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On-column refolding of recombinant chemokines for NMR studies and biological assays

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Abstract

We have applied an efficient solid-phase protein refolding method to the milligram scale production of natively folded recombinant chemokine proteins. Chemokines are intensely studied proteins because of their roles in immune system regulation, response to inflammation, fetal development, and numerous disease states including, but not limited to, HIV-1/AIDS, cancer metastasis, Crohn's disease, asthma and arthritis. Many investigators use recombinant chemokines for research purposes, however these proteins partition almost exclusively to the inclusion body fraction when produced in *E. coli*. A major hurdle is to correctly refold the chemokine and oxidize the two highly conserved disulfide bonds found in nearly all chemokines. Conventional methods for oxidation and refolding by dialysis or extreme dilution are effective but slow and yield large volumes of dilute chemokine. Here we use an on-column approach for rapid refolding and oxidation of four chemokines, CXCL12/SDF-1 α (stromal cell derived factor-1 α), CCL5/RANTES, XCL1/lymphotactin, and CX3CL1/fractalkine. NMR spectra of SDF-1 α , RANTES, lymphotactin, and fractalkine indicate these chemokines adopt native structures. On-column refolded SDF-1 α is fully active in an intracellular calcium flux assay. Our success with multiple SDF-1 α mutants and members of all four chemokine subfamilies suggests that on-column refolding is a robust method for preparative scale production of recombinant chemokine proteins.

Introduction

Chemokines are secreted chemoattractants that induce cell migration by activating seven transmembrane G-protein coupled receptors. Migrating cells follow a chemokine gradient that is thought to form through chemokine diffusion and binding to extra-cellular glycosaminoglycans. Chemokines are divided into four families, C, CC, CXC, and CX₃C, based on the spacing of two cysteines near the N-terminus that participate in a pair of conserved disulfide bonds[1]. These signaling proteins direct trafficking of leukocytes in normal inflammatory processes, but are also implicated in many disease states, including HIV-1/AIDS, arthritis, asthma, and Crohn's disease[2]. Hence chemokine signaling is extensively studied as an area for pharmaceutical intervention.

We are particularly interested in the CXC chemokine SDF-1 α /CXCL12 (stromal cell derived factor-1 α). SDF-1 α and its receptor CXCR4 are required for proper fetal development[3–5] and are involved in human disease states including HIV/AIDS[6] and cancer metastasis[7].

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CXCR4 $-/-$ or *SDF-1 α* $-/-$ mice have a perinatal lethal phenotype with defects in B-cell lymphopoiesis, bone marrow myelopoiesis, vascularization of the gastrointestinal tract, cardiac ventral septum formation, and cerebellar formation[3–5]. *CXCR4* is a co-receptor for X4 tropic and R5/X4 dual tropic HIV-1 and SDF-1 α can inhibit HIV-1 entry into cells[6]. Also, SDF-1 α can attract metastatic breast cancer cells circulating in the blood stream or lymph system to tissues and organs that constitutively express SDF-1 α [7].

Chemokine proteins are typically produced by chemical peptide synthesis or overexpressed in *E. coli* as insoluble inclusion bodies[8–11]. In each case the protein must be refolded and the cysteines must be oxidized in the correct disulfide combination. Refolding has most often been performed in dilute solution, in order to discourage intermolecular disulfide formation[8–11]. For preparative-scale production of chemokines, this process can be time consuming and laborious, since large volumes of dilute protein solution must be subsequently concentrated and purified. A more efficient method for refolding of biologically active chemokine proteins would be valuable for a broad range of research and industrial applications.

Our laboratory has previously employed a time-consuming protocol to refold and oxidize SDF-1 α that is similar to methods used by other groups. We solubilized bacterially expressed hexahistidine-tagged SDF-1 α from inclusion bodies in guanidine hydrochloride for metal affinity purification under denaturing conditions. Then we used dialysis to remove the denaturant which is followed by cleavage of the N-terminal hexahistidine tag using tobacco etch virus (TEV) protease under reducing conditions to prevent incorrect SDF-1 α disulfide bond formation. Finally, we oxidized the disulfide bonds and refolded SDF-1 α using a combination of dilution and dialysis. Others have used up to five separate dialysis steps against various buffers[11] or dilution to volumes as large as 8 L[9] to refold SDF-1 α .

We endeavored to develop a more efficient method for producing recombinant SDF-1 α and other chemokines under investigation on our laboratory. Rozema and Gellman have shown that the addition of “artificial chaperones” like detergents, cyclodextrin, and oxidizing or reducing agents during solution base protein refolding are helpful in renaturation of proteins from inclusion bodies[12,13]. Oganessian *et al.* combined the artificial chaperone approach of Rozema and Gellman with metal affinity chromatography for refolding of insoluble, recombinant structural genomics target proteins[14]. Using this combined approach Oganessian *et al.* successfully refolded 7 out of 10 insoluble targets. Using a similar approach we find that artificial chaperone and metal affinity chromatography assisted refolding rapidly catalyzes the formation of the correct disulfide pairing for SDF-1 α from *E. coli* inclusion bodies. This one-step on-column refolding, oxidation, and chromatographic purification eliminates the slow dialysis or dilution steps previously used to obtain folded recombinant chemokines. Moreover, we show that the CC chemokine RANTES/CCL5, the C chemokine lymphotactin/XCL1, and the CX₃C chemokine fractalkine/CX3CL1 can be produced by the same method. The chemokines produced using on-column refolding are correctly structured, as demonstrated by 2D NMR. Calcium flux assays using a monocyte cell line expressing *CXCR4* indicate that on-column refolded SDF-1 α is biologically active.

Methods

Expression Plasmid

The SDF-1 α expression plasmid has been previously described[10]. Briefly it is a pQE30 plasmid (Qiagen) containing an N-terminal hexahistidine tag and modified tobacco etch virus protease site (ENLFYQ/GM) followed by the coding sequence for mature SDF-1 α . The glycine residue is required for efficient TEV protease digestion, and the adjacent methionine provides for optional CNBr cleavage, which generates a native SDF-1 α amino terminus.

Protein expression

E. coli strain SG13009[pREP4] containing the SDF-1 α expression plasmid was grown at 37 °C in either 1 L of Luria-Bertani or M9 minimal media containing ¹⁵NH₄Cl as the sole nitrogen source. Once an OD₆₀₀ of ~ 0.7 was reached expression was induced through the addition of isopropyl- β -D-thiogalactopyranoside (IPTG) to the culture at a final concentration of 1mM. After 6 hours cells were harvested by centrifugation at 5,000 $\times$ g and stored at -80°C.

Protein Purification and Solution Based Dialysis Refolding

Cell pellets were resuspended in 10 mL of buffer A (50 mM sodium phosphate pH 7.4, 300 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, and 0.1% (v/v) 2-mercaptoethanol). Cells were lysed by two to three passages through a French pressure cell, 16,000 psi. Inclusion bodies containing SDF-1 α were isolated through centrifugation at 15,000 $\times$ g and the supernatant was discarded. The insoluble inclusion body pellet was solubilized using buffer AD (50 mM sodium phosphate pH 7.4, 300 mM NaCl, 10 mM imidazole, and 6 M guanidinium hydrochloride) and batch loaded onto 5 mL disposable columns containing 2 mL of Ni sepharose™ 6 fast flow resin (Amersham/GE Healthcare). After a 30 minute incubation the column was washed four times with 10 mL of buffer AD and eluted with buffer BD (50 mM sodium acetate pH 4.5, 300 mM NaCl, and 10 mM imidazole). The eluted SDF-1 α was dialyzed twice against 4 L of 0.3% (v/v) acetic acid. 2-Mercaptoethanol was added to the dialyzed SDF-1 α to a final concentration of 0.1% (v/v). NaH₂PO₄ and NaCl to a final concentration of 50 mM were also added and the pH was raised to 6.5–6.75 with NaOH. Tobacco etch virus protease (TEV) was then added to this solution to remove the hexahistidine tag (1:1,000 w/w). The disulfide bonds in SDF-1 α were formed through diluting protease digestion solution from 30–40 to 150 mL with 20 mM Tris pH 8.0 to raise the pH and is followed by dialysis against 4 L of the same buffer. Dialysis based SDF1- α oxidation fails if the hexahistidine tag is not removed before oxidation. After disulfide bond formation the SDF-1 α was concentrated to ~30 mL using ultrafiltration and acidified with HCl (pH < 3.0). SDF-1 α was then purified to greater than 98% homogeneity using reverse phase (RP) HPLC in 0.1% aqueous trifluoroacetic acid (TFA) with a 30 minute CH₃CN gradient from 21–42%. Purified SDF-1 α was lyophilized and then dissolved in 80% aqueous TFA with ~100-fold molar excess of CNBr overnight. Removal of the residual N-terminal Gly-Met dipeptide (which remained after digestion with TEV protease) was verified by MALDI-TOF mass spectrometry. Cleaved SDF-1 α was lyophilized and repurified by RP HPLC. Solution refolding and purification of RANTES[15], lymphotactin[16], and fractalkine[17,18] were performed as described elsewhere.

Protein Purification and On-column Refolding

The procedures for solution and on-column refolding of SDF-1 α are the same up to the point of batch loading the Ni sepharose™ 6 column and the 30 min. incubation. After incubation, the column was washed with 100 mL of detergent buffer (20 mM Tris pH 8.0, 100 mM NaCl, 1% Triton X-100 (v/v), 10 mM 2-mercaptoethanol) followed by 100 mL of oxidation buffer (20 mM Tris pH 8.0, 100 mM NaCl, 5 mM β -cyclodextrin, 1 mM reduced glutathione, 0.5 mM oxidized glutathione) and 70 mL of wash buffer (20 mM Tris pH 8.0, 500 mM NaCl). The refolded SDF-1 α containing correctly oxidized disulfide bonds was eluted with elution buffer (25 mM HEPES pH 7.4, 300 mM NaCl, 1 M imidazole). Fractions containing SDF-1 α were pooled and dialyzed against 4 L of 0.3% acetic acid and then lyophilized for CNBr cleavage. MALDI-TOF MS analysis of the SDF-1 α was done before and after the CNBr cleavage to check for hexahistidine tag removal. After the CNBr cleavage the refolded, native N-terminal SDF-1 α was lyophilized and purified to >98% homogeneity by RP HPLC. On-column refolding and purification of RANTES/CCL5, lymphotactin/XCL1 and fractalkine/CX₃CL1 were performed in a similar fashion.

NMR spectroscopy

NMR measurements were performed on a Bruker DRX 600 equipped with a $^1\text{H}/^{15}\text{N}/^{13}\text{C}$ Cryoprobe® or a conventional $^1\text{H}/^{15}\text{N}/^{13}\text{C}$ probe equipped with three axis gradients. NMR samples contained 90% H_2O (v/v), 10 % D_2O (v/v), and 0.02 % NaN_3 (w/v) in 20 mM deuterated MES buffer at pH 6.8 unless otherwise noted. Two-dimensional ^{15}N - ^1H HSQC spectra[19] were collected with 512 and 150 complex points in the ^1H and ^{15}N dimensions, respectively, and 4 or 16 transients per free induction decay. One HSQC spectrum performed on the conventional $^1\text{H}/^{15}\text{N}/^{13}\text{C}$ probe was acquired using 384 transients (on-column refolded fractalkine at a concentration of 50 μM).

SDF-1 α calcium flux assay

THP-1 cells (ATCC) were grown in RPMI 1640 media supplemented with 2 mM glutamine, 1 mM sodium pyruvate, and 10% fetal bovine serum and maintained between 0.2 and 1.0×10^6 cells per mL at 37°C and 5% CO_2 . Cells were collected by centrifugation at $500 \times g$, washed twice with EBSSH buffer, and resuspended at 1.25×10^6 cells per mL in EBSSH buffer (1 mM sodium phosphate, 26 mM HEPES pH 7.4, 1 mM MgSO_4 , 5 mM KCl, 125 mM NaCl, 5.6 mM D-glucose, 2 mM CaCl_2 , 2.5 mM Probenecid, and 0.1% w/v bovine serum albumin). Dye loading was performed by rocking cells with Fluo-3 AM (Invitrogen) (2.4 μg per 10^6 cells) for 1 hour at room temperature. After incubation, cells were washed twice with EBSSH buffer and divided into 1 mL aliquots of 1.25×10^6 cells.

Calcium-dependent Fluo-3 emission was measured at 25°C using a PTI spectrofluorometer with excitation at 505 nm and detection at 525 nm. Immediately before measurement an aliquot of cells was washed and resuspended in 1.5 mL of EBSSH and allowed to equilibrate at 25°C for five minutes in the cuvette. After establishing a baseline (~ 100 s) chemokine was added and the calcium flux response was monitored for ~ 300 seconds. Total fluorescence intensity was measured after lysing cells with Triton X-100 (1%), followed by the addition of EDTA (50 mM). Calcium flux signals are reported as the ratio of the chemokine-induced fluorescence intensity maximum and the fluorescence intensity after cell lysis.

Results

SDF-1 α refolding in solution and solid phase

Table 1 summarizes the procedures for solution and on-column refolding of SDF-1 α . To compare the relative rates of solution and solid-phase refolding, SDF-1 α samples were collected at different time points during dialysis and while refolding on the metal affinity column. The extent of SDF-1 α refolding was determined by analytical HPLC. As the SDF-1 α disulfide bonds are formed, the retention time is reduced by ~ 3.5 minutes.

For solution refolding, fully reduced, TEV protease digested SDF-1 α is diluted into 150 mL of oxidation buffer (20 mM Tris pH 8.0) and dialyzed against 4 L of the same buffer. Figure 1A shows the progress of SDF-1 α refolding in solution over a period of 24 h. After one hour in dialysis, most of the protein is still in the reduced state, with a retention time of 41.5 min. By 24 hours the majority of the SDF-1 α is completely oxidized, corresponding to a retention time of 38 min (Figure 1A).

To monitor the kinetics on-column refolding, a series of aliquots (250 μL) of resin were removed after loading with solubilized SDF-1 α (0 h), during washes with the detergent buffer (0.25 h), the oxidation buffer (0.5 h), and the wash buffer (0.75 h), and immediately before elution (1 h). Each sample of resin was then eluted using elution buffer and immediately acidified with concentrated HCl to prevent further oxidation. The 0 hour sample was washed with buffer AD before elution to remove any contaminating proteins before HPLC analysis.

The extent of SDF-1 α refolding was again monitored using analytical HPLC. Since there is no reducing agent present when SDF-1 α is initially loaded onto the column, disulfide bonds (either inter- or intramolecular) may begin forming immediately. The data for time 0 seems to reflect this, since peaks with retention times corresponding to fully oxidized His-tagged SDF-1 α , (37 min), fully reduced His-tagged SDF-1 α (40.2 min), and other species are all present (Figure 1B). After exposure to the detergent buffer, which contains 2-mercaptoethanol, and oxidation buffer, which contains oxidized and reduced glutathione, disulfide exchange quickly yields a homogeneous product corresponding to native SDF-1 α .

The native N-terminus of SDF-1 α is required for CXCR4 activation, and removal of extra residues is accomplished by cyanogen bromide (CNBr) treatment. SDF-1 α refolded in solution is purified by preparative HPLC followed by lyophilization, while on-column refolded SDF-1 α is simply lyophilized (see Table 1). SDF-1 α powder is then dissolved in 80% TFA and digested with CNBr. MALDI-TOF mass spectral results give a m/z ratio of 7,961.7 for solution refolding and 7,961.9 for on-column refolding with a theoretical value of 7962.7. The theoretical values for pre-CNBr cleaved solution refolded gly-met SDF-1 α and on-column refolded hexahistidine tagged SDF-1 α also corresponded well with measured m/z values (Solution refolding theoretical m/z: 8150.98, measured m/z: 8152.9; On-column refolding theoretical m/z: 10344.2, measured m/z: 10342.8). The theoretical and measured mass values for ^{15}N labeled SDF-1 α were also in close agreement. Final purification after the CNBr treatment is identical for both methods (Table 1). On-column refolding of SDF-1 α reduces by roughly two days the time required by our previous approach that used dilution and dialysis for refolding. The final yield for each protocol is ~5 mg of pure SDF-1 α from a 1 L bacterial cell culture grown in M9 medium. However, on occasion we have observed yields up to 15 mg with on-column refolding.

SDF-1 α is correctly folded

The ^{15}N - ^1H HSQC spectrum of on-column refolded SDF-1 α shows the broad chemical shift dispersion characteristic of a folded protein (Figure 2). Two solution structures [8,11] and two crystal structures [20,21] of SDF-1 α have been deposited in the PDB. We have also solved the structure of SDF-1 α using solution-refolded protein (C.T. Veldkamp, F.C. Peterson and B.F. Volkman, unpublished results) and the HSQC spectrum of on-column refolded SDF-1 α is indistinguishable from spectra acquired with solution refolded material (Figure 2). Based on these results, we conclude that on-column refolded SDF-1 α adopts the native chemokine structure. We also used this method successfully in the production of a series of SDF-1 α variants containing the single amino acid substitutions R41A, R47A, K27A, Q48A, and K64A (data not shown). Since on-column refolding appeared to be a robust method for refolding wild-type and variant SDF-1 α proteins, we tested the same approach on other classes of chemokines.

On-column refolding of other chemokines

Nearly 50 different human chemokines are known. SDF-1 α is member of the CXC subfamily. Most of the other chemokines belong to the CC class, with the C and CX3C classes each defined by a single member. We expressed RANTES (CCL5), lymphotactin (XCL1), and fractalkine (CX₃CL1) in order to test on-column refolding on representative proteins from all four chemokine subfamilies. Applying the same protocol we used for SDF-1 α , we successfully refolded RANTES, lymphotactin, and fractalkine. Figure 3A–C compares the ^{15}N - ^1H HSQC spectra of on-column refolded RANTES, lymphotactin, and fractalkine respectively with spectra of the corresponding proteins refolded by the solution method. The structures of RANTES and fractalkine were solved previously by NMR [15, 17]. We characterized the structure of lymphotactin and its conformational heterogeneity as a function of temperature and solution conditions [22, 23]. At 200 mM NaCl and 10°C lymphotactin adopts the canonical

chemokine fold and has a large unstructured C-terminus that gives rise to the numerous peaks around 8.1 ppm and explains the heterogeneous peak intensities in the ^{15}N - ^1H HSQC spectra. Additional peaks in the spectra for on-column refolded RANTES and lymphotactin arise from the uncleaved N-terminal histidine tag. Aside from these minor differences, the ^{15}N - ^1H HSQC spectra of our solution refolded and on-column refolded chemokines match the published ^{15}N - ^1H HSQC spectra [15, 17, 22]. Hence, both the on-column and solution refolded proteins contain fully and correctly oxidized disulfide bonds and have adopted the canonical chemokine fold. Solid-phase refolding produced smaller amounts of RANTES, lymphotactin, and fractalkine than we obtained in parallel preparations refolded in solution, however no attempt was made to improve yields by optimizing the on-column procedure for each individual chemokine.

SDF-1 α is biologically active

Calcium is a second messenger released upon activation of CXCR4 by SDF-1 α . Using cells loaded with a calcium-sensitive dye like Fluo-3, changes in fluorescence emission intensity can be used to measure the CXCR4 agonist activity of SDF-1 α . Figure 4A shows the experimental results for stimulation of Fluo-3 loaded THP-1 cells with 10 nM SDF-1 α . We measured CXCR4 activation using an intracellular calcium flux assay to determine if on-column refolded SDF-1 α is biologically active and obtained an EC_{50} value of 3.6 ± 1.4 nM (Figure 4B). Previous studies reported an EC_{30} of 3.6 ± 1.6 nM in a calcium flux assay [8] and an EC_{50} of 5 ± 1 nM for SDF-1 α in a chemotaxis assay [24]. On-column refolded SDF-1 α also induced a potent chemotactic response in a transwell migration assay (data not shown).

Discussion

On-column refolding appears to be a powerful approach for tackling difficult refolding problems and in some instances can be more efficient than refolding in solution. For example, bovine serum albumin, which contains 17 disulfide bonds, was recently refolded on an ion exchange column [25]. Middelberg has reviewed refolding of proteins produced as inclusion bodies in *E. coli* with a particular emphasis on on-column refolding [26]. Protein refolding has practical importance for structure-function studies of chemokine signaling, since commercially available chemokine proteins can be prohibitively expensive and chemokines containing mutations cannot be ordered. Bacterially expressed or chemically synthesized chemokines are produced as reduced, unfolded, biologically inactive polypeptides. To obtain larger quantities of biologically active chemokines, the proteins must be refolded and oxidized to the correct pattern of conserved disulfide bonds. Preparative-scale refolding of recombinant and synthetic chemokine proteins is typically performed in dilute solution, requiring cumbersome methods for handling large volumes [8–11].

We have devised a more efficient method for refolding chemokines produced recombinantly as inclusion bodies in *E. coli* using an on-column refolding and oxidation procedure. This procedure requires only 2 mL of metal affinity chromatography resin and small volumes of buffer for refolding and oxidation of ≥ 5 mg of chemokine protein. We showed using 2D NMR that members of all four chemokine subfamilies can be produced in the native, folded state using this rapid solid phase approach. SDF-1 α produced by this method exhibits the same activity in a calcium-flux assay for CXCR4 activation as SDF-1 α from other sources.

In our experience, the N-terminal affinity tag interferes with SDF-1 α folding and must be proteolytically removed before disulfide oxidation in the solution-based approach. Elimination of the TEV protease digestion step shortens the process by ~24 h. We speculate that at pH 8 the neutrally charged hexahistidine tag may interact and interfere with the hydrophobic collapse during the refolding of SDF-1 α in the solution phase. In on-column refolding the His-tag is sequestered by its interaction with the resin-bound metal ion and thereby prevented from

interfering with the folding of the attached protein. In the solid-phase strategy, the slow refolding and oxidation steps in solution were reduced from 24 h to 1 h and combined with metal-affinity chromatographic purification. On-column refolding of SDF-1 α thus reduces by roughly two days the time required by our previous solution-based approach.

Yields for solution refolding and on-column refolding of SDF-1 α are comparable, producing ~5 mg per 1-liter culture of ¹⁵N-enriched M9 minimal media, but up to 15 mg has occasionally been observed for on-column refolded SDF-1 α . While solid-phase refolding yields for the other chemokines (RANTES/CCL5, lymphotactin/XCL1, and fractalkine/CX3CL1) were lower than solution refolding, the protocol was not individually optimized for those proteins. Rozema and Gellman have shown that tailoring “artificial chaperones”, especially detergents, to individual proteins is important for successful refolding[13]. Likewise, Proudfoot and Borlat reported that certain oxidation buffers are more effective than others for RANTES and suggested that oxidation buffers should be customized for individual chemokines[27]. Thus, on-column refolding yields could probably be improved for RANTES, lymphotactin, and fractalkine through empirical optimization. Based on our results with SDF-1 α and other chemokines, on-column refolding should provide a more efficient method of producing biologically active material for research or industrial production.

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References

1. Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. *Immunity* 2000;12:121–127. [PubMed: 10714678]
2. Baggiolini M. Chemokines in pathology and medicine. *J Intern Med* 2001;250:91–104. [PubMed: 11489059]
3. Nagasawa T, Hirota S, Tachibana K, Takakura N, Nishikawa S, Kitamura Y, Yoshida N, Kikutani H, Kishimoto T. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* 1996;382:635–638. [PubMed: 8757135]
4. Tachibana K, Hirota S, Iizasa H, Yoshida H, Kawabata K, Kataoka Y, Kitamura Y, Matsushima K, Yoshida N, Nishikawa S, Kishimoto T, Nagasawa T. The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature* 1998;393:591–594. [PubMed: 9634237]
5. Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* 1998;393:595–599. [PubMed: 9634238]
6. Bleul CC, Farzan M, Choe H, Parolin C, Clark-Lewis I, Sodroski J, Springer TA. The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature* 1996;382:829–833. [PubMed: 8752280]
7. Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verastegui E, Zlotnik A. Involvement of chemokine receptors in breast cancer metastasis. *Nature* 2001;410:50–56. [PubMed: 11242036]
8. Crump MP, Gong JH, Loetscher P, Rajarathnam K, Amara A, Arenzana-Seisdedos F, Virelizier JL, Baggiolini M, Sykes BD, Clark-Lewis I. Solution structure and basis for functional activity of stromal cell-derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1. *EMBO J* 1997;16:6996–7007. [PubMed: 9384579]
9. Holmes WD, Consler TG, Dallas WS, Rocque WJ, Willard DH. Solution studies of recombinant human stromal-cell-derived factor-1. *Protein Expr Purif* 2001;21:367–377. [PubMed: 11281710]
10. Veldkamp CT, Peterson FC, Pelzek AJ, Volkman BF. The monomer-dimer equilibrium of stromal cell-derived factor-1 (CXCL 12) is altered by pH, phosphate, sulfate, and heparin. *Protein Sci* 2005;14:1071–1081. [PubMed: 15741341]

11. Gozansky EK, Louis JM, Caffrey M, Clore GM. Mapping the binding of the N-terminal extracellular tail of the CXCR4 receptor to stromal cell-derived factor-1 α . *J Mol Biol* 2005;345:651–658. [PubMed: 15588815]
12. Rozema D, Gellman SH. Artificial chaperone-assisted refolding of denatured-reduced lysozyme: modulation of the competition between renaturation and aggregation. *Biochemistry* 1996;35:15760–15771. [PubMed: 8961939]
13. Rozema D, Gellman SH. Artificial chaperone-assisted refolding of carbonic anhydrase B. *J Biol Chem* 1996;271:3478–3487. [PubMed: 8631951]
14. Oganessian N, Kim SH, Kim R. On-column protein refolding for crystallization. *J Struct Funct Genomics*. 2005;6:177–182.
15. Chung CW, Cooke RM, Proudfoot AE, Wells TN. The three-dimensional solution structure of RANTES. *Biochemistry* 1995;34:9307–9314. [PubMed: 7542919]
16. Peterson FC, Elgin ES, Nelson TJ, Zhang F, Hoeger TJ, Linhardt RJ, Volkman BF. Identification and characterization of a glycosaminoglycan recognition element of the C chemokine lymphotactin. *J Biol Chem* 2004;279:12598–12604. [PubMed: 14707146]
17. Mizoue LS, Bazan JF, Johnson EC, Handel TM. Solution structure and dynamics of the CX3C chemokine domain of fractalkine and its interaction with an N-terminal fragment of CX3CR1. *Biochemistry* 1999;38:1402–1414. [PubMed: 9931005]
18. Mizoue LS, Sullivan SK, King DS, Kledal TN, Schwartz TW, Bacon KB, Handel TM. Molecular determinants of receptor binding and signaling by the CX3C chemokine fractalkine. *J Biol Chem* 2001;276:33906–33914. [PubMed: 11432858]
19. Mori S, Abeygunawardana C, Johnson MO, van Zijl PCM. Improved sensitivity of HSQC spectra of exchanging protons at short interscan delays using a new fast HSQC (FHSQC) detection scheme that avoids water saturation. *J Magn Reson Ser B* 1995;105:94–98. [PubMed: 7627436]
20. Dealwis C, Fernandez EJ, Thompson DA, Simon RJ, Siani MA, Lolis E. Crystal structure of chemically synthesized [N33A] stromal cell-derived factor 1 α , a potent ligand for the HIV-1 "fusin" coreceptor. *Proc Natl Acad Sci U S A* 1998;95:6941–6946. [PubMed: 9618518]
21. Ohnishi Y, Senda T, Nandhagopal N, Sugimoto K, Shioda T, Nagai Y, Mitsui Y. Crystal structure of recombinant native SDF-1 α with additional mutagenesis studies: an attempt at a more comprehensive interpretation of accumulated structure-activity relationship data. *J Interferon Cytokine Res* 2000;20:691–700. [PubMed: 10954912]
22. Kuloglu ES, McCaslin DR, Kitabwalla M, Pauza CD, Markley JL, Volkman BF. Monomeric solution structure of the prototypical 'C' chemokine lymphotactin. *Biochemistry* 2001;40:12486–12496. [PubMed: 11601972]
23. Kuloglu ES, McCaslin DR, Markley JL, Volkman BF. Structural rearrangement of human lymphotactin, a C chemokine, under physiological solution conditions. *J Biol Chem* 2002;277:17863–17870. [PubMed: 11889129]
24. Loetscher P, Gong JH, Dewald B, Baggiolini M, Clark-Lewis I. N-terminal peptides of stromal cell-derived factor-1 with CXC chemokine receptor 4 agonist and antagonist activities. *J Biol Chem* 1998;273:22279–22283. [PubMed: 9712844]
25. Langenhof M, Leong SS, Pattenden LK, Middelberg AP. Controlled oxidative protein refolding using an ion-exchange column. *J Chromatogr A* 2005;1069:195–201. [PubMed: 15830945]
26. Middelberg AP. Preparative protein refolding. *TRENDS Biotechnol* 2002;20:437–443. [PubMed: 12220907]
27. Proudfoot AE, Borlat F. Purification of recombinant chemokines from *E. coli*. *Methods Mol Biol* 2000;138:75–87. [PubMed: 10840744]

Figure 1.

On-column refolding is faster than solution based dialysis refolding. Refolding can be monitored using RP HPLC. As the disulfide bonds form in SDF-1 α the retention time shift. A) RP HPLC traces of solution refolding SDF-1 α taken from dialysis at the indicated time points. Solution refolding of SDF-1 α after proteolytic removal of the hexahistidine tag is accomplished by diluting the TEV digestion reaction to 150 mL with 20 mM Tris pH 8.0 and dialysis against 4 L of the same buffer. B) RP HPLC traces of on-column refolding SDF-1 α with time points as indicated. On-column refolding time points correspond to removal of metal affinity resin after binding solubilized SDF-1 α inclusion bodies to the metal affinity resin (0 hours) and flowing a detergent buffer (0.25 hours), a oxidation buffer (0.5 hours), a wash buffer (0.75 hours), and an elution buffer (1.0 hours) over the column. The removed resin was eluted and the elution acidified to prevent further oxidation. Then refolding was monitored using RP HPLC.

Figure 2.

On-column refolded SDF-1 α adopts the native chemokine fold. Overlaid ^{15}N - ^1H HSQC spectra of solution refolded SDF-1 α (black) and on-column refolded SDF-1 α (red). 250 μM SDF-1 α .

Figure 3.

On-column refolded RANTES, lymphotoxin and fractalkine adopt the native chemokine fold. ^{15}N - ^1H HSQC spectra of on-column (red contours) and solution refolded (black contours) chemokines are shown for each protein. A) RANTES (25°C; 20 mM sodium phosphate, pH 3.2, 500 μM on-column protein) Spectrum on left contains additional peaks due to the unremoved N-terminal His tag. B) Lymphotoxin (10°C; 20 mM sodium phosphate, 200 mM sodium chloride, pH 6.0, 500 μM on-column protein) Spectrum on left contains additional peaks due to the unremoved N-terminal His₈ tag. C) Fractalkine (25°C; 20 mM deuterated acetate, pH 5.0, 50 μM on-column protein).

Figure 4.

On-column refolded SDF-1 α is biologically active. A) Fluorescence at 525 nm for Fluo-3 loaded THP-1 cells stimulated with 10 nM SDF-1 α at 100 seconds. The THP-1 calcium flux is the maximum absorbance after SDF-1 α addition minus the initial baseline intensity. B) Dose-dependent calcium flux measurements were analyzed to obtain an EC_{50} of 3.6 ± 1.4 nM for CXCR4 activation in THP-1 cells by on-column refolded SDF-1 α . The average THP-1 calcium flux normalized by total fluorescence intensity after cell lysis for each SDF-1 α concentration is shown. Error bars represent the standard deviation for 2–5 measurements performed at each SDF-1 α concentration.

Table 1Comparison of SDF-1 α refolding protocols

Day	Solution refolding	SDF-1 α (mg)	On-column refolding	SDF-1 α (mg)
1	Express and harvest inclusion bodies. Total estimated SDF-1 α ~20 mg/L			6–17 [*]
2	Nickel column and dialysis.	6–8 [*]	Refolding on-column, dialysis, and refolding check	
3	Cleavage with TEV protease.	6–7	Lyophilization	5–15
4	Refolding via dialysis and refolding check	5–6	CNBr cleavage and MALDI-TOF MS analysis	
5	Purification and lyophilization	5	Lyophilization and purification	5–15
6	CNBr cleavage and MALDI-TOF MS analysis		Lyophilization	
7	Lyophilization and purification	5	NMR analysis	5–15
8	Lyophilization			
9	NMR analysis			

^{*} Chromatographic yields vary widely because SDF-1 α binds very tightly, and to some extent irreversibly, to the metal affinity column in either protocol. No SDF-1 α is observed in the column flow through after loading the column with solubilized SDF-1 α from inclusion bodies (data not shown). Boiling the affinity resin in the presence of SDS and DTT consistently releases significant quantities of uneluted SDF-1 α (data not shown). Elution from the metal affinity column is at times much more efficient for SDF-1 α refolded by the on-column method, resulting in higher yields, but we have not yet found a reproducible solution to this problem.