The Crystal Structure of the Chemokine Domain of Fractalkine Shows a Novel Quaternary Arrangement*

Received for publication, March 27, 2000, and in revised form, April 14, 2000
Published, JBC Papers in Press, April 17, 2000, DOI 10.1074/jbc.M002584200

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Fractalkine, or neurotactin, is a chemokine that is present in endothelial cells from several tissues, including brain, liver, and kidney. It is the only member of the CX3C class of chemokines. Fractalkine contains a chemokine domain (CDF) attached to a membrane-spanning domain via a mucin-like stalk. However, fractalkine can also be proteolytically cleaved from its membrane-spanning domain to release a freely diffusible form. Fractalkine attracts and immobilizes leukocytes by binding to its receptor, CX3CR1. The x-ray crystal structure of CDF has been solved and refined to 2.0 Å resolution. The CDF monomers form a dimer through an intermolecular β-sheet. This interaction is somewhat similar to that seen in other dimeric CC chemokine crystal structures. However, the displacement of the first disulfide in CDF causes the dimer to assume a more compact quaternary structure relative to CC chemokines, which is unique to CX3C chemokines. Although fractalkine can bind to heparin in vitro, as shown by comparison of electrostatic surface plots with other chemokines and by heparin chromatography, the role of this property in vivo is not well understood.

Chemokines, or chemoattractant cytokines, are small (5–20 kDa), basic, heparin-binding proteins that show 20–70% identity in their amino acid sequences (1). Chemokines provide a chemoattractant gradient toward the site of inflammation as they direct the trafficking of a number of leukocytes, accomplishing this by binding to and activating 7-transmembrane-spanning G-protein-coupled receptors on the surface of the leukocytes. Activation of these receptors then leads to an increase in integrin adhesiveness and stable arrest (2–4). Chemokines are secreted onto the cell surface and immobilized by binding to GAGs (5). This immobilization is accompanied by oligomerization, which seems to enhance the affinity of the chemokines for their receptors (6, 7).

To date, more than 40 different human chemokines have been identified (8). They are mainly characterized by the presence and conserved relative position of the first two cysteine residues. The intramolecular disulfide bridges stabilize the fold of the peptide, ensuring its binding to specific receptors and its functional activity. Chemokines are classified into four categories: C, CC, CXC, and CX3C. CC chemokines contain two N-terminal adjacent cysteines, whereas the same cysteines in CXC chemokines are separated by one residue. C chemokines contain only one N-terminal cysteine residue, and only one member, lymphotactin, has been identified so far (9).

Recently, a new chemokine was discovered that contains the cysteine motif CX3C. This 372-residue protein, known as fractalkine (10, 11) or neurotactin (12), contains an extracellular 76-residue chemokine domain (CDF or chemokine domain of fractalkine) tethered to a membrane-spanning domain via a mucin-like stalk. Because of this natural immobilization, fractalkine can withstand vascular flow to capture and activate leukocytes (13, 14). An extracellular fragment of fractalkine (containing both the chemokine domain and the mucin-like stalk) can be released by proteolysis, and this form of the protein has activities that are different from those of the membrane-bound form (10, 12, 15). The fractalkine receptor, CX3CR1, is expressed in leukocytes and binds tightly (Kd ≈ 1–4 nM) to CDF (15–18); the complex seems to play an anti-inflammatory role (19).

A detailed analysis of the structure of CDF would elucidate the differences between CX3C and other chemokines. Here, we describe the x-ray crystal structure of CDF, solved at 2.0 Å resolution. The crystal structure shows a novel quaternary structure for chemokines, which is directly dependent on the bulge produced by the introduction of three residues between the N-terminal cysteines. CDF can bind to heparin, as shown by heparin-agarose chromatography, with an affinity similar to MIP-1α. A comparison of the calculated electrostatic surface maps for CDF with those for other chemokines shows similarities of charge distribution, which is indicative of heparin-binding capability.

EXPERIMENTAL PROCEDURES

Sample Preparation and Crystallization—CDF (residues 1–76 of fractalkine, with the N-terminal methionine numbered 0) and its selenomethionine form were overexpressed using the plasmid pLSM103 in Escherichia coli strain BL21/pLysS (20). Both proteins were lyophilized after reverse phase-high pressure liquid chromatography and were dissolved in water just before crystallization. Crystals were grown by vapor diffusion, mixing 4 µl of protein (17 mg/ml) with 2 µl of well solution (4 mM sodium formate, 100 mM disodium citrate, pH 5.0) and 10 µl of water. Hexagonal prisms formed quickly (within 1–2 days) and continued to grow for about 1 week. Although very large crystals could be grown (1–2-mm diameter), they diffracted poorly when exposed to a conventional x-ray source (a rotating anode powered by a Rigaku RU200 generator), on average showing a maximum resolution of 2.8–
Crystal Structure of the Chemokine Domain of Fractalkine

| Table I | Crystal data and refinement statistics |
|---------|--------------------------------------|
|         | Native | SeMet λ1 | SeMet λ2 | SeMet λ3 |
| Wavelength (Å) | 0.98 | 0.97915 | 0.97946 | 0.96483 |
| Resolution range (Å) | 20–2.0 | 20–3.0 | 20–3.0 | 20–3.0 |
| Number of reflections (total/unique) | 345,321/30,366 | 53,284/9,248 | 42,954/9,228 | 43,047/9,245 |
| Unit cell (Å) | a = 110.47 | c = 123.99 | a = 110.27 | c = 121.86 |
| Space group | P6122 | P6222 | P6122 | P6222 |
| Complete (overall/last shell) | 98.8%/98.4% | 100%/100% | 99.9%/99.9% | 99.9%/99.9% |
| Rmerge (overall/last shell) | 3.9%/49.1% | 9.8%/50.0% | 8.7%/41.0% | 8.9%/50.3% |
| I/B (overall/last shell) | 61.5/3.9 | 14.1/3.3 | 13.8/3.7 | 14.9/3.0 |
| Number of waters | 264 |
| Number of residues | 262 |

a Using only those reflections within the shell of 2.07–2.0 Å (native) or 3.11–3.0 Å resolution (SeMet).

RESULTS

Crystal Packing and Space Group Dependences—The interactions between monomers of CDF in the crystal are limited, and as a result the average B-factor of the molecule is relatively high (46 Å²) for a frozen crystal. This is not the first such example for chemokines; however, for instance, the crystal structure of viral MIP-2 solved using multiple anomalous diffraction also shows a high average B-factor (49 Å²) (PDB accession code 1cm9). The loose packing of the molecules reflects the high solvent content of 65–70%. Four monomers of CDF are found within the asymmetric unit arranged as layers along the c-parameter (Fig. 1). This arrangement gives rise to large (70 Å) channels along this direction.

The four monomers in the asymmetric unit are arranged as two asymmetric dimers related by a noncrystallographic 2-fold rotation. This 2-fold axis lies along the (1 -1 0) vector, translated to approximately c = 1/6. Because of slight translational misalignments and packing anomalies between the two dimers (see below), the exact symmetry for P66, with c = 62 Å (the pseudo half-cell), is broken, forcing four monomers rather than two into the asymmetric unit of P66,22, with c = 124 Å. When the data were scaled as the pseudo half-cell (l = 2n+1 removed) and the model was refined against these data, the free R-factor did not drop below 0.40, confirming the correct space group as P66,22. There is no direct contact seen between these two created dimers, but rather through bound solvent molecules. The closest contact (−4 Å) between the dimers is at the C terminus of monomer D, the α-helix of which is ordered for an extra 1½ turns (residues 68–74), and of the symmetry-related monomer B. There is a presumed contact between the disordered C termini of monomers A and C, whose helices, if ordered past residue 68, would overlap at the site of the noncrystallographic 2-fold axis in the crystal.

Dimerization—The two monomers that create the dimers (monomers A and D, and B and C) make close contact with each other through an asymmetric interaction of residues 8–14. The dimer is stabilized by a β-sheet formed by residues Cys²⁶ to Thr11 (monomers A and C) and residues Thr11 to Lys14 (monomers B and D) and additional hydrogen bonds between loops 26–28 and 46–48. Note that CDF has not been found to dimerize in solution at any concentration (20, 28). However, this does not rule out dimer formation in vivo, in the presence of the receptor and other participating molecules.

It has been observed that at high concentrations, as well as...
in crystalline environments, chemokines frequently form well defined dimers (or tetramers) but remain monomeric at lower concentrations in solution. The modes of dimerization of chemokines can be classified into two general categories, one observed for most of the CXC chemokines and the other found for CC chemokines. The dimer formed by CDF does not resemble either of these modes, except that the dimeric interaction is formed via an intermolecular \( \beta \)-sheet, similar to CC chemokines. However, the monomers in CDF form a more compact dimer than that of CC chemokine dimers. This motion is facilitated by the 3-residue insertion between the disulfides. Although the second disulfide of CDF (Cys12-Cys50) can be superimposed onto the second disulfide of two CC chemokines (MCP-1 and RANTES), the first disulfide of CDF (Cys8-Cys34) forces the N terminus to remain close to the core of the monomer. This arrangement causes the second monomer to twist around the peptide Ser13-Lys14 to form the intramolecular \( \beta \)-sheet. The first disulfide also causes a bulge in the \( \beta \)-strands formed between the two CDF monomers, further distorting the dimeric interface and possibly weakening the dimer formation. The asymmetry of the dimerization is evident when the monomers are superimposed onto each other (Fig. 2).

**Description of the Structure**—Overall, the structure of the CDF monomer (Fig. 3) is similar to that of other chemokine monomers, particularly MCP-1 and RANTES. This similarity is not surprising, because CDF has the highest identities with these two chemokines (35% and 21%, respectively). The C-terminal \( \alpha \)-helix on monomer D, however, extends out to Ala71, with the remaining residues in an extended conformation (Leu72-Arg74). The extension of the helical conformation is probably weakly stable, because there are no direct contacts between this extension and other protein atoms. Also, the extension of C termini on the other three monomers is blocked by the presence of protein crystal contacts. Almost all residues are clearly defined by electron density. Residues 42–47, which form a loop and are completely solvent accessible, are the most disordered within the chain, except for the extreme N and C termini. Several solvent-accessible side chains also appear to be disordered, such as Lys14 and Arg44. Met15, although clearly visible in the electron density, has a higher \( B \)-factor than Met32, which might be the reason why only one selenium site was determined by the multiple anomalous diffraction experiment.

Superpositions of CC chemokine monomers MCP-1 and RANTES onto the CDF monomer show significant differences where the sequence alignment deviates (Fig. 4). The crystal structures of MCP-1 and RANTES superimpose on the crystal structure of CDF with fewer deviations than the superposition of the CDF NMR model on the crystal structure of CDF. This is seen clearly within the 40s loop (Thr43-Arg47) and in the bulge between Cys8 and Cys12. The N terminus, from Val5-Cys12, and the loop encompassing residues 28–38 are shifted unidirectionally relative to the crystal structure of CDF. This shift is considerable, because the superpositions show a maximal difference of 7 Å between the NMR model and crystal structure of CDF. This shift is partly because of crystal contacts at the edge of the 30s loop (Ser33), stabilizing this region of the molecule and perhaps twisting the chain fragments away. However, the crystal structures of MCP-1 and RANTES, although determined in completely different crystallographic conditions, are closer in structural alignment to CDF.

**Charge Distribution and Heparin Binding**—Most chemokines are highly positively charged. This property facilitates the binding to GAGs on the surface of cells, which immobilizes and presents the chemokines for interaction with their receptors on mobile lymphocytes. The free, proteolytically cleaved fractalkine form can chemoattract neutrophils and T lymphocytes, whereas the tethered fractalkine form can only chemoattract neutrophils after posttranslational processing in vivo (12). This finding raises the question of whether free or tethered CDF can interact and bind to GAGs similar to other freely diffusible chemokines. There are several highly conserved, positively charged residues present in most chemokines, which can be grouped into two sites based on sequence proximity, mu-

![Fig. 1. Stereo ribbon drawing of the CDF asymmetric unit.](image-url)

Note the extension of the \( \alpha \)-helix for monomer D. \( \beta \)-Strands are shown as arrows; \( \alpha \)- and 3\( _{10} \)-helices are shown as coils. This figure was made using the program RIBBONS.
tagenesis, and structural proximity. Site 1 comprises residues within the C-terminal helix (which usually begins after a conserved proline, Pro54, in CDF). Site 2 comprises residues from the loop between the second and third β-strands of the main β-sheet (residues 44–47 in CDF). These two sites have been found to be important in binding chemokines to GAGs (32–34).

The electric charge distribution on the molecular surface of CDF shows clustered positive patches in three regions (Fig. 5). Two of the patches overlap with sites 1 and 2, as described above. It is not known whether CDF can bind to GAGs or whether binding to GAGs is required for CDF to chemotactically attract lymphocytes or neutrophils, but the free form of fractalkine might require some other type of immobilization to create a haptotactic gradient. To investigate whether CDF binds to GAGs with an affinity similar to other chemokines, we compared CDF with an assortment of chemokines using heparin affinity chromatography (Table II). The binding of CDF to heparin-Sepharose is closest in affinity to MIP-1α. Although the theoretical pI, number of charged residues, and protein sequence of CDF are most similar to those of RANTES and MCP-1, the binding of CDF to heparin-Sepharose is lower. MIP-1α has fewer positive charges on its surface than CDF, RANTES, or MCP-1. Thus, the affinity of CDF for binding to GAGs is not dependent strictly on total net charge but on charge localization on the surface of the protein.

**DISCUSSION**

Although it is known CDF does not oligomerize in solution, it does in the crystal. This phenomenon has been seen for the chemokines stromal cell-derived factor-1α (35) and viral MIP-2 (36). The driving forces in forming oligomers within a crystal lattice probably include the high concentrations of protein available in the crystal, as well as the unusual electrostatic environment that would be present under such conditions. Similar conditions might occur in vivo, because chemokines are not fully diffusible in simple buffered solutions but are immobilized within a two-dimensional area on the cell surface. It is unknown what local concentrations of the chemokine could be achieved under such conditions.

There are other factors known to drive oligomerization. A key structural determinant for aggregation of CC chemokines has been identified as two key acidic residues, one within the 20s loop (Glu26 in RANTES), the other within the C-terminal helix (Asp67 in RANTES) (29). Although there is no acidic residue in CDF corresponding to Glu26, CDF residue Asp67 does correspond to the second acidic residue in RANTES. Because the aggregation of MIP-1α, MIP-1β, and RANTES is pH-dependent, the oligomers are likely to be held primarily by electrostatic forces. Also, it was found that only one mutation is required to abolish all oligomerization in MIP-1α, whereas single mutations lower the amount of oligomerization in RANTES from large, nonspecific aggregates to dimers and tetramers (29). Because CDF has a more disperse positive charge on
its surface than RANTES, similar to MIP-1α, as well as only a single acidic residue, it seems to be in agreement with data for MIP-1α and RANTES that CDF does not oligomerize in solution (20, 28).

Because the CDF dimer formed within the crystal is both asymmetric and distinct from CC chemokines in form, a comparison may not totally explain the forces behind oligomerization. Although it is known that CDF does not oligomerize in solution, it cannot be ruled out that fractalkine might dimerize while tethered to a membrane. An analysis of the electrostatic surface maps and structure shows that this dimerization is possible. The dimeric interface is relatively devoid of charges, whereas the side opposite the interface is highly charged (data not shown). Also, both C termini for the two monomers within the dimer are on the same side (Fig. 1), unlike the dimers seen in CC chemokine structures (37). The C-terminal helix is likely to extend farther from the main β-sheet, as seen in monomer D. Thus, while bound to the membrane by a flexible mucin-like tether, CDFs might form dimers.

CDF binds to heparin with roughly the same affinity as MIP-1α (20, 32). The electrostatic surface of CDF is most similar to MIP-1α. The electrostatic surface maps and structure shows that this dimerization is possible. The dimeric interface is relatively devoid of charges, whereas the side opposite the interface is highly charged (data not shown). Also, both C termini for the two monomers within the dimer are on the same side (Fig. 1), unlike the dimers seen in CC chemokine structures (37). The C-terminal helix is likely to extend farther from the main β-sheet, as seen in monomer D. Thus, while bound to the membrane by a flexible mucin-like tether, CDFs might form dimers.

CDF binds to heparin with roughly the same affinity as MIP-1α (20, 32). The electrostatic surface of CDF is most similar to MIP-1α. We expect that the binding of CDF to heparin in vivo would be similar to that of MIP-1α. Mutation studies have shown that a cationic cleft composed of residues Arg18, Arg46, and Arg48 (Lys19, Arg45, and Arg48 in CDF) is required for binding MIP-1α to heparin; the neutralization of any one of

Fig. 4. Stereo drawings showing the superposition of CDF monomer D on RANTES, MCP-1, and the NMR model of CDF. A, RANTES (28) (green) and MCP-1 (30) (blue) superimposed onto CDF (gray). B, the NMR model of CDF (20) (red) superimposed onto CDF (gray). Disulfide bonds are shown in the same color as the models, with sulfurs colored yellow. C, 2Fo − Fc electron density (1.5 o) contoured around residues Cys8-Cys12 and Ala22-Lys26 of the D monomer of CDF. Superpositions were done using the program ALIGN (40), with only backbone atoms included. This figure was made using the program MOLSCRIPT (41).
FIG. 5. Charge distribution of chemokines showing potential GAG binding sites. A, CDF; B, RANTES; C, MCP-1; D, MIP-1α; and E, interleukin-8. Site 1 contains Arg44 and Arg67, and site 2 contains Lys14, Lys18, and Arg47. A third patch is present, encompassing residues Lys36, Arg74 (not shown), Lys54, and Lys59. Arg74 is only ordered in monomer D and is isolated from the three sites. Charges were calculated using GRASP (42), and surface maps were calculated and displayed using INSIGHT (Molecular Simulations, Inc.).

TABLE II

Comparison of the concentration of NaCl required to elute various chemokines from heparin-Sepharose and theoretical isoelectric points

| Protein                        | [NaCl], M | pH | No. K, R | No. D, E |
|--------------------------------|-----------|----|----------|----------|
| MIP-1α                         | 0.34, 0.39 | 5.14 | 7        | 8        |
| CDF                            | 0.37, 0.22 | 9.56 | 11       | 5        |
| MIP-1β                         | 0.49      | 4.77 | 6        | 8        |
| Neutrophil-activating peptide-2 | 0.56<sup>∗</sup> | 6.91 | 13       | 13       |
| Interleukin-8                   | 0.60      | 9.02 | 14       | 10       |
| MCP-1                          | 0.60      | 9.39 | 13       | 7        |
| MCP-3                          | 0.76, 0.80 | 9.74 | 16       | 7        |
| MCP-4                          | 0.78      | 9.98 | 15       | 4        |
| Interferon-inducible protein-10 | 0.85<sup>∗</sup> | 10.1 | 16       | 6        |
| Lymphotaxin                    | 0.86<sup>∗</sup> | 10.6 | 15       | 6        |
| RANTES                         | 0.95      | 9.24 | 10       | 5        |

<sup>a</sup> Theoretical.

<sup>b</sup> Number of lysine and arginine or aspartate and glutamate residues within the chemokine.

<sup>c</sup> From Ref. 43.

<sup>d</sup> From Ref. 20.

<sup>e</sup> From Ref. 6.
these positive charges eliminates binding to heparin (32). The same is probably true for CDF. Although disruption of MIP-1a-GAG interactions has little or no effect on binding to and signaling through CCR1 on Chinese hamster ovary cells, it impairs the ability of MIP-1a to induce both a change in the shape of monocytes and chemotaxis, presumably through disruption of interactions with CCR5 (32, 38). MIP-1a, as opposed to interleukin-8, MCP-1, or RANTES, does not oligomerize on the surface of human umbilical vein endothelial cells, and its activation of CXCXR1, CCR1, and CCR2-transfected Chinese hamster ovary cells is only slightly weakened by deglycosylation (6). Thus, although CDF might bind to GAGs, this binding probably only affects the chemotactrant and activation activities of CDF with specific receptors.

In summary, we have shown that CDF forms a novel quaternary structure relative to other chemokines. CDF binds to heparin and is most similar to MIP-1a in terms of electrostatic properties, although it is most similar to MCP-1 in terms of sequence homology. The coordinates for CDF were deposited at the Protein Data Bank, accession code 1f2l.

Acknowledgments—We thank Dr. Alexander Wlodawer for support of the work presented here, Dr. Zbigniew Dauter for suggestions on data collection, and Dr. Amanda Proudfoot for the gift of RANTES. We also thank Anne Arthur for editorial assistance.

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