Dimer Dissociation Is Essential for Interleukin-8 (IL-8) Binding to CXCR1 Receptor*

Received for publication, June 17, 2004, and in revised form, July 8, 2004
Published, JBC Papers in Press, July 12, 2004, DOI 10.1074/jbc.C400283200

Harshica Fernando, Christopher Chin, Jörg Rösgen, and Krishna Rajarathnam‡
From the Department of Human Biological Chemistry and Genetics and Sealy Center for Structural Biology, University of Texas Medical Branch, Galveston, Texas 77555-1055

Chemokines play a fundamental role in trafficking of immune cells and in host defense against infection. The role of chemokines in the recruitment process is highly regulated spatially and temporally and involves interactions with G protein-coupled receptors and cell surface glycosaminoglycans. The dynamic equilibrium between chemokine monomers and dimers, both free in solution and in cell surface-bound forms, regulates different components of recruitment such as chemotaxis and receptor signaling. The binding and activity of the chemokine interleukin-8 (IL-8) for its receptors, previously studied using “trapped” non-associating monomers and non-dissociating dimers, show that the monomer has a native-like function but support conflicting roles for the dimer. We have measured the binding of native IL-8 to the CXCR1 N-domain, using isothermal titration calorimetry and sedimentation equilibrium techniques. The N-domain constitutes a critical binding site, and IL-8 binding affinity to the receptor N-domain is in the same concentration range as the IL-8 monomer-dimer equilibrium. We observed that only the IL-8 monomer, and not the dimer, is competent in binding the receptor N-domain. Based on our results, we propose that IL-8 dimerization functions as a negative regulator for the receptor function and as a positive regulator for binding to glycosaminoglycans and that both play a role in the neutrophil recruitment process.

Chemokines, the largest subfamily of cytokines, play a fundamental role in diverse biological processes from organogenesis and leukocyte trafficking to host immune response against infection (1–3). These processes involve movement of cells from one site to another, and an imbalance in the chemokine response is a major causative factor in the pathophysiology of various autoimmune and inflammatory diseases and cancer. Moreover, chemokine receptors also function as co-receptors for human immunodeficiency virus infection (4). Therefore, knowledge of how chemokines mediate function is of significant interest for therapeutic intervention. Chemokines exert their function by binding to G protein-coupled receptors (GPCRs)1 on target cells, evoking such biological responses as cell shape change and downstream signaling, and by binding to cell surface glycosaminoglycans (GAG) for rolling and extravasation of the target cells.

Chemokines are classified into CXC, CC, CX₃C, and C families on the basis of conserved cysteines near the N terminus. A characteristic feature of chemokines is the observation that they form dimers. Even chemokines observed as monomers in solution at millimolar concentration in NMR studies are observed as dimers under crystallization conditions. These observations suggest that all chemokines dimerize and that dimerization plays a fundamental role in some aspect(s) of chemokine function. Under transient conditions that favor leukocyte recruitment, the chemokine concentration could reach levels high enough so that they form dimers. Recent studies also show that chemokines either bind as dimers or dimerize on binding to GAGs on endothelial cells, and such binding is important for leukocyte recruitment. Clearly, chemokines exist in a dynamic equilibrium between the solution and cell surface-bound monomer and dimer forms, and this equilibrium regulates interaction with GPCRs and GAGs in recruiting leukocytes. Therefore, knowledge of the similarities and the differences in the binding of the monomers and dimers for GPCRs and GAGs is critical for understanding their function.

Interleukin-8 (IL-8; also called CXCL8), a CXC chemokine that recruits neutrophils during inflammatory events by binding the receptors CXCR1 and CXCR2, is one of the best studied members of the chemokine family. Both NMR and x-ray structures show it to be a dimer (5, 6). IL-8 dimerization is sensitive to solution conditions, and the reported dissociation constants ($K_d$) vary by as much as 160-fold (0.1–16 μM), indicating that IL-8 could easily dimerize under in vivo conditions (7, 8). A variety of in vitro cell-based and in vivo studies also suggest dimerization of IL-8 on binding to GAGs (9, 10). IL-8 is active for GPCR function at nanomolar concentrations in in vitro cell-based assays, suggesting that IL-8 binds its receptor as a monomer. Indeed, we and others (8, 11) have shown that obligate IL-8 monomers have the same in vitro receptor activity as the native protein. Our trapped monomer was generated by modifying a dimer-interface backbone amide proton with a bulky methyl group; the observation that this monomer had the native-like function but support conflicting roles for the dimer. We have measured the binding of native IL-8 to the CXCR1 N-domain, using isothermal titration calorimetry and sedimentation equilibrium techniques. The N-domain constitutes a critical binding site, and IL-8 binding affinity to the receptor N-domain is in the same concentration range as the IL-8 monomer-dimer equilibrium. We observed that only the IL-8 monomer, and not the dimer, is competent in binding the receptor N-domain. Based on our results, we propose that IL-8 dimerization functions as a negative regulator for the receptor function and as a positive regulator for binding to glycosaminoglycans and that both play a role in the neutrophil recruitment process.

† To whom correspondence should be addressed. Tel.: 409-772-2238; Fax: 409-772-1790; E-mail: krrajara@utmb.edu.

‡ To whom correspondence should be addressed. Tel.: 409-772-2238; Fax: 409-772-1790; E-mail: krrajara@utmb.edu.

This work was supported by American Heart Association Texas Affiliate Grant 0356112Y (to K. R.) and by a training fellowship from the W. M. Keck Foundation (to J. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: GPCR, G protein-coupled receptor; GAG, glycosaminoglycan; IL-8, interleukin-8; ITC, isothermal titration calorimetry; CXCR1, CXC chemokine receptor-1; HPLC, high performance liquid chromatography.

© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.

Printed in U.S.A.
linked dimers are not known, so it is not clear whether these cross-linked dimers are the same as "native" dimer or linked at locations that allow the protein to function as a monomer or modify the protein structure and so affect function.

We have now studied the binding of native IL-8 to the CXCR1 N-domain peptide using titration calorimetry and sedimentation equilibrium methods. The receptor N-domain consists of a critical binding site, and structure-function studies have shown that IL-8 binds the isolated N-domain with an affinity similar to that for the N-domain in the intact receptor, and also this affinity is in the same range as the dimer dissociation constant (15–17). These properties allow studying binding under conditions where substantial populations of both IL-8 monomer and dimer exist. We also used a "trapped" monomer as a control in all these experiments. In an isothermal titration calorimetry (ITC) experiment, change in heat during the titration is measured and is the composite of all binding interactions in solution; in the sedimentation equilibrium experiment, average mass is measured and is the composite of the different species present in solution. Both experiments show that only the IL-8 monomer, and not the dimer, binds the receptor N-domain and further that the dissociation of dimeric IL-8 precedes binding. Based on our results, we propose that IL-8 dimerization functions as a negative regulator for receptor function.

MATERIALS AND METHODS

Protein and Peptide Synthesis—The native IL-8, trapped IL-8 L25NMe monomer, and rabbit CXCR1 N-domain peptide were synthesized using solid-phase peptide synthesis as described previously (11, 17). All were purified by reversed-phase HPLC, and the correctness of the synthesis was verified using electrospray mass spectrometry. The L25NMe monomer was a kind gift of Dr. Ian Clark-Lewis.

Analytical Ultracentrifugation Studies—Sedimentation equilibrium experiments were carried out using a Beckman-Coulter XL-A analytical ultracentrifuge with An-60 titanium rotors at 25 °C using absorbance optics at 280 nm. The average molecular weight (M_{av}) was calculated using the following equation.

\[ M_{av} = \frac{2RT}{(1-r)^2} \times \frac{d \ln C}{d r^2} \quad (\text{Eq. 1}) \]

For all experiments, the total concentration was calculated so that the A_{280} of the samples was between 0.5 and 1. In different samples, the concentration of IL-8 varied from 5 to 120 μM (in monomer) and of the N-domain peptide varied from 6 to 66 μM. The average molecular weights determined for the different mixtures were observed to be essentially independent for experimental variables such as rotor speeds, starting concentration, and buffers.

ITC—Isothermal titration calorimetry experiments were performed using the VP-ITC system (MicroCal) at 25 °C. All proteins were extensively dialyzed for 8–12 h against the buffer, centrifuged, filtered, and degassed before use. In a typical experiment, ~0.6 mM IL-8 was injected into the 1.42-ml sample cell containing ~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. Data were then fitted using a nonlinear least-squares routine using a single-site binding model in Origin for ITC v.5.0 (Microcal), varying the stoichiometry (n), binding constant (K_{b}), and binding enthalpy (ΔH^r).

RESULTS AND DISCUSSION

IL-8 binding and receptor activation involve two interactions, between the ligand N-terminal loop and the receptor N-domain residues (site-I) and between the ligand N-terminal and the receptor juxtamembrane domain residues (site-II). Structure-function studies show that site-I is a high affinity interaction and serves to dock the ligand on the receptor, and site-II is a low affinity high specificity interaction and is involved in activating the receptor (14). IL-8 binds the CXCR1 receptor with μM affinity (K_{d} ~ 3 μM), and IL-8 N-terminal mutants bind the receptor with micromolar affinity, indicating that site-I interaction corresponds to micromolar affinity. IL-8 binds the isolated CXCR1 N-domain with an affinity similar to that for the N-domain in the intact receptor, indicating that the structural requirements for the site-I interaction can be studied outside the context of the intact receptor. As IL-8 monomer-dimer equilibrium is in the same μM range, we have exploited these properties to study binding using ultracentrifugation and ITC techniques under conditions where native IL-8 is at monomer-dimer equilibrium. We have used the L25NMe monomer that has native activity as a control. The trapped L25NMe has been well characterized and has been used in a number of functional studies (9–11). We have solved its structure by solution NMR spectroscopy and observed its structure to be similar to that of the monomer in the dimeric structure (18).

Sedimentation equilibrium is the technique of choice for determining dimerization constants and can also be used to detect binding between proteins and is the only technique that measures binding interactions on the basis of molecular weights of the different species in solution. Therefore, this technique is ideally suited to detect binding interactions of the native IL-8 under conditions where multiple species could exist in solution. Sedimentation equilibrium profiles of the individual species and of the mixtures plotted as ln C versus r^2 are shown in Fig. 1. Such plots are easy to interpret, as the slopes are proportional to the average molecular weight of the species in solution. The data for the receptor N-domain and trapped L25NMe monomer could be fitted to a single species, and the experimental molecular weights corresponded to the calculated molecular weights (N-domain, 3.4 kDa; trapped monomer, 8.0 kDa). The data for native IL-8 could not be fitted to a single-species model but fit to a monomer-dimer equilibrium model with a dissociation constant (K_{d}) of 4 μM. For native IL-8: receptor N-domain mixture, the sedimentation profile showed a smaller slope than that for the IL-8 dimer, indicating dissociation of the dimer and not binding to the dimer; the observed molecular weight of ~11 kDa suggested the formation of a 1:1 complex between IL-8 monomer and the N-domain peptide. Experiments carried out at different molar ratios, including at excess N-domain or excess IL-8, showed no evidence of higher molecular weight species that could be attributed to N-domain...
binding to the native dimer (2:1 or 2:2 complexes). Indeed, sedimentation profiles for the monomer IL-8:receptor N-domain mixture were similar to that observed that for native IL-8, indicating formation of a 1:1 complex. We also carried out sedimentation velocity experiments that can provide insight into the stoichiometry of the complexes, and the data indicated interactions similar to that observed in the sedimentation equilibrium experiments and showed no evidence of binding to an IL-8 dimer (data not shown). In summary, these experiments indicate that it is the monomer that binds to the receptor N-domain and that dimer dissociation is essential for binding.

Isothermal titration calorimetry is the most direct method to accurately measure the thermodynamics of protein-protein interactions and can further provide the stoichiometry of the complex. In an ITC experiment, the net change in heat released or absorbed is measured and is the composite of all interactions that occur during the titration. ITC data for the native IL-8 and trapped monomer are shown in Fig. 2. The upper panels show thermograms recorded as a function of IL-8 (L) added to the N-domain peptide (R) that have been integrated with respect to time and normalized per mole of added ligand, and the lower panels show the integrated heats plotted as a function of molar L/R ratio. The IL-8 concentration in the syringe is high and exists as a dimer (≈0.6 mM), and the receptor peptide in the sample cell is a monomer. We discuss various possible binding interactions and the binding stoichiometry (n = L/R) on titrating IL-8 dimer into the receptor N-domain peptide. In model A, IL-8 dimer does not dissociate and binds two moles of receptor N-domain (n = 0.5); in model B, IL-8 dimer does not dissociate and binds 1 mol of receptor N-domain (n = 1); in model C, IL-8 dimer dissociates, and each monomer binds one receptor N-domain (n = 0.5); and in model D, IL-8 dimer dissociates, and each monomer binds 2 mol of receptor N-domain (n = 0.25).

\[
\begin{align*}
L_2 + 2R &\leftrightarrow L_2R_2 (n = 0.5) \quad \text{(Model A)} \\
L_2 + R &\leftrightarrow L_2R (n = 1) \quad \text{(Model B)} \\
1/2L_2 + R &\leftrightarrow LR (n = 0.5) \quad \text{(Model C)} \\
1/2L_2 + 2R &\leftrightarrow LR_2 (n = 0.25) \quad \text{(Model D)}
\end{align*}
\]

Accordingly, fitting the data, an "n" value of 1, 0.5, or 0.25 would indicate binding according to one of the above models, whereas an "n" value in between would indicate multiple binding events. An "n" value of 0.5 was obtained, ruling out multiple binding events and also models B and D. Model A can be ruled out on the basis that sedimentation equilibrium experiments showed no evidence of molecular weights corresponding to an L2R2 complex. Only model C is in agreement with the experimental data, which are also consistent with the molecular weights determined in sedimentation experiments. Furthermore, the ITC data for the IL-8 monomer could be fitted to a single-site model with an "n" value of 1 that corresponds to a single monomer binding to a single N-domain peptide (panel B). The observed enthalpies, stoichiometry, and binding constants for native IL-8 and monomer are summarized in Table I.

**Structural Basis of IL-8 Dimer Binding.**—Our ITC and sedimentation equilibrium studies on IL-8 binding to the receptor N-domain unambiguously demonstrate that only the IL-8 monomer is competent to bind the receptor N-domain. On the basis of our data, we provide a thermodynamic linkage scheme (Fig. 3). According to this model, the dimer does not bind the receptor N-domain, and binding of the monomer is coupled to dimer dissociation. The receptor N-domain constitutes a critical binding site and is responsible for most of the binding energy, so our results have direct relevance to IL-8 binding to the intact receptor, suggesting that the IL-8 dimer does not bind the intact receptor with the same affinity as the monomer. It has been reported previously from competitive inhibition experiments that CXCR1 N-domain peptides bind IL-8 with a binding constant (\(K_D\)) of ≈15 \(\mu\)M (15, 16) and from IL-8 chemical shift changes with a \(K_D\) of 170 \(\mu\)M (19). In the former experiments, ≈1 \(\mu\)M of radiolabeled IL-8 was competed with increasing concentrations of unlabeled N-domain peptide, whereas in the

### Table I

Thermodynamic parameters for the binding of native IL-8 and L25NMe monomer to CXCR1-N-domain as measured by isothermal titration calorimetry in 50 mM NaCl, 50 mM HEPES, pH 8.0, buffer at 25 °C

| Ligand     | n   | \(K_D\) | \(\Delta H\) | \(-T\Delta S\) | \(\Delta G\) |
|------------|-----|--------|-------------|--------------|-------------|
| Native IL-8| 0.55| 7.1    | -7.0        | -0.3         | -7.3        |
| Monomer    | 1.12| 6.0    | -10.5       | 3.4          | -7.1        |

Values reported are from an average of two experiments. The estimated average errors are ±0.2 kcal/mol for \(\Delta G\) and ±5% for \(\Delta H\).
latter NMR experiment, unlabeled peptide was added to a 0.75 mm \(^{15}\)N-labeled IL-8 sample. IL-8 is a monomer in the competitive binding experiments, and its \(K_d\) is in agreement with our \(K_d\) of \(~8\ \mu M\) from ITC and our observation that only the monomer binds in the presence of both monomer and dimer. Under NMR experimental conditions, IL-8 is a dimer, and the observed \(K_d\) of 170 \(\mu M\) suggests that the dimer binds the N-domain peptide with much lower affinity. This observation is also consistent with our observation that the monomeric state is critical for high affinity binding.

The importance of IL-8 dimer dissociation for receptor binding and function has been studied previously using disulfide-linked IL-8 dimers (12–14). Leong et al. (12) designed a single chain dimer by using a linker to join the C terminus of one monomer to the N terminus of another and further introduced different disulfides (E29C/A69C, E29C/S72C, T37C/A69C, T37C/S72C) to stabilize the dimer interface. These dimer mimics showed native IL-8 binding and functional response both in neutrophils and mammalian cells expressing the individual receptors (12). Williams et al. (13) also observed native activity for the E29C/A69C disulfide mutant and furthermore also provided a qualitative description of the structure on the basis of NMR chemical shift assignments. Chemical shift differences were observed for the helical residues 61, 62, 65, 66, 70, and 71, suggesting that the helix packing is perturbed. Therefore it is possible that the newly introduced disulfides actually destabilize the dimer interface, so these dimer mimics in effect have the functional properties of a monomer. In contrast to these studies, a R26C disulfide mutant that contains a single disulfide across the interface has been reported to be \(~15\)-fold less active (14). Unlike other dimer constructs, R26C has only one single disulfide about the 2-fold symmetry axis. Examination of the IL-8 dimer structure shows that mutating Arg to Cys and the formation of the disulfide is unlikely to perturb the native structure, so the R26C disulfide dimer could function as native dimer. The decreased activity of the R26C dimer is in agreement with our observation that native dimer does not bind to the receptor N-domain peptide.

We propose the lower binding affinity of the dimer is due to structural constraints but due to its reduced conformational flexibility and that dimerization locks the protein into a low-affinity conformation. The IL-8 dimer structure shows that the binding interfaces are on the opposite ends of the protein. Amide proton exchange rates, which are diagnostic of conformational flexibility, are observed to be slower in the dimer compared with the monomer. This observation strongly suggests that the decreased binding of the dimer is due to conformational constraints and that the greater flexibility observed in the monomer is essential for high affinity binding (11).

**Role of Monomer-Dimer Equilibrium for in Vivo Function**—A remarkable number of protein classes form reversible homodimers, and such an association has been observed to regulate diverse biological functions from protein folding and stability to ligand binding, catalysis, and signaling (20). Structure determination indicates that many chemokines dimerize, and the observation that chemokine dimerization is also coupled to binding to GAGs suggests that all chemokines form dimers and/or higher order oligomers (21, 22). Monomeric mutants of CXC and CC chemokines observed as dimers and tetramers in NMR and x-ray structures show the same activity as the native protein, indicating unambiguously that a monomer is competent for receptor function (11, 23–25). Whether the dimeric form is competent for receptor function has been less clear. Our observation that the IL-8 dimer does not bind the receptor N-domain indicates that the IL-8 dimer compared with the monomer is less competent in binding the receptor and that the dimer is a negative regulator for CXCR1 function.

Our observation that IL-8 functions as a negative regulator for receptor function has direct relevance for in vivo function. It is very likely that in vivo, IL-8 exists in dynamic equilibrium between the solution and cell surface-bound monomer and dimer forms. Under conditions of insult such as bacterial infection, IL-8 mediates neutrophil recruitment from the blood stream to the underlying tissue, and it is believed that IL-8 continues to play a role in directing the neutrophils to their destination in the tissue. Once reaching their destination, neutrophils release proteases and oxygen radicals for destroying the pathogens. All these steps involve interactions with GAGs and GPCR, and the ability to exist in two forms, both in the free and cell-bound form, allows fine-tuning the recruitment process. Self-association provides a mechanism by which IL-8 dimerization functions as a negative regulator for the receptor function and as a positive regulator for binding to GAGs, and both play a role in the neutrophil recruitment process. We propose that the dimeric forms of all chemokines function as negative regulators for their GPCR functions.
