁹F label on the p51 (p66/p51_{tfmF}) chain, reveals that the lowfield resonance in p66_{tfmF}/p66_{tfmF} appears identical in chemical shift to the resonance associated with the inactive NNRTI binding site on the p51 subunit in p66/p51, suggesting a similar structural and electronic environment. The high-field signal is much sharper than was previously observed for the tfmF amino acid located in the equivalent position in the active NNRTI binding site on p66 in heterodimeric p66_{tfmF}/p51. The latter is extremely broadened due to dynamic conformational heterogeneity³⁰.

NNRTI binding to p66_{tfmF}/p66_{tfmF}

Titration of p66_{tfmF}/p66_{tfmF} with increasing amounts of NVP, EFV, and RPV, monitored by ¹⁹F NMR, are displayed in Fig. 3. New resonances are observed at -59.7 , -60.4 , and -60.7 ppm, respectively (Fig. 3A, 3B and 3C, dotted lines), and the intensity of one of the apo-form resonances, at -61.9 ppm, decreases. For EFV binding, a second new resonance emerges at -61.7 ppm for the highest EFV concentrations (Fig. 3B). This signal may arise from EFV-bound monomeric p66_{tfmF} (see below in “*Modeling NNRTI binding to p66_{tfmF}/p66_{tfmF}*”).

To elucidate the origin of the new ¹⁹F resonances of p66_{tfmF}/p66_{tfmF} that appear in the presence of each of the three NNRTIs, the p66_{tfmF}/p66_{tfmF} spectra were compared with those of mature p66_{tfmF}/p51 RT with the equivalent NNRTI and with apo p66/p51_{tfmF}. Single resonances are observed in the ¹⁹F spectra of NNRTI-bound p66_{tfmF}/p51, whose frequencies differ for the three NNRTI-bound forms³⁰. The resonance frequencies of the new signals in the NNRTI-bound p66_{tfmF}/p66_{tfmF} spectra (Fig. 4) are essentially identical to those of the p66_{tfmF}/p51 RT spectra. In contrast, the other resonances in the spectra of p66_{tfmF}/p66_{tfmF} in the presence of NNRTI, which did not change in position upon NNRTI binding, are located at positions similar to the resonances of the p66/p51_{tfmF} protein.

A comparison of the p66/p51_{tfmF} spectra in the absence and presence of EFV (Fig. S1) revealed no differences for the free and bound resonances, demonstrating that the inhibitor interaction with the p66 subunit does not affect the tfmF residue at position 181 in the p51 subunit, which is $\sim 50 \text{ \AA}$ away from the NNRTI binding site. The spectra of p66_{tfmF}/p66_{tfmF} in the presence of NNRTIs comprise a superposition of two spectra, those of p66_{tfmF}/p51 and p66/p51_{tfmF} (Fig. S1 and 4). Taken together, for all three inhibitors, based on the comparison of the spectra of p66_{tfmF}/p66_{tfmF} in the presence of NVP, EFV, and RPV with those of p66_{tfmF}/p51 and p66/p51_{tfmF} bound to the same inhibitors, the bound resonances at

–59.7, –60.4, and –60.7 ppm are at the same positions as those of NNRTI-bound p66_{tfmF}/p51 at –59.7, –60.3, and –60.7 ppm (Fig. 4, black spectra). The “unchanged” resonances in the bound state line up with the equivalent inactive NNRTI binding site resonances of p66/p51_{tfmF} NNRTI complexes at –61.0 ppm (Fig. 4, cyan spectra).

Modeling NNRTI binding to p66_{tfmF}/p66_{tfmF}

Having identified the origin of the various resonances in the NNRTI-bound and free homodimeric p66_{tfmF}/p66_{tfmF} precursor allowed us to analyze the binding isotherms for stoichiometric 1:1 binding of NVP (Fig. 5A) or RPV (Fig. 5B) to p66/p66, based on the observed intensity changes that are depicted in Fig. 3A and Fig. 3B, respectively. For NVP binding, the decrease in signal intensity of the upfield free resonance (–61.9 ppm) is concomitant with an increase in the intensity of the downfield NVP-bound resonance (–59.7 ppm) (Fig. 5A). A similar observation holds for RPV binding, although the shift occurs at lower NNRTI/protein ratios, consistent with the higher affinity of RPV to RT compared to NVP^{33,34} (Fig. 5B). Assuming that dimeric p66_{tfmF}/p66_{tfmF} is in equilibrium between NNRTI-free and bound forms, with two resonances essentially reporting on the binding, NVP and RPV dissociation constants, K_D , were estimated to be $32. \pm 4.4$ and 0.86 ± 0.064 μ M, respectively. Thus, RPV interacts with p66_{tfmF}/p66_{tfmF} 40-fold tighter than NVP, essentially recapitulating the affinity difference that was previously observed for the interaction with the mature heterodimeric p66/p51 RT, despite binding to a less tight homodimer ($K_D \sim 4$ μ M), compared to the p66/p51 heterodimer^{23,35,36}. This implies that NNRTI-binding to the p66/p66 homodimeric precursor and mature p66/p51 is essentially identical, only involving the binding-competent site on p66 in both proteins.

In contrast to the NMR spectra of p66_{tfmF}/p66_{tfmF} with NVP and RPV, in the presence of 8-fold molar excess of EFV, an additional signal is observed at –61.7 ppm (Fig. 4B; red asterisk). This signal increases with higher EFV concentrations, while a decrease of the low field EFV-bound p66_{tfmF}/p66_{tfmF} signal (–60.4 ppm) is observed (Fig. 3B). An equivalent signal is also observed in the p51_{tfmF} titration with EFV (Fig. S2). It is more intense for p51_{tfmF}, compared to p66_{tfmF} (Fig. 3), at identical EFV concentrations. Since the p51/p51 dimer dissociation constant is known to be much higher ($K_D > 200$ μ M) than that of the p66/p66 homodimer^{23,35,36}, this additional signal has to be associated with EFV-bound monomeric p66_{tfmF}. Based on this interpretation, we generated theoretical binding isotherms based on data from the literature²⁴. Gratifyingly, the associated intensities for the different species are qualitatively consistent with our data (Fig. 5C). In particular, the build-up curve of the EFV-bound p66_{tfmF} monomeric species agrees with the experimentally observed intensity change of the –61.7 ppm resonance, supporting the above interpretation.

Discussion

NNRTI interactions with the p66 homodimer are known to affect viral maturation^{19,21}, although knowledge about the details of these interactions is lacking. In order to fill this gap, we generated singly ¹⁹F labeled p66/p66 at position 181, which is located in the NNRTI binding site. We observed two ¹⁹F resonances for the homodimer, possibly akin to the conformational heterogeneity observed in the crystal structure of apo-RT (Fig. 1B), which

contains an active binding site - the site on the p66 subunit in mature RT - and an “inactive” site - the site on the p51 subunit in mature RT -, with residue 181 clearly experiencing two distinct conformations in solution. Since the NNRTI binding pocket encompassing residue 181 in p66/p51 is highly plastic^{30, 37–40}, it is not surprising that structural and dynamic heterogeneity is also present in the p66/p66 homodimer.

Biochemical and biophysical studies have shown that NNRTIs are chemical enhancers of p66 dimerization^{22, 23}, and tighter dimerization of the p66/p51 heterodimer compared to the p66/p66 homodimer is consistently described in the literature^{23, 35, 36}. Upon interaction with NNRTIs, the ¹⁹F resonances of the tfmF side chains change and report on the different environments around these sites. Most interestingly, the resonance frequencies in NNRTI-bound p66/p66 become very similar to those in the NNRTI-bound heterodimeric p66/p51 (Fig. 3), suggesting that the NNRTI binding-competent site in the p66/p66 homodimer, when interacting with the inhibitor, exhibits a similar conformation as is seen in the p66 subunit of mature p66/p51 RT (Fig. 1C). The conformational change observed in the NNRTI binding site may be associated with a change in the dimer interface towards what is observed in p66/p51 RT, possibly explaining the enhancement of dimer formation by NNRTIs. Gratifyingly, the EFV titration data are consistent with the thermodynamic linkage model of EFV binding and subunit dimerization proposed by Braz et al.²⁴. These structural changes are possibly also associated with the increase in dimer affinities and conformational changes at the dimer interface, mediated by the NNRTI interaction.

In summary, our data provide new insights into NNRTI interactions with the p66/p66 RT precursor. Our data show that 1) two conformational states exist in the p66 protein in solution as evidenced by distinct resonances of the ¹⁹F probe at position 181; 2) Only one of the available sites in the p66/p66 homodimer around position 181 is competent for NNRTI binding; 3) NNRTIs modulate the structural environment in the NNRTI active binding site in one of the two subunits of p66/p66, similar to the active binding site in the p66 subunit of mature p66/p51 RT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

tfmF	4-trifluoromethyl phenylalanine
NNRTIs	non-nucleoside reverse transcriptase inhibitors
RT	HIV-1 reverse transcriptase
NVP	Nevirapine

EFV	Efavirenz
RPV	Rilpivirine

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Fig. 1.

(A) Schematic diagram of the domain organization of HIV-1 p66/p66 and p66/p51 RT. The locations of the catalytic Asp residues, D110, D185, and D186 are indicated by green bars and the location of Y181 in the allosteric binding site is shown by a red bar. (B) and (C) Backbone representation of apo-RT (PDB: 1DLO) and NVP-bound RT (PDB: 3HVT), indicating the active (p66) and inactive (p51) NNRTI-binding sites. Expansions of the backbone regions around β -strands β_4 , β_7 and β_8 in the p66 (blue) and p51 (yellow) chains of apo-RT and p66 (green) of NVP-bound RT are shown at the bottom. The side chains of Y181, Y183 and Y188 are depicted in grey ball-and-stick representation and the molecular volume occupied by the inhibitor in (C) is shown in red.

Fig. 2. 564.65 MHz 1D ^{19}F NMR spectra of $\text{p66}_{\text{tfmF}}/\text{p66}_{\text{tfmF}}$, $\text{p66}/\text{p51}_{\text{tfmF}}$, and $\text{p66}_{\text{tfmF}}/\text{p51}$ labeled with 4-tfmF at position 181.

Fig. 3. 564.65 MHz 1D ^{19}F NMR spectra of p66_{tfmF}/p66_{tfmF} in the absence (black traces) or presence of NVP (A, pink), EFV (B, green), and RPV (C, orange). The ^{19}F spectra in the presence of the NNRTI were recorded at [p66/p66]:[NNRTI] ratios of 1:1, 1:2, and 1:8 and are colored from light to dark for increasing NNRTI concentrations. NNRTI-bound resonances of p66/p66 are labeled in the spectra.

Fig. 4. Comparison of the 564.65 MHz ^{19}F spectra of p66_{t_{fm}F}/p66_{t_{fm}F} in presence of 1:8 equivalent of NVP (A, pink), 1:2 equivalent of EFV (B, green), and 1:2 equivalent of RPV (C, orange), compared with spectra of NNRTI-bound p66_{t_{fm}F}/p51 (black) and apo p66/p51_{t_{fm}F} (cyan). In (B) an additional resonance, most likely arising from EFV-bound monomer, is labeled by the asterisk. Resonances are labeled as in Fig. 3.

Fig. 5.

(A) NVP, (B) RPV, and (C) EFV titration data analysis. In (A) and (B), relative intensities (normalized with respect to the spectrum at 1:8 NNRTI:p66_{tfmF}/p66_{tfmF} ratio) of the apo protein signal at -61.9 ppm are represented by triangles and those of the NNRTI-bound signals at -59.7 and -60.7 ppm by circles. In (A) and (B), the fits of both free and bound intensities for K_D estimations are shown by the lines. In (C), resonance intensities for increasing amounts of EFV were compared to theoretical curves derived from a model in the literature that takes into account both EFV-monomer and EFV-dimer binding²⁴. The intensity of the resonance at -60.4 ppm, reporting on the binding-competent subunit of p66/p66, is represented by the black circles; the intensity of the “middle resonance” at -61 ppm, arising from apo-p66, apo-p66/p66, and the binding-incompetent subunit of EFV-bound p66/p66, is represented by the green triangles; and the intensity of the resonance at -61.7, associated with the EFV-bound p66 monomer, is represented by the red circle.