The Connection Domain Is Implicated in Metalloporphyrin Binding and Inhibition of HIV Reverse Transcriptase*

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We have shown that heme and zinc protoporphyrin inhibit both human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) reverse transcriptases (RTs) and, in combination with other nucleoside and non-nucleoside inhibitors, exert an additive effect on HIV-1 RT inhibition. Screening of a phage peptide library against heme resulted in the isolation of a peptide with sequence similarity to sequence 398–407 from the connection subdomain of both HIV-1 and HIV-2 RTs, suggesting that this highly conserved region of HIV RTs corresponds to the binding site for metalloporphyrins and a new site for inhibition of enzyme activity. Inclusion of a synthetic peptide corresponding to the exact sequence 398–407 of HIV-1 RT in RT inhibition assays had a protective effect on metalloporphyrin inhibition, as it was able to reverse the inhibitory effect of both metalloporphyrins on HIV-1 RT activity. Furthermore, intrinsic fluorescence assays indicated that these metalloporphyrins bind to synthetic peptide 398–407 as well as to intact dimeric HIV-1 RT. The identification of this novel inhibition site will help to expand our understanding of the mode of action of metalloporphyrins in RT inhibition and will assist in the design and development of more potent metalloporphyrin RT inhibitors for the management of HIV infection.

Human immunodeficiency virus type 1 (HIV-1)3 reverse transcriptase (RT) is a major target for chemotherapeutic agents used in the treatment of AIDS. Currently, two major classes of anti-AIDS chemotherapeutic drugs have been developed that target the enzyme: the nucleoside RT inhibitors (NIs), which are chain-terminating nucleoside analogs, and the non-nucleoside inhibitors (NNIs), which are chemically and structurally diverse and appear to inhibit HIV-1 RT by binding to a common site distinct from the binding site of the nucleoside compounds (for a review, see Refs. 1 and 2).

Recently, it has been shown that heme (iron protoporphyrin IX) has an additive effect on murine hematopoietic recovery after treatment with the nucleoside analog AZT-TP (3). Furthermore, heme and certain other metalloporphyrins stimulate the immune system and enhance erythropoiesis (4, 5). Heme, as well as the synthetic heme analog tin protoporphyrin, significantly enhances bone marrow erythroid progenitor proliferation and differentiation in lymphoproliferative disorders (6). It has also been shown that heme and other synthetic heme analogs significantly inhibit HIV-1 RT activity in a noncompetitive manner with respect to deoxynucleoside triphosphate and markedly enhance the inhibitory effect of AZT-TP on HIV-1 RT (7). We have extended these studies and demonstrated that heme and zinc protoporphyrin (ZnP) inhibit both HIV-1 and HIV-2 RTs in a concentration-dependent manner, using in vitro RT inhibition assays. Furthermore, these metalloporphyrins, in combination with other nucleoside (AZT-TP) and non-nucleoside (BHAP) HIV-1 RT inhibitors, exert an additive effect on HIV-1 RT inhibition, indicating an independent mode of action by binding to a distinct inhibition site. We thus used a novel approach to investigate the sites on HIV RT and eventually other proteins that might be the target of heme binding and modulation. In recent years, random peptide libraries displayed on the surface of filamentous bacteriophage M13 have been used successfully for the identification of peptide ligands that interact with molecular targets. Phage libraries have been generated to display peptides of variable lengths ranging from 6 to 38 random amino acids, protein fragments, or fully active folded proteins (for a review, see Ref. 8). Such libraries have been used to identify peptide epitopes for monoclonal (9–12) and polyclonal (13–15) antibodies as well as ligands for a variety of other molecules, both proteinaceous and non-proteinaceous, including those that are known to interact with small peptide ligands (HLA-DR1 (16), BiP (17), and calmodulin (18)) and those that had previously undefined specificity for peptides (concanavalin A (19), streptavidin (20), and others).

Here we report the use of a 12-amino acid residue random peptide library displayed on bacteriophage M13 to identify peptide ligands that interact with heme. Sequence determinations of the displayed peptides, found to be specific for heme, revealed that they lack amino acid sequence similarity, yet are highly enriched in Trp and Tyr residues and have homology to a wide variety of proteins. Among the different Trp-rich peptides displayed by heme-selected clones, one in particular was found to be partially homologous to sequence 398–407 from the connection domain of both HIV-1 and HIV-2 RTs as well as SIV RT, suggesting that this sequence corresponds to the distinct binding site for heme and a site for inhibition of enzyme activity. Intrinsic fluorescence assays indicated that heme and
ZnPP bind to a synthetic peptide corresponding to the exact sequence 398–407 of HIV-1 RT as well as to intact dimeric HIV-1 RT. We have also demonstrated the ability of synthetic peptide 398–407 to reverse the inhibitory effect of both metalloporphins on HIV-1 RT activity. Molecular modeling indicated that sequence 398–407 from the connection subdomain of HIV-1 RT is sufficiently exposed to accommodate heme binding. These studies have thus identified a new site for inhibition on HIV-1 and HIV-2 RTs that facilitates the design and preparation of new porphyrin-based inhibitors of RT that may have clinical potential in the management of AIDS.

**EXPERIMENTAL PROCEDURES**

**Materials**—Heme was purchased as the chloride salt, hemin chloride (Calbiochem), and prepared as described (21). In certain experiments, hemin chloride was conjugated through its propionyl groups to BSA by EDC-mediated hapten carrier protein formation (Imject Immunogen EDC Conjugation kit, Pierce), and the conjugated protein was purified by gel filtration. In other experiments, heme was directly solubilized in methanol. Bovine hemin (type I) immobilized on cross-linked 4% beaded agarose was purchased from Sigma. ZnPP was obtained from Porphyrin Products (Logan, UT) and prepared as described (21).

**Peptide Library**—The construction of the phage peptide library is described elsewhere (12, 22). The total complexity of the peptide library was 2 × 1013. The combinatorial gene sequences displayed at the N terminus of the M13 phl gene were of the form TCG AGN (NNK)12 TCT AGA, where N represents all four nucleotides (A, C, G, and T) in an equal molar ratio, and K represents an equal molar ratio of G and T. The encoded amino acid scheme was SI(S/R)(X)SR. The library had a working titer of 1 × 1015 pfu/ml.

**Affinity Purification of Binding Phage**—Affinity purification was performed utilizing three different attachment strategies for the heme. In the first strategy, hemin chloride, EDC-coupled to BSA (20 μg), was directly bound on microtiter wells of a 96-well microtiter plate (Corning Inc., Oneonta, NY) in 100 mM NaHCO3, pH 8.5, at ambient temperature for 2–3 h at 4°C overnight. Wells were then blocked with 30 mg/ml BSA in 100 mM NaHCO3 (blocking solution) for 2 h at ambient temperature and finally washed three to five times with washing buffer (phosphate-buffered saline and 0.5% (v/v) Tween 20, pH 7.4). Phage (1011 pfu) were added to each well and allowed to bind for 3 h at ambient temperature. Unbound phage were removed by extensive washing of the wells, and adherent phage were eluted with 50 mM glycine HCl, pH 1. In the second strategy, where hemin chloride was directly dissolved in methanol at a concentration of 1 mg/ml (50 μg) of the solution were added to the microtiter wells and dried out after incubation for 1–2 h at 37°C. The wells were then blocked, and screening of the library was performed as described above. In the third strategy, commercially available heme (bovine, type I) immobilized on cross-linked 4% beaded agarose was used as a target for screening the CW peptide library. In brief, 500 μg of target-coupled beads were incubated in a microcentrifuge tube in 1 ml of phosphate-buffered saline/Tween 20 with phage (1010 pfu) for 2 h at ambient temperature. Beads were then washed extensively, and bound phage were eluted by resuspending the beads in 300 μl of elution buffer, pH 2.2, for 15 min at ambient temperature. The beads were pelleted, and eluted phage were neutralized by transferring the solution to a new tube containing 300 μl of neutralization buffer, pH 7.5. The recovered phage were amplified, and the affinity purification was repeated for a total of three rounds.

**Evaluation of the Specificity of Selected Clones**—To evaluate the specificity of each selected clone against heme, we performed plaque assays as described (23). In brief, equal titers (1010 input pfu) of each phage clone were incubated with the heme target, immobilized on a solid support by the three different attachment strategies. In parallel, equal titers of phage stock were added to microtiter wells coated with blocking solution or to cross-linked beaded agarose in tubes, which served as negative controls. Microtiter wells and tubes were incubated and washed as described above. Eluted and neutralized phage were serially diluted and plated onto LB plates. The binding specificity was determined by comparing the calculated percentage of input pfu recovered from the phage bound to heme with that bound to BSA or to cross-linked beaded agarose.

**Phage Sequencing**—Aliquots of the heme-bound phage output, after the third round of selection, were plated to isolate individual plaques (clones), from which phage stocks were produced for the purification of double-stranded DNA (Wizard Plus Miniprep DNA Purification system, Promega, Madison, WI). Purified double-stranded DNA was sequenced by automated cycle sequencing (ABI 377 or 375A Stretch sequencers, with T7 FS dye terminator or dye primer chemistry). An oligonucleotide primer with the sequence 5-ACC GTA ACG TCT AAT A-3’ was used to determine the nucleotide sequence of the unique region of the M13 phage.

**Peptide Synthesis and Purification**—The P1 peptide (WETTW-TEYWQ), which corresponds to sequence 398–407 from the connection domain of HIV-1 RT (based on the HIV-1 BH10 molecular clone sequence), was purchased as a crude cleavage product from the Biopolymer Synthesis Center, California Institute of Technology (Pasadena, CA). The P2 control peptide (ASQEVKNWM, from HIV-1 glycosylated protein) was synthesized in our laboratory by using the RAmPS multiple peptide synthesis system procedure on Wang resin (Du Pont) using Fmoc (N-(9-fluorenylmethoxycarbonyl) nitrogen terminal protected amino acids (BACHM Biotech Co., Philadelphia, PA). Both peptides were purified in our laboratory by reverse-phase high performance liquid chromatography (Perkin-Elmer Series 400) using a Vydac C18 column, and their identity and purity were verified by matrix-assisted laser desorption ionization/mass spectroscopy (24).

**Reverse Transcriptase Assays**—Recombinant HIV-1 and HIV-2 RTs were donated by the Center for AIDS Research at Case Western Reserve University. The N1 AZT-TP was obtained from Moravek Biochemicals, Inc. (Brea, CA), and the NNI U-90152T, which is a BHP analog, from Pharmacia Upjohn Co. [methyl-3H]Deoxythymidine triphosphate ([3H]dTTP), with a specific activity of 17.5 Ci/mmol, was purchased from NEN Life Science Products, and the primer-template poly(A)rp(dT)12-18 was from Pharmacia Biotech. The reaction mixture for measuring polymerase activity, using poly(rA)rp(dT)12-18 (10 units/ml), contained 100 mM Tris-HCl, pH 8.5, 5 mM MgCl2, 1 mM dithiothreitol, 2.5 units HIV-1 RT, 5 μCi [3H]dTTP, and 1 μCi of [3H]dTPP as a substrate in a total volume of 200 μl. Reaction mixtures were incubated for 1 h at 37°C and terminated by the addition of 0.5 ml of 5% trichloroacetic acid with 2% (w/v) sodium pyrophosphate. Then 100 μl of a solution containing BSA (2.5%) and calf thymus DNA (5%) were added. Acid-precipitable material was collected on glass-fiber filters and washed with 5% trichloroacetic acid and 95% ethanol, and radioactivity retained on the filters was measured by scintillation counting.

**Fluorescence Assays**—Conventional fluorescence spectra were recorded with a Perkin-Elmer LS-5 luminescence spectrometer. HIV-1 RT and the peptides were routinely excited at 280 nm, and the fluorescence emission spectrum was integrated between 300 and 460 nm. The maximum emission intensity for the RT was measured at 340 nm and for the peptides at 360 nm. The intrinsic fluorescence emission of HIV-1 RT (200 nM protein) and of peptides P1 and P2 (1 μM) was measured in fluorescence buffer containing 0.1 Tris-HCl, pH 8.3. The average intrinsic association constant or affinity (Ka) was calculated on the basis of the logarithmic form of the Sips distribution function as described (25).

**Analytical Ultracentrifugation**—Sedimentation equilibrium measurements were made using a Beckman XL-I analytical ultracentrifuge with interference optics. Samples of RT alone and RT with heme were prepared using the same mixture composition as described under "Reverse Transcriptase Assay," but all reagents were 50-fold more concentrated. Sedimentation of the samples was measured against buffer blanks in standard 1.2-cm path length, six-channel cells with sapphire windows. Data were obtained at 10°C at 15,000 rpm for 36 h, followed by another 30 h at 12,000 rpm. Equilibrium concentration profiles at each speed were analyzed using equilibrium sedimentation equations for reversible heterodimerization with Igor-Pro® programs (Wave metrics, Lake Oswego, OR).

**Molecular Graphics Representations of HIV-1 RT and Heme**—The structure of HIV-1 RT (BH10 isolate) was obtained from the Protein Data Bank at Brookhaven National Laboratory (PDB file 3HVT). The heme structure was extracted from PDB file 1MYG. Model building was performed using the SYBYL molecular modeling package (Tripos Associates, St. Louis, MO) on a Silicon Graphics display system as well as the molecular visualization program RasMol Version 2.5 (50) on a Macintosh display system.
ZnPP in combination with the well characterized NNI, BHAP, compared the inhibitory effect of heme, ZnPP, and heme or ZnPP in combination with the well characterized NNI, BHAP. To investigate the sites of action of metalloporphyrins in HIV-1 RT inhibition, we conducted RT inhibition assays as described under “Experimental Procedures.”

### RESULTS

**Heme and ZnPP Inhibit HIV-1 RT in a Noncompetitive/Nonexclusive Manner with NIs and NNIs and Exert an Additive Effect on Enzyme Inhibition**—To confirm the findings of other investigators and to further our understanding of the mode of action of metalloporphyrins in HIV-1 RT inhibition, we conducted RT inhibition assays as described under “Experimental Procedures.” As shown in Table I, both metalloporphyrins inhibited RT in a concentration-dependent manner. Heme or ZnPP (both at 10 μM) resulted in >80% inhibition, which is significantly greater than the findings of other workers (7). Our results also indicate that ZnPP is a slightly better inhibitor than heme. The calculated IC50 values for inhibition of HIV-1 RT were 6 μM for heme and 4.8 μM for ZnPP. In addition, we compared the inhibitory effects on HIV-1 RT of heme, ZnPP, and heme or ZnPP in combination with AZT-TP with those of AZT-TP alone. As shown in Table I, AZT-TP (10 nM) inhibited the enzyme by 19%. The inclusion of heme or ZnPP markedly enhanced the inhibition of RT by AZT-TP, thus confirming the findings of Staudinger et al. (7) that the combination of heme and AZT-TP synergistically inhibits the activity of HIV-1 RT. Furthermore, our results indicate that metalloporphyrins in combination with the chain terminator AZT-TP exert a strictly additive effect on the inhibition of the enzyme. The studies of Staudinger et al. have also suggested a distinct but allosterically linked binding site for heme and its analogs on HIV-1 RT with respect to the nucleoside-binding site. To investigate whether metalloporphyrins bind to the other known sites of inhibition on RT, the common binding site of other NIs, BHAP (26–30). As shown in Table I, heme and ZnPP in combination with the NNI inhibited RT in a noncompetitive and nonexclusive manner. Indeed, they enhanced the inhibitory effect of BHAP by wielding an additive effect on the enzyme inhibition, indicating that metalloporphyrins bind to a distinct binding site on HIV-1 RT that is not shared by either NIs or NNIs.

**Identification of a Heme-binding Site on HIV-1 and HIV-2 RTs Using a Phage Peptide Library**—To investigate the sites on HIV-1 RT that might be the target of heme binding, we screened a phage-displayed 12-mer peptide library against heme. After three rounds of selection, a total of 43 heme-specific clones were isolated, which represented 40 different amino acid sequences. Table II shows the results of the selection experiments. The selected clones were sequenced and the nucleotide sequences were determined. The peptide sequences of the selected clones were then compared to the amino acid sequences of known binding sites.

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**Table I**

**Inhibitory effects of metalloporphyrins alone and in combination with AZT-TP and BHAP on HIV-1 RT activity**

| Inhibition | % |
|------------|---|
| Heme       |   |
| 2 μM       | 15 (±2) |
| 8 μM       | 66 (±4) |
| 10 μM      | 50 (±4) |
| AZT-TP     |   |
| 10 nM + 0 μM heme | 19 (±2) |
| 10 nM + 2 μM heme | 36 (±4) |
| 10 nM + 5 μM heme | 61 (±3) |
| 10 nM + 10 μM heme | 100 (±1) |
| BHAP       |   |
| 250 nM + 0 μM heme | 51 (±2) |
| 250 nM + 2 μM heme | 62 (±4) |
| 250 nM + 5 μM heme | 83 (±2) |
| 250 nM + 10 μM heme | 100 (±1) |
| ZnPP       |   |
| 2 μM       | 23 (±4) |
| 8 μM       | 85 (±6) |
| 10 μM      | 100 (±1) |

**Table II**

**Deduced amino acid sequences of peptides displayed by heme-selected phages after three rounds of selection**

| Designation | Peptide sequence | No. of isolated clones |
|-------------|-----------------|------------------------|
| A-1         | SS MFRAPGDHIKGV | SR 1                   |
| A-2         | SR SPWODWYSNLTP | SR 1                   |
| A-3         | SS MVGLSSDFRSGV | SR 1                   |
| A-4         | SS WLPWTYSQYGSM | SR 1                   |
| A-5         | SR YDWGSWGTDIA | SR 1                   |
| A-6         | SR YWWWNGITNVVE | SR 1                   |
| A-7         | SS WGEWWNNRDQW | SR 1                   |
| A-8         | SS ESWRNYVSAA | SR 1                   |
| A-9         | SR AVLQQWTVDVS | SR 1                   |
| A-10        | SS LMQRTSWVSQHL | SR 1                   |
| A-11        | SS WHGWPPWYSKH | SR 1                   |
| A-12        | SR DASTTYYSRKWG | SR 1                   |
| A-13        | SR WENGREGRWHWL | SR 1                   |
| A-14        | SS WGEFYRMQGYLL | SR 1                   |
| A-15        | SS NAVSLRAVTPFEA | SR 1                   |
| A-16        | SS WD WrLTHWWQG | SR 1                   |
| A-17        | SR WDWVWYGTYGATPSR 1 | |
| A-18        | SS WEAWMYDWRMSV | SR 1                   |
| A-19        | SS KNWNSWTDVSLSR 1 | |
| A-20        | SR GYFVEGAGSWEDSR 1 | |
| A-21        | SS YARGDWDFANGV | SR 1                   |
| A-22        | SS AWTQLQKYIGQY | SR 1                   |
| A-23        | SS HEDYAWFGWSSAR 1 | |
| A-24        | SS WDTRGWNWVWLYL | SR 1                   |
| A-25        | SS WSSLYWLQSHQQSK | SR 1                   |
| B-1         | SS WWSVNGLRWDSH | SR 3                   |
| B-2         | SS ASMYESHYARWNY | SR 1                   |
| B-3         | SS VHQWLWWGGYQW | SR 1                   |
| B-4         | SR PWVDQWEHQGQSRSR 1 | |
| B-5         | SR WDDLRSTKFWBV | SR 1                   |
| B-6         | SS LGRWGEYYWSSAG | SR 1                   |
| B-7         | SS RDRHRWVGDSILL | SR 1                   |
| B-8         | SS HRTDWSSRRYHLH | SR 1                   |
| B-9         | SS WDRWRNYVENVT | SR 1                   |
| B-10        | SS AQGNWKRREWSAH | SR 1                   |
| C-1         | SS SDEWWREPEQTS | SR 1                   |
| C-2         | SS WDGREYRWVTSRH | SR 1                   |
| C-3         | SS AEWSPMWHRSASA | SR 1                   |
| C-4         | SS VYNWFDDAQNG | SR 1                   |
| C-5         | SS WCARSWDFKLA | SR 1                   |

A-1–35, sequences selected from the screening of the phage library against heme coupled to BSA; B-1–10, sequences selected from screening against heme dissolved in methanol and dried on microtiter plates; C-1–5, sequences selected from screening against heme immobilized on 4% beaded agarose.

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residue. No significant differences were found in the amino acid composition and frequency among the peptides displayed by heme-selected phage from the different attachment strategies for heme. The evaluation of the specificity of the 40 recovered clones by the plaque assay showed that all phage clones were heme-specific, with a typical $10^{-1}$ to $10^{3}\%$ recovery of input pfu as compared with the negative controls, which gave $10^{-6}$ to $10^{-7}\%$ recovery of input pfu. Overall, all 40 different clones bound at least $10^4$ times better to heme targets than to the negative controls (wells coated with BSA alone or with cross-linked beaded agarose).

To determine any homology between our peptide sequences and registered protein sequences, including HIV-1 RT, we performed a search of protein data bases using the advanced BLAST Sequencing Similarity Searching, from the National Center for Biotechnology Information Services. The advanced searching revealed complete or partial homology between almost all of our peptide sequences and a large number of different protein sequences registered in protein data bases, indicating that the random library did generate authentic protein sequences. It is noteworthy that the advanced searching also partially matched several of our heme-specific peptide sequences with registered heme-binding proteins (our clone A-8 with cytochrome $b_5$, clone A-14 with heme exporter protein D, clone A-16 with cytochrome $c$ oxidase, and clone B-3 with probable cytochrome $c$ biosynthesis protein). Since our objective was to identify peptide ligands reflecting regions of HIV-1 RT that might be the target of heme binding, we were gratified to find that heme-specific clone A-7 (WGEWWRNGWQRD) partially matched a sequence from the connection domain of HIV-1 RT (sequence 398–407, WETWWTEYWQ) as well as WEQWWDNYWQ (corresponding to sequence 398–407 of HIV-2 RT) and WEQWWADYWQ (sequence 398–407 of SIV RT). The partial homology of the Trp-rich clone A-7, from the screening of the phage library against heme, with sequence 398–407 from the connection domain of the HIV and SIV RTs suggested that this sequence corresponds to at least a part of the binding site for metalloporphyrins on RT. It should be noted that clone A-7, which was originally selected from the screening of the phage library against heme coupled to BSA, also bound $10^4$ times better to heme immobilized on cross-linked agarose than to the negative control, cross-linked beaded agarose (data not shown).

**A Synthetic Peptide (P1) Corresponding to the Exact Sequence 398–407 of HIV-1 RT (WETWWTEYWQ) Protects HIV-1 RT from Metalloporphyrin Inhibition**—To test our hypothesis that sequence 398–407 of HIV-1 RT corresponds to the binding site for heme and eventually other heme analogs, we examined the ability of the P1 peptide to reverse the inhibitory effect of heme and ZnPP on RT activity, perhaps by competing with HIV-1 RT for binding to the porphyrin compounds, using *in vitro* inhibition assays. For this purpose, we included the peptide in the reaction mixture with the metalloporphyrins at different molar ratios prior to the addition of the enzyme and initiation of the reaction. To control for the direct effects of the P1 peptide on the activity of HIV-1 RT, we also tested the peptide alone. In addition, we tested a peptide (P2) from HIV-1 glycosaminoglycan (ASQEVKNWM), as a negative control, in combination with the metalloporphyrins and alone. The effects of the P1 and P2 peptides on the enzyme activity, either in the presence or absence of heme and/or ZnPP, are shown in Fig. 1. Inclusion of the P1 peptide at an equimolar ratio (5 $\mu$M) with heme did not alter the inhibitory effect of heme on the enzyme activity. However, at a peptide concentration of 50 $\mu$M, which translates to a molar ratio of 10:1 for P1 peptide/heme, the enzyme activity was almost completely restored. The P1 peptide exerted a similar “protective” effect on the enzyme activity when used in combination with ZnPP, although to a lesser extent. As expected, the P2 peptide at similar concentrations in the presence of heme and/or ZnPP did not have any effect on the enzyme activity. Both peptides, when tested alone, did not show any effect on the activity of HIV-1 RT. Our results strongly suggest that sequence 398–407 of HIV-1 RT is implicated as the binding site for heme and eventually other heme analogs.

**Metalloporphyrins Are Potent Inhibitors of HIV-2 RT**—Given that the heme-specific phage clone A-7 matched not only sequence 398–407 of HIV-1 RT, but also the same highly conserved region in HIV-2 and SIV RTs, we wished to examine the effect of heme and ZnPP on HIV-2 and SIV RT activities. The ability of metalloporphyrins to inhibit other immunodeficiency viral RTs has not previously been investigated. Because of limited availability of these reagents (recombinant HIV-2 and SIV RTs), we were able to test the effect of heme and ZnPP only on HIV-2 RT. As shown in Table III, heme and ZnPP were also potent inhibitors of HIV-2 RT. Our results indicate that ZnPP is a better inhibitor than heme. The calculated IC$_{50}$ values for inhibition of HIV-2 RT were 23 $\mu$M for heme and 7 $\mu$M for ZnPP. Our findings strongly support our suggestion that metalloporphyrins inhibit both HIV-1 and HIV-2 (and perhaps SIV) RTs by binding to the highly conserved region 398–407 from the...
connection domain of these enzymes.

**Intrinsic Fluorescence Assays Confirm the Binding of Metalloporphyrins to Synthetic Peptide P1 and Intact Dimeric HIV-1 RT**—To verify the specific binding of metalloporphyrins to the HIV-1 P1 peptide (WETWTEYEWQ), we used fluorescence quenching spectroscopy. We measured changes in tryptophan fluorescence on metalloporphyrin binding to examine the binding of the P1 peptide to heme and ZnPP using a fixed peptide concentration (1 μM) and increasing concentrations of heme and ZnPP. The results of the intrinsic tryptophan fluorescence changes are shown in Fig. 2A and demonstrate that both porphyrin derivatives bind to the peptide, with ZnPP binding slightly better ($K_o = 0.14 \times 10^8 \text{ M}^{-1}$) than heme ($K_o = 0.10 \times 10^8 \text{ M}^{-1}$). Overall, our data confirm that the HIV-1 RT sequence 398–407 binds to heme and ZnPP and support our suggestion that this sequence corresponds to the binding site for metalloporphyrins on HIV-1 RT. To examine the RT-heme and RT-ZnPP interactions, we measured the fluorescence changes using a fixed enzyme concentration of 200 nM and increasing concentrations of heme and ZnPP (Fig. 2B). The binding of ZnPP to the enzyme was greater ($K_o = 0.36 \times 10^8 \text{ M}^{-1}$) than that of heme ($K_o = 0.15 \times 10^8 \text{ M}^{-1}$). These results are consistent with the RT inhibition assays, in which we showed that ZnPP is a more effective inhibitor of HIV-1 RT than heme. They are also consistent with the requirement for high concentrations of peptide to abrogate heme inhibition of RT (Fig. 1) in that the affinity of peptide for heme is 2 logs lower than that of heme for RT.

**Heme Does Not Inhibit RT by Promoting Dissociation of the Dimer**—It should be noted that the addition of heme or ZnPP to HIV-1 RT did not alter the maximum of the emission spectrum, suggesting that no new Trp residues are exposed when metalloporphyrins bind to the enzyme. This argues against a mechanism for heme inhibition in which the metalloporphyrin promotes dissociation of the RT dimer by binding to the connection domain. To test this hypothesis, we also performed sedimentation equilibrium measurements by analytical ultracentrifugation, and no dissociation of the RT dimer in the presence of heme was observed (data not shown).

**The Crystal Structure of HIV-1 RT Indicates That Sequence 398–407 Is Sufficiently Exposed to Provide a Site for Heme**—The recent availability of several crystallographic structures of the enzyme complexed with double-stranded DNA as well as with non-nucleoside inhibitors has promoted our understanding of the structure-function relationship of the biochemical reaction catalyzed by HIV-1 RT and facilitated the rational design of more specific and potent inhibitors. Crystal structures of non-nucleoside inhibitors such as nevirapine (31), 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (32), o-anilinophenylacetamide (32), and tetrabromoimidazo[4,5,1-\(\text{jb}\)1,4-benzodiazepin-2\(^{1\text{H}}\)-one (33) complexed with HIV-1 RT revealed that these structurally diverse molecules all bind at a common site in the p66 subdomain, although each exhibits slightly different interactions with the enzyme. The binding site for the NNIs is located close to the p66 polymerase active site and is more of a hydrophobic cavity than a pocket. All inhibitors are able to bind to this site in a “butterfly” shape that is a native structural feature of the molecule or one that the molecule can adopt with little energy penalty. In the native enzyme, the drug-binding cavity does not exist; it is created by the movement of the side chains of Trp229, Tyr181, and Tyr 188 in the enzyme, the drug-binding cavity does not exist; it is created by the movement of the side chains of Trp229, Tyr181, and Tyr 188 in the native enzyme, the drug-binding cavity does not exist; it is created by the movement of the side chains of Trp229, Tyr181, and Tyr 188 in the native enzyme, the drug-binding cavity does not exist; it is created by the movement of the side chains of Trp229, Tyr181, and Tyr 188 in the native enzyme, the drug-binding cavity does not exist; it is created by the movement of the side chains of Trp229, Tyr181, and Tyr 188 in the native enzyme, the drug-binding cavity does not exist; it is created by the movement of the side chains of Trp229, Tyr181, and Tyr 188 in the native enzyme, the drug-binding cavity does not exist; it is created by the movement of the side chains of Trp229, Tyr181, and Tyr 188. Our study indicates that heme has a completely different binding site on HIV-1 RT as compared with the known binding site of the NNIs. As shown in Figs. 3 and 4, molecular modeling indicates that the highly conserved sequence 398–407 from the connection domain of HIV-1 RT is sufficiently exposed to provide surfaces of appropriate size for heme binding. Given that the two subunits have the same amino acid sequence throughout the polymerase region, sequence 398–407 occurs twice in the RT dimer and once in each of the p66 and p51 subunits. This sequence overlaps \(\alpha\)-helix L from the connection domains, sequence 395–404 in p66 and sequence

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**TABLE III**

Inhibitory effects of metalloporphyrins on HIV-2 RT activity

|          | Inhibition % |
|----------|--------------|
| Heme     |              |
| 5 μM     | 10           |
| 10 μM    | 21           |
| 20 μM    | 44           |
| 40 μM    | 88           |
| 80 μM    | 100          |
| ZnPP     |              |
| 5 μM     | 40           |
| 10 μM    | 72           |
| 20 μM    | 100          |

Results are the mean of two experiments.

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**FIG. 2.** Fluorescence properties of synthetic P1 and control P2 peptide (A) and HIV-1 RT (B) binding to heme and ZnPP.
1395–1404 in p51, as defined in the RT three-dimensional structure (37). Each sequence 398–407 is partially exposed on the front surface, passes through the interface of the p66 and p51 dimer, and has another exposed surface on the other side. The largest exposed area, which could accommodate a heme molecule bound with the tetrapyrrole groups planar to the RT surface, occurs on the back surface of the p66 domain (Fig. 3C and Fig. 4B). The other three exposed surfaces are narrower and would require heme to bind perpendicular to the RT surface (Fig. 3, A, B, and D; and Fig. 4, A and B) perhaps through hydrophobic contact with the vinyl groups.

**DISCUSSION**

At present, RT represents one of the major targets in the development of chemotherapeutic drugs against HIV, the etiologic agent responsible for the development of AIDS. Inhibitors that have already been developed can be divided into nucleoside analogs that are active in the triphosphorylated form and non-nucleosides that interact directly with RT (1, 2). There have also been attempts to develop other classes of RT inhibitors, such as antisense RNA and sense oligonucleotides (38–40), ribozymes (41, 42), aptamers (43), and oligopeptides (44), that exert an inhibitory effect by interfering with the dimer-
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ization process of RT.

Metalloporphyrins have been shown to represent potent non-nucleoside inhibitors of HIV-1 RT and viral replication. In a recent study, noncompetitive inhibition of HIV-1 RT activity with respect to the nucleoside-binding site was demonstrated (7), suggesting a distinct but allosterically linked binding site for heme and its analogs as compared with that for AZT. In the present work, we have further investigated the mode of heme and ZnPP inhibition of HIV-1 RT. Using in vitro RT inhibition assays, we have shown that metalloporphyrins, in combination with the well characterized NNI, BHAP, inhibit the enzyme in a noncompetitive and nonexclusive manner and enhance the inhibitory effect of BHAP by wielding an additive effect on the enzyme inhibition, which indicates that heme and its analogs inhibit HIV-1 RT by binding to a distinct site as compared with the common binding site of NNIs.

To investigate the sites on HIV-1 RT that might be the target of heme binding, we screened a phage peptide library against heme and isolated heme-specific peptides that were highly enriched in Trp and Tyr residues, indicating that these residues are important for heme binding. It is well documented that the specific structure of heme enables this small molecule to interact with proteins and peptides in various ways. The fifth and sixth coordination sites of the iron can ligand with various amino acids, whereas the propionyl and vinyl side chains of the tetrapyrrole ring are able to interact with appropriate amino acids via ionic and hydrophobic interactions, respectively (45). We believe that the high frequency of Trp and Tyr residues in the peptides identified by phage display arises due to the interaction of the Trp and Tyr residues in the heme-specific displayed peptides with the heme molecule via π-π aromatic stacking. Nevertheless, comparison of our heme-specific peptide sequences with those in protein sequence databases revealed complete or partial homology between almost all of our peptide sequences and a large number of different protein sequences, including known heme-binding proteins. It was also not surprising that we found a match between one clone, A-7, and the highly conserved sequence 398–407 from the connection domain of HIV-1, HIV-2, and SIV RTs, indicating that this region corresponds to the binding site for heme and for other heme analogs on RT. It should be noted that the identification of a ligand-binding site in a protein by random peptide libraries is not unprecedented. Similar studies have described the isolation of phage-displayed peptides specific for different proteinaceous targets, which led to the identification of ligand-binding sites in proteins known to interact with those targets (46, 47).

This paper also provides the first demonstration that heme and its synthetic analog, ZnPP, are potent inhibitors of HIV-2 RT, thus strongly supporting our suggestion that metalloporphyrins inhibit both HIV-1 and HIV-2 (and perhaps SIV) RTs by binding to region 398–407 from the connection domain of the enzymes, which is conserved between these species. The ability of a synthetic peptide corresponding to the exact sequence 398–407 of HIV-1 RT to reverse the inhibitory effect of both heme and ZnPP on HIV-1 RT activity is consistent with our findings. Intrinsic fluorescence assays also indicated that metalloporphyrins bind to the HIV-1 RT synthetic peptide 398–407, although with much lower affinity than to intact dimeric enzyme. In addition, sedimentation equilibrium measurements by analytical ultracentrifugation also demonstrated that heme binds to the dimeric form of RT, which argues against a mechanism for heme inhibition in which porphyrin promotes dissociation of the RT dimer. Support of our suggestion that metalloporphyrins inhibit RT from HIV-1 and HIV-2, by binding to region 398–407 from the connection domain of the enzymes, is provided by the finding that heme does not inhibit RT from feline immunodeficiency virus, where region 398–407 (WESNLINSPY) (48) from the connection domain of the enzyme is dissimilar to that of HIV-1, HIV-2, and SIV.2

As indicated by molecular modeling, the highly conserved sequence 398–407 from the connection domain of HIV-1 RT is sufficiently exposed to provide surfaces of appropriate size for heme binding (Figs. 3 and 4). There are several possible modes for heme binding to the RT heterodimer. Heme may bind to sequence 398–407 either at the front or back of the p66 or p51 connection domain at either exposed site. Alternatively, it may bind to both subunits on a larger site including surface residues not identified by the phage-displayed peptide library, such as Trp110, which is also highly exposed and links the p66 and p51 peptide sites on the back of the molecule (Fig. 3B). Only one of these four potential heme-binding sites could obviously interfere with RT enzyme activity. This is the p51 front exposed surface shown in Fig. 3A, in which Trp398, Glu399, Thr400, and Trp402 are partially exposed. Given that α-helix L of the p51 subunit has been shown to make contact with the primer strand (37), binding to this site could interfere with the binding of the primer-template. If the region shown in Fig. 3A is indeed the heme-binding site, then this is a rare example of a site of inhibition in the p51 monomer of RT. To our knowledge, the only other residue in the p51 monomer implicated in a site of inhibition is Glu138, which may be involved in inhibition by NNIs (49). Heme binding to the other three sites, however, could only inhibit RT activity by indirectly disturbing the conformation of the molecule in the active-site region.

The mechanism of heme inhibition clearly merits further study. The identification, here, of a possible site or sites of inhibition will facilitate this process. Site-directed mutagenesis to generate single or extended amino acid substitutions within the coding region of HIV-1 RT, with specific emphasis on region 398–407 from the connection subdomains of the enzyme, will facilitate the determination of the mechanism of heme binding to HIV-1 RT and its inhibitory effect on the enzyme. In addition, p66 and/or p51 could be subjected to subunit-specific mutagenesis to distinguish which of the four possible heme-binding sites on RT (Fig. 3, A and B) are sites of inhibition. This work demonstrates that heme and ZnPP are indeed true HIV-1 and HIV-2 RT inhibitors by binding to a distinct binding site on the enzyme and encourages the design and preparation of a new class of porphyrin-based systems that will have a more potent inhibitory effect on HIV-1 RT, especially in soluble forms.

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