Monomeric structure of the cardioprotective chemokine SDF-1/CXCL12

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Received 22 March 2009; Accepted 7 May 2009

DOI: 10.1002/pro.167

Published online 20 May 2009 proteinscience.org

Abstract: The chemokine stromal cell-derived factor-1 (SDF-1/CXCL12) directs leukocyte migration, stem cell homing, and cancer metastasis through activation of CXCR4, which is also a coreceptor for T-tropic HIV-1. Recently, SDF-1 was shown to play a protective role after myocardial infarction, and the protein is a candidate for development of new anti-ischemic compounds. SDF-1 is monomeric at nanomolar concentrations but binding partners promote self-association at higher concentrations to form a typical CXC chemokine homodimer. Two NMR structures have been reported for the SDF-1 monomer, but only one matches the conformation observed in a series of dimeric crystal structures. In the other model, the C-terminal helix is tilted at an angle incompatible with SDF-1 dimerization. Using a rat heart explant model for ischemia/reperfusion injury, we found that dimeric SDF-1 exerts no cardioprotective effect, suggesting that the active species is monomeric. To resolve the discrepancy between existing models, we solved the NMR structure of the SDF-1 monomer in different solution conditions. Irrespective of pH and buffer composition, the C-terminal helix remains tilted at an angle with no evidence for the perpendicular arrangement. Furthermore, we find that phospholipid bicelles promote dimerization that necessarily shifts the helix to the perpendicular orientation, yielding dipolar couplings that are incompatible with the NOE distance constraints. We conclude that interactions with the alignment medium biased the previous structure, masking flexibility in the helix position that may be essential for the distinct functional properties of the SDF-1 monomer.

Keywords: NMR; bicelle; dipolar coupling; chemokine; monomer–dimer equilibrium; myocardial infarction

Introduction

Chemokines are a family of ~50 small, secreted proteins that function as chemoattractants, directing leu-

kocyte trafficking in normal immune function and a variety of disease states.^{1–3} They target a family of ~20 G protein-coupled receptors (GPCRs), which enable cells to migrate in response to a chemokine concentration gradient.^{1–3} Beyond their normal roles in the innate immune system, chemokines and chemokine receptors participate in inflammatory diseases, HIV infection, and cancer progression, prompting numerous efforts to develop novel chemokine receptor antagonists for therapeutic use. For example, the CXC chemokine stromal cell-derived factor-1 (SDF-1, also known as CXCL12) and its receptor CXCR4 are essential for proper fetal development.^{4–6} CXCR4 is also the

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: NIH; Grant number: R01 AI058072; Grant sponsor: SpinOdyssey American Cancer Society Postdoctoral Fellowship; State of Wisconsin Breast Cancer Research Tax Check Off Grant.

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major coreceptor for T-tropic (X4) strains of human immunodeficiency virus-1 (HIV-1), and SDF-1 inhibits HIV-1 infection.^{7–10} Additionally, SDF-1 signaling mediates cancer cell migration and metastasis, and CXCR4 inhibition reduces metastatic tumor formation in a mouse model for human breast cancer.¹¹

Following myocardial ischemia/reperfusion injury, it has recently been observed that there is an increased expression of SDF-1 in cardiac myocytes, endothelial cells, smooth muscle, and fibroblasts.^{12–14} The cardioprotective properties of SDF-1, which are observed both pre- and postischemic event, include reduced infarct zone, decreased scar tissue, increased angiogenesis, resistance to hypoxia/reoxygenation damage, and improved cardiac function.^{13–18} Cardioprotection by SDF-1 is evident *in vitro* and *in vivo* and two mechanisms of action have been proposed: SDF-1 may activate growth and survival signaling pathways in cardiomyocytes directly^{13,14} or enhance cardiac regeneration by recruiting hematopoietic stem and progenitor cells into the heart.^{12,18–22} Various methods for SDF-1 administration in ischemic tissue are currently in development. These include: SDF-1 attached to poly (ethylene glycol)-fibrin patches,¹⁸ self-assembling nanofibers with a timed SDF-1 release mechanism,¹⁵ and viruses engineered to produce SDF-1.¹² In each case, SDF-1 treatment is effective, but variations in the protective effect may be dose-related.

SDF-1 exists in a monomer–dimer equilibrium that shifts toward dimer upon crystallization^{23–25} and in the presence of various binding partners,²⁶ including an extracellular domain of its receptor CXCR4.^{27,28} To investigate the structure–function relationships of the two oligomeric states, we generated a symmetric disulfide-linked dimer [SDF-1(L36C/A65C)]²⁷ and a preferentially monomeric variant (H25R).²⁶ Surprisingly, the preferentially monomeric SDF-1 variant showed enhanced activity relative to the wild-type protein, whereas the constitutive dimer inhibited chemotactic responses to the wild-type chemokine.²⁷ In this work, measurements of cardioprotection using the same SDF-1 variants also suggest that the monomeric species is primarily responsible for this effect. However, differences in the 3D structures of the SDF-1 monomer solved previously by NMR spectroscopy create uncertainty in the correct model for the CXCR4 agonist.²⁷

Chemokine tertiary structure consists of a flexible N-terminus connected by an extended “N-loop” and a turn of 3_{10} -helix to a three-stranded β -sheet and a C-terminal α -helix. Four chemokine subclasses, C, CC, CXC, and CX₃C, are distinguished by the spacing of conserved cysteines linking the N-terminus to the β -sheet. Most chemokines self-associate, with CC dimerization typically mediated by residues near the N-terminus. In contrast, CXC dimers are linked by the β 1 strands to form a six-stranded sheet with the C-terminal helices crossing the dimer interface in an anti-parallel arrangement.²

Figure 1. Comparisons of SDF-1 solution and crystal structures. (A) NMR structure of SDF-1 solved at pH 4.9 in acetate buffer (PDB ID 1SDF). The C-terminal α -helix and the strands of the β -sheet lie at an angle of $\sim 55^\circ$. (B) Three X-ray crystal structures (1A15, black; 1QG7, gray; 2J7Z, white) display the same SDF-1 dimeric arrangement. (C) Two copies of the SDF-1 monomer from (A) (cyan) superimposed on the SDF-1 dimer (white). (D) The SDF-1 NMR structure solved at pH 5.5 in phosphate buffer (PDB ID 1VMC; blue) superimposes with a C α r.m.s.d. of 1.0 Å on the SDF-1 crystallographic dimer 2J7Z.

In the original SDF-1 structure solved by 2D NMR at pH 4.9, the α -helix and β -sheet are oriented at a $\sim 55^\circ$ angle²⁹ [Fig. 1(A)]. This configuration is incompatible with the CXC-type dimer observed in subsequent SDF-1 crystal structures^{25,30,31} [Fig. 1(B)], because the helices from each subunit of the dimer would clash with each other [Fig. 1(C)]. In a newer structure of the SDF-1 monomer solved at pH 5.5 using 3D heteronuclear NMR methods,³² the helix and sheet are perpendicular and superimposable on a subunit of the crystal structures [Fig. 1(D)].³² Noting this agreement with the SDF-1 dimer, Gozansky *et al.*³² argued that their model is more accurate than the original structure by Crump *et al.* owing to the use of [U - ^{13}C , ^{15}N]-enriched protein and refinement against residual dipolar coupling (RDC) constraints. Until this discrepancy is resolved with additional data showing that one of the NMR structures is incorrect or by identifying differences in solution conditions that alter the protein conformation, key aspects of the SDF-1 structure remain unclear.

Concentration-dependent ^{15}N relaxation measurements by Baryshnikova and Sykes³³ indicated that even in the absence of monomer–dimer exchange the SDF-1 monomer undergoes millisecond–microsecond conformational fluctuations. Flexibility at the base of the SDF-1 helix would permit exchange between a unique conformation compatible with dimer formation

Figure 2. Monomeric SDF-1 is cardioprotective. Perfused explanted rat hearts were pretreated (15 min) with (A) wild-type SDF-1, (B) a preferentially monomeric SDF-1 variant [SDF-1(H25R)], or (C) disulfide-locked SDF-1 dimer [SDF-1(L36C/A65C)]. Left ventricle developed pressure (LVDP) (left) and infarct size (right) were measured after global, no-flow ischemia (25 min) and subsequent reperfusion (180 min).

(helix angle = 90°) and others that are not (helix angle < 90°). We speculated that the distinct helix positions in the two published structures reflect intrinsic conformational dynamics of the SDF-1 monomer and that the range of accessible conformations might be sensitive to changes in solution conditions.

Our previous work showed that the SDF-1 dimer is stabilized in solution by phosphate, sulfate, and other binding partners.^{26,28} One of the NMR structures (1SDF) was solved in acetate buffer at pH 4.9, whereas the 1VMC structure used 50 mM phosphate at pH 5.5. To understand why this important chemokine structure remains in dispute, we measured histidine pK_a values and determined the solution structure of the SDF-1 monomer in the presence and absence of phosphate. We also investigated the effect of phospholipid bicelles on the SDF-1 conformation and oligo-

meric state. Our results show that NOE-based refinement of the SDF-1 monomer at pH 5.5 yields a helix position intermediate to the previous models. Furthermore, we find that RDC constraints bias the SDF-1 monomer structure toward a conformation consistent with the crystallographic dimers because bicelles promote SDF-1 dimer formation.

Results

Monomeric SDF-1 is cardioprotective

We used an isolated, buffer-perfused rat heart explant model to assess the direct cardioprotective effects of pretreatment with wild-type SDF-1, preferentially monomeric SDF-1(H25R)²⁶ and a constitutive SDF-1 dimer, SDF-1(L36C/A65C) (SDF1₂).²⁷ Aerobic cardiac function (heart rate, coronary flow rate, and developed

Figure 3. Electrostatic repulsion alters the His 25 pK_a . $H^{\epsilon 1}$ chemical shifts from His 17 (triangles) and His 25 (circles) plotted as a function of pH. Equation (1) was used to fit the deprotonated chemical shift, protonated chemical shift, and pK_a of the respective nuclei to changes in the monitored chemical shift as a function of pH. Solution conditions were 1 mM SDF-1 in either 0 mM sodium phosphate (open) or 50 mM sodium phosphate (filled). The lower pK_a of His 25 reflects its proximity to Lys 27 of the opposing subunit (dark spheres) across the dimer interface, in contrast to the unperturbed pK_a of His 17 located far from the interface.

pressure) was measured before ischemia, and increased resistance to injury from myocardial ischemia/reperfusion was determined from reduction in infarct size and increased recovery of developed pressure relative to untreated control hearts. Wild-type SDF-1 [Fig. 2(A)] and the H25R mutant [Fig. 2(B)] show significant dose-dependent cardioprotection at 25 and 50 nM concentrations as indicated by infarct size and left ventricle diastolic pressure (LVDP). In contrast, the SDF₁₂ dimer was less active at the tested concentrations [Fig. 2(C)], suggesting that monomeric SDF-1 is required for resistance to ischemia/reperfusion injury.

Histidine pK_a values for SDF-1

The quaternary structure of SDF-1 is highly sensitive to changes in solution conditions. SDF-1 self-association occurs when electrostatic repulsion at the dimer interface is balanced by ions like phosphate or sulfate, combined with deprotonation of His 25 to the neutral species.²⁶ Both of the previous NMR structures were solved at acidic pH (4.9 and 5.5), where His 25 is likely to be positively charged because the histidine pK_a values are typically six or higher. However, histidine pK_a values in proteins vary widely, and we speculated that proximity to other positively charged residues (Lys 24, Lys 27, and Arg 41) or the presence of 50 mM phosphate could depress the His 25 pK_a . To determine whether differences in solution conditions could alter the structure of SDF-1, we monitored the protonation state of His 25 as a function of pH and phosphate concentration.

We determined histidine pK_a values by nonlinear fitting of 1H and ^{15}N chemical shifts to a modified Henderson–Hasselbalch equation (see Fig. 3). Chemical shift and pK_a values measured in 0 and 50 mM phosphate for both histidines are listed in Table I. His 17, located far from the SDF-1 dimer interface, has a

pK_a of 6.6 in the presence and absence of phosphate. In contrast, the pK_a of His 25, which participates in the dimer interface, is lower by nearly a full pH unit (5.7). At the pH values used for previous NMR structures, the fraction of positively charged His 25 ranges from ~90% at pH 4.9 (1SDF) to only ~50% at pH 5.5 (1VMC). Thus, the His 25 protonation state may contribute to structural differences, but our previous work suggested that phosphate could have an even more pronounced effect on conformation.

Monomeric SDF-1 structures with and without phosphate

Phosphate, sulfate, and other SDF-1 binding partners have a dramatic stabilizing effect on the dimeric state.²⁶ We speculated that the presence of 50 mM phosphate in the 1VMC solution conditions and its absence in the 1SDF solution could be responsible for the structural difference. To test this hypothesis, we solved the NMR structure of SDF-1 at pH 5.5 in the presence and absence of 50 mM sodium phosphate. Although we expected to see significant differences in helix orientation, our structures of SDF-1 in 0 or 50 mM sodium phosphate (pH 5.5) superimpose with a C α RMSD of 0.3 Å for residues 9–66 [Fig. 4(A)]. Refinement statistics for each structure are summarized in Supporting Information Tables I and II. In comparison with the Gozansky *et al.* (blue) and Crump *et al.* (cyan) structures, the helix is tilted at an intermediate angle [Fig. 4(B,C)].

NOEs define the SDF-1 helix position

To determine why our structure of SDF-1 in 50 mM sodium phosphate (pH 5.5) disagrees with the 1VMC structure solved under identical solution conditions, we compared the restraints used in calculating the structures and found a key difference. A set of NOEs in the 3D ^{13}C -edited NOESY-HSQC links Tyr 61 to the methyl groups of Ile 28 and Ile 38 [Fig. 5(A)]. Restraints between Tyr 61 and Ile 28 or Ile 38 were

Table I. Histidine Side Chain 1H and ^{15}N Chemical Shifts and pK_a Values in 0 and 50 mM Phosphate

Residue	Atom	Sodium phosphate (mM)	pK_a	δ_{AH+} (ppm)	δ_A (ppm)
His 25	$H^{\epsilon 1}$	0	5.7	8.6988	7.8069
		50	5.9	8.6604	7.8069
	$H^{\delta 2}$	0	5.6	7.3276	6.9035
		50	5.8	7.2817	6.9239
	$N^{\epsilon 2}$	0	5.7	189.5488	194.7319
		50	5.7	189.5682	194.3376
His 17	$H^{\epsilon 1}$	0	6.6	8.6791	7.7668
		50	6.7	8.6586	7.7599
	$H^{\delta 2}$	0	6.5	7.4307	7.060
		50	6.7	7.4061	7.0288
	$N^{\epsilon 2}$	0	6.7	189.1804	193.0362
		50	6.6	189.2218	192.720

Figure 4. Solution structures of SDF-1 at 0 and 50 mM sodium phosphate. (A) Structures of SDF-1 in 0 mM (salmon; PDB ID 2KED) and 50 mM (red; PDB ID 2KEC) sodium phosphate at pH 5.5. (B) Solution structure of SDF-1 at pH 5.5 in 0 mM sodium phosphate (salmon) overlaid onto the SDF-1 structures 1VMC (blue) and 1SDF (cyan). (C) Solution structure of SDF-1 at pH 5.5 in 50 mM sodium phosphate (red) overlaid onto the SDF-1 structures 1VMC (blue) and 1SDF (cyan).

not present in the 1VMC restraint list*, and inspection of the 1VMC structure shows that the corresponding distances between the aromatic protons of Tyr 61 and the H^{δ1} methyl protons of either Ile 28 or Ile 38 are >5.5 Å [Fig. 5(B)], whereas our NOE-derived restraints require distances <5.5 Å. Structures computed without the Tyr 61-Ile 28 and Tyr 61-Ile 38 restraints more closely resembled the 1VMC structure [Fig. 5(C)]. We concluded that these NOEs linking hydrophobic core residues of the C-terminal α-helix and β-sheet define a different helix position than the one reported by Gozansky *et al.* [Fig. 5(B,C)].

RDCs constrain the SDF-1 helix in the 1VMC structure

Restraints from RDCs were used to solve the 1VMC structure.³² We measured ¹H-¹⁵N RDCs for SDF-1 under the same conditions using 5% DMPC/DHPC bicelles and compared the observed couplings with each SDF-1 solution structure. Figure 6 shows correlation plots of observed RDC values (D_{obs}) and calculated RDC values (D_{calc}) for the 1VMC and 1SDF structures. As expected, D_{calc} values from the 1VMC structure had the highest correlation with D_{obs} values ($r = 0.913$), whereas 1SDF had the lowest correlation ($r = 0.772$). Our SDF-1 structures determined in 0 and 50 mM sodium phosphate (pH 5.5) had intermediate correlation coefficients ($r = 0.838$ and 0.846 , respectively). Overall, the relationship between correlation coefficient and helix angle suggests that RDC restraints contributed to the perpendicular orientation observed in the 1VMC structure.

Bicelles promote SDF-1 dimer formation

DMPC/DHPC bicelles form a liquid crystalline medium that orients with the magnetic field that is meant to induce a small degree of molecular alignment without altering the structure of the protein under investigation.^{34–37} To examine whether phospholipids used for RDC measurement could affect the helix con-

figuration, we looked for evidence of changes in the SDF-1 structure in the presence of bicelles. A comparison of ¹⁵N-¹H HSQC spectra of SDF-1 in the presence and absence of bicelles revealed noticeable chemical shift changes [Fig. 7(A)], suggesting that the DMPC/DHPC bicelles may influence the SDF-1 structure. We previously demonstrated that ¹H and ¹⁵N shifts of K24 and K27, which lie in the β1-strand and contribute to the dimer interface, report on the SDF-1 oligomeric state.²⁶ Addition of DMPC/DHPC bicelles shifted the peaks for K24 and K27 in a direction consistent with an increasing population of SDF-1 dimer [Fig. 7(B)].

Pulse field gradient measurements of translational self-diffusion coefficients (D_s) confirmed the observation that DMPC/DHPC bicelles promote SDF-1 dimer formation. A decrease in the D_s value from $1.74 \times$

Figure 5. NOEs linking Y61 to I28 and I38 define the SDF-1 helix position. (A) Strips from a 3D ¹³C-edited NOESY-HSQC showing NOEs between the H^{δ1} and H^{ε1} protons of Y61 and the H^δ methyl protons of I28 and I38. (B) NOE-derived distance restraints between the Y61 H^δ and H^ε protons and the H^δ methyl protons of I28 and I38 (light red) define a different helix position (red) than that observed in 1VMC (blue) where the Y61-I28 and Y61-I38 distances exceed 5 Å (light blue). (C) Comparison of SDF-1 structures in 50 mM sodium phosphate, pH 5.5 computed with (red) or without (pink) distance restraints derived from the Y61-I28 and Y61-I38 NOEs in (A).

*<http://tang.bmr.b.wisc.edu:8080/WebModule/MRGridServlet?pdb_id=1VMC&show_blocks=true&min_items=0>.

Figure 6. Correlation of measured RDC values with **1VMC** and **1SDF**. Residual dipolar couplings (RDCs) were measured in 5% DMPC/DHPC bicelles and in isotropic (water) solution on 0.25 mM SDF-1 at 35°C. Singular value decomposition of the 35 measured RDC values was then used to calculate RDCs for the **1VMC** and **1SDF** structures and best-fit alignment tensors. Plots of the measured RDCs (D_{obs}) versus the calculated RDCs (D_{calc}), and the respective structures are shown for (A) **1VMC** and (B) **1SDF**. Pearson's correlation coefficients for the **1VMC** and **1SDF** structures are 0.913 and 0.772, respectively.

10^{-6} to $1.42 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ accompanied the addition of 5% DMPC/DHPC (2.8:1) bicelles to a sample of 0.25 mM SDF-1 at pH 7 [Fig. 7(C)]. To estimate the contribution of self-aggregation to the SDF-1 self-diffusion coefficient, we also measured diffusion in 5% DMPC/DHPC (2.8:1) bicelles for constitutively dimeric SDF-1(L36C/A65C) (15.9 kDa; $D_s = 1.04 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$) and ubiquitin (8.6 kDa; $D_s = 1.70 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$). Using $D_{s,\text{ubiquitin}}$ and $D_{s,\text{SDF-1}\alpha(\text{L36C/A65C})}$ as representative standards for monomer and dimer self-diffusion coefficients in the presence of DMPC/DHPC bicelles, we estimated the contribution of the dimeric species to the measured D_s value to be $\sim 40\%$ at 0.250 mM SDF-1. At higher SDF-1 concentrations, most of the protein would be dimeric. Hence, restraints derived from RDCs measured in DMPC/DHPC bicelles will reflect significant contributions from the SDF-1 dimer.

In DMPC/DHPC bicelles, RDC values could be determined for only 35 residues because of selective line broadening of many signals including four residues in the C-terminal helix. To discourage electrostatic interactions between SDF-1 (calculated pI = 9.9) and the negatively charged DMPC/DHPC bicelles, we measured ^1H - ^{15}N RDCs in a fresh preparation of DMPC/DHPC bicelles doped with tetradecyltrimethylammonium bromide (TTAB), a cationic lipid, at lower pH (5.5 instead of 7.0) in an effort to prevent SDF-1 dimer formation. Addition of TTAB restored most of the broadened signals yielding RDCs for 55 residues, including all 12 residues of the helix (57–68). Comparison of D_{obs} measured in the TTAB/DMPC/DHPC bicelles with D_{calc} shows the best correlation with our structures with D_{calc} shows the best correlation with our structures in 0 and 50 mM sodium phosphate ($r = 0.882$ and 0.877 , respectively) compared with the **1VMC** structure ($r = 0.833$) [Fig. 8(A–C)]. Although these RDC values agreed best with our SDF-1

Figure 7. DMPC/DHPC bicelles promote SDF-1 dimer formation. (A) ^{15}N - ^1H HSQC spectra of SDF-1 in the absence (gray) or presence (black) of 5% DMPC/DHPC bicelles (2.8:1 molar ratio). (B) Chemical shifts of residues at the dimer interface report on the oligomeric state of SDF-1. In the absence of bicelles, chemical shifts for K24 and K27 are consistent with monomer (M) but shift toward dimer (D) in the presence of DMPC/DHPC bicelles (2.8:1 molar ratio). (C) Differences in translational self-diffusion coefficients measured for 0.25 mM SDF-1 suggest that oligomerization is favored in the presence of bicelles. Nonlinear fitting of peak intensities from a series of 1D ^1H spectra acquired in the absence (gray) and presence of DMPC/DHPC bicelles (black) using a longitudinal encode-decode pulse field gradient diffusion scheme (diffusion delay $\Delta = 80 \text{ ms}$; gradient pulse $\Delta = 5 \text{ ms}$) yielded self diffusion coefficients (D_s) of 1.74×10^{-6} and $1.42 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$.

Figure 8. RDCs in TTAB/DMPC/DHPC bicelles are consistent with monomeric structure. RDC values measured in 0.8 mM TTAB, 5% DMPC/DHPC, and 10% $^2\text{H}_2\text{O}$ (pH 5.5). TTAB was added to minimize electrostatic interactions between bicelles and SDF-1, and the pH was lowered to shift the monomer–dimer equilibrium toward monomer. Plots of the RDCs measured in TTAB/DMPC/DHPC versus the calculated RDCs for 1VMC (A), 2KED (B), and 2KEC (C), and the respective structures are shown. A total of 55 RDC values were measured. The Pearson’s correlation coefficients for the 1VMC, 2KED, and 2KEC structures are 0.833, 0.882, and 0.877. (D) Overlay of SDF-1 structures solved in 20 mM MES at pH 6.8 (gray; PDB ID 2KEE), 0 mM sodium phosphate at pH 5.5 (salmon; PDB ID 2KED), and 50 mM sodium phosphate at pH 5.5 (red; PDB ID 2KEC). (E) NMR structure of the SDF-1 locked dimer in complex with the CXCR4 N-terminus (orange). One SDF-1 subunit is illustrated with electrostatic potential surface and the opposing monomer is represented by a transparent ribbon. Tyrosine 7 of CXCR4 occupies a pocket at the dimer interface, but Ile 4 and Ile 6 do not make specific contacts with the SDF-1 dimer surface. Dissociation of the dimer reveals a hydrophobic surface that could be occupied by Ile 4, Ile 6, and Tyr 7 upon rearrangement of the CXCR4 N-terminus (orange dots).

structures, PFG diffusion measurements suggest that the addition of TTAB does not eliminate SDF-1 interactions with the phospholipid bicelles. To avoid bias or distortion from bicelle-induced conformational changes, we omitted RDC restraints from the refinement of SDF-1 NMR structures presented here.

SDF-1 structure at pH 6.8

Previous NMR structures were solved at acidic pH in an effort to prevent complications from the SDF-1 monomer–dimer equilibrium. However, we showed previously that SDF-1 is monomeric at near-neutral pH in organic buffers like MES and TRIS.²⁶ To define the monomeric state at a more physiologically relevant pH, we solved the structure of SDF-1 at pH 6.8 in 20 mM MES buffer, conditions where His 25 is uncharged. The SDF-1 structure at pH 6.8 superimposes on the structures solved in 0 and 50 mM sodium phosphate at pH

5.5 with C α r.m.s.d. values of 0.7 and 0.6 Å, respectively [Fig. 8(D)]. In each case, overlays of the NMR monomers on the SDF-1 dimer indicate that overlap between C-terminal helices of the opposing subunits would prevent self-association (data not shown).

Discussion

Full activation of chemokine receptors is thought to depend exclusively on binding of the monomeric ligand.^{38,39} Yet oligomerization is essential for *in vivo* chemokine function,^{39–42} presumably to support immobilization on cell-surface glycosaminoglycans (GAG). Interference with chemokine oligomerization and GAG binding can be used to create anti-inflammatory molecules, as demonstrated by CC chemokine variants that block leukocyte recruitment by the wild-type chemokine *in vivo*.^{43,44} Here, we show that a constitutively dimeric form of SDF-1 exerts none of the

cardioprotective activity conferred by the wild-type chemokine or a preferentially monomeric mutant (H25R). Interestingly, SDF-1 is less effective at the highest concentration tested (100 nM) than at 10–50 nM, consistent with the bell-shaped activity profile observed in previous measurements of leukocyte chemotaxis.²⁷ This reinforces and extends our recent discovery that, although monomeric SDF-1 fully activates CXCR4 to induce chemotaxis, dimerization of SDF-1 creates a partial CXCR4 agonist that inhibits chemotaxis toward wild-type SDF-1.²⁷ In addition, because our measurements of ischemia/reperfusion injury were performed using explanted hearts, these results suggest that monomeric SDF-1 exerts a direct, prosurvival effect on cardiomyocytes, in addition to mobilization of cardiac progenitor cells that may occur *in vivo*. Interactions that promote SDF-1 dimerization may reduce its beneficial effects, and we speculate that SDF-1 variants that resist dimerization may be useful anti-ischemic compounds. Accurate three-dimensional structures of chemokines are invaluable tools in the design of novel mimics or inhibitors and are necessary to establish structure–function relationships for the monomeric and oligomeric species. To understand how the SDF-1 monomer binds and activates CXCR4, it was necessary to resolve a discrepancy between the published **1SDF** and **1VMC** structural models in the orientation of the C-terminal α -helix.

We solved the NMR structure of monomeric SDF-1 at pH 5.5 in buffers containing either 0 or 50 mM phosphate and found close agreement between the two structures (see Fig. 4). However, the intermediate helix angle in our models (75° relative to the β -sheet) was distinct from both published structures, which show the helix at either 90° (**1VMC**) or 55° (**1SDF**) (see Fig. 1). In NOESY spectra acquired under identical conditions, we assigned long-range NOEs that are incompatible with the **1VMC** structure and found that distance restraints corresponding to those NOEs were not used to calculate that model. Chemical shift differences and self-diffusion measurements revealed that phospholipid bicelles used for RDC measurements in the Gozansky study³² promote SDF-1 dimerization (see Fig. 7). As illustrated in Figure 1(C), SDF-1 dimerization is incompatible with the monomeric conformations observed in the original NMR structure of Crump *et al.*,²⁹ as well as the new structures we present here. We concluded that RDC restraints used in the **1VMC** refinement do not describe the structure of monomeric SDF-1 but instead bias the helix toward the perpendicular orientation found in crystal structures of the dimer. RDC restraints can improve the accuracy of NMR structures. However, as others have cautioned, a suitable alignment medium must not interact strongly with the molecule under study to avoid distortions or errors in the resulting structural model.³⁷ In this instance, we were alerted to bicelle-SDF-1 binding by chemical shift perturbations and line broadening in

the HSQC spectrum. Doping the negatively charged DMPC/DHPC bicelles with the cationic lipid TTAB restored the majority of missing peaks in the HSQC, presumably by weakening electrostatic interactions with the highly basic SDF-1 protein.

The difference in helix angle between the original model from Crump *et al.*²⁹ and our solution structures of SDF-1 may reflect real variations in the SDF-1 conformation, particularly if a fully charged His 25 at pH 4.9 shifts the helix to a more acute angle than the other structures measured at or above the pK_a of His 25. NMR relaxation measurements by Baryshnikova and Sykes³³ suggest that the SDF-1 monomer exists in a conformational equilibrium between two states, only one of which is compatible with dimer formation. We hypothesize that the helix position responds to interactions with SDF-1 binding partners, which can in turn shift the monomer–dimer equilibrium.

In particular, we showed previously that the CXCR4 N-terminal domain promotes SDF-1 dimerization by forming specific contacts with both subunits of the dimer.^{27,28} However, residues at the extreme CXCR4 N-terminus participate in unique interactions with monomeric SDF-1 that are not accounted for in the structure of a dimeric 2:2 complex.²⁷ Residues 3–9 show the largest chemical shift perturbations in a titration of ¹⁵N-labeled N-terminal CXCR4 peptide with wild-type SDF-1,²⁸ but Tyr 7 is the only residue in this region that makes a specific contact with the locked SDF-1 dimer.²⁷ Figure 8(E) shows how the dimer interface conceals a hydrophobic surface on the SDF-1 monomer that could interact with apolar residues at the CXCR4 N-terminus. We hypothesize that when monomeric SDF-1 binds to CXCR4, Ile 4 and Ile 6 of the receptor dock into the hydrophobic cleft exposed upon dissociation of the SDF-1 dimer. This hydrophobic surface is largest when the helix angle is 90° and smaller when the helix pivots to a position incompatible with dimer formation. When monomeric SDF-1 encounters CXCR4, recognition by the receptor N-terminus may shift the helix angle toward the perpendicular arrangement. SDF-1 dimerization and rearrangement of the first few residues of CXCR4 would then be permitted, depending on the concentration of the chemokine. By defining the three-dimensional structure of monomeric SDF-1, the results presented here lay the foundation for future studies of the dynamics of molecular recognition in the SDF-1/CXCR4 signaling system, as well as efforts to design SDF-1 variants with novel functional properties.

Methods

Protein expression and purification

SDF-1 was produced as previously described.^{26–28,45} Briefly, N-terminal hexa-His-tagged SDF-1 was expressed as insoluble inclusion bodies in *E. coli* and purified using metal affinity chromatography.

Refolding of SDF-1 was done either while immobilized to a metal affinity column or in solution as described previously.⁴⁵ Tobacco etch virus protease cleavage of the N-terminal hexa-His tag was followed by reverse phase HPLC purification to >98% homogeneity.

Cyanogen bromide cleavage to generate biologically active SDF-1^{27,28,45} was omitted for these structural studies because the flexible SDF-1 N-terminus does not interact with the folded chemokine domain or influence the monomer–dimer equilibrium.^{24–27,32} Hence, SDF-1 used for structural studies contained an N-terminal Gly-Ser dipeptide for the structure solved in 25 mM MES at pH 6.8 or an N-terminal gly-met dipeptide for the structures solved in either 0 mM sodium phosphate at pH 5.5 or 50 mM sodium phosphate at pH 5.5.

Cardioprotection assay

Isolated rat hearts were perfused for 15 min, followed by aerobic perfusion with SDF-1 variants 15 min prior to 25 min global, no-flow ischemia and 180 min reperfusion. Hearts subjected to the perfusion sequence in the absence of SDF-1 served as ischemic controls. Hearts perfused continuously for 245 min served as nonischemic controls. The perfusion sequence was administered as previously described in Baker *et al.*^{46,47} Each dosage group consisted of six replicates. Resistance to injury from myocardial infarction/reperfusion was determined by a reduction in infarct size and increase in recovery of developed pressure.

Following 3-h reperfusion, hearts were removed from the perfusion apparatus and prepared for infarct size measurement as previously described.^{46,47} Briefly, hearts were sliced across the long axis of the left ventricle, from apex to base, into 2-mm-thick transverse sections and then incubated in 1% triphenyltetrazolium chloride in phosphate buffer (pH 7.3) at 38°C for 20 min. Infarcted tissue was visually enhanced by storage in 10% formaldehyde solution for 24 h before analysis. Infarcted tissue and the area at risk were surgically removed and measured in a blinded manner; tissue volumes were calculated by multiplying the weight of planimetered area by cross-section thickness and expressed as a percentage of left ventricular volume. Cardiac function was recorded continuously throughout each experiment as previously described.^{46,47} End-diastolic pressure was initially set at 3 mmHg for 2 min. The balloons were progressively inflated with a microsyringe to set end-diastolic pressures to 8 mmHg for the left ventricle and 3 mmHg for the right ventricle. Developed pressure was recorded during steady-state conditions. Coronary flow rate was measured throughout the experiment by timed collections of the coronary effluent from the right side of the heart into a graduated cylinder.

Determination of histidine pK_a values

NMR experiments were performed on 0.5 mL solutions of 10% ²H₂O, 1 mM protein, and 0 or 50 mM sodium

phosphate. The pH was adjusted by adding either HCl or NaOH and was measured before each experiment. The protonation state of the nitrogen atoms of the imidazole ring of His 17 and His 25 was measured by a modified HCN experiment.⁴⁸ The HCN technique utilizes the scalar coupling ¹J_{CH} and ¹J_{CN} for sequential magnetization transfer from imidazole ring ¹H to ¹³C to ¹⁵N and reverse, resulting in a 2D spectrum correlating carbon-bonded protons to imidazole nitrogens. Changes in H^{ε1}, H^{δ2}, N^{ε2}, and N^{δ1} chemical shifts as a function of pH were used for pK_a determination. NMR data were analyzed with the program pro Fit 5.6 (QuantumSoft, Switzerland) and pH titration data were fitted to a modified Henderson–Hasselbalch equation:

$$\delta_{\text{obs}} = \frac{\delta_{\text{AH}^+} + \delta_{\text{A}} 10^{(\text{pH}-\text{pK}_a)}}{1 + 10^{(\text{pH}-\text{pK}_a)}}, \quad (1)$$

in which δ_{obs} is the observed chemical shift at each pH value, and δ_{AH^+} and δ_{A} are the chemical shifts when the imidazole nitrogens are fully protonated or deprotonated, respectively.

Structure determination

Samples for structure determination contained [^U-¹⁵N,¹³C]-SDF-1 in either 0 mM sodium phosphate at pH 5.5, 50 mM sodium phosphate at pH 5.5, or 25 mM MES at pH 6.8 at protein concentrations of 1.6, 1.6, and 1.2 mM, respectively. Standard NMR techniques were used for generating chemical shift assignments for SDF-1.⁴⁹ 3D ¹⁵N-edited NOESY-HSQC, ¹³C-edited NOESY-HSQC, and ¹³C(aromatic)-edited NOESY-HSQC spectra ($\tau_{\text{mix}} = 80$ ms) were used to generate distance constraints. TALOS was used to generate backbone dihedral angle constraints from ¹H^α, ¹³C^α, ¹³C^β, ¹³C^γ, and ¹⁵N chemical shifts.⁵⁰ The NOEASSIGN module of the torsion angle dynamics program CYANA was used to determine initial structures and was followed by iterative manual refinement to eliminate constraint violations.⁵¹ X-PLOR was used for further refinement, in which physical force field terms and explicit water solvent molecules were added to the experimental constraints.⁵² Refinement and validation statistics for each ensemble of 20 conformers are given in Supporting Information Tables I–III, and they also list the statistics for Procheck-NMR validation of the final 20 conformers.

Self-diffusion measurements

Measurements of the self-diffusion coefficients, D_s , for SDF-1, SDF-1(L36C/A65C), and ubiquitin were acquired using a water-suppressed longitudinal encode-decode (water-sLED) experiment.⁵³ A 1% (w/v) sample of β-cyclodextrin in 90% H₂O and 10% ²H₂O, with a diffusion coefficient 3.239×10^{-6} cm² s^{−1} at 25°C,⁵⁴ was used as a standard for gradient strength calibration. The diffusion delay was 80 ms, and the gradient pulse length was 5 ms. The gradient strength was varied from 10 to 80% in 1% intervals with 58.97

$G \text{ cm}^{-1}$ as the maximum (100%) gradient strength. Nonlinear least-squares fitting was used to obtain self-diffusion coefficients (D_s) from the following equation:

$$A(2\tau) = A(0) \exp[-(\gamma\delta G)^2(\Delta - \delta/3)D_s] \quad (2)$$

where γ is the magnetogyric ratio of ^1H , δ is the gradient pulse length, G is the gradient intensity, and Δ is the diffusion delay.

RDC measurements

^1H - ^{15}N RDCs were measured using an in-phase, anti-phase pulse scheme.⁵⁵ The difference between $^1J_{\text{NH}} + D_{\text{NH}}$ splittings recorded in anisotropic (5% bicelles, 2.8:1 DMPC/DHPC) and isotropic (water) solvent yields the RDC. Samples in both anisotropic and isotropic solvents contained 0.250 mM SDF-1 in 10% $^2\text{H}_2\text{O}$ (pH 7.0) at 35°C; bicelles were suspended and aligned according to Fleming and Matthews.³⁷ Alignment was confirmed by monitoring the quadrupolar splitting of the HDO ^2H resonance. Using the DC module of the NMRPipe program,⁵⁶ alignment parameters were fitted via singular value decomposition with the dipolar interaction held constant at $-21,585 \text{ Hz}$. Thirty-four RDCs were extracted and compared to various SDF-1 structures. Additional RDC measurements were performed in 5% DHPC/DMPC bicelles doped with 0.8 mM TTAB at pH 5.5. Under these conditions, 55 RDC values were extracted for comparison with SDF-1 structures.

Acknowledgments

NMR parameters for each SDF-1 structure have been deposited in the BioMagResBank with accession numbers 16142 (SDF-1 50 mM phosphate, pH 5.5), 16143 (SDF-1 0 mM phosphate, pH 5.5), and 16145 (SDF-1, pH 6.8).

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