Probing Receptor Binding Activity of Interleukin-8 Dimer Using a Disulfide Trap[†]

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ABSTRACT: Interleukin-8 (IL-8), a member of the chemokine superfamily, exists as both monomers and dimers, and mediates its function by binding to neutrophil CXCR1 and CXCR2 receptors that belong to the G protein-coupled receptor class. It is now well established that the monomer functions as a high-affinity ligand, but the binding affinity of the dimer remains controversial. The ∼1000-fold difference between monomer−dimer equilibrium constant (μM) and receptor binding constant (nM) of IL-8 does not allow receptor-binding affinity measurements of the native IL-8 dimer. In this study, we overcame this roadblock by creating a "trapped" nondissociating dimer that contains a disulfide bond across the dimer interface at the 2-fold symmetry point. The NMR studies show that the structure of this trapped dimer is indistinguishable from the native dimer. The trapped dimer, compared to a trapped monomer, bound CXCR1 with ∼70-fold and CXCR2 with ∼20-fold lower affinities. Receptor binding involves two interactions, between the IL-8 N-loop and receptor N-domain residues, and between IL-8 N-terminal and receptor extracellular loop residues. In contrast to a trapped monomer that bound an isolated CXCR1 N-domain peptide with μM affinity, the trapped dimer failed to show any binding, indicating that dimerization predominantly perturbs the binding of only the N-loop residues. These results demonstrate that only the monomer is a high-affinity ligand for both receptors, and also provide a structural basis for the lower binding affinity of the dimer.

Interleukin-8 (IL-8,[1] also known as CXCL8), a member of the chemokine superfamily, plays a pivotal role in recruiting neutrophils under conditions such as tissue injury, wound healing, and bacterial infection (*1*). IL-8 belongs to the class of proteins now identified as "weak" transient homodimers that exist as both monomers and dimers under physiological conditions (*2*). During active neutrophil recruitment, IL-8 is translocated from the injury site in the tissue to the vasculature, and so its concentration will vary spatially and temporally and cannot be defined by a single physiological concentration. Local concentration under these conditions could reach levels high enough so that both monomers and dimers exist. IL-8 function involves binding to neutrophil G protein-coupled receptors (GPCR) and cell-surface glycosaminoglycans (GAG) (*3*, *4*). Therefore, knowledge of the relative binding affinities and functional activities of the monomer and dimer for their GPCR receptors and GAGs is critical for understanding the role of monomer−dimer equilibrium in neutrophil recruitment.

IL-8 dimerizes at μM concentrations ($K_d \sim 10$ μM) and binds its receptors (CXCR1 and CXCR2) at nM concentrations ($K_d \sim 1$ nM), suggesting that a monomer is sufficient for receptor function (*4*−*6*). An IL-8 "trapped" monomer and also monomer mutants show native binding affinities (*7*, *8*), and various other structure−function studies have also shown that mutating dimer-interface residues does not affect binding, indicating that dimer-interface residues are not essential for receptor function (*4*, *8*, *9*). However, measuring binding affinities of the dimer is less straightforward, as the ∼1000-fold difference between the IL-8 monomer−dimer equilibrium constant (μM) and receptor binding constant (nM) precludes binding studies of the native IL-8 dimer. In principle, the IL-8 dimer, compared to the monomer, could bind with higher, lower, or the same affinity, or it could be completely inactive, and also the IL-8 dimer could bind the two receptors with different affinities.

Previous studies have used the strategy of designing nondissociating disulfide-linked IL-8 dimers, but these variants show both nativelike and reduced receptor function (*10*−*12*). All these studies used the criterion of proximity in the IL-8 dimer structure for mutating a pair of residues to cysteine for introducing disulfides across the dimer interface (*13*). Leong et al. designed a single chain fusion protein by joining the C-terminus of one monomer to the N-terminus of another monomer using a flexible linker, and created four different variants by introducing a pair of disulfides across the dimer interface between Glu29 or Thr37 in one monomer and Ala69 or Ser72 in the other monomer (*10*). Compared to native IL-8, they observed the disulfide variants to bind

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[1] Abbreviations: IL-8, interleukin-8; CXCR1, CXC chemokine receptor 1; GAG, glycosaminoglycan; GPCR, G protein-coupled receptor; ITC, isothermal titration calorimetry; NMR, nuclear magnetic resonance; N-domain, N-terminal domain.

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CXCR1 with 6- to 21-fold lower affinities and CXCR2 with native to 4-fold lower affinities, and concluded that dimer dissociation is not essential for IL-8 biological activity. Williams et al. observed that a disulfide-linked dimer created by introducing a pair of disulfides (E29C/A69C) across the dimer interface was as active as the native protein in a neutrophil Ca^{2+} release assay (*11*). We had reported in a preliminary study that a R26C disulfide-linked dimer, produced by introducing a single disulfide at the site of 2-fold symmetry, was 15-fold less active in a neutrophil elastase activity (*12*). The latter two studies did not report binding affinities, and also neutrophils express both CXCR1 and CXCR2 receptors, and so reduced function could be due to reduced binding to one or both receptors. Most importantly, the consequence of introducing disulfides in these variants is not known, except Williams et al. have characterized their E29C/A69C mutant by nuclear magnetic resonance (NMR) spectroscopy (*11*). They observed chemical shift changes for a number of helical residues, including those of Phe65 and Leu66 that play an important role in stabilizing the native dimer, suggesting that the packing interactions in the dimer interface are perturbed; so its functional properties do not correspond to that of the native dimer. Whereas Arg26 is solvent exposed, residues Glu29, Thr37, and Ala69 are largely buried in the dimer structure (*13*); so mutating the latter set of residues for introducing new disulfides, if not optimally accommodated, would perturb the dimer interface structure, as observed by Williams et al. for the E29/A69 disulfide mutant. Therefore, these disulfide-linked dimers could have differential structural perturbations of the dimer interface and so have varied function, and therefore it is not clear which of these disulfide-linked variants mimic native dimer structure.

We recently exploited the knowledge that IL-8 binding to the CXCR1 N-domain peptide is in the same μM range as the dimer dissociation constant, and observed that in the presence of both monomers and dimers, only the monomer preferentially binds a receptor N-domain peptide (*14*, *15*). We measured the binding constant of the trapped monomer for the receptor N-domain using ITC to be $\sim$5 μM, and a NMR study has shown that the native dimer binds the N-domain with much lower affinity $\sim$170 μM (*16*). Though these studies show that the dimer has a lower binding potency, it can be argued that these results do not exclude the possibility of native dimer binding to the intact receptor with the same affinity as the monomer. Further, we could not carry out similar binding studies with a CXCR2 N-domain peptide due to experimental constraints, and so the relative binding affinity of the native dimer for CXCR2 remains unanswered. In this report, we have addressed some of these questions by studying the structural and receptor binding properties of the "trapped" R26C IL-8 dimer containing a single disulfide at the 2-fold symmetry axis. NMR studies indicate that mutating $Arg^{26} \rightarrow$ Cys and introducing a new disulfide does not perturb the native dimer interface, and that the structure of this disulfide-linked trapped dimer is indistinguishable from the native dimer. Therefore, the binding affinity of the trapped dimer should reflect the binding affinity of the native dimer. The R26C trapped dimer, compared to a trapped monomer, binds CXCR1 and CXCR2 receptors with $\sim$70-fold and $\sim$20-fold lower affinities, respectively. We could not detect any binding of the trapped dimer to a CXCR1 N-domain peptide, indicating that the significantly reduced affinity of the dimer should be due to reduced binding of the N-loop residues. These results emphasize that only the monomer is a high-affinity ligand for both CXCR1 and CXCR2 receptors, and also provide a structural mechanism for the lower binding affinity of the dimer.

EXPERIMENTAL PROCEDURES

Protein Synthesis. Both the R26C disulfide-linked dimer and L25NMe monomer were synthesized using solid-phase peptide synthesis (SPPS), and purified by reversed-phase HPLC as described previously (*17*). Both proteins were synthesized in the 4−72 form, as previous studies have shown that the 4−72 form has the same binding affinity and function as the full-length form (*4*). The rabbit CXCR1 N-domain used in this study was also synthesized by SPPS, and has been extensively characterized previously (*14*, *15*, *20*).

IL-8 Receptor Binding Assays. The binding measurements in human neutrophils and Rat-1 cells expressing either CXCR1 or CXCR2 receptors were carried out as described previously (*18*). Human neutrophils were suspended at a concentration of 1×10^7 cells/mL in phosphate-buffered saline (PBS) containing 0.1% (w/v) bovine serum albumin and 20 mM HEPES (pH 7.4) and incubated at 4 °C for 2 h in the presence of 2 nM ^{125}I-IL-8 and increasing concentrations of unlabeled IL-8. The binding was terminated by centrifuging the incubation mixture after overlaying it on top of 10% (w/v) sucrose solution. Radioactivity in the pellet was measured in a γ counter. For stably transfected IL-8 receptors, cells were incubated in the presence of 2 nM of ^{125}IL-8 and increasing concentrations of IL-8 ligand up to 1 μM for 2 h at 4 °C. Cells were then washed twice with PBS, lysed with 0.2% SDS, and measured in a γ counter. Curve fit and IC_{50} determination were acquired using the Sigma plot program (SPSS Science, Chicago, IL) from the nonlinear regression analysis. Binding affinities are from at least two independent experiments, and each experiment was performed either in duplicates or triplicates.

Isothermal Titration Calorimetry (ITC). Isothermal titration calorimetry experiments were performed using the VP-ITC system (MicroCal) at 25 °C. The trapped IL-8 dimer and monomer proteins were extensively dialyzed for 8−12 h against the buffer, and in a typical experiment, $\sim$0.6 mM IL-8 was injected into the 1.42-mL sample cell containing $\sim$0.06 mM receptor N-domain peptide. The heats of dilution of the peptide and the buffer were small compared with the heat of binding, and were subtracted from the experimental titration results.

NMR Studies. ^{1}H NMR NOESY (mixing time 150 ms) and TOCSY (mixing time 70 ms) spectra for chemical shift assignments and structure determination were acquired on a Varian Unity 750 spectrometer at 30 and 40 °C as described previously (*19*). The protein concentration was $\sim$ 1 mM in a buffer containing 50 mM sodium acetate, 1 mM sodium azide, 1 mM sodium 2,2-dimethyl-2-silapentane sulfonate, pH 5.5 in 90% H_2O/10% ^2H_2O (v/v).

RESULTS

Design and Structural Characteristics of Trapped R26C Dimer. In this study, we have characterized a trapped

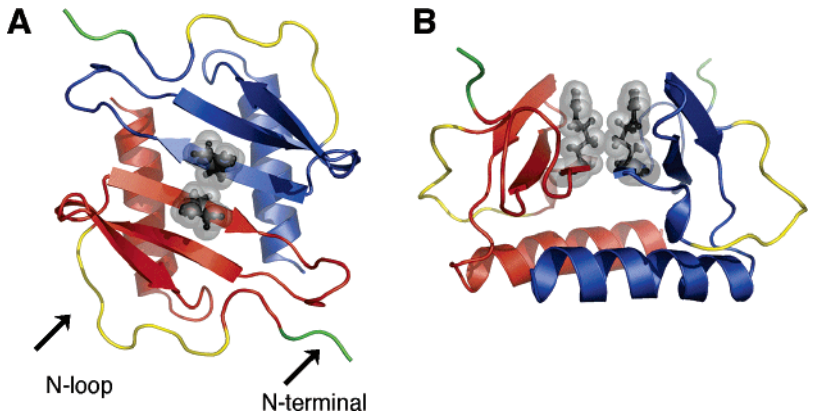

FIGURE 1: A schematic of the native IL-8 dimer. The structure is shown in two different orientations to highlight the orientation of the solvent exposed Arg26 side chain. The individual monomers in the dimer structure (PDB ID 1IL8; ref 13) are shown in red and blue. The structure reveals that the Arg26 side chains (shown in black) in the individual monomers are solvent exposed and are spatially proximal to each other. The functionally important N-terminal region (green) and the N-loop region (yellow) are highlighted, and labeled in one of the monomers.

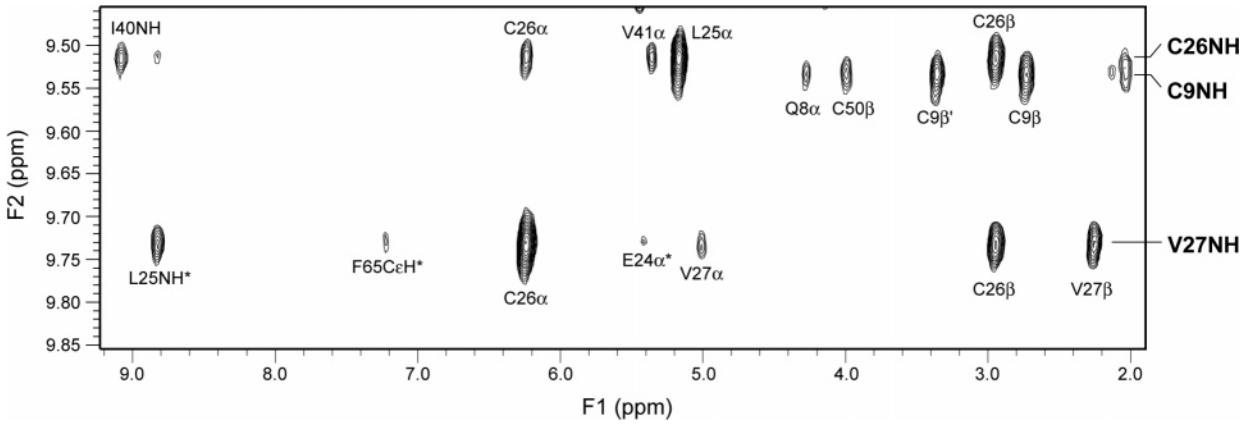

FIGURE 2: NMR NOESY data of the trapped IL-8 dimer. A section of the 750-MHz 2D NOESY ^{1}H NMR spectrum of the R26C trapped IL-8 dimer showing NOEs from Cys26, Val27, and Cys9 amide protons. The NOE pattern is similar to that observed in the native dimer. NOEs across the dimer interface are indicated by an asterisk (*).

nondissociating dimer that contains a single disulfide link (R26C) across the dimer interface (Figure 1). The native IL-8 dimer structure reveals that the Arg26 of the first β-strand constitutes the 2-fold symmetry axis, its side chain is solvent exposed and is not involved in any interactions across the dimer interface, and its backbone amide is H-bonded to the Ile40 carbonyl of the adjacent β-strand within the monomer (*13*). Introducing a disulfide at any other location involves mutating a pair of residues, resulting in two disulfides across the dimer interface about the 2-fold symmetry point. These observations suggested to us that substituting Arg with a Cys should not perturb the native structure. Further, in contrast to residues mutated in other studies such as Glu29 and Thr37 (*10*, *11*), Arg26 is the farthest from the functionally important N-terminal and N-loop residues. Previous studies have also shown that even a charge reversal mutation to Glu (R26E) had no effect on function, indicating that the Arg side chain is not involved in receptor function (*4*).

The R26C trapped dimer was synthesized by SPPS, and its structure was characterized by solution NMR spectroscopy. The nuclear Overhauser effect (NOE) data, which is converted to semiquantitative proton−proton distances, are the major experimental distance restraints in NMR structure calculations. The NOE data of the R26C trapped dimer showed a pattern similar to that observed for the native dimer, indicating that the structure of the R26C dimer is essentially the same as the native dimer. For example, in the native IL-8 dimer, Val27 shows NOEs to Arg26, Ile39, Leu25*, Glu24*, and Phe65* (NOEs across the dimer interface are indicated by an asterisk (*)); and Arg26 to Leu25, Val27, Ile40, and Val41. Figure 2 shows a region of the NOESY spectra of the R26C trapped dimer showing NOEs from Cys26 and Val27. Characteristic sequential and long-range NOEs, within the monomer and across the dimer interface observed in the native dimer, are also observed in the trapped dimer, indicating that the disulfide across the dimer interface is accommodated into the protein structure without affecting the native fold.

Chemical shifts are sensitive to secondary, tertiary, and quaternary structure, and characteristic chemical shifts observed in the native dimer, including those of highly downfield and upfield shifted residues (such as Gln8, Val58, Lys15, Pro16, Phe17, and Cys50), are also observed in the trapped dimer. The exact relationship between chemical shifts and structure/dynamics is not known, but it is well established that backbone $C_\alpha H$ shifts are sensitive to structure and NH shifts are sensitive to both structure and dynamics. Identical backbone chemical shifts, for instance, for less structured and unstructured residues both in the native and in the trapped R26C dimer would mean that they have identical structures/dynamics, and that differences in chemical shifts can be interpreted as being due to structural/dynamic

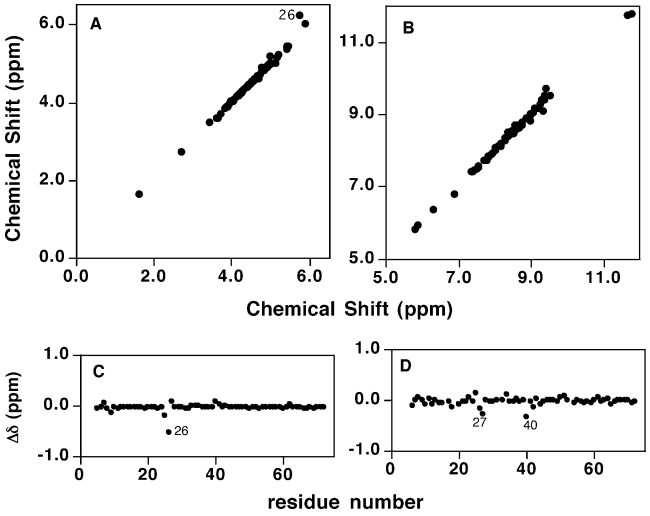

FIGURE 3: Plots of the ^{1}H NMR (A) C_αH and (B) NH chemical shifts of the native and the trapped IL-8 dimer. The chemical shifts of the native protein are plotted along the *y*-axis. The chemical shift differences between the native and the R26C dimer for C_αH and NH chemical shifts are shown in panels C and D, respectively. Residues that show relatively large shift differences are labeled.

changes. Therefore, we feel comparing chemical shifts provides better insight and is more sensitive to structural changes (or lack of) than comparing NOE-derived structures. Chemical shifts of the backbone NH and C_αH of the trapped and native dimer and the differences in chemical shift are plotted in Figure 3. Shifts are essentially identical, despite large variation in C_αH (∼ 4.5 ppm) and NH (∼6 ppm) chemical shifts of individual residues. Slightly higher shifts for mutated residue Cys26 can be accounted for by larger random coil shifts of Cys compared to Arg, and differences in Val27 and Val40 NH protons to small changes in H-bonding. Therefore, the NMR NOE and chemical shift data unambiguously indicate that introducing a disulfide does not perturb the dimer interface structure, and is essentially the same as that of the native dimer.

Receptor Binding of the R26C Trapped Dimer. We measured the binding affinity of trapped dimer and monomer in neutrophils, and observed that the trapped dimer compared to the monomer binds with ∼25-fold lower affinity (Figure 4, Table 1). As neutrophils coexpress two IL-8 receptors (CXCR1 and CXCR2), reduced binding affinity could be due to reduced binding to one or both receptors. Therefore, we also measured the binding affinity to the individual receptors transfected in rat fibroblast cells. We observed that

Table 1: Binding Affinities of Trapped CXCL8 Dimer and Monomer[a]

	neutrophils		CXCR1		CXCR2	
ligand	K_d (nM)	fold	K_d (nM)	fold	K_d (nM)	fold
monomer[b]	0.4 ± 0.1	1	0.8 ± 0.3	1	0.5 ± 0.3	1
dimer[b]	10.4 ± 1.2	26	55.0 ± 6.7	69	8.5 ± 2.0	17

[a] Fold is the relative dissociation constant of the dimer compared to the monomer ($K_{d,dimer}/K_{d,monomer}$). [b] Monomer and dimer correspond to the trapped L25NMe monomer and trapped R26C dimer.

the trapped dimer binds the CXCR1 and CXCR2 receptors with ∼70- and ∼20-fold lower affinities, respectively, indicating that only the monomeric form of the protein is a high-affinity ligand for both receptors (Figure 4, Table 1). As shown previously, the trapped monomer and the native IL-8 had similar binding affinities (data not shown).

IL-8 binds both CXCR1 and CXCR2 receptors with nM affinity (Table 1). Receptor binding involves interactions between the IL-8 N-terminal loop and the receptor N-domain residues (site I), and between the IL-8 N-terminus and the receptor extracellular loop residues (site II). IL-8 binds the isolated CXCR1 N-domain with an affinity (μM) similar to that for the N-domain in the intact receptor, indicating that the structural requirements for site-I interaction can be studied outside the context of the intact receptor (*20*, *21*). These observations also suggest that site-I interaction provides most of the binding affinity. As IL-8 monomer−dimer equilibrium is in the same μM range, we studied the binding of native IL-8 and trapped monomer to a CXCR1 N-domain peptide using ITC, and observed that, in the presence of both monomers and dimers, only the monomer preferentially binds the receptor N-domain peptide (*14*). We have now carried out ITC studies of the trapped dimer, and failed to detect any binding to the CXCR1 N-domain peptide (Figure 5).

DISCUSSION

Design of nonassociating trapped monomers and nondissociating trapped dimers provides powerful tools for studying the structural and functional role of the individual species, and to gain insights into how monomer−dimer equilibrium regulates function. Ideal design of a trapped monomer/dimer should not perturb the native monomer/dimer structure, so that the observed function can be unambiguously attributed to the monomeric/dimeric state of the protein, and not to the structural change as a consequence of the modification. Previously, we had shown that such a trapped nonassociating

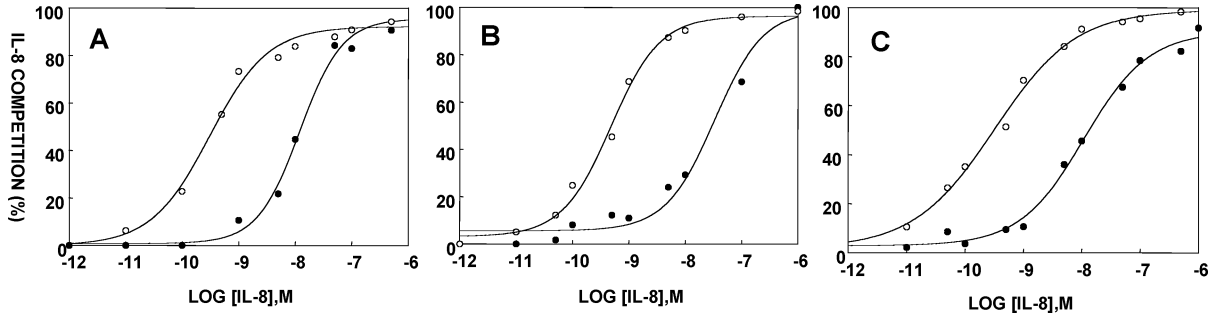

FIGURE 4: Binding of the trapped IL-8 dimer for CXCR1 and CXCR2 receptors. Plots of the competitive binding curves of the trapped IL-8 dimer (●) and trapped monomer (○) to neutrophils expressing both receptors (A), and to Rat-1 fibroblast cells expressing either CXCR1 (B) or CXCR2 receptors (C). The results shown are representative of two to five independent experiments each performed in duplicate or triplicate.

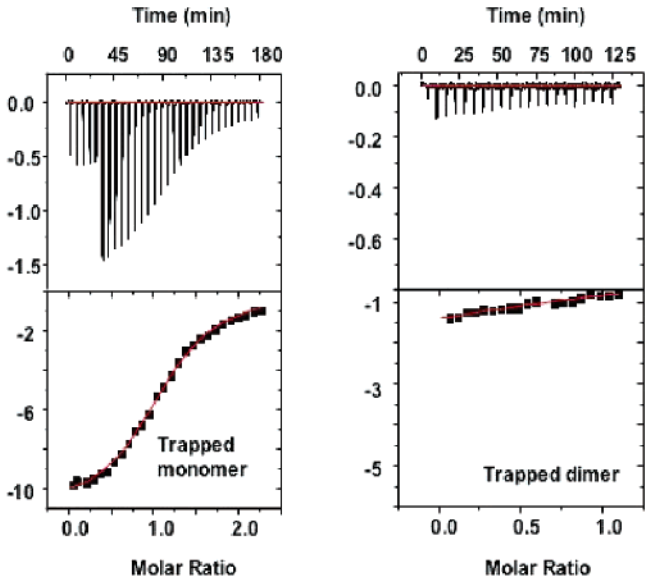

FIGURE 5: Binding of the trapped IL-8 dimer and monomer to CXCR1 N-domain peptide. The isothermal titration calorimetry (ITC) titration profiles for the trapped dimer and trapped monomer are shown in panels A and B, respectively. The data were collected at 25 °C in 50 mM Hepes, 50 mM NaCl, pH 8.0. The upper panels represent the ITC thermograms, and the lower panels represent the fitted binding isotherms.

IL-8 monomer is as active as the native protein, indicating that a monomer is sufficient for receptor function. The protein was trapped in the monomeric state by introducing a non-natural *N*-methyl amino acid that disrupts H-bonding interactions about the 2-fold symmetry axis and functions as a β-sheet breaker (*7*, *19*). Subsequently, it has been demonstrated for various chemokines that form dimers and higher order oligomers, using *N*-methyl amino acids as described here and conventional molecular biology approaches, that a monomer is sufficient for receptor function (*22*−*26*). It is now generally accepted that all chemokines are active as monomers for their receptor function, providing proof of principle that such designed trapped proteins can provide valuable information on structure−function.

Unlike designing a trapped monomer where the objective is to disrupt dimer interactions and to preserve native monomer structure, designing a trapped dimer is more challenging as the objective is to preserve the native dimer structure and not introduce new interactions. The trapped R26C dimer used in this study has a disulfide at the 2-fold symmetry point, and our NMR data indicate that the dimer interface is not perturbed due to the newly introduced disulfide, and the structure of the trapped dimer is essentially the same as the native dimer; so its receptor binding characteristics reflect the binding affinity of the native dimer. Our binding studies to the intact receptors and to the isolated CXCR1 N-domain together indicate that the lower binding affinity of the dimer is due to reduced binding of N-loop residues to the receptor CXCR1 N-domain. The observation that the dimer binds CXCR1, compared to CXCR2, with lower affinity (Table 1), was also made by Leong et al., though their disulfide variants had overall higher binding affinities (*11*). The higher binding affinities of their disulfide variants are very likely due to perturbation of the interface packing and stability as a consequence of newly introduced disulfides, and so show binding properties that do not reflect the native dimer.

What could be the structural basis for the differential binding of the monomers and dimers? The solution structure of the trapped IL-8 monomer has been determined by NMR spectroscopy, and is observed to be essentially similar to that of a monomer in the dimer structure except that the C-terminal helical residues are unstructured in the monomer and structured and helical in the dimer (*13*, *19*). N-Terminal and N-loop residues mediate receptor binding, and these residues are located away from the dimer interface. Binding to the receptor N-domain peptide shows that the reduced binding affinity of the dimer is due to the altered binding properties of the N-loop residues. Structure and the topology of the N-loop residues are preserved between the monomer and dimer, and so the reduced binding of the dimer is not due to structural changes. On the other hand, NMR amide exchange experiments reveal that the dimer is conformationally more constrained compared to the monomer, suggesting that the overall reduced conformational flexibility inhibits binding of the N-loop residues to the receptor N-domain (*7*, *19*). Greater loss of binding to CXCR1 is consistent with the observation from structure−function studies that have shown that mutating IL-8 N-loop residues results in greater loss of CXCR1 binding, indicating that properties of N-loop residues are more stringent for CXCR1 than CXCR2 binding (*27*).

IL-8 function involves binding to GPCRs on target cells evoking signaling events such as cell shape change and Ca^{2+} release, and binding to endothelial cell surface syndecans which are heparan sulfate proteoglycans for rolling, adhesion, and extravasation of the target cells (*28*). Various studies have shown that IL-8 promotes neutrophil recruitment at low concentrations, but actually attenuates recruitment at high concentrations, suggesting that high IL-8 concentrations negatively regulate receptor function (*29*−*31*). Therefore, it is possible that, at high concentrations, IL-8 exists as a dimer, and the observed lower activity is due to lower receptor affinity of the dimer. A variety of in vitro and ex vivo studies using both native and monomeric mutants of IL-8 also suggest that cell surface proteoglycan-bound IL-8 dimer plays an essential role for neutrophil recruitment (*4*, *32*−*34*). Under conditions of active neutrophil recruitment, self-association could provide a mechanism by which in vivo IL-8 dimerization negatively regulates receptor binding and positively regulates GAG binding, and this regulation could play an important role in the neutrophil recruitment process. The observation that dimerization results in more pronounced loss of binding affinity for CXCR1 compared to CXCR2 also provides a tantalizing possibility of selective in vivo activation of CXCR2 over CXCR1. There is recent evidence that both CXCR1 and CXCR2 can form homodimers and heterodimers (*35*, *36*), but the relevance of this observation for binding of monomer and dimer ligands remains to be studied.

Chemokines, the largest subfamily of cytokines, with more than 40 members identified to date, play very fundamental and diverse roles from immune surveillance and leukocyte trafficking to organogenesis. Structural and functional studies indicate that all chemokines share a common protein fold, all bind their receptors with the same two-site mechanism, and all bind GAGs. Structure determination and solution

characterization indicate that dimerization is a fundamental property shared by all chemokines. The dimerization potency varies from weak (mM) to strong (nM), and is sensitive to solution conditions such as pH and ionic strength (*6*, *8*, *37*). Even chemokines observed as monomers in solution NMR studies at mM concentration are observed as dimers under crystallization conditions (*38*, *39*). Nevertheless, structure−function data consistently indicate, including for chemokines such as CCL4 and CCL5 that form strong dimers, that a monomer is sufficient for receptor binding, and a dimer or tetramer formation is coupled to GAG binding (*23*, *40*, *41*). In CC chemokines, the dimer interface and the receptor-binding interface overlap, and there is evidence that dimerization in these chemokines also reduces receptor binding potency (*23*). Recently, various chemokines including IL-8 have been shown to form heterodimers with other chemokines, and such heterodimers show distinctly different in vitro activities compared to monomers, indicating that heterodimerization could also play a role in vivo (*42*). Therefore, our results on the role of IL-8 dimerization are directly relevant for all chemokines, and we propose that the fundamental property of chemokines to form reversible homodimers plays a crucial role in mediating in vivo function.

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SUPPORTING INFORMATION AVAILABLE

NOESY figure of the native dimer, showing NOEs from Arg26 and Val27 amide protons. This material is available free of charge via the Internet at http://pubs.acs.org.

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