Metal Ion Enhanced Binding of AMD3100 to Asp^{262} in the CXCR4 Receptor†

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ABSTRACT: The affinity of AMD3100, a symmetrical nonpeptide antagonist composed of two 1,4,8,11-tetraazacyclotetradecane (cyclam) rings connected through a 1,4-dimethylene(phenylene) linker to the CXCR4 chemokine receptor was increased 7, 36, and 50-fold, respectively, by incorporation of the following: Cu^{2+}, Zn^{2+}, or Ni^{2+} into the cyclam rings of the compound. The rank order of the transition metal ions correlated with the calculated binding energy between free acetate and the metal ions coordinated in a cyclam ring. Construction of AMD3100 substituted with only a single Cu^{2+} or Ni^{2+} ion demonstrated that the increase in binding affinity of the metal ion substituted bicyclam is achieved through an enhanced interaction of just one of the ring systems. Mutational analysis of potential metal ion binding residues in the main ligand binding crevice of the CXCR4 receptor showed that although binding of the bicyclam is dependent on both Asp^{171} and Asp^{262}, the enhancing effect of the metal ion was selectively eliminated by substitution of Asp^{262} located at the extracellular end of TM-VI. It is concluded that the increased binding affinity of the metal ion substituted AMD3100 is obtained through enhanced interaction of one of the cyclam ring systems with the carboxylate group of Asp^{262}. It is suggested that this occurs through a strong concomitant interaction of one of the oxygen's directly with the metal ion and the other oxygen to one of the nitrogens of the cyclam ring through a hydrogen bond.

The CXCR4 chemokine receptor is expressed on a wide variety of not only leukocytes but also cells outside the immune system. The receptor and its ligand SDF (stromal cell derived factor) are involved in control of migration and tissue targeting, homing of leukocytes (*1*) and apparently even in, for example, control of the metastatic spread and survival of several different forms of cancer cells (*2*). The currently available, most potent, and specific CXCR4 antagonists are the nonpeptide bicyclam derivatives, which are composed of two 1,4,8,11-tetrazazcyclotetradecane (cyclam) moieties connected by a conformationally constraining linker (*3*). These were originally developed as antiviral agents blocking the cell entry of T cell tropic HIV[1] strains. The prototype bicyclam, AMD3100, is a highly specific CXCR4 antagonist that inhibits binding and function of the natural chemokine ligand SDF-1α with high affinity (*4*, *5*) and potency (*4*, *6*). In anti-HIV studies, AMD3100 has been shown to block the outgrowth of all X4 HIV as well as dual-tropic (T cell- and macrophage-tropic) variants that use CXCR4 for entering its target cells (*7*).

The cyclam ring can function as a tetradentate coordination ring for transition metals, and it has been shown that chelation of such metal ions by the macrocyclic rings of AMD3100 alters its binding affinity to the CXCR4 receptor (*8*, *9*). Thus, the Zn^{2+} complex of AMD3100 (Figure 1A) binds with a 10-fold higher affinity to the receptor as compared to AMD3100 alone and has an up to 6-fold increased potency as an anti-HIV agent (*8*, *10*, *11*).

Zn^{2+} is located in the center of the cyclam ring, coordinating the four nitrogens in a planar fashion (*12*). Since Zn^{2+} does not coordinate in a square planar conformation, it either obtains a square pyramidal or an octahedral geometry with one or two vacant coordination sites (*13*). Zn^{2+} has the option to make strong interactions with both histidine and cysteine residues, as well as acidic residues such as aspartates (*14*, *15*). The CXCR4 receptor does not contain any free extracellular cysteines (*16*); however, the main interaction points for AMD3100 are two aspartates, and furthermore, several histidine residues are located in TM-III, TM-V, and TM-VII pointing toward the main ligand binding crevice (Figure 3). Thus, metal ion coordination could either improve the binding mode of AMD3100 to one or more of the two aspartates, Asp^{171} and Asp^{262}, or it could pick up interaction with one or more of the His residues that potentially could serve as partners in the coordination of Zn^{2+} bound by the bicyclam. Prior to the identification of the importance of these aspartate residues, it had been predicted that the improved HIV potency of the Zn^{2+} complex of AMD3100 probably was caused by an improved, optimal interaction of the metal ion cyclam complex with a carboxylate group

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[1] Abbreviations: HIV, human immunodeficiency virus; SDF, stomal cell-derived factor; TM, transmembrane domain; X4, T cell-tropic (CXCR4 dependent).

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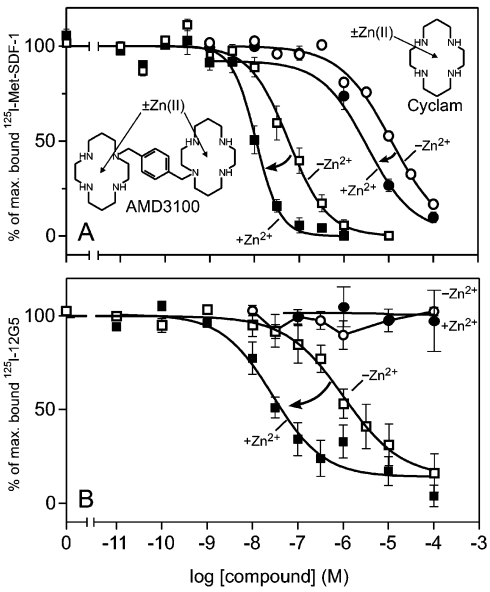

FIGURE 1: Zn^{2+} increases the affinity of AMD3100 and 1,4,8,11-tetrazacyclotetradecane (cyclam) using either ^{125}I-SDF-1α (A) or ^{125}I-12G5 monoclonal receptor antibody (B) as radiotracer. Whole cell competition binding was performed on wild-type CXCR4 receptor expressed in COS-7 cells. Ligands used are AMD3100 (□), AMD3100$(Zn)_2$ (■), cyclam (○), and cyclam(Zn) (●). Data are shown as mean ±SE (n = 3−11).

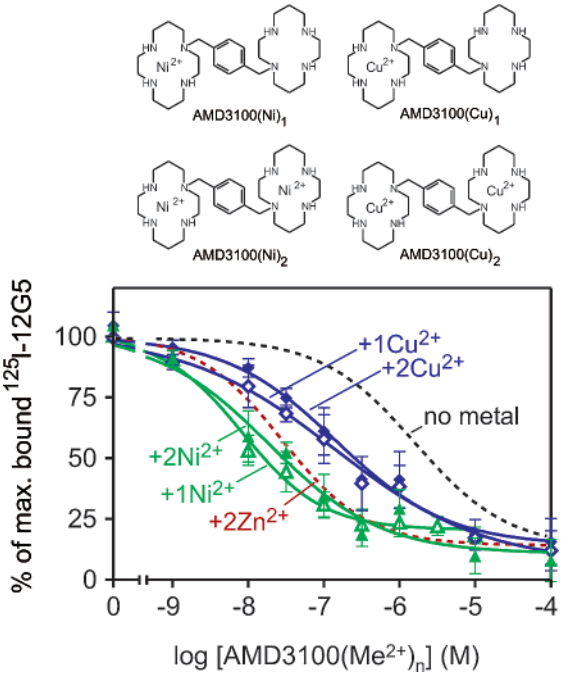

FIGURE 2: Effect of incorporation of one (open symbols) or two (closed symbols) Ni^{2+} or Cu^{2+} ions in AMD3100 on the wild-type CXCR4 receptor. Whole cell competition binding using ^{125}I-12G5 monoclonal receptor antibody as radiotracer. Ligands used are AMD3100 (black dashed line), AMD3100$(Zn)_2$ (orange dashed line), AMD3100$(Ni)_1$ (△), AMD3100$(Ni)_2$ (▲), AMD3100$(Cu)_1$ (◇), and AMD3100$(Cu)_2$ (◆). Data are shown as mean ±SE (n = 3−4).

somewhere in the CXCR4 receptor in analogy with the interaction seen in structural analysis of such complexes with various carboxylates (*9*). It should be noted that X-ray structural analysis has also demonstrated that metal ions can function as bridges between small molecule inhibitors (i.e., metal ion chelators and enzymes, ref *17*). In the latter case,

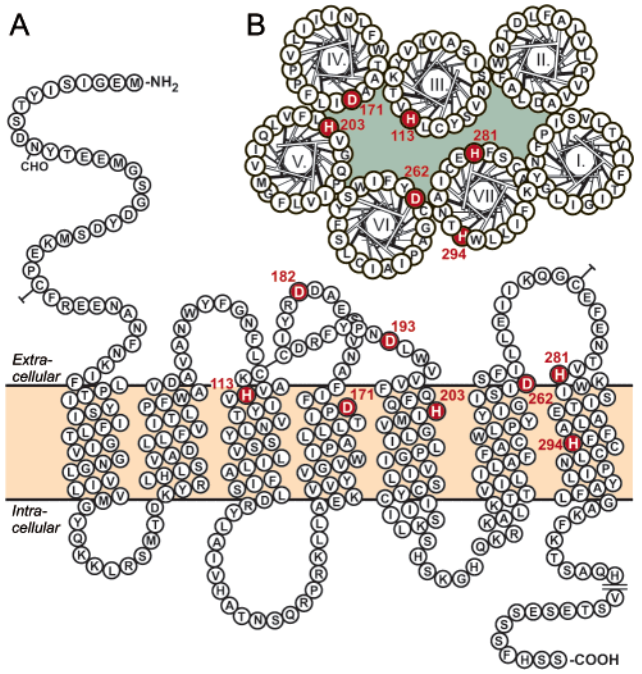

FIGURE 3: Serpentine (A) and helical wheel diagram (B) of the CXCR4 receptor. White letters in red circles represent residues substituted with Ala or Asn probing the amino acid residue responsible for the increased affinity for the Zn^{2+} complex of AMD3100.

the presence of Zn^{2+} enhanced the inhibition of trypsin by the chelator BABIM more than 4000-fold through coordination of two of the residues in the active site of the serine protease (*17*).

The present study is aimed at identifying the molecular basis for the metal ion mediated enhancement of the binding and function of bicyclam nonpeptide antagonists in the CXCR4 receptor through mutagenesis combined with the construction of a series of single and double metal ion substituted analogues of AMD3100. It is found that although AMD3100 is a symmetrical bicyclam, metal ion binding in only one of the rings is responsible for the enhanced binding, and the effect could be demonstrated to be mediated through interaction with a specific residue, Asp^{262} of the receptor.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. Point mutations were introduced in the receptor by the polymerase chain reaction overlap extension technique (*18*) using the human wild-type CXCR4 receptor cDNA as a template. All reactions were carried out using the Pfu polymerase (Strategene) under conditions recommended by the manufacturer. The generated fragments were subcloned into the pTEJ-8 eukaryotic expression vector (*19*) containing the wild-type CXCR4 receptor cDNA by substituting the wild-type cDNA fragment with the mutated cDNA fragment. The mutations were verified by restriction endonuclease digestion and DNA sequencing (ABI 310, Perkin-Elmer Inc.)

Expression of Mutant Receptors. COS-7 cells were grown at 10% CO_2 and 37 °C in Dulbecco's modified Eagle's medium 1885 supplemented with 10% fetal calf serum, 2 mM glutamine, and 10 μg/mL gentamicin. The wild-type CXCR4 receptor and mutant receptors were transiently transfected into COS-7 cells by the calcium phosphate precipitation method as described previously (*20*).

Ligands. The human chemokine Met-SDF-1α was kindly provided from Michael A. Luther, GlaxoSmithKline). This SDF-1α contains an additional NH_2-terminal methionine; however, the protein shows the same binding properties as the natural ligand SDF-1α (*21*, *22*). [^{125}I]Met-SDF-1α was prepared by oxidative iodination using Iodogen (Pierce Warriner Ltd.) followed by HPLC purification to separate unlabeled from labeled compound. The monoclonal antibody 12G5 was kindly provided from Jim Hoxie (University of Pennsylvania, Philadelphia). 12G5 was ^{125}I-labeled using Bolten and Hunter reagent (Amersham International) as described by Signoret et al. (*23*). AMD3100 ([1,1′-[1,4-phenylenebis(methylene)]-bis(1,4,8,11-tetraazacyclotetradecane)), AMD3100$(Cu)_2$, and AMD3100$(Zn)_2$ were synthesized as previously described (*24*). AMD3100$(Ni)_2$ (AMD3100$(Ni(ClO_4)_2)_2$) follows the same route as for AMD3100$(Zn)_2$ using $NiCl_2$ instead of $ZnCl_2$ (*10*). AMD3100-$(Ni)_1$ AMD3100$(Ni(ClO_4)_2)$ was prepared by dissolving 0.90 g (1.08 mmol) of free base AMD3100 in water/dilute HCl (30 mL). The pH was adjusted to 5 with sodium acetate, and 85 mg (0.36 mmol) of $NiCl_2 \cdot 6H_2O$ was dissolved in 10 mL of water and added dropwise to the stirred ligand solution. After 30 min, NaOH was added to adjust the pH to 13, and the mixture was extracted three times with chloroform (pH was checked after each extraction to make sure it was 13). The mixture was treated with dilute HCl to readjust the pH to 5, and excess $NaClO_4$ was added. After several hours, an orange solid was isolated, washed with methanol, and dried in vacuo. AMD3100$(Cu)_1$ (AMD3100-$(Cu(ClO_4)_2)$) was prepared similarly to AMD3100$(Ni)_1$ starting with $Cu(OAc)_2$. Cyclam (1,4,8,11-tetraazacyclotetradecane) was purchased from Aldrich. Cyclam$(Zn)_1$ was prepared by adding 0.27 g (2.0 mmol) of $ZnCl_2$ in 1 mL of water to a stirred solution of cyclam (free base) (0.40 g, 2.0 mmol) in 250 mL of 2-propanol. During this time, a white precipitate formed that was collected and washed three times with 2-propanol.

Receptor Binding Assays. The transfected COS-7 cells were transferred to culture plates 1 day after transfection. The number of cells seeded per well was determined by the apparent expression efficiency of the individual clones; the number of cells per well was adjusted, aiming at 5−10% binding of the added radioligand. Two days after transfection, cells were assayed by competition binding performed on whole cells for 3 h at 4 °C using 12 pM [^{125}I]Met-SDF-1α or 32 pM [^{125}I]12G5 plus variable amounts of unlabeled peptide or nonpeptide compounds in 400 μL of 50 mM HEPES buffer, pH 7.7, supplemented with 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.5% (w/v) bovine serum albumin (Sigma). After incubation, cells were washed quickly two times in 4 °C binding buffer (12G5 binding) or four times in 4 °C binding buffer supplemented with 0.5 M NaCl (SDF-1α binding). Determinations were made in duplicate.

Binding Analysis. IC_{50} values were determined by nonlinear regression using Prism 3.0 (GraphPad Software, San Diego, CA).

Quantum Chemistry Modeling. We have optimized the geometry of six complexes with cyclam as the tetradendate equatorial ligand, acetate as one axial ligand, and water as the second axial ligand. Acetate is the model of the Asp^{262} residues that have been proposed as binding sites in the CXCR4 receptor. The water ligand completes the first coordination sphere of the complex. We emphasize that the usual receptor is highly solvent accessible, so this is likely to be a reasonable choice of a model system. The central ions were (all in their ground state) as follows: Zn^{2+} as the closed-shell singlet state, Ni^{2+} as intermediate spin (M_S = 1), Cu^{2+} as low-spin (M_S = 1/2), Co^{2+} as low-spin (M_S = 1/2), Fe^{2+} as high-spin (M_S = 2), and finally Pd^{2+} as low-spin (M_S = 0). All complexes were optimized in the trans-III conformation, corresponding to an R,R,S,S configuration at the four asymmetric nitrogen atoms. This conformation has been found to be the most stable, both in theory (*25*) (although it is close in energy to the all-R conformation) and experiment (*26*).

Computational Details. All calculations were performed with the B3LYP hybrid functional (*27*). B3LYP is widely recognized as one of the most accurate density functional methods, in general terms, for structures, energies, and frequencies (*28*−*30*). The calculations were carried out with the Turbomole software, version 5.3. The basis set used for optimizing the geometries was the 6-31G(d) for all atoms except the metals. These were described by the DZ basis set of Schäfer et al. (*31*), augmented with two p, one d, and one f function (DZpdf). We applied the default (m3) grid size for sampling the density, and all optimizations were carried out in redundant internal coordinates. Energies were converged down to 10^{-6} hartree (2.6 J/mol) and internal degrees of freedom to 10^{-3} au (0.053 pm or 0.057°). Energies were calculated with the large triple-ζ 6-311+G(2d,2p) basis set, which includes diffuse functions on heavy atoms and two polarization functions on all atoms. The results obtained with this basis set should be close to the basis set limit. The relative binding energy (RBE) to acetate is given by

$$\mathrm{RBE} = \mathrm{E(complex)} - \mathrm{E(complex\ without\ acetate)} - \mathrm{E(reference)} \quad (1)$$

where E(Reference) is defined according to eq 2 since the Ni^{2+} complex has the lowest binding energy of the series of antagonistic models.

$$\mathrm{E(reference)} = \mathrm{E(Ni^{2+}\ complex)} - \mathrm{E(Ni^{2+}\ complex\ without\ acetate)} \quad (2)$$

We calculated this energy both for the

$$H_2O{-}Me{-}acetate \rightarrow H_2O{-}Me + acetate$$

and for the

$$H_2O{-}Me{-}acetate + H_2O \rightarrow H_2O{-}Me{-}H_2O + acetate$$

reactions, to evaluate the possibility of water reducing the binding energy by occupying the vacant coordination site of acetate. However, the energy effect of this possibility was ∼4 kJ/mol. The results of the latter, more general case, are presented in Table 3.

RESULTS

Metal Ion Enhanced Binding of AMD3100. In COS-7 cells transiently transfected with the human CXCR4 chemokine receptor, AMD3100 competed for binding both against the radiolabeled endogenous ligand ^{125}I-SDF-1α (K_i = 74 nM) and against the radiolabeled monoclonal receptor antibody

Table 1: Affinity of AMD3100 and Cyclam with or without Zn(II) for the Wild-Type CXCR4 Receptor and His- and Asp-Substituted CXCR4 Receptor Mutants[a]

	AMD3100			AMD3100(Zn)2				Cyclam			Cyclam (Zn)		
	log $K_i \pm$ SEM	K_i (nM)	$(n)^b$	log $K_i \pm$ SEM	K_i (nM)	(n)	fold to AMD3100	log $K_i \pm$ SEM	K_i (μM)	(n)	log $K_i \pm$ SEM	K_i (μM)	(n)
WT	-7.13 ± 0.10	74	(11)	-7.93 ± 0.08	12.0	(10)	6	-4.90 ± 0.11	13.0	(6)	-5.53 ± 0.13	3.0	(4)
H113A	-6.89 ± 0.01	130	(3)	-7.65 ± 0.14	23.0	(4)	6	-5.07 ± 0.25	8.5	(6)	-5.28 ± 0.19	5.3	(6)
D171N	-5.16 ± 0.03	6900	(4)	-5.78 ± 0.36	1700.0	(5)	4	-3.43 ± 0.11	370.0	(3)	-3.71 ± 0.06	200.0	(5)
D182N	-6.89 ± 0.10	130	(3)	-7.21 ± 0.20	62.0	(3)	2	-4.93 ± 0.09	12.0	(3)	-5.80 ± 0.16	1.6	(3)
D193N	-7.09 ± 0.13	81	(3)	-7.31 ± 0.17	49.0	(3)	2	-5.04 ± 0.11	9.2	(3)	-5.80 ± 0.14	1.6	(3)
H203A	-7.17 ± 0.16	68	(3)	-7.44 ± 0.18	36.0	(4)	2	-4.79 ± 0.16	16.0	(3)	-5.89 ± 0.30	1.3	(3)
D262N	-5.90 ± 0.08	1300	(5)	-6.00 ± 0.04	1000.0	(4)	1	-5.02 ± 0.22	9.6	(4)	-5.64 ± 0.29	2.3	(4)
H281A	-7.62 ± 0.17	24	(5)	-8.06 ± 0.07	8.7	(4)	3	-4.49 ± 0.15	33.0	(4)	-5.30 ± 0.14	5.0	(4)
H294A	-6.86 ± 0.05	140	(3)	-7.47 ± 0.16	34.0	(4)	4	-4.59 ± 0.13	26.0	(3)	-5.46 ± 0.07	3.4	(3)

[a] The data were obtained from competition binding in COS-7 cells expressing the wild-type and mutant receptors using 125I-SDF-1a as radioligand. [b] Values in parentheses represent number of experiments (n).

Table 2: Affinity of AMD3100 and AMD3100(Zn)2 for the Wild-Type CXCR4 Receptor and His- and Asp-Substituted CXCR4 Receptor Mutants[a]

	AMD3100			AMD3100(Zn)2			
	log $K_i \pm$ SEM	K_i (μM)	$(n)^b$	log $K_i \pm$ SEM	K_i (nM)	(n)	fold to AMD3100
WT	-6.00 ± 0.12	1.00	(5)	-7.55 ± 0.20	0.028	(3)	36
H113A	-5.84 ± 0.46	1.40	(3)	-7.00 ± 0.22	0.100	(4)	14
D171N	-4.92 ± 0.29	12.00	(4)	-6.28 ± 0.22	0.520	(4)	23
D182N	-5.49 ± 0.13	3.21	(3)	-7.14 ± 0.33	0.072	(3)	45
D193N	-6.04 ± 0.22	0.91	(3)	-7.16 ± 0.15	0.069	(3)	13
H203A	-5.69 ± 0.22	2.04	(2)	-7.28 ± 0.03	0.052	(2)	39
D262N	-4.76 ± 0.38	17.40	(4)	-4.89 ± 0.28	13.000	(3)	1
H281A	-5.97 ± 0.19	1.10	(2)	-7.29 ± 0.41	0.052	(2)	21
H294A	-6.29 ± 0.19	0.51	(2)	-7.65 ± 0.38	0.022	(2)	23

[a] The data were obtained from competition binding in COS-7 cells expressing the wild-type and mutant receptors using 125I-12G5 as radioligand. [b] Values in parentheses represent number of experiments (n).

Table 3: Calculated Properties of Water-acetato-Me(II)-cyclams

metal ion	Ni(II)	Zn(II)	Co(II)	Cu(II)	Pd(II)
spin of ground state	intermediate	low	low	low	low
M−O(acetate) (Å)	1.99	1.96	2.15	2.14	3.35/3.43
M−O(water) (Å)	3.22	3.06	2.9	3.27	4.02
RBE (kJ/mol)[a]	0	1	38	49	338

[a] Relative bond energy, where the Ni(II) complex is defined as reference. Higher energies correspond to weaker binding.

^{125}I-12G5 ($K_i = 1$ μM). Incorporation of Zn^{2+} into the two macrocyclic rings of AMD3100 (AMD3100$(Zn)_2$) increased the affinity of the bicyclam compound 6-fold as determined in competition against ^{125}I-Met-SDF-1α ($K_i = 12$ nM, Figure 1A, Table 1) and 36-fold as determined against ^{125}I-12G5 binding ($K_i = 28$ nM, Figure 1B, Table 2). It should be noted that the difference in the affinity determined in the two binding assays reflects the fact that we are not measuring the actual affinity of the ligands but rather their ability to displace the two different radiolabeled tracers from the CXCR4 receptor.

The key receptor recognition motifs of AMD3100 are the two identical 1,4,8,11-tetraazacyclotetradecane cyclam rings. Incorporation of Zn^{2+} into the free cyclam moiety increased its apparent affinity for the CXCR4 receptor in a similar manner as for AMD3100 (i.e., 4-fold as determined against ^{125}I-SDF-1α binding) (Figure 1A, Table 1). The empty cyclam moiety was, as previously observed, unable to displace ^{125}I-12G5 from the CXCR4 receptor even at millimolar concentrations (*5*). It is interesting to note that not only did Zn^{2+} binding increase the affinity of AMD3100, it also increased the Hill coefficient of the binding curve from 1 to 1.6, indicating a more complex interaction mode. This was not the case for the free cyclam compound (Figure 1).

Incorporation of Ni^{2+} and Cu^{2+} into the cyclam rings of AMD3100 also increased its affinity for the CXCR4 receptor with AMD3100$(Ni)_2$ showing a similar or slightly higher affinity than AMD3100$(Zn)_2$, whereas Cu^{2+} did not increase the affinity of the bicyclam as efficiently as did Zn^{2+} and Ni^{2+} (Figure 2, Table 2). For Ni^{2+} and Cu^{2+}, the monosubstituted analogues were also prepared (i.e., AMD3100 with a metal ion incorporated into only one of the cyclam moieties). In both cases, the monosubstituted analogues competed for binding to the CXCR4 receptor with an affinity identical to that of the corresponding bisubstituted analogues (Figure 2, Table 2). This indicates that only one of the chelated metal ions is responsible for the enhanced binding of the bisubstituted AMD3100 and that the other metal ion substituted cyclam ring conceivably is recognized in a similar manner as the unsubstituted ring.

Mutational Mapping of the Metal Ion Enhancement of AMD3100 Binding. Previously we have performed a mutational analysis of the binding site for AMD3100 and identified Asp^{171} and Asp^{262} as being key residues involved in the binding of the unsubstituted bicyclam compound (*5*). In principle, the metal ion enhancement could be due to either an improved binding to one of these residues or it could be caused by a metal ion interaction with another residue in the receptor. Carboxylic acids can both form complexes with metal containing cyclam rings (*9*) and bind the positively

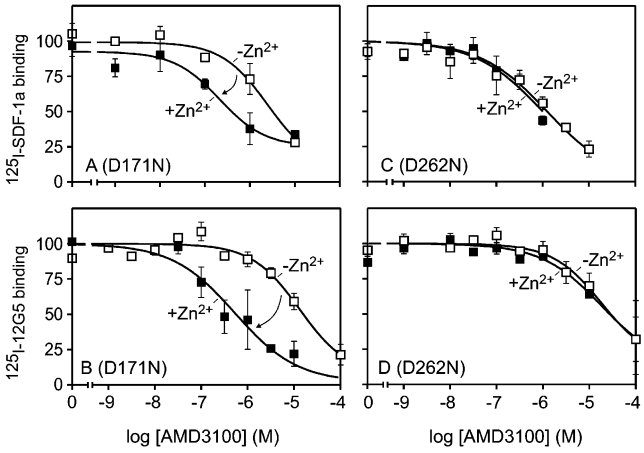

FIGURE 4: Effect of Asp-to-Asn substitution at position 171 (A and B) and 262 (C and D) in the CXCR4 receptor on the Zn^{2+} enhancing effect on AMD3100. Whole cell competition binding using either ^{125}I-SDF-1α (A and C) or ^{125}I-12G5 monoclonal receptor antibody (B and D) as radiotracer. Ligands used are AMD3100 (□) and AMD3100$(Zn)_2$ (■). Data are shown as mean ±SE (n = 3−5).

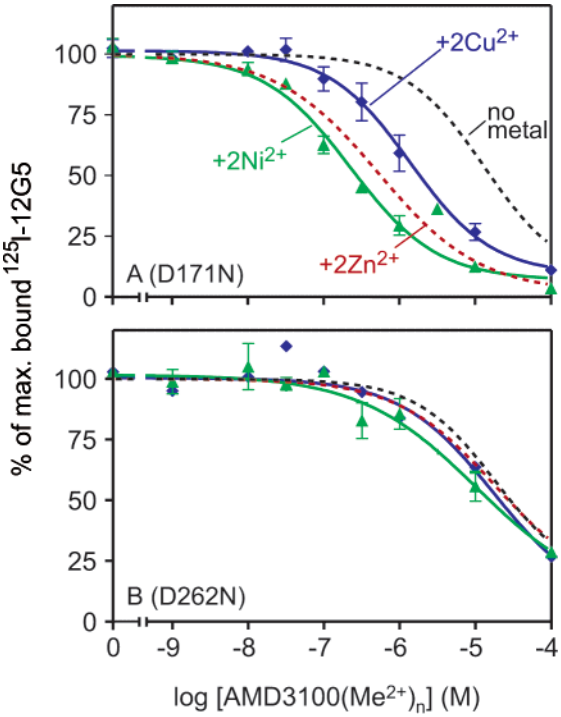

FIGURE 5: Effect of incoorporation of two Ni^{2+} or Cu^{2+} ions in AMD3100 on the D171 (A) and D262N (B) CXCR4 receptor. Whole cell competition binding using ^{125}I-12G5 monoclonal receptor antibody as radiotracer. Ligands used are AMD3100 (black dashed line), AMD3100$(Zn)_2$ (orange dashed line), AMD3100$(Ni)_2$ (▲), and AMD3100$(Cu)_2$ (◆). Data are shown as mean ±SE (n = 2−4).

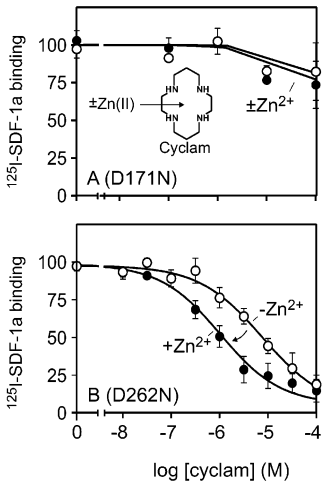

FIGURE 6: Effect of Asp-to-Asn substitution at position 171 (A) and 262 (B) in the CXCR4 receptor on the Zn^{2+} enhancing effect on cyclam. Whole cell competition binding using ^{125}I-SDF-1α as radiotracer. Ligands used are cyclam (○) and cyclam(Zn) (●). Data are shown as mean ±SE (n = 3−5).

charged macrocyclic ring by electrostatic interactions (*32*). Four Asp mutations (D171N, D182N, D193N, and D262N) (Figure 3) were tested for their ability to eliminate the affinity enhancing effect of incorporation of Zn^{2+} into AMD3100. In addition, the four His residues located in the extracellular or transmembrane domains (i.e., H113A, H203A, H281A, and H294A) (Figure 3) were tested as a histidyl could also coordinate the metal ion inserted into the macrocyclic ring of AMD3100.

As shown previously (*5*), all of the single mutations were expressed well in COS-7 cells, and all bound the chemokine ligand SDF-1α as well as the receptor antibody 12G5 with similar affinity as the wild-type CXCR4 receptor. In all receptor mutants—except the Asn substitution of Asp^{262}—a similar increase in affinity of AMD3100$(Zn)_2$ as compared to AMD3100 was observed in competition both against ^{125}I-SDF-1α and against ^{125}I-12G5 (Tables 1 and 2). As shown in Figure 4, although mutation of either Asp^{171} or Asp^{262} decreases the affinity of the unsubstituted AMD3100 as compared to the wild-type receptor, incorporation of Zn^{2+} into the cyclam rings still enhances the binding of AMD3100 to a similar degree in the Asn for Asp^{171} mutation as it does in the wild-type CXCR4 receptor. In contrast, the Zn^{2+} effect is totally eliminated by the Asn for Asp^{262} mutation. Similarly, the enhanced binding observed with Ni^{2+} and Cu^{2+} in the wild-type receptor was preserved in the Asn for Asp^{171} mutation but was eliminated in the Asn for the Asp^{262} mutant (Figure 5).

As previously shown, the effect of the free, unsubstituted cyclam moiety on SDF-1α binding is totally dependent on Asp^{171}, as was also observed for the Zn^{2+} substituted, free cyclam (Figure 6A). Interestingly, whereas no metal ion effects were observed for AMD3100 in the Asn for Asp^{262} mutant, a clear 4-fold increase in affinity was observed for the free cyclam when Zn^{2+} was incorporated into the ring (Figure 6B). This indicates that the free cyclam ring is able to bind with an increased affinity to Asp^{171} when Zn^{2+} is incorporated into the ring, which apparently is not the case when the cyclam ring is found in AMD3100.

Binding Modes for Metal Ion Cyclam Complexes with Carboxylates. To study the presumed receptor−ligand interaction in more detail, we constructed a local model of the interaction between a carboxylic acid and a cyclam chelating a transition metal described by quantum chemistry. The geometries and relative bond energies of Zn^{2+}, Ni^{2+}, or Cu^{2+} (coordinated in the cyclam ring system) to acetate were computed by density functional (DFT) methods. Earlier studies on 4-coordinate metal cyclams have shown that DFT structures for similar systems are in excellent agreement with

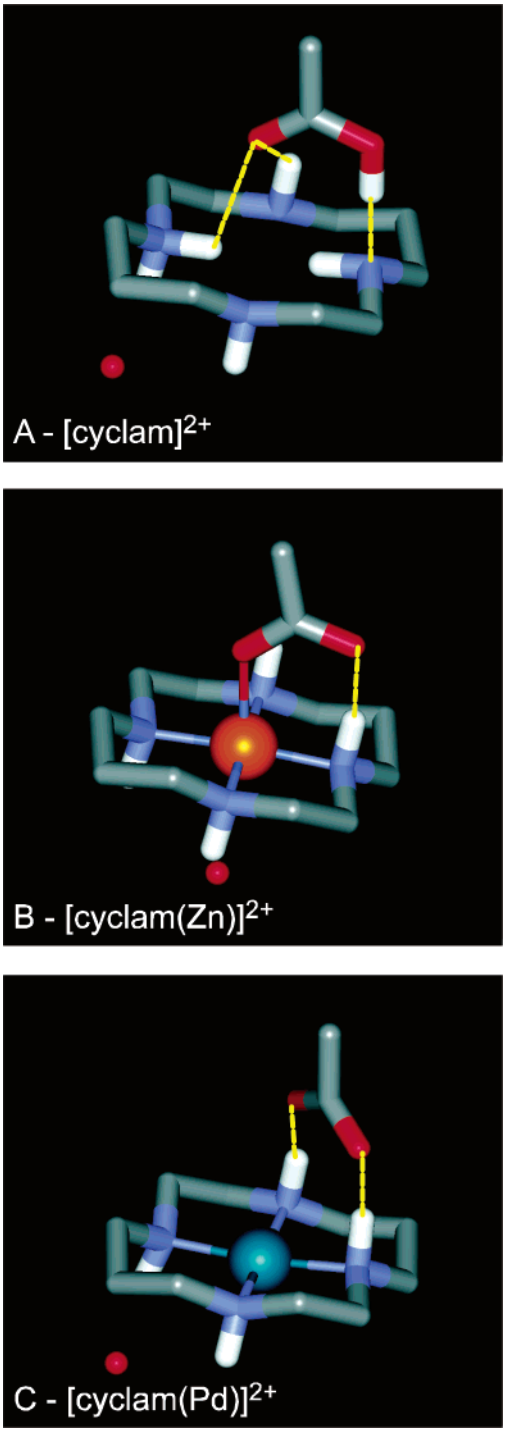

FIGURE 7: Structures of cyclam complexes optimized by quantum chemistry. (A) Acetate binding to cyclam by forming three hydrogen bonds, (B) acetate binding to cyclam(Zn) by forming one hydrogen bond and one metal ion coordination, and (C) acetate binding to cyclam(Pd) by forming two hydrogen bonds. Nonpolar hydrogen atoms are omitted for clarity.

X-ray structures (*25*). The obtained relative binding energies are given in Table 3. Of the three ions, the most stable bond was obtained for the Ni^{2+} complex. The Zn^{2+} complex was calculated to have similar binding energy to the Ni^{2+} complex, whereas the Cu^{2+} complex had the lowest binding energy of the three transition metals. This correlates well with the experimental data, where we observe the affinities of the metal ion complexes of AMD3100 in the following order: $Ni^{2+} = Zn^{2+} > Cu^{2+}$. As shown in Figure 7, acetate

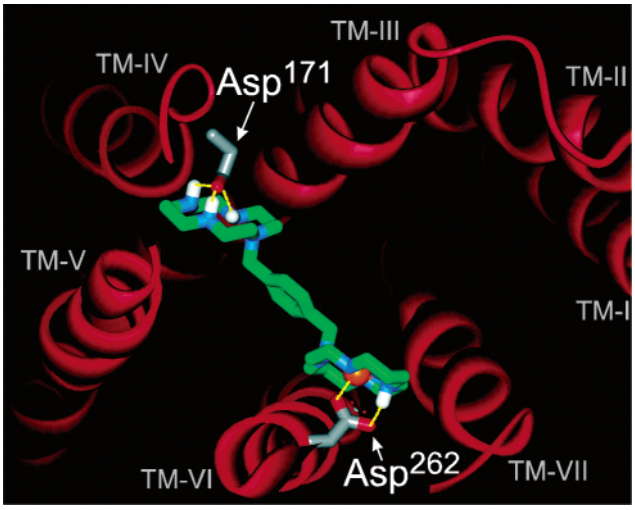

FIGURE 8: Molecular model of the main ligand-binding pocket of the CXCR4 receptor with AMD3100(Zn) manually docked into favorable interactions with Asp^{171} in TM-IV and Asp^{262} in TM-VI. The receptor model is built over the rhodopsin model of Palcewski et al. (*45*). The conformation of AMD3100 is based on structural requirements of high antiviral effects of AMD3100 (*10*, *11*) and the crystallographic X-ray structure of 6,6′-spirobis-(1,4,8,11-tetraazacyclotetradecane)-dinickel(II)tetraperchlorate (*46*), obtained from the Cambridge Structural Database.

makes three (one strong, one intermediate, and one weak) hydrogen bonds with protonated cyclam when there is no metal ion present (Figure 7A) (*33*). However, when Zn^{2+} is coordinated in the cyclam ring the acetate can make a strong coordination bond to the metal and one weaker hydrogen bond to a nitrogen in the cyclam ring (Figure 7B) (*12*).

DISCUSSION

In the present study, the enhancing effect of transition metals on the binding of the bicyclam AMD3100 to the chemokine CXCR4 receptor has been characterized. We find that incorporation of Zn^{2+} or other transition metals into the macrocyclic rings of AMD3100 enhances the binding affinity to the CXCR4 receptor 6−36-fold depending on the radiolabeled ligand used in the assay. By combining receptor mutagenesis with studies of singly metal substituted bi- and mono-cyclams, it is shown that the increased binding affinity of the metal ion substituted AMD3100 conceivably is obtained through an enhanced interaction of only one of the cyclam ring systems with the carboxylate group of Asp^{262}. It is suggested that this occurs through a strong concomitant interaction of one of the oxygens directly with the metal ion and the other oxygen to one of the nitrogens of the cyclam ring through a hydrogen bond (Figure 8). Our data is consistent with the original hypothesis that the protonated cyclam rings or certain metal complexes of these rings in AMD3100 can bind in a manner similar to carboxylate residues in the CXCR4 receptor or other proteins (*9*).

Mutational analysis has previously indicated that each of the two cyclam moieties of AMD3100 mainly interacts with Asp^{171} and Asp^{262}, which are located at the extracellular ends of TM-IV and TM-VI, respectively, in the CXCR4 receptor (*5*). The present study indicates that upon chelation of Zn^{2+} by AMD3100, the complex does not change binding mode, but more likely its interaction with Asp^{262} becomes stronger. It has previously been shown that carboxylic acids have the

option to bind a cyclam both with and without a chelated metal ion. An X-ray and neutron diffraction structure of a protonated cyclam complex with 4 equiv of 4-*tert*-butylbenzoic acid shows that the cyclam ring can form a direct complex with a carboxylic acid group (*33*). At physiological pH, the cyclam ring has an overall charge of +2 and can adopt the most stable so-called trans-III-type (R,R,S,S configuration at the four asymmetric nitrogen atoms) of conformation (*34*, *35*). This conformation can form three nonequivalent hydrogen bonds (one strong, one intermediate, and one weak) between the oxygens of the carboxylic acid and the amines of the cyclam ring (see Figure 7A) (*33*). Cyclam complexes of Zn^{2+}, Ni^{2+}, and Cu^{2+} also adopt the thermodynamically favored trans-III cyclam configuration to give the compound an overall charge of +2 (*12*, *36*, *37*). These metal complexes also form stable complexes with carboxylic acids where the metal ion coordinates the unidentate $C-O^-$, and a secondary amine proton forms a hydrogen-bond with the noncoordinated oxygen of the carboxylate group (Figure 7B) (*12*, *38*). More recently, Sadler et al. have reported NMR and crystallographic evidence for the complexation of carboxylates with the bis-zinc complex of AMD3100 (*39*). In aqueous solution, the macrocyclic rings of AMD3100(Zn)2 were found to exist in two major configurations, trans I and trans III. Interestingly, addition of acetate can induce a conformational change in the cyclam ring(s) to the unusual cis-V configuration, with bidentate coordination of acetate to Zn^{2+} and hydrogen-bond complexation of a second acetate group on the opposite side of the cyclam ring.

The metal-enhanced increase in binding affinity of cyclam for carboxylic acids may be a general property since we observe the same affinity increase with binding of a free, single cyclam ring to the cyclam binding site in the CXCR4 receptor located at the extracellular end of TM-IV (see Figure 3). Asp^{171} is known to be important for the ability of cyclam to compete for SDF-1α binding, whereas substitution of the other Asp residue in the main binding pocket, Asp^{262} in TM-VI, did not affect binding of the free cyclam as determined in competition against ^{125}I-SDF-1α (*5*). Mutation of Asp^{171} (D171N) removed the apparent binding of cyclam (i.e., its effect upon ^{125}I-SDF radioligand binding). From the present data, it appears that the Asp^{171}, which is involved in the binding of AMD3100, is not able to make an interaction with the chelated Zn^{2+} ion in AMD3100. The substitution of Asp^{171} with Asn has no effect on the Zn^{2+} enhanced effect, and binding experiments with two incorporated Cu^{2+} or Ni^{2+} ions also suggest that only Asp^{262} has the ability to favorably interact with both the metal and the macrocyclic ring of AMD3100. One explanation can be that a single cyclam ring with Zn^{2+} can position itself to obtain the favorable interaction with Asp^{171}, but the addition of a large moiety (*N*-(4-methyl)benzylcyclam) inhibits the correct arrangement of the cyclam ring to the Asp^{171} residue. The single positive Zn^{2+} interaction with Asp^{262} is further confirmed by testing AMD3100 carrying either one or two Cu^{2+} or Ni^{2+} ions in the macrocyclic rings on the wild-type receptor. Complexes of cyclam with Zn^{2+}, Ni^{2+}, and Cu^{2+} exhibit stability constants (log *K*) indicative of high thermodynamic stability. The stability constants are reported to be in the range of 15.0−15.3 (Zn^{2+}); 19.9−20.3 (Ni^{2+}); and 26.4−27.9 (Cu^{2+}) (*34*). The Ni^{2+} and Cu^{2+} complexes of cyclam are also reported to exhibit slow kinetics of dissociation, requiring highly acidic conditions to force the reaction (*40*, *41*). These combined observations suggest that metal complexes of AMD3100 (containing one or two metal ions) remain intact in the assay. The test competition binding experiment of AMD3100 carrying either one or two Cu^{2+} or Ni^{2+} ions in the macrocyclic rings showed that only metal ion chelation in one of the two cyclam rings of AMD3100 is required for the increased ligand affinity, indicating the existence of only one metal−cyclam site for AMD3100 (Figure 2). This is further supported by the total elimination of the positive metal effect by the D262N mutant (see Figure 5B).

The calculated difference in relative binding energy of Cu^{2+} and Zn^{2+} complexes is 71−23 kJ/mol = 48 kJ/mol. This difference is considerably larger than the observed effects in the competition binding assay where the $(\Delta\Delta G)_{Zn-Cu}$ is only approximately 4 kJ/mol. However, entropy effects, hydrogen bonding, and other interactions from residues other than Asp^{262} to AMD3100 would decrease the binding energy and increase the binding entropy, and this would result in a smaller relative binding energy between the aspartate residue and one cyclam moiety of AMD3100. Nevertheless, the important observation is that the calculated relative binding energies give a qualitatively right prediction of the order of binding affinities. Besides computing the relative binding energy of the Zn^{2+}, Ni^{2+}, and Cu^{2+} complexes, we have in addition also minimized the structure of cyclam chelating either Co^{2+} or Fe^{2+} with a bound acetate (Table 3). This revealed that the Fe^{2+}−cyclam complex had the most stable bond to acetate of the five transition metals complexes calculated; hence, AMD3100 chelating Fe^{2+} should be tested in the future for an increased affinity to the CXCR4 receptor.

The interactions of bicyclams with CXCR4 monitored by inhibition of 12G5 monoclonal antibody binding follows a similar structure−activity relationship as for inhibition of HIV-1 replication (*5*, *8*, *10*, *42*). Also, the Zn^{2+} complex of AMD3100 exhibits a slightly better antiviral activity as compared to the uncomplexed analogue, AMD3100 (*8*−*10*). However, the Zn^{2+} ion only had a 1−6-fold increase in potency on AMD3100 depending on the viral strain used in the assay as compared to the 36-fold increase observed in 12G5 binding, indicating one disadvantage of direct comparison between 12G5 binding and inhibition of HIV replication. One explanation could be that the AMD3100 inhibition of some HIV strains lack involvement of Asp^{262} in the CXCR4 receptor. However, Hatse et al. have recently shown that Asp^{262} is essential for the anti-HIV activity of AMD3100 against the NL4.3 virus (*43*). This particular virus strain has, on the other hand, been shown by Esté et al. not to be inhibited more potently by AMD3100$(Zn)_2$ than AMD3100 (*8*), indicating that AMD3100$(Zn)_2$ may have the option to bind Asp^{262} in different conformations. Since Asp^{171} has the ability to coordinate the Zn^{2+} ion in cyclam(Zn) but not in AMD3100$(Zn)_2$, it is possible that AMD3100 can bind to the chemokine CXCR4 in different conformations; one where the metal ion in the macrocyclic ring of AMD3100 can coordinate Asp^{262} and one that cannot coordinate Asp^{262} but can only make the regular carboxylic acid−cyclam interaction.

It has previously been shown that the Pd^{2+} complex of AMD3100 is virtually inactive against HIV-entry and has more than 1000-fold lower binding affinity for the CXCR4

receptor as compared to AMD3100 (*8*, *10*, *11*). We have tried to dock acetate to cyclam(Pd) by quantum chemistry to seek the reason for the low affinity of AMD3100$(Pd)_2$ to the CXCR4 receptor. As shown in Figure 7C, the coordination geometry of Pd^{2+} is strictly square planar, and this metal ion therefore does not have any vacant coordination sites available for acetate (*44*); thus, we find that the carboxylic acid compensates by making two weak hydrogen bonds with the cyclam ring.

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