Conformational Landscape of non-B Variants of HIV-1 Protease: A pulsed EPR Study

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Abstract

HIV infection is a global health epidemic with current FDA-approved HIV-1PRotease inhibitors (PIs) designed against subtype B protease, yet they are used in HIV treatment world-wide regardless of patient HIV classification. In this study, double electron-electron resonance (DEER) electron paramagnetic resonance (EPR) spectroscopy was utilized to gain insights in how natural polymorphisms in several African and Brazilian protease (PR) variants affect the conformational landscape both in the absence and presence of inhibitors. Findings show that Subtypes F and H HIV-1PR adopt a primarily closed conformation in the unbound state with two secondary mutations, D60E and I62V, postulated to be responsible for the increased probability for closed conformation. In contrast, subtype D, CRF_AG, and CRF_BF HIV-1PR adopt a primarily semi-open conformation, as observed for PI-naïve-subtype B when unbound by substrate or inhibitor. The impact that inhibitor binding has on shifting the conformational land scape of these variants is also characterized, where analysis provides classification of inhibitor induced shifts away from the semi-open state into weak, moderate and strong effects. The findings are compared to those for prior studies of inhibitor induced conformational shifts in PI-naïve Subtype B, C and CRF_AE.

Keywords: HIV-1PRotease, non-B subtypes, natural polymorphisms, DEER, flap distance, conformational landscape

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) protease (PR) is an essential enzyme in the life cycle of HIV virus yielding mature and infectious viruses by cleaving the viral *gag* and *gag-pol* polyproteins.[1,2] Due to this important role, HIV-1PR is a drug target in HAART therapy,[3]

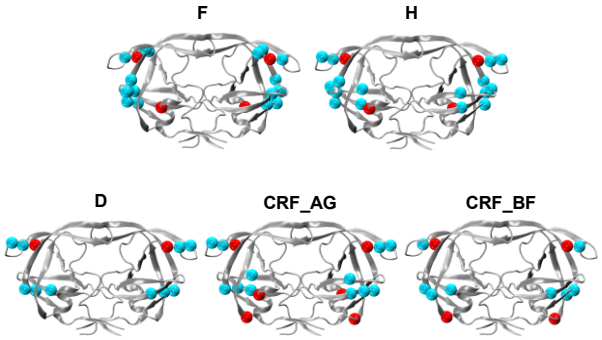
which is utilized to combat the global epidemic. During function, the extended glycine rich β-hairpin regions called the flaps allow access to the active site with the dimeric cavity containing the catalytic aspartic residues located at the bottom of the active site pocket.[4,5]

HIV-1 is categorized into 9 different subtypes or clades namely A, B, C, D, F, G, H, J, and K.[6–8] Combination of genetic mosaics of different subtypes are called circulating recombinant forms (CRFs) and unique recombinant forms (URFs). While accounting for only 12% of global infections, subtype B is dominant in advanced countries and receives the most attention in research and drug design,[9] with current FDA-approved PIs designed against subtype B. In contrast, subtype C is dominant in sub-Saharan Africa accounting for roughly 50% of global infections. Sub-Saharan Africa also has the most diversity of different HIV-1 subtypes and CRFs.[10] Natural polymorphisms (NPs) are mutations between subtypes due to genetic drifts. Certain drug-pressured mutations observed in subtype B are present in other subtypes or CRFs as NPs.[11] Thus, it is possible that treatment of non-B HIV-1 using current PIs encounters higher barrier and drug resistance in non-B variants can be acquired more rapidly.[12,13] In fact, studies have shown subtype C HIV-1PR has different conformational landscape compared to subtype B, with subtype C exhibiting an unusually high probability for the wide-open conformation.[14] Additionally compared to subtype B, the Ki values for current PIs to subtype C are 2-4 fold higher.[15] Some structural and kinetic studies have also been performed for subtype F and CRF_AE, whereas other Central African variants have received less attention.[16–19]

Site-directed spin labeling (SDSL) double electron-electron resonance (DEER), a pulsed electron paramagnetic resonance (EPR) spectroscopy technique, can be used to characterize a distance

distribution profile between two unpaired electrons, such as nitroxide spin-labels, commonly in proteins.[20,21] DEER has been utilized to monitor HIV-1PR flap distance distribution profiles, hence the conformational landscape,[22] where the flaps are defined to adopt four conformations with the following distances between spin-pairs: curled/tucked (24-30 Å), closed (~33 Å), semi-open (~36 Å), and wide-open (40-45 Å).[14,23–27] Here, DEER spectroscopy was utilized to characterize the conformational landscape defined by flap distance profiles; thus providing insights into how NPs affect the conformational landscape of the following non-B variants: F, H, D, CRF_AG, and CRF_BF found in Brazil and Central Africa. Figure 1 shows ribbon diagrams of the HIV-1PR dimeric structure, along with the amino acid sequences of constructs studied.

A



B

Residue Number	10	20	30	40	50
B	PQITLWKRPL	VTIKIGGQLK	EALLNTGADD	TVIEEMSLPG	RWKPKMIGGI
F	....	....V.....	....	....DIA...	....
H	....	..V..E...R	....	.....IN...	K.........
D	....	..V.....	....	.....IN...	K.........
CRF_AG	....	..VR.....I	....	.....IN...	K.........
CRF_BF	....	..V.....	....	.....IN...	....
Residue Number	**60**	**70**	**80**	**90**	
B	GGFICVRQYD	QIIIEIAGHK	AIGTVLVGPT	PVNIIGRNLL	TQIGATLNF
F	......K..E	NVSL......	....	........M.	....
H	.........E	.VA.....	....	........I.	....
D	....	..PL.....	....	....	....
CRF_AG	....	..L.....K.	....	........M.	....
CRF_BF	....	..P.....R.	.T........	....	....

Figure 1. (A) Ribbon diagrams of non-B variants with spheres showing the location of NPs relative to PI-naïve subtype B (cyan) with those corresponding to subtype B TPV-resistant mutations shown in red (spheres/text). (B) Sequences of HIV-1PR constructs.

2. Methods

2.1. Cloning

Escherichia coli codon-optimized genes of HIV-1PR subtypes, whose amino acid sequences are given in Figure 1B, were purchased from ATUM (Neward, CA), which were then cloned into pET-23a plasmid between NdeI and BamHI restriction sites. Constructs contain two stabilizing mutations (Q7K and L33I) to prevent autolysis of the protease,[28] as well as the D25N mutation

to inactivate the enzyme. The third stabilizing mutation, L63I, usually included in other works from our laboratory, is not included in this work for it is an NP in some constructs. All constructs contain K55C mutation for site-specific spin labeling for DEER experiments, with C67A and C95A mutations for eliminating native cysteines.[29,30]

2.2. Expression, Protein Purification and Spin Labeling.

Protein expression, purification, and spin labeling (SL) with (1-oxyl-2,2,5,5-tetramethyl-d3-pyrrolidine-3-methyl)methanethiosulfonate (MTSL) (Santa Cruz Biotechnology, Santa Cruz, CA) were carried out using protocols previously described, with the following modification.[22–27] The pH of the inclusion body resuspension buffer was adjusted according to the isoelectric point (pI) of each construct to be one pH unit below the protein pI. Calculated pI values for subtypes F, H, D, and CRF_AG, CRF_BF sequences are 9.33, 9.02, 9.33, 9.39, and 9.66, respectively (pH of resuspension buffers were 8.33, 8.02, 8.33, 8.39, and 8.66 accordingly). Excess free spin label was removed and protein was buffer exchanged to 2 mM NaOAc, pH 5.0 using HiPrep 26/10 desalting column (GE Healthcare). Sequence and spin-labeling were confirmed via electrospray ionization time-of-flight mass spectrometry (Table SI-1)

2.3. DEER experiments and Data Analysis

HIV-1PR samples were prepared similar to earlier reports with SL-protein prepared in 20 mM d_3-NaOAc in 30% v/v deuterated glycerol D_2O, pH 5.0 at 80-150 μM protein (dimer) concentration.[14,23–27] Inhibitors, including substrate mimic CaP2, were added to reach 4 molar excess and allowed to equilibrate for at least an hour prior to measurements. Protease inhibitors were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. CaP2 is a nonhydrolyzable substrate mimic with sequence H-Arg-Val-Leu-r-Phe-Glu-Ala-Nle/NH2 (r = reduced). Samples were transferred to a 4 mm quartz

EPR tube (Norell, Marion, NC), flash-frozen in liquid nitrogen, and then immediately inserted into the ER 4118X-MD-5 dielectric ring resonator within the ELEXSYS E580 EPR spectrometer (Bruker). DEER experiments were carried out at 65 K using a four-pulse DEER sequence.[20,21] DEER modulation curves were background-corrected and converted to distance distribution profiles via Tikhonov regularization using DeerAnalysis,[31] with population analysis performed via DEERconstruct,[32] a program developed in our laboratory. Sample data analysis provided in Supporting Information, Figures SI-1 and SI-2.

3. **Results and Discussion**

3.1. Brazilian/African NPs shift the unbound conformational ensemble to more closed and curled states.

The HIV-1PR sequences investigated here represent a set of diverse and sparse sequences from Brazil and Central African nations that were taken from the AIDS database.[33] Results reveal these accumulated NPs alter the conformational landscape to favor increases in the fractional occupancy of closed and curled conformations with decreases in the semi-open and wide-open states relative to PI-naïve Subtype B, even though kinetic investigations reveal K_m and k_{cat} values within 2-fold of WT values (Table SI-2).[11,18,25] Figure 2A plots the DEER distance profiles for all five HIV-1PR constructs in the absence of PIs. Figure 2B plots the relative percentage of the four conformations determined from deconstruction analysis of the DEER distance profiles (Figures SI1-2,Tables SI-3-SI-7) with Figure 2C showing the relative change of these populations compared to PI-naïve Subtype B. For all five constructs, a significant population with average distance below 30 Å associated with the curled/tucked conformation is observed, and the fractional occupancy of this state is 10-20% compared to PI-

naïve subtype B of ~ 0%.[23] We previously demonstrated that an increase in the relative occupancy of a curled/tucked conformation with average distances below 30 Å can arise from drug-pressure selected mutations[34] and select NPs.[24,25] The non-B variants in this study contain different NPs, but their conformational landscapes all show statistically significant increases in curled/tucked conformation population; thus, indicating that PI resistance may arise faster and in different patterns than in subtype B when patients receive inhibitor therapy.

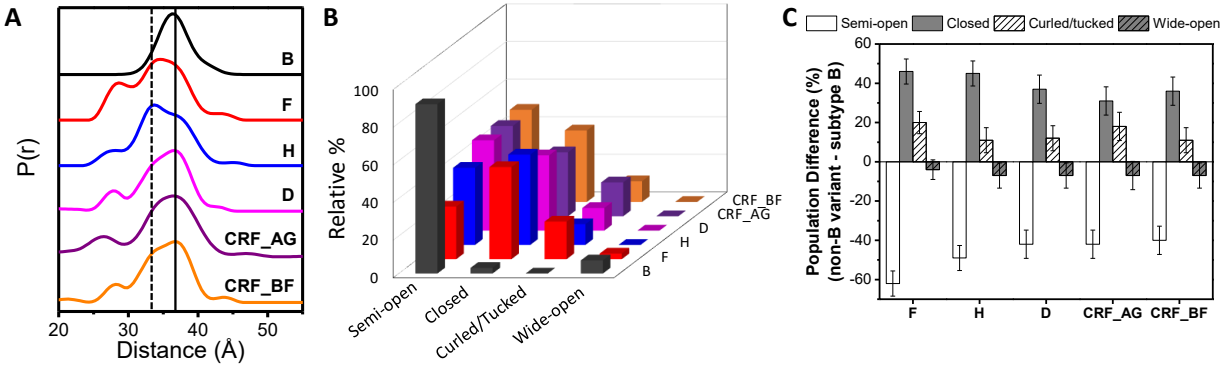


Figure 2. (A) Stack plot of DEER distance probability profiles in unbound form for each variant of HIV-1PR vertically offset for clarity. Lines placed at 33 Å and 36 Å represent distances for closed (dashed) and semi-open (solid) conformations; respectively. (B) Relative percentages of populations determined from DEER distance profile analysis. Error is ± 3-7% dependent upon data collection signal-to-noise ratio and peak suppressions as shown in Supporting information and described in detail elsewhere.[22,32,34] (C) Population difference for each conformation for non-B variants relative to PI-naïve subtype B.[23]

In the unbound state, subtype B predominantly adopts the semi-open conformation.[23] Similarly, subtype D, CRF_AG, and CRF_BF predominantly adopt semi-open conformation even though the probability for this state is decreased from 86% in PI-naïve subtype B to approximately only 50% in these constructs (Tables SI-3-SI-7 and Figure 2B). On the other hand, subtypes F and H, predominantly adopt the closed conformation (49% and 47% fractional occupancy; respectively) in the unbound state. But, in fact, compared to PI-naïve subtype B, which has only 3% fractional occupancy of the closed conformation, all constructs studied here

have higher percentages of this state (~> 30%) being sampled in the absence of inhibitor or substrate. The differences in conformational landscape of non-B variants in unbound form results from the combination of the NPs as well as the type of NPs each of these variants contains. We hypothesize that NPs such as D60E and I62V may contribute to shifting the conformational landscape due to altered salt-bridge and van der Waals contacts; respectively.[16,35]

3.2. PI binding DEER distance profiles for Brazilian and Central African HIV-1PR.

Current FDA-approved PIs compete with substrate for direct binding into the active site of HIV-1PR in the closed conformation.[36–40] We previously showed that the propensity of the ensemble to shift towards a closed conformation reflects tighter inhibitor binding even with the removal of the active site aspartic acids by inclusion of the D25N active site mutation.[24,26,34,41] Figure 3 plots DEER distance profiles obtained for each subtype with inhibitors. As done for other HIV-1PR constructs, such as PI-naïve subtypes B and C and CRF_AE, the data are grouped into those that minimally shift the conformational ensemble from the unbound state (left side) and those that have a marked perturbation on the conformation ensemble, resulting in a predominantly closed conformation (right side). For all constructs, IDV, NFV, and ATV have minimal to no impact on the conformational landscape. In all except in Subtype D, RTV also had a minimal shift on the conformational sampling of HIV-1PR. Note, RTV was previously found to have a strong impact on shifting the conformational ensemble of HIV-1PR PI-naïve Subtypes B and C.

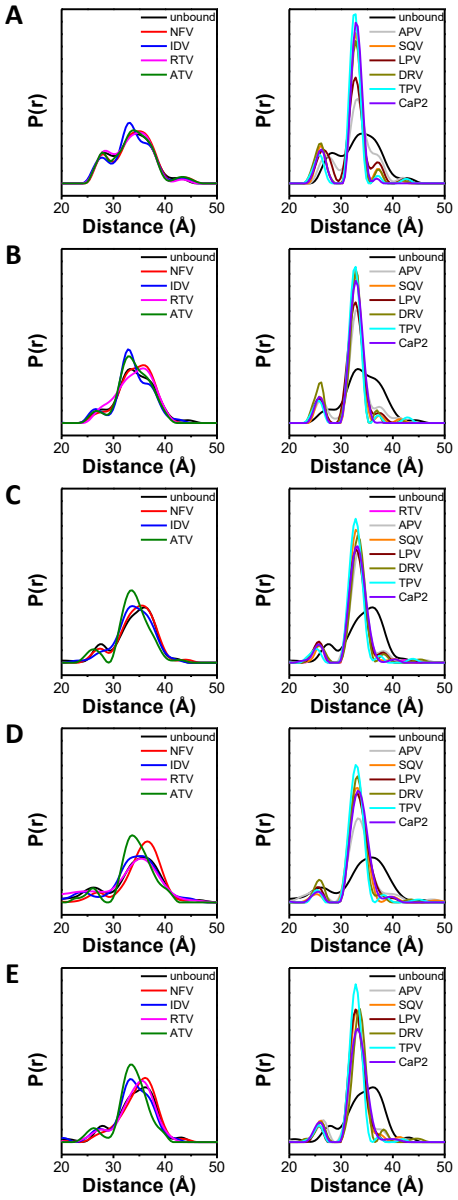


Figure 3. DEER distance probability profiles for HIV-1PR in the unbound state and in the presence of PIs that (left) have little or no significant change to the distance profile compared to (right) those that have marked change to a closed conformation similar to substrate mimic CaP2: (A) subtype F, (B) subtype H, (C) subtype D, (D) CRF_AG, (E) CRF_BF.

3.3. Characterizing Weak, Moderate and Strong conformational shifts for inhibitors.

In our earlier publications, we defined a parameter, Δc, that related the relative change in the closed conformation population of the PI-bound to unbound state to assess how well a PI

binds and closes the flaps, thus leading to inhibition.[24,26,41] However, because the conformational ensembles for the unbound non-B variants studied here show a significantly non-zero closed conformation percentage, with some constructs displaying a predominantly closed conformation (subtypes F and H), an alternative parameter was utilized to characterize how effective an inhibitor is at shifting the conformational ensemble. Given the semi-open conformation is posited to be the catalytically active conformation of HIV-1PR,[34] and that crystal structures of drug-resistant constructs can bind inhibitor in non-canonical closed form orientations (twisted/curled/asymmetric);[42] here, we are define the loss of semi-open (s.o.) population to be a better indicator of how well an inhibitor is binding and altering the conformational landscape.

The results of the deconstruction into the four populations for all DEER distance profiles for inhibitor HIV-PR variants are given in Figure SI-3. Figure 4 plots the relative percentage change of the semi-open conformation of the various HIV-1PR DEER distance profile conformational landscapes as a function of each inhibitor. DEER distance population analysis results for each construct with all inhibitors are given and tabulated in Supporting Information. Based upon prior work with PI-naïve subtype B investigations that combined inhibitor induced shifts to the DEER distance profiles with NMR HSQC investigations showing the time scale of interactions of inhibitors with HIV-1PR, we have defined three degrees of inhibitor interaction with HIV-1PR categorized as weak, moderate or strong.[41] Here these categories map well with changes in the DEER profiles as seen in Figure 3 and more quantitatively characterized in Figure 4A where a weak inhibitor is defined as one that reduces the relative percentage of the semi-open population from 0-30% it original value, moderate from 30-75% its original value, and strong those that reduce the semi-open population > 75% its original value. Figure 4B graphically

depicts these designations for the constructs investigated here as well as in our prior works. [23][43] Note, all of our DEER investigations have the D25 active site substituted with D25N, which has been shown to lower binding affinities by 100-1000 folds.[44] The significance of the DEER conformational landscape shifts with D25N lies in that the data reflect how the inhibitor is interacting with other regions of the binding pocket displaying subtle differences and indication of how/where mutations may more easily limit the effectiveness of inhibitors when drug resistance emerges.

For all constructs, NFV- and IDV-bound states show a minimal to undistinguishable change in the overall distance profile, and hence population of the semi-open conformation of compared to their respective unbound states. This result was also observed for DEER characterizations of PI-binding to PI-naïve inactive (D25N) subtype B[23] as well as PI-naïve subtype C and PI-naïve CRF_AE),[41] NMR spectroscopy showed that inhibitors classified as "weak" are in a fast-exchange regime when bound in the HIV-1PR binding pocket with the D25N mutation, likely meaning that during the freezing process of our samples in liquid nitrogen (required for DEER data collection), inhibitors do not bind and lock in the closed conformation due to the fast μS time scale observed with NMR.[43]

In contrast, for all constructs, inhibitors SQV, DRV, TPV and CaP2 all had very strong shifts to the conformational landscapes, nearly depleting the semi-open population in many cases. Prior NMR investigations showed that binding of "strong" inhibitors to HIV-1PR induced splitting in the HSQC spectra, indicating slow exchange on the NMR timescale ~mS exchange, where the splitting originates from asymmetry induced in the dimer NMR resonances because the inhibitors are not symmetric. LPV is characterized as a strong inhibitor in all constructs

except for Subtype F. It is noteworthy that the overall inhibitor profile for Subtype D is similar to subtype B.

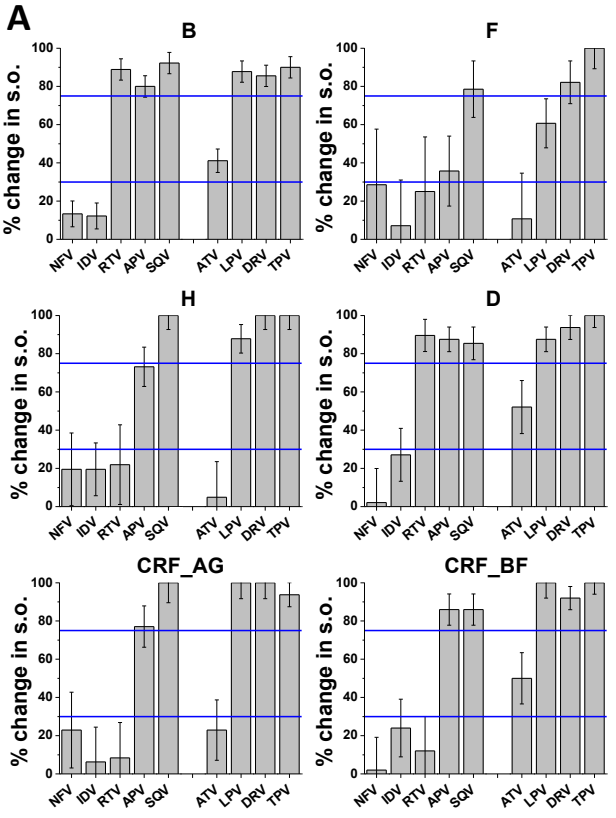


Figure 4. (A) Plots of relative percentage change in semi-open population for HIV-1PR as a function of inhibitor. % change = 100x[%s.o.(unbound)-%s.o.(inhibitor)]/%s.o (unbound). Solid blue lines indicate boundaries between weak, moderate and strong effects at 30% and 75%; respectively. (B) Tabular representation of inhibitors in the weak (orange), moderate (yellow) and strong (green) shifts on the semi-open population; with first and second generation inhibitors labeled in blue and black; respectively for constructs studied here and elsewhere.[26]

The greatest variability in inhibitor binding effects is observed in the moderate classifications comprising most often times inhibitors ATV and APV. ATV as an inhibitor is found to display both weak and moderate shifts to the conformational landscapes whereas APV, induced either moderate or strong effect. RTV, which displays strong interactions with Subtypes B, D and C, interestingly, has weak interactions with other constructs investigated here. Although this may indicate a propensity for a more robust PI-induced resistance to RTV in these other subtypes and CRFs, RTV is no longer recommended as a first line of treatment nor a standalone PI but as a booster drug in treatment plan not because it doesn't inhibit HIV-1PR but because of its numerous side effects including hepatotoxicity and blood lipid abnormalities.[45]

3.4. Comments on First Generation versus Second generation inhibitors.

Of the first generation inhibitors, NFV and IDV show weak interactions, with SQV showing strong, RTV showing a mix of weak and strong interactions, and with APV having a mix of moderate to strong interactions. Despite being the first FDA-approved PI, SQV possesses a strong ability to interact with and shift the HIV-1PR conformational landscape to the closed-inhibited state. Although this ability to shift the conformational ensemble with slow exchange kinetics would indicate effectiveness as an inhibitor, the antiviral activity of SQV is lower *in vivo* due to its poor bioavailability,[38] making SQV is no longer a recommended PI. RTV and APV appear to have weakened interactions with some constructs compared to subtype B.

Except for ATV, the second-generation PIs are found to have a strong effects on the conformational landscape of HIV-1PR. Even though ATV is a second-generation PI, this inhibitor is seen as weak or moderate in its ability to suppress the semi-open conformation. In fact, the conformational ensemble for Subtypes F and H in the presence of ATV did not change within error compared to their respective unbound states. Larger shifts in the semi-open

population are observed for subtype D, CRF_AG, and CRF_BF, but a significant non-zero fractional occupancy of the semi-open conformation is still present in these variants. The other three second-generation PIs, LPV, DRV, and TPV, induce a strong suppression of the semi-open conformation comparable to the results observed from binding with substrate mimic CaP2.

4. Conclusions

Combination of different NPs in central African non-B variants studied here alter the conformational ensembles to increase the relative occupancy of flap curling and closing compared to PI-naïve Subtype B. Most second generation inhibitors are found to induce dramatic changes in these conformational ensembles, lessening the presence of the semi-open population indicating effectiveness as inhibitors. Even though the current FDA-approved second-generation PIs, namely LPV, DRV, and TPV, effectively suppress the semi-open conformation of non-B HIV-1PR, the non-B variants with many NPs can more readily acquire drug resistance and altered drug resistant patterns[46] than observed in subtype B.

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