Protein recognition of macrocycles: Binding of anti-HIV metalloccyclams to lysozyme

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The macrocyclic antiviral drug xylyl-bicyclam blocks entry of HIV into cells by targeting the CXCR4 coreceptor, a seven-helix transmembrane G-protein-coupled receptor. Its affinity for CXCR4 is enhanced by binding to Cu2+, Ni2+, or Zn2+. Metalloccyclams have a rich configurational chemistry and proteins may bind selectively to specific metalloccyclam configurations. Our studies of lysozyme reveal structural details of protein–metalloccyclam interactions that are important for receptor recognition. Solution NMR studies show that Cu-cyclam interacts with specific tryptophan residues of lysozyme (Trp-62, Trp-63, and Trp-123). Two major binding sites for both Cu-cyclam and Cu2-xylyl-bicyclam were detected by x-ray crystallography. In the first site, Cu2+ in one cyclam ring of Cu2-xylyl-bicyclam adopts a trans configuration and is coordinated to a carboxylate oxygen of Asp-101, whereas for Cu-cyclam two ring NH groups form H bonds to the carboxylate oxygens of Asp-101, stabilizing an unusual cis (folded) cyclam configuration. For both complexes in this site, a cyclam ring is sandwiched between the indole side chains of two tryptophan residues (Trp-62 and Trp-63). In the second site, a trans cyclam ring is stacked on Trp-123 and H bonded to the backbone carbonyl of Gly-117. We show that there is a pocket in a model of the human CXCR4 coreceptor in which trans and cis configurations of metalloccyclams can bind by direct metal coordination to carboxylate side chains, cyclam-NH–carboxylate H bonding, together with hydrophobic interactions with tryptophan residues. These studies provide a structural basis for the design of macrocycles that bind stereospecifically to G-coupled and other protein receptors.

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Fig. 1. Structures of the major configurations of a metal cyclam and of xylyl-bicyclam (the hydrochloride salt is the drug AMD3100).
adducts of metal cyclams. In particular, we have suggested (11) that carboxylate groups in a protein positioned above the faces of a metallo-yclam might stabilize the unusual cis-V configuration. To explore the structures of protein–cyclam adducts, we have studied the binding of metal–cyclam and metal–bicyclam complexes to lysozyme, chosen as a model protein because it is readily crystallized (e.g., ref. 13) and the active site contains carboxylates from Glu-35 and Asp-52 in close proximity. These amino acid side chains are known to bind to metal ions such as Gd³⁺ (14). Our data provide structural insight into specific polar and nonpolar interactions involved in the recognition of metallo-yclams by proteins, features that can be found in a binding pocket for anti-HIV metallo-yclams on the human CXCR4 coreceptor.

Experimental Section

Materials. Hen egg white lysozyme (HEWL) and cyclam were purchased from Sigma. Xylyl-cyclam and ZnN(xylyl-bicyclam)-(ClO₄)₂ were prepared as previously described (11). (¹⁵N)Cy-cyclam was synthesized by using the route shown in Scheme 1 with (¹⁵N)phthalimide (98 atom % ¹⁵N) and (¹⁵N)glycine (99 atom % ¹⁵N), both from Aldrich, as sources of ¹⁵N, and the Zn complex was prepared and characterized as described in ref. 15. [Cu(cyclam)(H₂O)₂]ClO₄ (1). Cyclam (400.7 mg, 2 mmol) was dissolved in methanol (50 ml), and Cu(OAc)₂ (363.5 mg, 2 mmol) was added. The reaction mixture was heated to reflux, stirred for 2 h, and then filtered to give a clear, purple solution. The solvent was removed in vacuo, and the purple solid was recrystallized by slow diffusion of methanol into ether to give purple needle crystals of 1-2MeOH (m/z = 322.2 [CuCl₁₀N₄H₂₃O₂]⁺, 262.0 [CuCl₉N₄H₂₃]⁺). Samples of this complex used in NMR studies did not contain MeOH, which is readily lost on drying the complex. [Cu(xylyl-bicyclam)(OAc)₂] (2). Xylyl-bicyclam (50.2 mg, 0.1 mmol) was dissolved in methanol (5 ml) and Cu(OAc)₂ (36.9 mg, 0.2 mmol) was added. The dark blue solution was heated under reflux for 2 h, filtered, and concentrated on a rotary evaporator to give a dark blue crystalline material, which was recrystallized from methanol to give dark blue crystals (m/z = 805.1, [Cu₂C₁₂N₄H₂₇O₄]⁺).

Lysozyme Crystallization. The hanging-drop method was used. The reservoir solution contained 100 μl of 50 mM acetate buffer, pH 4.5, 200 μl of saturated NaCl solution, and 700 μl of distilled water, and the hanging drop contained 2.5 μl of HEWL (50 mg/ml) and 2.5 μl of the reservoir solution. Crystals suitable for x-ray diffraction were grown at 277 K within a period of 7 weeks. Crystals of HEWL complexes grew at 277 K within a period of 7 weeks. Crystals of HEWL complexes were grown at 277 K within a period of 7 weeks. Crystals of HEWL complexes were grown at 277 K within a period of 7 weeks.

Crystal Soaking. Soaking was carried out for 5 days at 288 K with HEWL crystals in drops to which either solid NiO or CuCl₂ had been added (to saturation) or 2 had been added as a saturated solution in the well solution. Soaked crystals became purple, and they were removed in a cryoloop and frozen in liquid nitrogen by using type A immersion oil as a cryoprotectant.

X-Ray Crystallography. Diffraction data for complex 1 were collected with Mo-Kα radiation at 150 K on a Bruker SMART APEX charge-coupled device diffractometer equipped with an Oxford Cryosystems low-temperature device. Systematic errors were treated with SADABS (16). The structure was solved by Patterson methods (DIRDIF (17)) and refined by least squares against F² (CRYSTALS (18)). H atoms were placed on C in calculated positions, but those attached to N and O were located in difference maps and refined. Crystal data: Orthorhombic, space group Pbca, a = 15.9421(7) Å, b = 7.0819(3) Å, and c = 20.9833(10) Å. The final conventional R factor was 0.0341; other data have been deposited in the Cambridge Structural Database.

Diffraction data for HEWL complexes were collected at Station 14.2 at the Daresbury Synchrotron Radiation Source and processed by using the programs MOSFLM and SCALA (19). The initial structure was solved by using a reported lysozyme structure (ref. 13, PDB code 193L). Refinement was performed by using the program REFMAC (20) with waters being added by ARP/WARP, and manual checking and correction were performed with the program O (21). Data collection and refinement are summarized in Table 1. The positions of the metal ions in the structures of the adducts were confirmed in anomalous difference maps produced from data on HEWL crystals soaked with Ni-cyclam at a wavelength of 1.488 Å (Daresbury Synchrotron Radiation Source Station 14.1; data not shown).

NMR Spectroscopy. ¹H NMR data were acquired over a range of 5 ppm at 298 K on a Bruker Avance 600 (¹H = 599.92 MHz) NMR spectrometer using a TXI ¹H, ¹³C, ¹⁵N cyroprobe, equipped with z-field gradients. ¹H chemical shifts were referenced internally to dioxane (3.75 ppm). All NMR experiments were performed in 90% H₂O/10% D₂O. The water resonance was suppressed by presaturation. Standard pulse sequences were used for 2D total correlation spectroscopy (TOCSY) and correlation spectroscopy (COSY) (mixing times of 60 and 80 ms, respectively).

The pH of 5 mM solutions of HEWL in 90% H₂O/10% D₂O used for NMR titrations was adjusted to 4.6 with 0.1 M HCl. Titrations were carried out by adding microliter aliquots of aqueous Cu-cyclam (125 mM, pH 4.6). The assignments for ¹H NMR resonances of HEWL are based on those given by Noda et al. (22).

Mass Spectrometry. Positive-ion electrospray ionization mass spectra were acquired on a Micromass Platform II mass spectrometer, collision energy 4 eV, source temperature 353 K, capillary voltage 1.5 kV, cone voltage 25 V (Cu complexes 1 and 2) or 90 V (protein). Aqueous solutions were diluted 1:1 with acetonitrile before injection.
Supporting Information. Scheme 1 showing the synthetic route to (15N)cyclam and Figs. 4–10 showing 1D and 2D NMR spectra, x-ray crystal structure of complex 1 in 2MeOH, lysozyme crystals, crystal packing, contact distances, and comparison of tryptophan sandwiches are published as supporting information on the PNAS web site. Crystallographic data have also been deposited as follows: complex 1 in the Cambridge Crystallographic Data Centre CCDC 260045, Cu-cyclam-HEWL in the Protein Data Bank 1YIK, and Cu2-bicyclam-HEWL in the Protein Data Bank 1YIL.

Results and Discussion
Interaction of Zn-Cyclams with Lysozyme. First we sought to demonstrate that Zn2-bicyclam binds to lysozyme. Electrospray ionization mass spectrometry of an aqueous solution containing lysozyme and Zn2(bicyclam)(ClO4)2 in a 1:1 molar ratio gave two well resolved series of charged states with masses in the transformed spectrum of 14,308 ± 1 and 14,943 ± 9 Da corresponding to lysozyme and an adduct with [Zn2(bicyclam)]2+, respectively. An interaction of Zn-cyclam with HEWL was detected by using Zn2([15N]cyclam)Cl2 and 2D 1H, 13C NMR spectroscopy (Fig. 4). Of the three cross-peaks for the trans-I, trans-III, and cis-V configurations of Zn-cyclam, those for trans-I and cis-V shifted by 0.04 and 0.13 ppm, respectively, in the presence of HEWL (calculated from [(ΔδRHN)2 + (∆δN × 0.17)]1/2 (23)), whereas that for trans-III was unaffected. These experiments show that both zinc cyclam and zinc bicyclam bind to HEWL, and thus suggest that HEWL is a useful model for identification of specific interactions relevant to the binding of metalloccyclams to the CXCR4 coreceptor.

Cu-xylel-bicyclam also exhibits anti-HIV activity, although it is less active than Zn2-xylel-bicyclam (8). We used Cu2+-cyclam as a paramagnetic probe to investigate interactions of metalloccyclams with the model target protein HEWL in solution. The structures of Cu-cyclam (1 in 2MeOH, Fig. 5) and Zn-cyclam ([Zn(cyclam)(H2O)2][OAc]2 (15)) are similar.

Cu-Cyclam–HEWL Interactions in Solution. Resonances from protons in the protein that are within a few ångstroms of Cu2+ in a bound Cu-cyclam would be expected to broaden significantly on account of the paramagnetism of Cu2+ [3d5; dipolar broadening ∝ r−6 (24), where r = Cu–H distance]. The main features of the 1D 1H NMR spectrum of HEWL were unchanged after addition of up to 10 mol equiv of the Cu-cyclam complex 1. The pH of the solution (4.6) was chosen so as to be close to that under which lysozyme readily crystalizes. As is evident from the plot of linewidth versus Cu-cyclam concentration (Upper), there are specific paramagnetic broadening effects on Trp-62, Trp-63, and Trp-73, implying that these residues are close to the bound Cu2+. The linewidth of the backbone NH resonance of Cys-76 is also shown for comparison.

In the structure of Cu2-bicyclam-HEWL, two cyclam rings were readily identified. Both adopt trans configurations (Fig. 3A). No electron density was present for the p-phenylenebis(methylene) group that links the two cyclam rings. Although the rings in the two sites are close (Cu–Cu distance 8.45 Å), it was not possible to model the two cyclam rings as part of a single bicyclam unit (therefore only monomicyclam rings were built and refined). The next closest cyclam ring in the crystal is too distant (Cu–Cu distance 14.87 Å) to be modeled as part of a single bicyclam. Hence it appears that the cyclam rings in sites 1 and 2 belong to two different Cu2-bicyclam molecules.

In Cu-cyclam-HEWL, the Cu in site 1 is in a slightly different position from that in the bicyclam structure, and the cyclam ring in this structure has strong density consistent with a cis configuration. Copper in site 2 is in a position similar to that found in the bicyclam structure, and the planarity of the cyclam ring is indicative of the presence of a trans configuration (Fig. 3B). Cu-cyclam itself in 2MeOH adopts the trans-III configuration even though carboxylate groups are present from acetate, the counter anion (Fig. 5). It is evident, therefore, that the protein plays an important role in stabilizing the bound cis configuration in site 1.

Metal Coordination, H Bonding, and Hydrophobic Interactions. In site 2, the cyclams in both the monomicyclam and bicyclam structures adopt trans configurations. The trans-III configuration is common for octahedral metal cyclam complexes, and trans-I is the backbone carbonyl of Gly-117. These residues are on different regions on the protein surface. The packing of the protein molecules in the crystal brings the binding sites close to each other (Fig. 8). The crystal packing and the interaction between the cyclam rings therefore appear to play a crucial role in the formation of the binding site in the crystal.

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This change appears to be a consequence of the difference in hydrogen bond is formed (Fig. 3). Cu$_2$ from that found in the bicyclam structure (Cu movement 1.61 Å; nate directly to Asp-101 and is in a significantly different position in the monocyclam structure is also very weakly coordinated to a water molecule (Cu–O 3.07 Å) on the opposite side of the ring (Fig. 9). In the monocyclam structure, the cyclam ring adopts a cis configuration. The Cu in this ring does not coordinate directly to Asp-101 and is in a significantly different position from that found in the bicyclam structure (Cu movement 1.61 Å; Fig. 3C). In this folded conformation, two NH–O hydrogen bonds are formed between the carboxylate oxygens of Asp-101 and two NH groups on one face of the cyclam ring (O$^{\text{cis}}$–N$^{\text{cis}}$ 2.70 Å, O$^{\text{cis}}$–N$^{\text{trans}}$ 2.93 Å, Fig. 9). The Cu in this ring coordinates weakly to two water molecules (Cu–O 2.76, 2.88 Å). In both the monocyclam and bicyclam structures, the cyclam in site 1 is sandwiched between the indole rings of Trp-62 and Trp-63 and stacks directly with Trp-62. This hydrophobic interaction with tryptophan would therefore appear to be important for the binding of these cyclams in both cis and trans configurations. Girard et al. (25), in their search for heavy atom derivatives useful in anomalous dispersion methods for solving protein structures, have studied adducts of HEWL with Gd-HPDO3A, the Gd$^{3+}$ complex of a derivative of the smaller [12]aneN$_4$ macrocycle cyclen (1,4,7,10-tetraazacyclododecane). They were unable to produce Gd-HPDO3A-HEWL by soaking, only by cocrystallization. Intriguingly Gd-HPDO3A binds in the same sites 1 and 2 as Cu–(bi)cyclam (Fig. 10C); the main protein interaction is between Trp-62 and the cyclen macrocyle with no direct metal coordination or involvement of H bonds with Asp-101 (which is turned away from the site).

The difference in the orientation of the cyclam plane in site 2 of the monocyclam and bicyclam structures is likely to be a consequence of the difference in conformation of the cyclam in site 1. In both structures, the cyclams interact weakly with each other (closest ring–ring distance 3.21 Å for bicyclam, 3.34 Å for monocyclam. Figs. 9 and 10). The overlay of the monocyclam and bicyclam structures (Fig. 3C) reveals that the different orientations of the plane conserve the tightness of the cyclam–cyclam interaction (which would otherwise increase to 3.62 Å). It is unclear why the cyclam in site 1 is in the trans configuration in the bicyclam structure compared to the cis configuration for monocyclam. The lack of difference between the protein structures does not provide any clues. It would therefore seem likely that this difference is related to the presence of a linker between the cyclam rings for bicyclam (although the linker is disordered).

What are the important factors in stabilization of the cis cyclam configuration? In crystals of the zinc complex of the drug xyllyl-bicyclam, [Zn$_2$(xyllyl-bicyclam)(OAc$_2$)$_2$](OAc)$_2$·2MeOH, one acetate is bidentate and the other forms double H bonds to the NH groups on the opposite face of the cyclam ring that adopts the folded cis-V configuration (11). In the Cu-cyclam-HEWL structure, there is no direct coordination of Cu$^{2+}$ to a carboxylate, only NH hydrogen bonding, and the carboxylate oxygen coordination sites appear to be occupied by weakly bound water molecules. cis-Metalocyclams with two bound aqua ligands are unusual, although Barefield et al. (26) prepared cis-[Ni(cyclam)(H$_2$O)$_3$]Cl$_2$·2H$_2$O by displacement of a chelated ethylenediamine by water under acidic conditions. In solution, the interaction of carboxylic groups with Zn$^{2+}$ cyclams readily leads to the formation of the folded cis-V configuration, equilibria being reached within 1 h at millimolar concentrations, at 298 K (11, 15).

**CXCR4 Binding Sites.** Previously (11) we built a model of the human CXCR4 co-receptor based on the x-ray structure of rhodopsin (27) and docked Zn$_3$-bicyclam such that there is direct coordination of Zn$^{2+}$ in one cyclam ring to the carboxylate of Asp-171, and of Zn$^{2+}$ in the other to Asp-262. In the latter site, Glu-288 on the opposite side of the ring can form double H bonds to two ring NH groups, and the macrocycle folds into the cis-V configuration. In the first site, a trans-I or trans-III configuration is more likely for the cyclam ring. Inspection of the same CXCR4 model now reveals that there are tryptophan indole rings close to each of the docked cyclams (Fig. 3D). Notable is the stacking of Trp-195 on the trans-cyclam ring that contains Zn$^{2+}$ bound to Asp-171. These are all features seen in the HEWL-(bi)cyclam structures. The hydrophobic interaction of the N(CH$_2$)$_3$N and N(CH$_2$)$_3$N backbone of cyclam with tryptophan indole rings has a parallel in the hydrophobic contact of the N(CH$_2$)$_3$CH cyclic side-chain of proline with tryptophan at some protein–protein interfaces, often as a Trp-Pro-Trp.

![Fig. 3. The two Cu-cyclam and Cu$_2$-bicyclam binding sites in crystals of HEWL. (A) Cu-bicyclam-HEWL. 2$F_o$ – $F_i$ density is shown around the cyclams at 0.9σ. No electron density is seen for the p-phenylenebis(methylene) linker. (B) Cu-cyclam-HEWL. 2$F_o$ – $F_i$ density is shown around the cyclams at 0.9σ. (C) Stereo view of an overlay of the cyclam positions in lysozyme. Color code: orange, Cu$_2$-bicyclam; green, Cu-cyclam. The sites are at the intersection of three lysozyme molecules in the crystal (Fig. 8). Molecule 1 is green and contains Asp-101, Trp-62, and Trp-63; molecule 2 is purple and contains Trp-123; and molecule 3 is yellow and contains Gly-117. (D) A model showing [Zn$_2$(xyllyl-bicyclam)]$^{4+}$ bound to the human coreceptor CXCR4 (11). One of the cyclam rings in a trans configuration is stacked on Trp-195 and its Zn is bound to Asp-171. The other cyclam ring has a folded configuration (cis) and is close to Trp-283; its Zn is bound to Asp-262, and two ring NH groups are H bonded to the oxygens of Glu-288.](image-url)
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de likely to crystallize. To date, the only member of this class of proteins for which the x-ray
crystal structure has been solved is bovine rhodopsin (27). We
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therefore a useful model for CXCR4.

The x-ray crystal structures of Cu-cyclam and Cu2-bicyclam
complexes of lysozyme establish that protein recognition can
involve a range of both polar and nonpolar interactions. These
include H bonding between the cyclam ring and protein carbox-
ylate groups, which, for the Cu-cyclam structure, anchors the
cyclam ring in the unusual folded cis configuration without direct
coordination of Cu2+ to the protein. The presence of an addi-
tional cyclam ring, as in a bicyclam, can influence the configu-
ration adopted by the bound macrocycle. Intriguingly, hydro-
phobic interactions between the cyclam ring and the indole ring
of tryptophan, reminiscent of tryptophan-proline sandwiches
often found at protein–protein interfaces, occur not only in
-crystals of HEWL but also in aqueous solution. Such tryptophan
stacking interactions are also possible in models of CXCR4
adducts of metallocyclams, which contain at the same time other
features important for metallocyclam docking, including metal
coordination to Asp-171 and Asp-262 (Fig. 3D). Tryptophan, an
amphipathic amino acid, is often located toward the surface
(interfacial regions) of membrane proteins (29), and may play a
crucial role in the recognition of antiviral metallocyclams and
other macrocycles by the protein coreceptor. Knowledge of these
diverse interactions will aid the design of new generations of
chemokine receptor antagonists.

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