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Monomeric variants of IL-8: Effects of side chain substitutions and solution conditions upon dimer formation

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Abstract

IL-8 dimers have been observed in NMR and X-ray structures of the protein. We have engineered IL-8 monomers by mutations of residues throughout the dimer interface, which introduce hindrance determinants to dimerization. These IL-8 variants are shown by NMR to have wild-type monomer folding, but by ultracentrifugation to have a range of dimerization constants from μM to mM, as compared with a dimerization constant of about 10 μM for wild-type IL-8, under physiological salt and temperature conditions. The monomeric variants of IL-8 bind the erythrocyte chemokine receptor DARC, as well as the neutrophil IL-8 receptors CXCR1 and CXCR2 with affinities similar to that of wild-type IL-8. In addition, the monomeric variants were shown to have agonist activity, with similar potency to wild-type, in both Ca^{2+}-flux assays on CXCR1 and CXCR2 transfected cells, and in chemotaxis assays on neutrophils. Thus, these variants confirm that monomeric IL-8 is functionally equivalent to wild-type in in vitro assays. We have also investigated the effects of various solution conditions upon IL-8 dimer formation using analytical ultracentrifugation. At salt concentrations, temperatures, and pH conditions lower than physiological, the dimerization affinity of IL-8 is greatly enhanced. This suggests that, under some conditions, IL-8 dimer formation may occur at concentrations of IL-8 considerably lower than 10 μM, with consequences in vivo that are yet to be determined.

Keywords: analytical ultracentrifugation; chemokine; mutagenesis; neutrophil; NMR

Homo-dimerization is a common property of many proteins (Jones & Thornton, 1995). In addition to the formation of covalent dimers and higher order associations, as in the immunoglobulins (Amzel & Poljak, 1979), many dimerization events occur through non-covalent associations that provide a means of functional regulation through the modulation of quaternary structure. Examples include hemoglobin (Perutz et al., 1987), certain cell-surface receptors responding to binding of a monomeric or dimeric ligand (Schlessinger & Ullrich, 1992), and various DNA binding proteins (Luisi, 1995). In other cases, dimerization may be critical to protein folding and stability (and, hence, ultimately essential for function), even though the association-dissociation equilibrium may not be exploited for purposes of regulation. Finally, for other proteins, the purpose of dimerization is not clear, although the evolutionary conservation of exquisitely specific dimer–interface interactions argues that there is some selective pressure to maintain the interface. Such appears to be the case among the chemokines, a group of small (MW approx. 8 kDa) proteins that dimerize, but for which no clear role of dimerization has been established (Burrows et al., 1994; Paolini et al., 1994; Rajarathnam et al., 1994, 1995; Clark-Lewis et al., 1995; Hébert & Lowman, 1996; Leong et al., 1997). We have further examined the characteristics and effects of dimer formation in one of these proteins, interleukin-8, by mutagenesis and biophysical studies.

Interleukin-8 (IL-8) is a small protein chemoattractant (Baggiolini & Clark-Lewis, 1992; Hébert & Baker, 1993) that recruits neutrophils to sites of inflammation through interactions with at least two types of receptor, CXCR1 and CXCR2, also known as IL-8 receptor types A and B, respectively (Holmes et al., 1991; Murphy & Tiffany, 1991; Lee et al., 1992), which activate known G proteins (Wu et al., 1993). IL-8 exists as a non-covalent homodimer of 8 kDa subunits both at high concentrations in solution and crystal structures (Clore et al., 1989, 1990; Baldwin et al., 1991; Clore & Gronenborn, 1991), raising the question of whether ligand dimerization is involved in signal transduction. The primary functional determinant for binding of IL-8 to its neutrophil receptors is

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a short peptide motif corresponding to the peptide sequence Glu-Leu-Arg ("ELR") near the amino terminus of IL-8 (Clark-Lewis et al., 1991, 1993; Hébert et al., 1991). Additionally, a region of more hydrophobic character has been identified in the residues 12–21 region of IL-8 (the N-loop) that clearly contributes to high-affinity receptor binding (Clubb et al., 1994; Schraufstätter et al., 1995; Lowman et al., 1996; Williams et al., 1996). Both of these regions, however, are rather distal to the dimer interface, and well exposed to solvent in either dimeric (Baldwin et al., 1991; Clore & Gronenborn, 1991) or monomeric forms (Rajarathnam et al., 1995) of the protein.

Additionally, IL-8 and other chemokines bind to a promiscuous receptor on erythrocytes, called DARC (Darbonne et al., 1991; Horuk et al., 1993), and to sulfated glycosaminoglycans, such as heparin and other saccharides (Webb et al., 1993). While there is reason to think that these interactions are physiologically relevant (Rot et al., 1996), the specific consequences of IL-8 binding to these targets in vivo has not been established. For the DARC receptor, however, chemokine-binding is mediated in part through interactions with the Arg residue of the ELR motif as well as through interactions elsewhere within the structure (Hesselgesser et al., 1995). Proteoglycan binding, on the other hand, has been partially localized to the C-terminal α-helices of the structure (Webb et al., 1993).

Earlier studies of IL-8 dimerization showed that in solution, under conditions approaching physiological, IL-8 dimerized with a K_{dd} (dimer dissociation constant) of about 10–20 μM (Burrows et al., 1994; Paolini et al., 1994). Because IL-8 binds its known receptors with nM affinities ($K_d \approx$ 1–2 nM for neutrophil receptors, and $K_d \approx$ 20 nM for DARC), and neutrophil chemotaxis and degranulation are induced over the same range, it was concluded that the monomeric form was the active species. On the assumption that IL-8 is present in soluble form, and at only low concentrations (e.g., 1 nM) in tissues, the monomer would indeed be predicted to be the active and most relevant species in vivo. In fact, a variant of IL-8 was produced by synthetic means (Rajarathnam et al., 1994) that effectively disrupted IL-8 dimer formation by altering the protein backbone through substitution of N-methyl Leu at position L25. This synthetic mutant was active in binding and activating neutrophils in vitro, providing evidence that the monomeric form of IL-8 is active. Certain side-chain substitutions within the β-sheet part of the dimer interface (namely at L25 and V27), on the other hand, apparently disrupted receptor binding (Lusti-Narasimhan et al., 1995, 1996), which might be interpreted as an indication that part of the dimer interface necessarily need be exposed in order for IL-8 to bind receptor. Nevertheless, other evidence has suggested that IL-8 dimerization may simply be irrelevant to receptor binding. For example, crosslinking experiments (Schnitzel et al., 1994) suggested that both monomeric and dimeric forms of IL-8 can, in fact, bind to neutrophil receptors.

In this article we describe a study of the contributions of side-chain packing to IL-8 dimer formation through side-chain substitutions within the dimer interface. The resulting variants provide forms of IL-8 with a range of dimerization affinities under various solution conditions, from μM to several mM. We further investigated the specific receptor-binding and activation properties of these variants, and found that all are capable of binding and activating both IL-8 CXCR1 and CXCR2, and are also capable of binding the DARC receptor. In addition, we measured the dimerization constants of IL-8 and mutants under conditions of varying ionic strength, pH, and temperature. We show that the dimerization affinities of IL-8, as well as some, but not all of the dimerization-defective mutants constructed by side-chain replacement, are highly sensitive to solution conditions. Thus, there may exist conditions under which IL-8 dimer formation is observed at concentrations considerably lower than previously reported.

Results

Design of weakly dimerizing IL-8 variants through side-chain substitutions

To develop reagents that could test the possible role(s) of IL-8 dimerization in vivo, we sought to engineer variants of IL-8 that are defective in dimerization, but otherwise largely maintain the structure of a native-like IL-8 subunit. Because single amino acid substitutions that might affect dimerization could also render the protein misfolded, we sampled a range of mutations of residues in the interface between IL-8 subunits (Fig. 1A), tested variants for dimerization, and then assayed for receptor binding and activity.

We first identified residues for mutagenesis by calculating the degree to which residues on the surface of a monomer of IL-8 would be buried upon formation of the dimer (Fig. 1B). In addition to the obvious main-chain interactions between subunits, including the backbone H-bonding along the first β-strands of each subunit, the results of this calculation show that significant changes in solvent accessibility are predicted upon dimerization for the side chains of residues 4, 23–30, 37, 42, 53, 54, 59, 62, 65, 66, 69, and

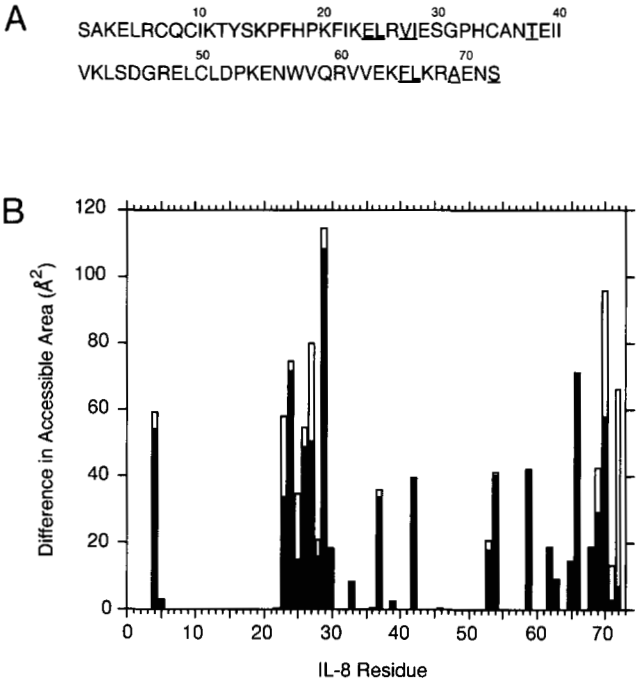


Fig. 1. Analysis of buried side chains in the IL-8 dimer interface. **A:** Sequence of residues 1–72 of IL-8 showing sites of side chain substitution (underlined) in IL-8 monomer variants. **B:** Solvent accessibility in monomeric versus dimeric IL-8. Accessibility to a 1.4 Å probe was calculated (Lee & Richards, 1971) for an IL-8 dimer and for a single IL-8 subunit based upon the X-ray structure of the dimer (3il8; Baldwin et al., 1991). The difference, (Monomer accessible area) − (Dimer accessible area), is shown for each residue's main chain (shaded bar) and side chain (open bar) contribution in Å^2.

72. Of these, E4 is a known binding determinant and was eliminated from consideration. S30 is involved in an unusual "kink" in the structure, and if substituted, might induce misfolding within a monomer subunit. Similarly, residue P53 starts a turn that is likely to be important for protein (monomer) folding. V62 is involved in intra-subunit contact between α-helix and β-sheet portions of the monomer structure (Clore et al., 1990). Of the remaining residues, K23, R26, K42, K54, and Q59 are both polar and rather solvent exposed in the dimer (solvent accessibility $\geq$ ca. 20% for each residue; Fig. 1B). Substitutions here might therefore not be expected to provide hindrance determinants to dimerization even if substituted non-conservatively.

Having eliminated side chains likely to be critical for monomer folding, our approach to engineering monomers was to introduce side chain "hindrance determinants" to the subunit interface at the remaining sites of side chain contact: E24, L25, V27, I28, E29, T37, F65, L66, A69, and S72 (Fig. 2A). Among these, E24 of the first subunit has van der Waals contacts with I28′ (residues of the second subunit are denoted by a primed residue number, e.g. 28′) and forms a salt bridge with R26′. The side chain of L25 contacts V27′, in a packing interaction near the twofold symmetry axis at the center of the dimer interface. Even more extensive side chain–side chain contacts are seen in the interface between the helix of one subunit and the β-sheet of the second (Fig. 2B). Here, T37 is closely packed with A69′ and S72′ of the second subunit. The side chain of E29 has ionic interaction with that of R68′. In addition to having some intra-subunit interactions, F65 and L66 make multiple contacts across the dimer interface. F65 makes contacts with V27′ and with E29′. L66 is the only mutually contacting residue in both subunits, packing with L66′ as well as with V62′. The latter residue is otherwise quite buried within the monomer subunit (14% solvent accessibility in the monomeric subunit) as noted above.

These residues were mutated in sets of two or three residues, grouped to take advantage of their proximity across the subunit interface. For example, the E24 side chain is in contact with the opposing subunit at I28′; therefore, residues E24 and I28 were mutated simultaneously, yielding four proximal changes in the subunit interface. Charged amino acid replacements were chosen to increase the hydrophilic nature of the interface. Glu was chosen over Asp substitution and Arg over Lys because of the larger respective side chains, providing more steric bulk. In some cases, His was used to introduce a polar/charged side chain to replace an aromatic residue of similar size (e.g., F65). The nature of the charge (Glu or Arg) was chosen to oppose the local charged residues seen in the dimer structure. For example, E24 of the first subunit forms an ion pair with R26′ of the second subunit (Baldwin et al., 1991). Therefore, E24R was selected to disrupt the ion pair and provide repulsive interactions with R26′ and I28′R. Additionally, substitutions were selected to alter the packing of the interface (van der Waals contacts), by selecting Glu or Arg to change the side-chain volume of the targeted residue.

Once mutations were selected, variants were constructed by recombinant DNA techniques, and expressed in *E. coli*. Because folded proteins are more resistant to proteolysis than non-folded or mis-folded forms, we used secretion of protein as a screen of conformational integrity (Shortle & Lin, 1985; Pakula et al., 1986). When we examined expression levels of IL-8 variants in non-suppressor *E. coli* strains, we found that expression was best for wild-type, E29R/A69R, F65E/L66E, F65H/L66E, and T37E/A69E/S72E (5–25 mg/L *E. coli* culture). The variants V27P and E24R/I28R expressed less well (1 mg/L culture). Variants L25P, L25E/V27E, and L25E/V27E/L66E failed to express at significant levels (<1 mg/L culture) and were omitted from further studies.

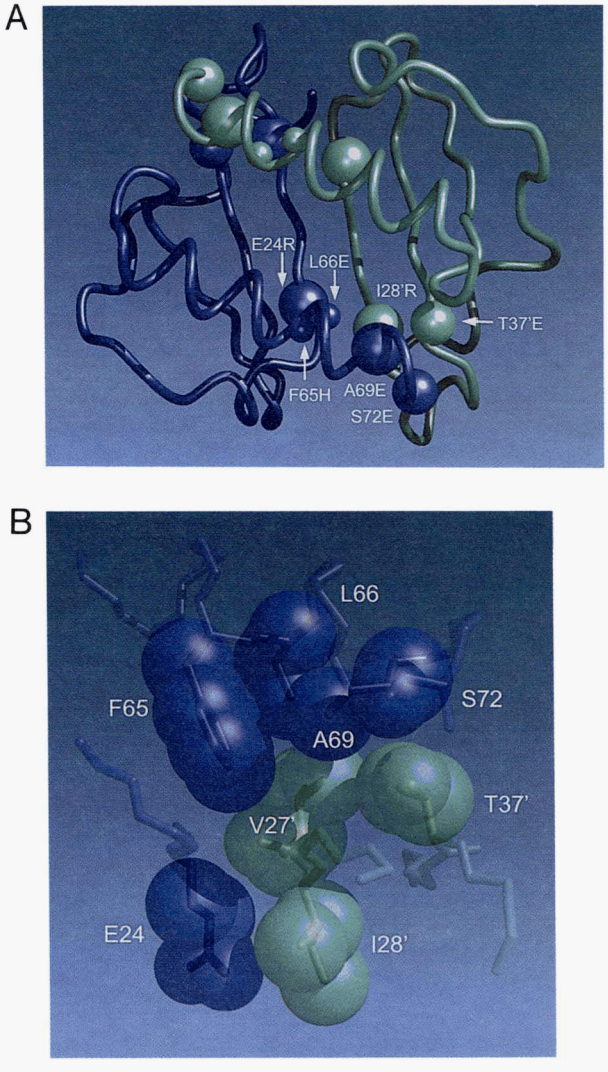


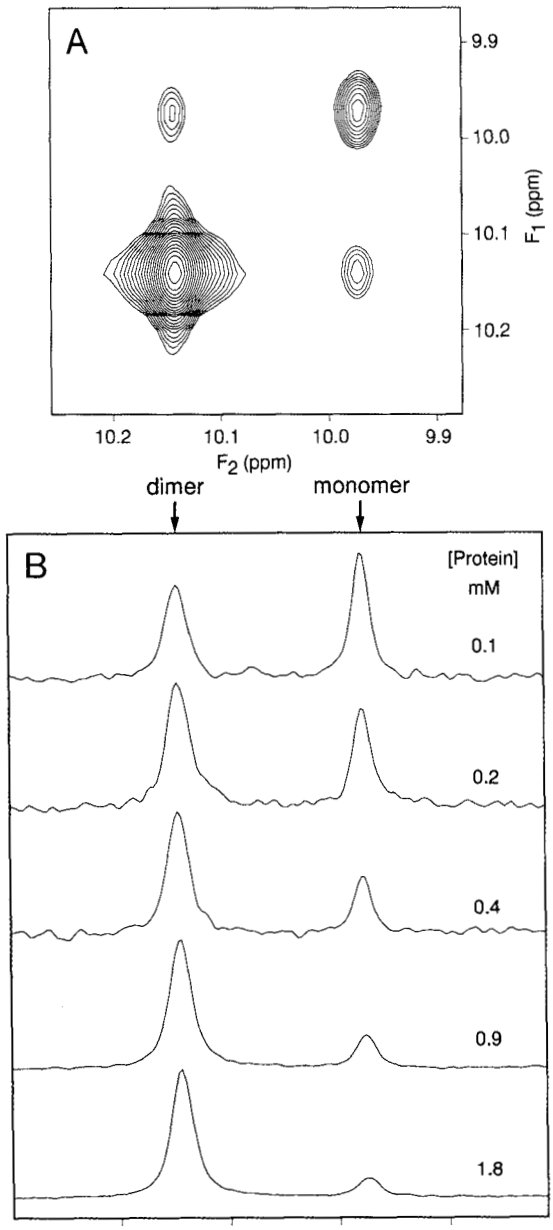
Fig. 2. Structural context of IL-8 mutations. **A:** Mutations in IL-8 monomeric variants are shown in the context of the native dimer (3il8; Baldwin et al., 1991). One of the two subunits is shown in blue, the other in cyan for clarity. Spheres indicate the positions of mutations inserted to engineer various non-dimerizing forms of IL-8. The size of each sphere corresponds to the change in side chain area for the indicated substitution. **B:** Detailed view of the region where mutations were introduced to destabilize the dimer shows van der Waals contacts between residues in the two subunits (colored as above) that were mutated. Mutations at residue V27 generally reduced expression levels and therefore are not depicted here.

The calculated molecular weight of wild-type IL-8 is 8.3 kDa. However, we found IL-8 run on Superose-12 sizing columns in either PBS buffer (137 mM NaCl, 2.7 mM KCl, 7.9 mM Na_3PO_4, and 1.14 mM K_3PO_4, pH 7.2) or Mes/salt buffer (pH 6.2, 0.25 M NaCl), eluted with an apparent molecular weight of 11.4 kDa, as compared with a set of molecular weight standards. This difference could be explained by non-specific binding effects, or by equilibration of monomeric and dimeric forms of wild-type. On the other hand, IL-8 variant I (E24R/I28R) was found to elute at an apparent molecular weight of 4.6 kDa in PBS buffer, suggesting that this

variant exists as a monomer in solution. At high concentrations of protein (>100 μM), both monomeric and dimeric forms of this variant could be seen by size-exclusion chromatography (data not shown).

Additive effects of dimerization hindrance determinants

The effects of mutations on a protein's function arising from single-point mutations often obey simple thermodynamic additivity rules when these mutations are combined into a single molecule (Wells, 1990). We, therefore, reasoned that combinations of mutations that weakened the dimer interface of IL-8 could lead to a variant that would exist almost entirely as a monomer in solution. We had previously found that IL-8 variant I (E24R/I28R) was significantly reduced in dimerization affinity. Therefore, we used variant I as a starting point for additional mutations that did not apparently alter folding on their own, but appeared likely to disrupt dimerization further. Mutations from variant I and II were combined to yield variant III (E24R/I28R/T37E/A69E/S72E), which expressed well in *E. coli* and appeared monomeric by size-exclusion chromatography (data not shown). Mutations at residues F65 and L66 were also predicted to affect dimerization. We, therefore, combined these with those of variant I or variant III. Among these, Variants (I + F65E/L66E), (I + F65H/L66E), and (III + F65E/L66E) failed to express at detectable levels. However, (III + F65H/L66E), called variant IV, expressed well and appeared monomeric by size-exclusion chromatography.



NMR of IL-8 variants indicates monomers are well folded

To evaluate the folding and dimerization of some variants, we examined their NMR spectra as a function of concentration. In particular, from two-dimensional NMR experiments, variant I appeared to form a stable dimer at high (1.8 mM) concentrations, with an intact β-sheet interface between subunits, as indicated by several d_{NN}, $d_{\alpha N}$, and $d_{\alpha\alpha}$ NOEs observed between residues across the dimer interface. Well-resolved upfield-shifted resonances, with chemical shifts similar to that of wild-type IL-8 (data not shown), together with many NOEs indicative of secondary structure, show that variant I is well folded and IL-8- like in solution. Exchange peaks observed in TOCSY, NOESY, and ROESY spectra (see, e.g., Fig. 3A) show that the major dimeric species exists in equilibrium with another (presumably the monomeric IL-8) species.

In a dilution series, variant I also showed a concentration-dependent transition to the second form, with concomitant loss of the starting form, based on the Trp-57 $N^{\epsilon 1}H$ resonance (Fig. 3B). In the NMR experiments, at 40 °C in phosphate buffer pH 5.7, the dimerization affinity of variant I was determined to be about 0.1 mM. Similar behavior was observed for other resonances, including the downfield shifted backbone amide protons of residues Gln 8 (11.28 ppm in the homodimeric form) and Lys 20 (11.83 ppm in the homodimeric form).

Variant IV, which combined mutations E24R/I28R with T37E/A69E/S72E, was also examined by NMR and found to be well folded. NOEs consistent with an IL-8–like dimer interface were not observed at 40 °C; however, NOEs consistent with IL-8–like secondary structure were observed, although the C-terminal helix appears shorter by at least one turn relative to native IL-8 (data not shown).

Fig. 3. NMR shows dissociation of a weakly dimerizing IL-8 variant. **A:** A portion of the downfield region of a 100 ms NOESY spectrum (see Materials and methods) of a 1.8 mM solution of IL-8 variant I (i.e., E24R/I28R) shows exchange peaks between resonances assigned to Trp-57 $N^{\epsilon 1}H$ in two different forms of the protein. The resonance at 10.15 ppm corresponds to the homodimeric form, as judged from analysis of the 2D NMR spectra. **B:** A dilution series shows the concentration dependence of the Trp-57 $N^{\epsilon 1}H$ resonances. With decreasing concentration, the resonance at 10.15 ppm decreases in intensity with a concomitant increase in the intensity of its exchange partner at 9.98 ppm. The estimated dimerization constant for variant I, based on this experiment at 40 °C, is ~0.1 mM (see Results).

Sedimentation analysis

Dimerization constants were determined at 20 °C and 37 °C in phosphate and PBS buffers, respectively, by equilibrium sedimentation (see Materials and methods). As an example, data are shown for wild-type IL-8 (Fig. 4A). We initially chose to perform the sedimentation experiments in the buffer used for NMR experiments (50 mM phosphate, pH 5.7). The mean K_{dd} from two experiments with two concentrations of protein were determined by fitting to a monomer-dimer equilibrium (Table 1). Dimerization of variant I appears tighter under these conditions than in NMR experiments at 40 °C (cf. Fig. 3B). The dimerization affinities, measured by sedimentation in phosphate buffer, pH 5.7, at 20 °C, were 10^2- to 10^6-fold weaker than that of wild type, ranging from 7 μM for variant I (E24R/I28R), to 1.8 mM for variant IV (Table 1).

Noting that the wild-type IL-8 dimerized with high affinity under these conditions, we next examined dimerization as a function of temperature, pH, and ionic strength. The results of these sedimentation experiments showed that wild-type dimerization affinity significantly decreased as temperature was increased from 5 °C to 40 °C (Fig. 4B), or as ionic strength was raised from 100 mM to 860 mM NaCl (Fig. 4C). Dimerization affinity also decreased slightly when the solution pH was raised from 5.7 to 7.4.

Binding of monomeric variants to three IL-8 receptor types

CXCR1 or CXCR2 binding by several weakly dimerizing variants (I, II, III, or IV) showed affinities ranging from slightly tighter than wild-type IL-8 to only slightly weaker (Table 2). Indeed, the weakest affinity measured was only ~ sixfold weaker for variant IV binding to CXCR1, even though this variant was at least 10^4- to 10^6-fold weaker in dimerization. This slightly lower affinity is an indirect result of the mutations rather than dimerization because variant III, with tighter affinity, had the same dimerization constant (Table 2). Thus, weakly dimerizing forms of IL-8 are able to bind these receptors nearly as tightly as native IL-8. In contrast, a negative control variant (IV + R6K), which had the ELR sequence altered, produced ~1000-fold weaker binding to each receptor (Table 2).

In binding assays to the receptor DARC on human erythrocytes, monomer variants I, II, III, and IV all bound with affinities within threefold of that for wild-type IL-8 (Table 3). As a control, variant V, containing the R6K mutation in addition to the monomer-IV mutations, was greatly reduced (about 200-fold) in affinity for DARC.

Bioactivity of monomeric IL-8 through CXCR1 and CXCR2

In vitro biological activity of the IL-8 monomer variants (I, III, and IV) was compared to that of wild-type IL-8 in two different assays, both widely used as a readout for chemokine-stimulating activity on leukocytes: chemotaxis and calcium flux. Figure 5 shows that the neutrophil chemotactic activity of the monomer variants was comparable to that of wild-type IL-8, with the measured neutrophil chemotaxis peaking in all four cases in the 11 to 110 nM range of protein concentration.

One of the many responses of neutrophils to IL-8 stimulation is the release of calcium into the cytoplasm of responsive cells (Naccache et al., 1989). Calcium flux assays were used to demonstrate the activation of CXCR1 or CXCR2 separately on 293 cells stably expressing these IL-8 receptors (Fig. 6). The calculated relative activities [i.e., EC_{50}(mutant)/EC_{50}(wild-type)] on CXCR1 were 1.0 for variant I; 0.9 for variant III; and 8.0 for variant IV. On

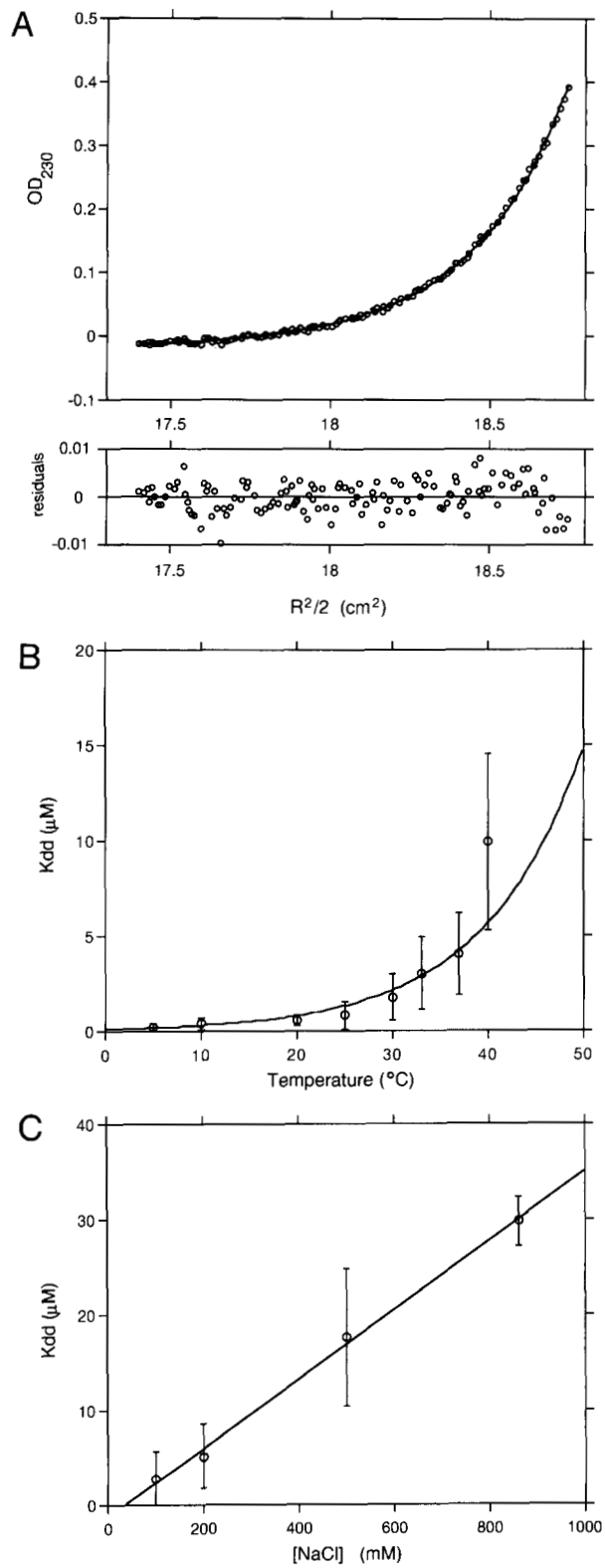


Fig. 4. Sedimentation analysis of IL-8. **A:** Ultracentrifugation data from wild-type IL-8 in PBS buffer at 37 °C. Protein concentration is plotted as optical density (at 230 nm) as a function of $R^2/2$, where R is the radial distance in the centrifuge. The data were fit to a simple monomer-dimer equilibrium model in order to determine the dimer-dissociation equilibrium constant, K_{dd}, as described (see Materials and methods). **B:** Dependence of the calculated wild-type IL-8 dimer dissociation (K_{dd}) upon temperature. **C:** Dependence of K_{dd} for wild-type IL-8, determined by sedimentation, upon concentration of NaCl.

Table 1. *Dimerization constants of weakly dimerizing IL-8 variants*

IL-8 variant	Dimerization affinity K_{dd} (μM)[a]	K_{dd} (μM)[b]
Wild-type	<0.1[c]	4.0 ± 2.2
I = E24R/I28R	7.6 ± 4.2	64 ± 17
II = T37E/ A69E /S72E	n.d.[d]	1,600 ± 860
III = I+II	1,800 ± 550	1,200 ± 800
IV = III+ F65H/L66E	1,800 ± 500	1,900 ± 1,000

[a]In 50 mM phosphate buffer, pH 5.7, 20 °C.
[b]In PBS buffer, pH 7.2, 37 °C.
[c]Dissociation of wild-type was not detected under these conditions (see Fig. 3).
[d]n.d., not determined.

CXCR2, relative activities were 1.4 for variant I; 5.3 for variant III; and 2.2 for variant IV (Fig. 6). Thus, all the variants show Ca^{2+} flux activity, with potencies similar to that of wild-type IL-8.

Discussion

Engineering monomers through side-chain substitutions

Sedimentation analysis showed that IL-8 monomer variants, having a range of dimerization constants from μM to mM, were produced by the introduction of side-chain hindrance determinants. One variant, called variant I (E24R/I28R), was further analyzed by NMR spectroscopy over a concentration range of 0.1 to 1.8 mM total protein. The results (Fig. 3) showed a concentration-dependent appearance of a new molecular species (i.e., monomer) as protein concentration was lowered. When we fit the NMR-determined amount of dimer as a function of protein concentration to a simple dimerization model, we find that variant I dissociates with a K_{dd} of approximately 0.1 mM at 40 °C in phosphate buffer, pH 5.7. This corresponds to a >1,000-fold reduction in dimerization affinity as compared to wild-type IL-8 (K_{dd} < 0.1 μM) as measured by sedimentation in the same buffer at 20 °C. In fact, both ionic strength and temperature dramatically affected the dimerization of wild-type IL-8 (Table 1; Fig. 4).

When the dimerization affinities of monomer mutants were examined as a function of solution conditions, version I showed about 10-fold enhanced dimer formation at lower salt and temperature, similar to the effect seen for wild-type. However, variants III and IV, which included mutations in the helix-sheet interface (T37E/A69E/S72E in variant III; and T37E/F65H/L66E/A69E/S72E in variant IV), had dimerization affinities that were equivalent (within experimental error) under both sets of conditions. This suggests that the dominant effect of salt concentration and temperature is localized at the site of packing around T37 (Fig. 2B).

While the dimerization affinities of mutants were clearly quite weak, especially when compared with the concentration at which they bind and activate known receptors, they do show self-association at very high concentrations. For monomer variant I, NMR data shows that this association corresponds to formation of a native-like IL-8 dimer. However, for the other variants (II, III, and IV), we do not know whether the very weak self-associations represent native-like dimer formation in which the side-chain alterations are accommodated through local rearrangements, or whether this self-association represents a novel form of association not previously observed for wild type. To our knowledge, even in the mM range, no higher order association of IL-8 dimers in solution has been observed by NMR that could suggest an alternative interface for association of IL-8 subunits. Efforts in progress to crystallize the dimeric form of these "monomeric" mutants should answer the question of how IL-8 monomers, extensively modified in their native–dimer interface, can still associate to form dimers at high (mM) concentrations.

The analysis of several IL-8 monomers stabilized through multiple side-chain substitutions shows that calculation of buried surface area combined with inspection of the IL-8 structure can correctly identify sites for introducing hindrance determinants of IL-8 dimerization. Substitutions at residues having the greatest side-chain packing between subunits (e.g., T37, A69, and S72; Fig. 2A) had the greatest effect on dimerization affinity (Table 1). These determinants should be equally applicable to other molecules with similar sequence and structure to IL-8, such as MGSA (Fairbrother et al., 1994).

Dimerization properties of wild-type IL-8

The dimerization state of IL-8 in vivo is not known. However, estimates of dimerization affinity in vitro under conditions approaching physiological have previously been reported as K_{dd} =

Table 2. *Binding affinities of IL-8 variants to transfected 293 cells*[a]

IL-8 variant	CXCR1 K_d (nM)	CXCR1 K_d(mut)/K_d(IL-8)	CXCR2 K_d (nM)	CXCR2 K_d(mut)/K_d(IL-8)
Wild-type	2.3 ± 1.5[b]	1	0.84 ± 0.42[b]	1
I = E24R/I28R	2.1 ± 0.37	0.92	0.34 ± 0.09	0.40
II = T37E + A69E/S72E	8.7 ± 1.9	3.8	1.5 ± 0.34	1.8
III = I+II	4.4 ± 1.9	1.9	3.2 ± 1.1	3.8
IV = III + F65H/L66E	14.7 ± 6.3	6.4	3.9 ± 1.4	4.6
V = IV + R6K	1,700 ± 600	760	860 ± 320	1,020

[a]Apparent receptor binding K_ds were determined in parallel with and normalized to wild-type controls, representing an average of duplicates or triplicates (±SD).
[b]Values are from Lowman et al. (1996).

Table 3. *Binding affinities of IL-8 variants to erythrocytes*[a]

IL-8 variant	Erythrocytes K_d (nM)	Erythrocytes K_d(mut)/K_d(IL-8)
Wild-type	20.8 ± 3.7	1
I = E24R/I28R	16.1 ± 5.5	0.84
II = T37E + A69E/S72E	45.0 ± 12	2.4
III = I + II	18.7 ± 5.1	0.98
IV = III + F65H/L66E	48.8 ± 9.6	2.6
V = IV + R6K	3,770 ± 299	197

[a]Binding affinities of IL-8 variants to erythrocytes were determined in parallel with and normalized to wild-type controls, representing an average of duplicates or triplicates (±SD).

14–18 μM (pH 7.4, 150 mM NaCl, 20 mM phosphate; Burrows et al., 1994); 21 ± 10 μM (conditions not specified, Paolini et al., 1994); and <60 μM (100 mM sodium acetate pH 6.0; Rajarathnam et al., 1994). NMR studies, on the other hand, had reportedly shown no evidence of dimer dissociation at concentrations as low as 40 μM at pH 6.5 under low ionic strength conditions (Clore & Gronenborn, 1991). Because evidence of a monomer species is apparent in the NMR spectra of an IL-8 variant having $K_{dd} \approx$ 100 μM at protein concentrations of >1 mM, the NMR data on wild-type IL-8 suggest that dimerization under the specified conditions occurs with $K_{dd} < 1$ μM.

Our data provide an explanation for the range of different dimerization affinities observed. Namely, the dimerization of wild-type IL-8 is acutely dependent upon temperature and ionic strength. The dimerization affinity of wild-type IL-8 is clearly highly sensitive to solution conditions. For wild-type IL-8, we measured $K_{dd} = 4.0 \pm 2.2$ μM at 37 °C in PBS buffer, pH 7.2, in reasonable agreement with previous reports (Burrows et al., 1994; Paolini et al., 1994). At 25 °C, 50 mM sodium phosphate, pH 5.9, we find no detectable dissociation of the wild-type IL-8 dimer ($K_{dd} <$ 0.1 μM) by sedimentation. We also made the direct comparison of our IL-8 preparation with the one that was used by Burrows' group. We found that both molecules have very similar dissociation constants ($K_{dd} = 0.26$–1.12 μM for our preparation and 0.23–1.08 μM for that provided by the Burrows group) in PBS buffer at 25 °C. This again supports our observation that the different dimerization affinities observed are mainly caused by the change of solution conditions.

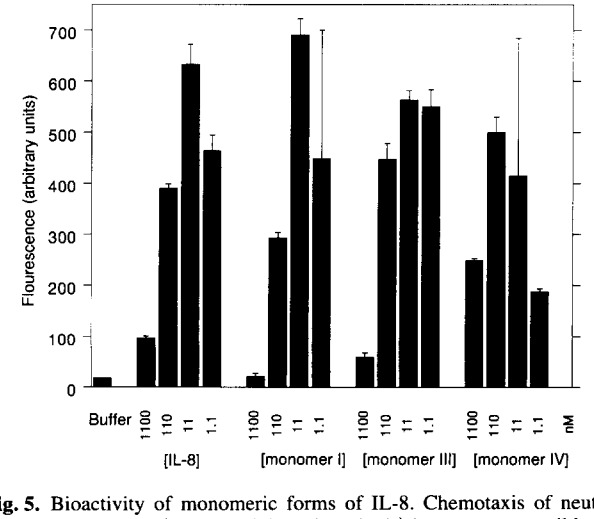


Fig. 5. Bioactivity of monomeric forms of IL-8. Chemotaxis of neutrophils was measured (see Materials and methods) in response to wild-type and monomeric variants of IL-8 (see Fig. 1A). The magnitude of response (corresponding to the number of cells migrating) is shown in arbitrary fluorescence units. The relative responses were typical in duplicate experiments, including a "bell-shaped" dose–response curve in each case.

Role of IL-8 dimerization in biological activity

Given the large effects of solution conditions on dimerization affinity, it seems plausible that under in vivo conditions, IL-8 dimerization may be modulated by local conditions, such as the binding of IL-8 to heparan sulfate proteoglycans (Webb et al., 1993). In fact, the dissociation kinetics of IL-8 dimers fixed on a biosensor dextran surface suggest that IL-8 dimerization affinity is indeed increased by such a surface-presentation environment (Lowman, unpub. obs.). The relative affinity of IL-8 variants for proteoglycans has been estimated by the salt concentration at which they elute from a heparin-sepharose column (Webb et al., 1993). We observed that the monomeric variants I, II, III, and IV, each eluted at lower salt concentration than wild-type IL-8 during purifications (see Materials and methods). However, the reduction in salt concentration did not correlate with the measured dimerization affinities of these variants (Table 1). It is interesting to note that variant I, containing only mutations E24R/I28R, elutes at a slightly lower salt concentration than wild type, even though the added positive charges might be expected to increase (non-specific) heparin binding. This may reflect a slightly reduced affinity of the monomeric form for heparin. We cannot attribute the lower heparin-binding affinity of the remaining variants entirely to their dimerization state because each contains charge substitutions within the α-helical region, which is believed to be important for heparin binding, based upon truncation studies of IL-8 (Webb et al., 1993) as well as mutational studies of PF4 (Maione et al., 1991).

IL-8 has been shown to cause granule enzyme (β-glucuronidase; elastase) release in neutrophils pre-treated with cytochalasin-B. By measuring the amount of β-glucuronidase released into the medium, one can therefore assess the extent of neutrophil degranulation induced by wild-type and variant IL-8. We have previously reported degranulation activity of monomeric forms of IL-8 (Lowman et al., 1996). The data from these and other experiments (data not shown) demonstrate that several monomeric variants have essentially the same activity as wild-type IL-8, and this conclusion is supported by findings for single-chain heterodimers of IL-8 (Leong et al., 1997), which contain only one "active" subunit.

IL-8 is predicted to be monomeric in solution in the range of the K_d for receptor binding and the EC_{50}s for calcium-flux, degranulation, and chemotactic activity. But if IL-8 dimerization is not required for receptor binding or activation, what, if any, biological role might it serve? One possibility is that the relatively tight dimer interface merely contributes to protein stability. We noted far lower yields from *E. coli* expression of monomers than that found for wild-type IL-8. Expression levels in *E. coli* have been interpreted as indicative of protein conformational stability. A more intriguing possibility is that the dimeric form may be involved in additional

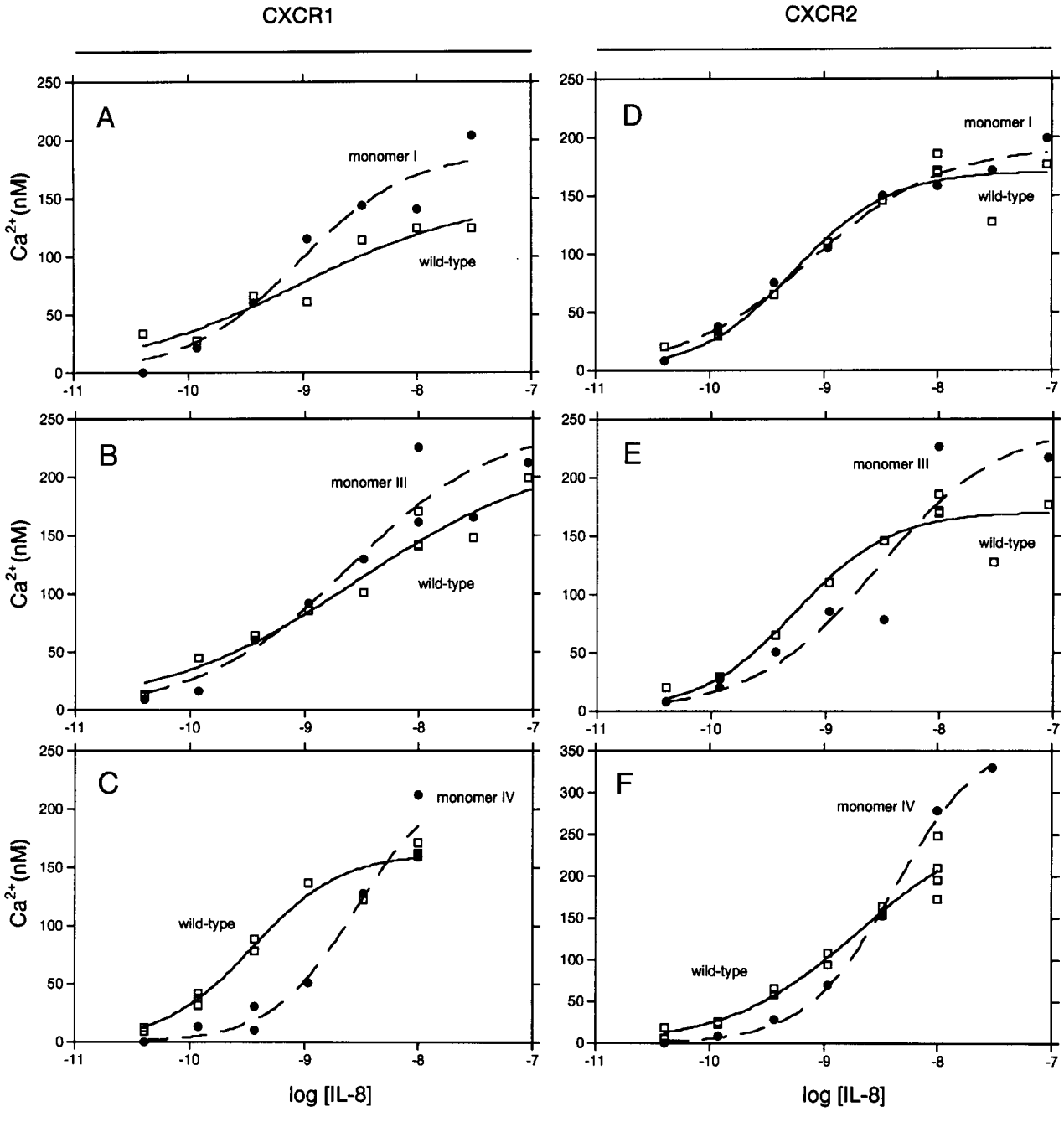


Fig. 6. Calcium-flux activity of monomeric IL-8 variants on 293 cells expressing IL-8 receptor A or receptor B. Fluorescence assays, using indo-1/AM as indicator, were carried out to follow the release of intracellular calcium upon activation by IL-8 as described (see Materials and methods). For CXCR1 cells, calcium flux is shown as a function of the dose of IL-8 variant for monomer variant I (**A**); variant III (**B**); and for variant IV (**C**). On CXCR2 cells, activity is shown for monomer variant I (**D**); for variant III (**E**); and for variant IV (**F**).

macromolecule interactions, such as the binding of IL-8 to endothelial surface proteoglycans (Tanaka et al., 1993; Webb et al., 1993). The decoration of the endothelial cell membrane with IL-8, recently demonstrated by Rot and co-workers (Rot et al., 1996), is a step that may be critical for the initial presentation of IL-8 to rolling neutrophils in the microenvironment of the inflamed endothelium.

Materials and methods

Construction of IL-8 variants

A protein-expression and phage-display (Lowman & Wells, 1991) plasmid for IL-8 variants was constructed by PCR using Taq polymerase (Perkin-Elmer) of the IL-8 gene from an existing plasmid, pAPSTII.IL8.72 (Hébert et al., 1991) containing the wild-type

IL-8 gene under control of the P_{phoA} promoter (Chang et al., 1987). The upstream PCR primer was 5′-TCA CGA GGC CCT TTC GT-3′ (which primes upstream of an extant EcoRI site) and the downstream primer was 5′CAC GGC AAG CTA GCT ATT CTC AGC CCT CTT CAA-3′ (NheI site underlined). The IL-8 gene-containing fragment was PCR amplified, then digested with restriction enzymes EcoRI and NheI.

The hGHam-g3 plasmid (Lowman & Wells, 1991) was digested with EcoRI and NheI and ligated with the IL-8 fragment. Ligation products were transformed into *E. coli* (strain XL1-Blue™), and isolated colonies were picked for sequencing. The resulting construct, called pPS0170, has the P_{phoA} promoter controlling IL-8 expression, with secretion into the periplasm directed by the stII signal sequence. The IL-8 gene (residues 1–72) is followed by an amber (TAG) stop codon and the C-terminal fragment of the bacteriophage M13 gene, g3 (Lowman & Wells, 1991). Thus, in amber-suppressor strains of *E. coli* (such as XL1-Blue) this construct produces a fusion protein between IL-8 and g3p. However, in non-suppressor strains of *E. coli* translation terminates at the amber codon, and full-length IL-8 is produced.

Preparation of IL-8 variants

IL-8 variants L25P, V27P, L25E/V27E, L25E/V27E/L66E, T37E/A69E/S72E (variant II), E29R/A69R, E24R/I28R, F65E/L66E, and F65H/L66E were each constructed by site-directed mutagenesis of plasmid pPS0170 using the method of Kunkel (Kunkel et al., 1987). Mutations were confirmed by single-stranded DNA sequencing using Sequenase® (US Biochemical).

IL-8 and variants were expressed in *E. coli* in low-phosphate media (Chang et al., 1987) at 27–37 °C, and purified using a variation (Lowman et al., 1996) of the procedure for wild-type IL-8 (Hébert et al., 1990). Monomer variants were purified chromatographically, initially on an S-sepharose column equilibrated with 10 mM Mes (pH 6) buffer. After a step-elution with 1 M NaCl, the protein was dialyzed into 10 mM phosphate buffer (pH 7.2) and loaded onto a Mono-S (Pharmacia) column. A gradient elution of 0–1 M NaCl was used to elute the protein. Thereafter, peak fractions were pooled, and α2-macroglobulin (Boehringer-Mannheim) was added to reduce proteolysis. Saturated $(NH_4)_2SO_4$ was added to a final concentration of 60–70% saturated $(NH_4)_2SO_4$ for loading onto an alkyl-superose (Pharmacia) column, and a reverse $(NH_4)_2SO_4$ gradient was used to elute the protein. Samples were desalted into PBS buffer using NAP-5 gel filtration columns (Pharmacia). Some samples of monomer variants were further purified on Hitrap-heparin columns in 10 mM phosphate (pH 7.2) buffer, with a linear gradient of 0-1 M NaCl for elution. Each of the monomeric IL-8 variants described here bound to the heparin column, eluting at salt concentrations of 0.42 M, 0.18 M, 0.32 M, or 0.28 M NaCl for variants I, II, III, and IV, respectively, as compared with 0.54 M NaCl for wild-type IL-8 (data not shown).

An additional sample of wild-type IL-8 was also kindly provided by Dr. Preston Hensley, SmithKline Beecham Pharmaceuticals.

Proteins were assayed by SDS-PAGE, using a 16% polyacrylamide gel (Novex, Inc.) and tricine-SDS buffer. In some cases, purified proteins were further subjected to mass spectrometry analysis, which confirmed the mutations present as well as lack of significant proteolysis. Mass spectrometry was carried out by running samples on an HPLC followed by electrospray spectrometry on a PE-SCIEX API III spectrometer. The measured masses were variant I, 8,453 ± 0.7 Da vs. 8,453 theoretical; variant III, 8,580 ± 0.6 Da vs. 8,580 theoretical; variant IV, 8,589 ± 0.8 Da vs. 8,586 theoretical.

NMR spectroscopy

All NMR spectra were acquired at 40 °C on a Bruker AMX-500 spectrometer. The protein samples were dialyzed into 90% H_2O/10% D_2O, 50 mM sodium phosphate, pH 5.7, containing 0.01% sodium azide. The following homonuclear two-dimensional (2D) NMR spectra were recorded using standard pulse sequences and phase cycling (Cavanagh et al., 1995): pre-TOCSY-COSY (Otting & Wüthrich, 1987), TOCSY (Braunschweiler & Ernst, 1983), NOESY (Kumar et al., 1980), and jump-and-return NOESY (Plateau & Guéron, 1982). All 2D spectra were acquired in the phase-sensitive mode using time-proportional phase incrementation for quadrature detection in the t1 dimension (Marion & Wüthrich, 1983). With the exception of the JR-NOESY experiments the solvent signal was suppressed by low-power phase-locked presaturation for 1.3 s. TOCSY spectra were acquired using a 68 ms "clean" DIPSI-2rc isotropic mixing sequence (Cavanagh & Rance, 1992). NOESY spectra were acquired with irradiation of solvent during the 100 ms mixing times. JR-NOESY spectra were acquired with mixing times of 150 ms.

All spectra were processed and analyzed on Silicon Graphics workstations using the program FELIX (Biosym Technologies). Resonance assignments were obtained using standard methods utilizing the 2D spectra discussed above (Wüthrich, 1986).

Analytical ultracentrifugation

Equilibrium sedimentation analysis was carried out in a Beckman XL-A analytical ultracentrifuge. Initially, these assays were performed in a buffered solution identical to that used for NMR: 50 mM phosphate buffer, pH 5.7 containing 0.01% sodium azide. The sedimentation was carried out at 20 °C.

To test dimerization under physiological salt conditions or higher salt conditions, samples of IL-8 and its variants were dialyzed into PBS (137 mM NaCl, 2.7 mM KCl, 7.9 mM Na_3PO_4, 1.14 mM K_3PO_4, pH 7.2) or 50 mM phosphate buffer with different concentrations of NaCl (100 mM, 200 mM, 500 mM, and 860 mM NaCl) with a 1,000 Da cutoff membrane. The dimerization of IL-8 in PBS was also examined at temperatures ranging from 5 °C to 40 °C.

Samples of IL-8 wild-type and monomer variants I, II, III, and IV were run at concentrations from 0.5 mg/mL to 0.02 mg/mL. Optical density (280 or 230 nm) was used to measure concentration as a function of radial position at 35,000, 40,000, and 45,000 rpm. All data were then fit to a simple monomer-dimer model with a nonlinear least-squares fitting program, NONLIN (Johnson et al., 1981) to obtain dimer dissociation constants, K_{dd}.

IL-8 binding to CXCR1, CXCR2, and DARC receptors

Binding assays were carried out on stably transfected 293 cells expressing CXCR1 or CXCR2 receptor essentially as described (Lowman et al., 1996). Cells were incubated with ^{125}I-labeled IL-8 (0.25– 0.5 nM) and serial dilutions of cold competitor at 4 °C for one hour. Thereafter, cells were layered onto a sucrose solution and pelleted. The supernatant was removed and the cells counted on a gamma counter.

For binding to the DARC receptor, competition binding assays were performed on human erythrocytes as described (Horuk et al.,

1993). Briefly, ^{125}I-labeled IL-8 (New England Nuclear) was mixed with unlabeled competitor (as above) in 96-well plates (Nunc), and erythrocytes were added. After incubation for one hour at 4 °C, the cells were pelleted through a paraffin–oil mixture. The tips of the tubes, containing the cell pellet, were then excised and counted on an Isodata-120 gamma counter (ICN Pharmaceuticals).

All data from binding assays were analyzed by Scatchard analysis to determine the ratio of mutant affinity to wild-type affinity, K_d(mut)/K_d(wt).

Neutrophil chemotaxis assays

Wild-type IL-8 and the variants were serially diluted in chemotaxis buffer (0.1% BSA in Ca^{2+} and Mg^{2+}-free HBSS) (BioWhittaker) and each sample tested in quadruplicate on a standard 96-well chemotaxis chamber (Neuro Probe, Inc., Cabin John, MD) set with a 5 μm filter. Freshly isolated human neutrophils were labeled with 10 μg/mL Calcein AM (Molecular Probes, Inc., Eugene, OR) for 15 min at 37 °C and 5×10^5 cells were loaded per well and allowed to migrate for 40 min at 37 °C. Neutrophil chemotaxis was measured by reading relative fluorescence in a CytoFluor 2350 Fluorescence Measurement System (Millipore Corp., Bedford, MA).

Calcium flux assays using 293 cells

Each experiment consisted of parallel dilutions of wild-type and variant IL-8 using a single batch of 293 cells, expressing CXCR1 or CXCR2, which had been loaded with the calcium-binding dye INDO-1AM (Molecular Probes, Inc., Eugene, OR). Serial dilutions of wild-type and IL-8 variants were performed and aliquots injected into the equilibrated cuvette. These assays were performed in a stirred cell as described (Naccache et al., 1989; Neote et al., 1993). The fluorescence (excitation = 350 nm; emission = 405 nm) versus time curves were analyzed by converting the fluorescence values to nM Ca^{2+} concentrations (Naccache et al., 1989). An average baseline was calculated and subtracted from the calcium value at peak signal to yield the amplitude of response plotted as a function of IL-8 or variant concentration.

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