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Targeting a Dark Excited State of HIV-1 Nucleocapsid by Antiretroviral Thioesters Revealed by NMR Spectroscopy

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Abstract

HIV-1 nucleocapsid (NCp7) is a two Cys₂HisCys zinc knuckle (N-Zn and C-Zn) protein that plays a key role in viral replication. NCp7 conformational dynamics is characterized by NMR relaxation dispersion and chemical exchange saturation transfer measurements. While the N-Zn knuckle is conformationally stable, the C-Zn knuckle interconverts on the millisecond timescale between the major state, in which the zinc is coordinated by three cysteines and a histidine, and two folded minor species (with populations around 1%) in which one of the coordination bonds (Cys413-Sγ-Zn or His421-Ne2-Zn) is hydrolyzed. These findings explain why antiretroviral thioesters specifically disrupt the C-Zn knuckle by initial acylation of Cys413, and show that transient, sparsely-populated (“dark”), excited states of proteins can present effective targets for rational drug design.

Keywords

antiretrovirals; conformational exchange; HIV-1 nucleocapsid; relaxation dispersion; zinc

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Conflict of interest

The authors declare no conflict of interest.

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HIV-1 nucleocapsid (NCp7) plays a central role in the viral replication cycle, including RNA packaging, viral assembly and release, integration, and reverse transcription.^[1] NCp7 contains two zinc knuckles of the Cys-X₂-Cys-X₄-His-X₄-Cys variety with 55% sequence identity between them (Figure 1A), and essentially identical three-dimensional structures.^[2] NCp7 is conserved in all viral strains and is therefore a potential target for next-generation anti-HIV therapy.^[3] Inhibition of NCp7 results in the production of immature, non-infectious virions that are unable to infect the host.^[3] Mercaptobenzamide thioesters have been shown to specifically target the C-terminal zinc (C-Zn) knuckle of NCp7^[4] by initial acylation of Cys413,^[5] but the reason for the susceptibility of the C-Zn knuckle rather than the N-terminal (N-Zn) one is not clear. Mercaptobenzamide thioesters display low cellular toxicity and inhibit viral activity at concentrations less than 50 nM, suggesting that they may provide a useful avenue for anti-HIV-1 drug design.^[6]

NCp7 is expressed as part of the Gag polyprotein and subsequently released during retroviral maturation by proteolytic cleavage. During the course of our previous work on the kinetics of the interactions of HIV-1 protease with Gag by relaxation-based NMR methods,^[7] including Carr–Purcell–Meiboom–Gill (CPMG) dispersion^[8] and chemical exchange saturation transfer (CEST),^[9] we noticed that the C-Zn knuckle undergoes exchange dynamics independent of the presence of protease. Here we investigate in detail the conformational dynamics within the C-Zn knuckle of NCp7 by ¹⁵N-CPMG and ¹⁵N- and ¹³C'-CEST experiments.

Figure 1B shows ¹H–¹⁵N correlation spectra of NCp7 before and after addition of a 20-fold molar excess of mercaptobenzamide-1, a representative of the mercaptobenzamide thioester class of NCp7 inhibitors. The corresponding time courses of the normalized cumulative cross-peak heights and volumes for the N-Zn and C-Zn knuckles are shown in Figure 1C. The intensities of cross-peaks arising from the C-Zn knuckle are reduced by over 50% after 3 h. After 6 h, almost no cross-peaks are observed for the C-Zn knuckle, whereas those of the N-Zn knuckle are still present. Only after about 9 hours are the cross-peak intensities for the N-Zn knuckle significantly reduced, and at 12 h only a few weak cross-peaks at positions characteristic of random coil remain visible (Supporting Information, Figure S1). As the time courses of the normalized cross-peak heights and volumes are superimposable (Figure 1C), the reduction in cross-peak intensities cannot be attributed to an intermediate exchange process involving the folded state, but rather to the formation of large amorphous aggregates, seen by negative staining electron microscopy (Supporting Information, Figure S2), driven by the ejection of zinc from the coordination site and concomitant unfolding of the C-Zn knuckle triggered by the acylation of Cys413, followed by additional acylations at other sites.^[5,6b] Note that intermediate exchange would result in line broadening leading to a reduction in cross-peak heights while leaving the corresponding volumes unaltered^[8].

CPMG relaxation dispersion and CEST experiments probe exchange between a major NMR visible state and invisible, sparsely-populated states (as low as about 0.5%), characterized by different chemical shifts, on time scales ranging from about 0.1 to about 10 ms^[8] and from about 5 to about 50 ms, respectively.^[9] In relaxation dispersion experiments, chemical exchange line-broadening is progressively suppressed by increasingly strong CPMG field strengths. In CEST, the application of a weak radiofrequency (RF) field at the resonance of a

minor invisible state(s) produces a loss of intensity at the resonance frequency of the major visible species resulting in a characteristic pair of intensity dips providing the exchanging states have significantly different chemical shifts. Quantitative analysis (best-fitting) of CPMG and/or CEST profiles via propagation of the Bloch–McConnell equations^[10] yields the kinetic rate constants for the exchange process(es) and the chemical shifts of the invisible minor state(s).

Representative ¹⁵N-CPMG relaxation dispersion profiles are shown in Figure 2A. All residues within the C-Zn knuckle (411–428) exhibit significant dispersions (Supporting Information, Table S1). In contrast, the dispersion profiles for all residues of the N-Zn knuckle are flat (insets, Figure 2A). In favorable cases (when the chemical shift differences between the major and minor states, ω , are sufficiently large), ¹⁵N- and ¹³C'-CEST profiles of C-Zn residues show characteristic second dips in intensity at the resonance frequencies of the minor species (Figure 2B), indicating that the timescale of exchange is sufficiently slow to be accessible to both CPMG-based and exchange saturation transfer techniques ($\tau_{\text{ex}} \approx 2$ –10 ms).

Several lines of evidence point to the existence of multiple minor conformational states for the C-Zn knuckle. First, not all relaxation dispersion profiles could be adequately fit to a two-state (A $\leftrightarrow$ B) exchange model, with the poorest fits observed for residues with the largest resulting ω values. Second, fits of ¹⁵N-CEST data to a two-state exchange model for a subset of residues with well resolved minor intensity dips yielded very high values for the transverse relaxation rates ($R_2 \approx 100$ –400 s⁻¹ at 600 MHz) of the minor species, indicative of the presence of at least two minor states in intermediate exchange with one another. This finding is similar to previously observed line-broadening of the minor intensity dips in CEST profiles arising from an intermediate exchange process between folding intermediates of an FF domain.^[11] Indeed, a pair of minor intensity dips is distinguishable in the 600 MHz ¹³C'-CEST (Figure 2B, right) and 800 MHz ¹⁵N-CEST (Supporting Information, Figure S3 at 800 MHz) profiles for Gln422. We note that the field-dependence (800 vs. 600 MHz) of exchange line-broadening of the minor species, R_{ex} (approximately R_2^{B} from the fits to a two-state model), for ¹⁵N-CEST profiles with either completely separated or completely coalesced minor dips, falls significantly short of the ratio of fields squared (1.78), indicating that interconversion between the minor states lies in the slow-to-intermediate regime.

As it seemed likely that the imidazole side-chain of His421 was directly implicated in one of the exchange processes, we also looked for evidence of exchange at the N ϵ 2 site coordinated to zinc.^[2a–c] A variant of the ¹⁵N-CEST experiment that exploits two-bond scalar couplings between the imidazole ring nitrogen atoms (¹⁵N δ 1 and ¹⁵N ϵ 2) and the protons attached to the ring C ϵ 1/C δ 2 carbons ($^2J_{\text{HN}} \approx 6$ –10 Hz),^[12] was recorded (Figure 2C). No evidence for any CEST effect was observed for the ¹⁵N ϵ 2 atom of His400 in the N-Zn knuckle. In the C-Zn knuckle, however, the chemical shift of the ¹⁵N ϵ 2 atom of His421 changes from 220.4 ppm, typical of a Zn²⁺-coordinated state,^[13] to 170.3 ppm in the minor state, characteristic of protonation accompanying the breaking of the His421-N ϵ 2–Zn bond. The chemical shift of the ¹⁵N δ 1 atom of His421 (172.5 ppm) in the major species is also indicative of a

protonated state.^[14] It is therefore likely that one of the forms of His421 in minor species **B** corresponds to a protonated, positively charged imidazole ring (Figure 3). Because the value of $|\omega_N|$ is so large in this case (>50 ppm), the exchange regime for His421-Ne2 is shifted far to the slow extreme ($|\omega_N| \tau_{ex} \gg 1$), making it undetectable by CPMG relaxation dispersion. Further, quantitative analysis of the His421-Ne2 CEST profile is complicated by rapid interconversion between different tautomeric states of the imidazole ring,^[15] leading to line-broadening of state **B** that is not accounted for by the three-state exchange model of Figure 3.

The kinetic model that quantitatively accounts for all the ¹⁵N-CPMG relaxation dispersion and ¹⁵N/¹³C'-CEST data for the C-Zn knuckle is shown in Figure 3, and represents a three-state exchanging system where the major state (**A**; with all Zn coordinate bonds intact) interconverts with two sparsely populated species: **B** in which the His421-Ne2-Zn coordination bond is hydrolyzed, and **C** where a Cys-Sγ-Zn coordination bond is hydrolyzed. The two minor states **B** and **C** interconvert through the concomitant loss of water and recreation of the corresponding Zn-coordination bonds (Figure 3). As only 5 residues of the C-Zn knuckle provide well separated minor intensity dips in ¹⁵N-CEST profiles, whereas all 18 sites show significant relaxation dispersion ($R_{ex} > 3 \text{ s}^{-1}$), we supplemented the ¹⁵N-CPMG (at 600 and 800 MHz) and ¹⁵N-CEST (600 MHz) data with 1) ¹⁵N-CEST profiles acquired at 800 MHz at a single RF field, and 2) ¹³C'-CEST profiles at two RF fields (Supporting Information, Table S1). The rationale for this approach is that CEST data acquired at another spectrometer field or for another type of nucleus increases the sensitivity of the data towards the exchange rates between the minor states **B** and **C**, as the exchange regime on the chemical shift timescale dictates the extent of line-broadening experienced by the nuclei of each minor state. Simultaneous fitting of the data to the three-state model (Figure 3) yields rate constants for the interconversion processes, as well as the populations and chemical shifts (Figure 2D; Supporting Information, Table S2) of the minor species. The two minor states **B** and **C** are approximately equally populated (ca. 1.4% and ca. 1.2%, respectively; Figure 3) and interconvert on the slow or intermediate timescale depending on the differences in their chemical shifts ($|\omega_{AB} - \omega_{AC}|$).

The chemical shift differences between the observable and invisible states help clarify the chemical nature of the minor species (Figure 2D). ¹⁵N- ω values are localized in the vicinity of Cys413 and His421: specifically, His421 and Gln422, and to a lesser extent Gly417, for the A↔B process (Figure 2D, blue), and Trp414, Cys416, His421, and Gln422 for the A↔C process (Figure 2D, red). The largest ¹³C'- ω values, manifested by discrete minor dips in the ¹³C'-CEST profiles, are observed for Trp414, Cys416, Gln422, Met423, and Lys424 (Supporting Information, Table S2). State **B**, therefore, likely corresponds to the state in which the His421-Ne2-Zn bond is hydrolyzed, consistent with the ¹⁵N-Ne2-CEST profile in Figure 2C. State **C** corresponds to the state in which the Cys413-Sγ-Zn is hydrolyzed, consistent with the mass spectrometry data indicating that the Cys413-Sγ is the site of initial acylation by mercaptobenzamide thioesters.^[5] Note that it would be difficult to distinguish with certainty between Cys413 and Cys416 as the site of hydrolysis of the Sγ-Zn bond in the absence of any prior structural or mass spectrometry data.

The values of ^{15}N - ω_{AC} can be rationalized from the structure of the C-Zn knuckle^[2e] as follows: no ^{15}N shift change is observed for Cys413 owing to preservation of the backbone Cys413(N)–Lys418(O) bond; the large ^{15}N shift difference for Cys416 is likely due to an alteration in the electronic state of the S γ atom of Cys413 which is within hydrogen bonding distance (ca. 3.2 Å) of the backbone nitrogen of Cys416; and the large ^{15}N shift for Trp414 can be attributed to the altered state of the sidechain of the preceding residue,^[16] Cys413. The backbone nitrogen of Gly417 is within 4.5 Å of the S γ atoms of both Cys413 and Cys416, and the significant changes in ^{15}N shift for Gly417 in state **B** may be due to small changes in these distances as a consequence of hydrolysis of the His421-N ϵ 2–Zn bond. Finally, the slightly larger ^{15}N - ω values of His421 and Gln422 for the A $\leftrightarrow$ C than A $\leftrightarrow$ B interconversion may perhaps be attributable to the larger electronegativity of S γ relative to N ϵ 2.

In terms of backbone conformation, it would be predicted that a loss of only a single protein-Zn coordinating bond would result in only minimal backbone perturbations relative to the major **A** state. Since $^{13}\text{C}\alpha$ shifts are good reporters of backbone ϕ/ψ angles,^[17] we recorded $^{13}\text{C}\alpha$ -CPMG relaxation dispersions^[18] on a selectively $^{13}\text{C}\alpha$ -labeled^[19] NCp7 sample (Supporting Information, Figure S4). The $^{13}\text{C}\alpha$ -dispersions are relatively small with exchange line broadening R_{ex} values of $\sim 5 \text{ s}^{-1}$. The $^{13}\text{C}\alpha$ -| ω_{AC} | values are less than 1.5 ppm; three residues have $^{13}\text{C}\alpha$ -| ω_{AB} | values in excess of 1.5 ppm (Lys415, Met423 and Cys426) but none greater than about 3 ppm. One can therefore conclude that any changes in backbone torsion angles in the minor species are likely confined to the same preferred region of the Ramachandran map in which they are located in the major species.^[17]

The rate constant for hydrolysis of the His421-N ϵ 2–Zn bond ($k_{\text{AB}} \approx 5.2 \text{ s}^{-1}$) is about double that for the Cys413-S γ –Zn bond ($k_{\text{AC}} \approx 2.5 \text{ s}^{-1}$) implying a lower activation energy for the former process, a finding consistent with the lower force required to break a His–Zn bond (ca. 30 pN) compared to a Cys–Zn bond (ca. 170 pN) determined by single-molecule atomic force microscopy measurements.^[20] Further, the rate constants for coordinate bond formation (k_{BA} and k_{CA} in Figure 3) are not significantly different from those for interconversion between the two minor species **B** and **C** (k_{BC} and k_{CB}), as might be expected as they involve similar underlying processes.

In conclusion, the C-Zn knuckle of HIV-1 NCp7, but not the N-Zn knuckle, undergoes millisecond exchange at the zinc coordination site between a major species in which the zinc is coordinated to three cysteines and a histidine, and two minor excited dark states, populated at about 1%, in which one of the protein-Zn coordinating bonds, involving either His421 or Cys413, is hydrolyzed. Transient exposure of a reactive sulphhydryl group renders the C-Zn knuckle susceptible to acylation by mercaptobenzamide thioesters, and provides proof of concept that a sparsely populated transient state of a protein can be targeted for drug design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Mercaptobenzamide thioesters specifically target the C-Zn knuckle of NCp7. A) HIV-1 NCp7. Numbering is based on the Gag polypeptide precursor from strain HXB2. B) Three regions of the ^1H - ^{15}N correlation spectrum of 50 μM NCp7 alone (green), and 3 (red) and 6 hours (blue) after addition of a 20-fold molar excess of mercaptobenzamide-1 at 35°C and pH 6.5. Cross-peaks of the C-Zn are encircled and labeled in black, while those of the N-Zn knuckle are labeled in gray. The chemical structure of S-(2-((3-amino-3-oxopropyl)-carbamoyl)phenyl)pent-4-ynethioate (referred to as mercaptobenzamide-1) is shown as an inset. C) Time course of the cumulative normalized cross-peak heights and volumes for the N-Zn (blue, residues 390–405) and C-Zn (red, residues 413–428) knuckles following addition of mercaptobenzamide-1.

Figure 2.

CPMG relaxation dispersion and CEST on NCp7. Representative backbone A) ^{15}N -CPMG relaxation dispersion and B) ^{15}N - and $^{13}\text{C}'$ -CEST profiles observed for the C-Zn knuckle. C) ^{15}N -CEST profiles for the N ϵ 2 atom of the imidazole ring of His421. Relaxation dispersions were recorded at 600 (red) and 800 MHz (blue). CEST profiles were recorded at 600 MHz. For ^{15}N -CEST constant-wave (CW) saturation was applied for $T_{\text{CEST}} = 200$ ms at RF field strengths of 15 (blue) and 30 (red) Hz for backbone ^{15}N and 50 Hz for histidine side chain $^{15}\text{N}\epsilon 2$. For $^{13}\text{C}'$ -CEST, $T_{\text{CEST}} = 300$ ms with RF field strengths of 30 (blue) and 40 (red) Hz were used. The insets in (A)–(C) show ^{15}N -CPMG and ^{15}N -/ $^{13}\text{C}'$ -CEST profiles for the corresponding residues of N-Zn. The experimental data are displayed as circles, and the global best-fits to a three-state exchange model (see Figure 3) as solid lines. For N-Zn residues (insets) and the $^{15}\text{N}\epsilon 2$ -CEST profile (C), dashed lines are drawn purely to guide the eye. D) ^{15}N chemical shift differences, ω_{AB} (blue) and ω_{AC} (red) between major and minor species derived from the fits to the experimental CPMG and CEST data. Note the Gly420 $^1\text{H}_\text{N}/^{15}\text{N}$ cross-peak is not observed due to line-broadening from water/amide proton exchange. Experiments were conducted at 30 °C and pH 6.5 in H_2O on

deuterated ^{15}N or $^{15}\text{N}/^{13}\text{C}'$ -labeled samples. Details of experiments and data fitting are described in the Supporting Information.

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Figure 3.

Kinetics and rate constants for exchange between native and sparsely-populated states of the C-Zn knuckle of NCp7. See the Supporting Information for the details of global fitting of the ^{15}N -CPMG relaxation dispersion and $^{15}\text{N}/^{13}\text{C}'$ -CEST data.