Determination of the Monomer–Dimer Equilibrium of Interleukin-8 Reveals It Is a Monomer at Physiological Concentrations

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ABSTRACT: Interleukin-8 has been shown by X-ray crystallography and NMR to be a homodimer, suggesting that this is the form which binds to its receptor. Here we measure, for the first time, the monomer–dimer equilibrium of interleukin-8 using analytical ultracentrifugation and titration microcalorimetry and find that it dissociates readily to monomers with an equilibrium dissociation constant of 18 ± 6 μM at 37 °C. The present findings suggest that the monomer is the form which binds to the receptor. Comparison of experimental and structure-based calculated thermodynamics of interleukin-8 dimerization argues for limited subunit conformational changes upon dissociation to monomer.

Interleukin-8 (IL-8)[1] is a potent chemoattractant of neutrophils and certain T cells. It is a member of a family of proteins, called chemokines, all of which act as chemoattractants for various leukocytes (Oppenheim et al., 1991). IL-8 is secreted by monocytes and endothelial cells in response to inflammatory stimuli and has been implicated in a number of inflammatory diseases (Baggiolini & Clark-Lewis, 1992; Hebert & Baker, 1993). The biological effects of IL-8 are mediated through binding to G-protein-coupled seven-transmembrane receptors (Holmes et al., 1991; Murphy & Tiffany, 1991).

IL-8 is secreted predominantly as an 8-kDa, 72 amino acid species with two internal disulfide bridges (Baggiolini & Clark-Lewis, 1992; Oppenheim et al., 1991). NMR and X-ray crystallography show that IL-8 is a homodimer (Baldwin et al., 1991; Clore et al., 1990) and reveal a structure related to platelet factor-4, another member of the chemokine family, which is tetrameric (Charles et al., 1989). Other members of the chemokine family, such as MIP-1β (Lodi et al., 1994), are also structurally similar and tend to self-associate (Graham et al., 1994).

Therefore, a key question that remains to be answered about the molecular basis of IL-8 and other chemokines binding to their receptors is whether they bind as monomers or self-associated forms. To date, interpretation of structure–activity studies of IL-8 (Clark-Lewis et al., 1991, 1993; Hebert et al., 1991; Moser et al., 1993; Schraufstatter et al., 1993) has been guided by structural studies (Baldwin et al., 1991; Clore et al., 1990) which are inherently done at much higher concentrations than those required for biological activity. However, the recent finding of a chemically modified IL-8 which does not dimerize in solution yet retains full biological activity suggests that the monomer may be the active form (Rajarathnam et al., 1994).

Here we report results on direct measurements of the monomer–dimer equilibrium of IL-8 and show by analytical ultracentrifugation, time-resolved fluorescence anisotropy, and titration microcalorimetry that IL-8 is monomeric at nanomolar physiological concentrations.

EXPERIMENTAL PROCEDURES

Expression and Purification of IL-8. A 230-bp *Bsp*MI/*Bam*HI fragment of synthetic IL-8 cDNA (British Biotechnology) encoding the mature 72 amino acid human IL-8 was cloned into the *Escherichia coli* λp_L-dependent expression vector pOTS207Nco between *Nco*I and *Bam*HI sites. IL-8 was expressed by thermal induction of the λp_L promoter in a lysogenic strain of *E. coli* containing the temperature-sensitive $\lambda C1_{857}$ repressor AR58 (Shatzman & Rosenberg, 1986).

E. coli cell paste was resuspended in 0.1 M NaCl and 50 mM MES, pH 6.5, to 1 g/10 mL (w/v) using a Tekmar Tissuemizer, and processed through a Rannie Mini-Lab type 8.30H high-pressure homogenizer (APV Rannie, St. Paul, MN) at a minimum pressure of 8000 psi. The lysate was centrifuged at 14300*g* for 30 min and the supernatant decanted, recentrifuged, and filtered. IL-8 was found only in the soluble fraction, permitting a higher yield than that found by previous methods (Furuta et al., 1989) where soluble and insoluble fractions were found. The soluble fraction was chromatographed with a SP Sepharose Fast Flow matrix equilibrated with 0.1 M NaCl and 50 mM MES, pH 6.5, and eluted at pH 6.5 with a gradient from 0.1 to 1 M NaCl. IL-8 was concentrated and then purified on a Superdex 75 column in PBS, pH 7.8, with a final yield of 1.98 mg/g of cell paste (yield >95% of the expressed protein). The amino acid composition and amino-terminal sequence confirmed the identity of IL-8 and removal of the amino-terminal Met.

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; IL-8, interleukin-8; MIP-1α, macrophage inflammatory protein-1α; MIP-1β, macrophage inflammatory protein-1β; PF4, platelet factor-4.

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Chemotaxis Assays. Polymorphonuclear leukocytes were separated from whole blood of healthy volunteers by the one-step Hypaque–Ficoll method (Ferrante & Thong, 1982), washed twice in phosphate buffer, and resuspended at 5×10^6 cells/mL in phosphate buffer containing 1 mM $MgCl_2$ and $CaCl_2$. Cell motility was determined in a modified Boyden chamber as described by Bignold (1987).

Stimulation of Intracellular Calcium. Neutrophils were loaded with Fura-2 by the method of McCarthy et al. (1988), placed in a magnetically stirred cuvette, and kept in suspension at 37 °C. IL-8 was added to the stirred cuvette, and changes in fluorescence were monitored for 5 min using a counting spectrofluorometer (Biomedical Instruments Group, University of Pennsylvania) with fluorescent excitation at 340 nm and emission measured at 510 nm.

Receptor Binding. Neutrophil membranes were made by lysing 1×10^8 freshly isolated and purified human neutrophils in lysis buffer (20 mM Tris·HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, 0.5 mg/L leupeptin, 0.01 mg/mL aprotinin). After homogenization, nuclei were removed by low-speed centrifugation (1000*g*, 5 min), and crude membranes were centrifuged at 100000*g* for 60 min at 4 °C in a Beckman ultracentrifuge. Membranes were resuspended in lysis buffer at 500 μg/mL and used either directly or frozen at −70 °C. To measure binding via displacement, 10 μL of binding buffer (100 mM Bis-tris–propane, pH 8, 5 mM $MgCl_2$, 750 mM NaCl) was added to 10-μL volumes of 500 μg/mL membrane, followed by 10 μL of H_2O with or without various concentrations of recombinant IL-8 and 20 μL of 1 nM ^{125}I-IL-8 (Amersham, 2000 Ci/mmol) resuspended in H_2O. After incubation at room temperature for 60 min, the mixture was harvested onto Whatman GF-B fiberglass filters using an automated harvester. Each filter was washed with 2 mL of filter washing solution (10 mM Tris·HCl, 1 mM $MgSO_4$, 0.5 mM EDTA, 150 mM NaCl, pH 7.4), dried, and counted in a Beckman γ counter for 1 min.

Analytical Ultracentrifugation. Measurements of IL-8 distribution were determined on a Beckman XL-A analytical ultracentrifuge at rotor speeds of 45 000 rpm. The distribution of IL-8 was best-fit to

$$A_{226,r} = c_m\epsilon_{226}\exp[\sigma] + (c_m c_m/K_D)2\epsilon_{226}\exp[2\sigma] + \text{base} \quad (1)$$

where $\sigma = M(1 - \bar{v}\rho)\omega^2(r^2 - r_m^2)/2RT$ and K_D is the dimer equilibrium dissociation constant. $A_{226,r}$ is absorbance at 226 nm at the radial position r, c_m is the concentration of monomer at the meniscus, and ϵ_{226} is the molar extinction coefficient of the monomer at 226 nm. M and $\bar{v}$ are the molecular weight and partial specific volume of the monomer, ρ is solvent density, ω is angular velocity, r_m is the reference radial position in centimeters, R is the universal gas constant, T is absolute temperature, and base is the baseline. Data were fit using nonlinear least squares methods (Brooks et al., 1994). K_D was calculated by a global analysis of the results from three ultracentrifugation runs with different initial IL-8 concentrations.

Time-Resolved Fluorescence Anisotropy. Measurements of fluorescence anisotropy were made using the sum and difference method with an excitation pulse at 295 nm (Badea & Brand, 1979; Knutson et al., 1986). The decay in fluorescence anisotropy was fitted to $r(t) = r_0\exp(-t/\phi)$, where r_0 is the anisotropy at zero time and ϕ is the rotational correlation time. Here, $r(t) = I_\parallel(t) - I_\perp(t)/[I_\parallel(t) + 2I_\perp(t)]$, where $I_\parallel(t)$ and $I_\perp(t)$ are time-dependent changes in fluorescence intensity from polarizers in the parallel and perpendicular position, respectively.

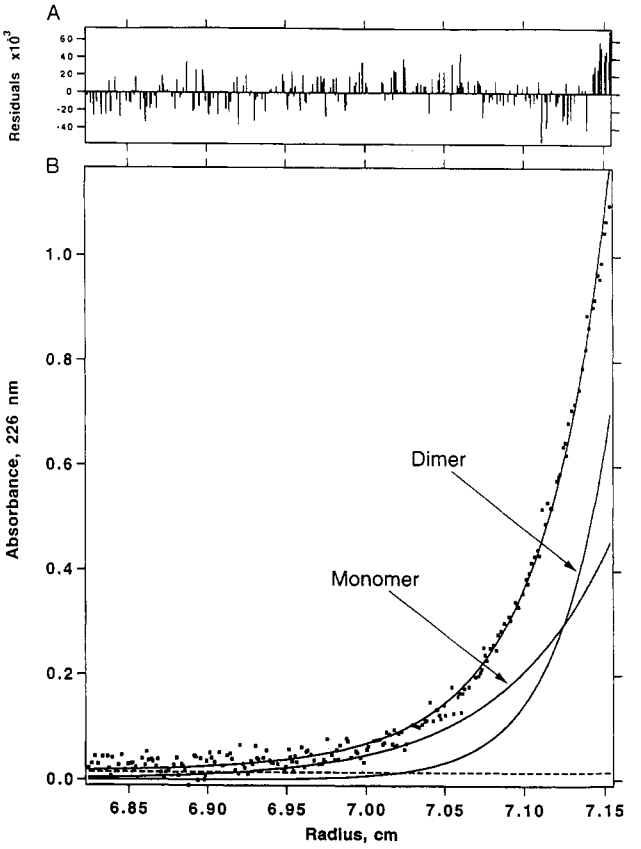

FIGURE 1: Fitting of IL-8 equilibrium analytical ultracentrifuge data to a monomer–dimer equilibrium: (A) residuals for the best-fit curve versus radial position; (B) absorbance (226 nm) versus radial position. The best-fit curve from a global analysis of three different starting concentrations yields $K_D = 14 \pm 4$ μM (Brooks et al., 1994). Curves below the data show the relative amounts of monomer and dimer. The dashed line is the baseline absorbance. The concentration range of IL-8 was 0.03–30 μM in 20 mM sodium phosphate and 150 mM NaCl, pH 7.4, at 25 °C.

Titration Microcalorimetry. Heats of dissociation of IL-8 dimers to monomers were measured with a Microcal, Inc., Omega microcalorimeter (Wiseman et al., 1989). IL-8 was diluted inside the calorimeter from a concentration where it was predominantly dimeric to concentrations where it was predominantly monomeric. Data were fit to the following monomer–dimer model where the heat at the ith injection is

$$q_{obs}(i) = {}^1/_2\Delta H^\circ[n(i)f_d(i) - n(i-1)f_d(i-1) - n(\text{inj})f_d(\text{inj})] \quad (2)$$

Here ΔH° is the enthalpy change for dimerization, and the indices i, $i-1$, and inj refer to the number of moles of IL-8 subunits (n) and the fraction as dimer (f_d) in the reaction cell after the ith injection, after the $i-1$ injection, and the amount injected into the cell at the ith injection, respectively. The fraction of dimer is equal to $f_d = 1 - [(1 + 8KC)^{1/2} - 1]/4KC$, where K is the monomer–dimer equilibrium association constant and C is the total concentration of IL-8 subunits.

Thermodynamic Calculations. The general methods for correlating the thermodynamics of protein–protein interactions with changes in buried polar and nonpolar surface area have been described previously (Murphy & Freire, 1992). Structure-based computation of the solvent-accessible surface area was by the method of Lee and Richards (1971), using a 1.4-Å

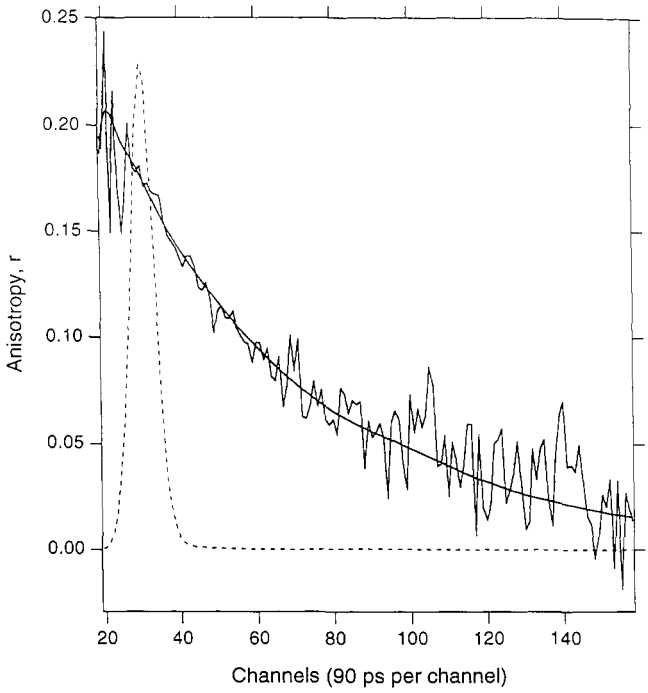

FIGURE 2: Time-resolved fluorescence anisotropy decay for 7 μM IL-8. The best-fit curve (Badea & Brand, 1979) yields a rotational correlation time ϕ = 4.7 ns (smooth curve). The dashed curve is the excitation pulse (295 nm). Conditions: 150 mM NaCl and 20 mM sodium phosphate, pH 7.4, at 25 °C.

probe and a slice width of 0.25 Å. Structural coordinates were from Brookhaven Protein Data Bank file PDB1IL8.ent.

RESULTS

Human IL-8 was expressed in *E. coli*, purified from the soluble fraction without refolding, and shown to behave identically to natural IL-8 in receptor binding (IC_{50} of 1.2 nM), stimulation of Ca^{2+} influx (ED_{50} = 0.2 nM), and chemotaxis of neutrophils (ED_{50} = 0.4 nM).

The monomer–dimer equilibrium of IL-8 was investigated by equilibrium analytical ultracentrifugation, and the results are shown in Figure 1. The IL-8 equilibrium distribution could not be fitted by either a homogeneous monomer or dimer but did fit well to a monomer–dimer equilibrium with a dissociation constant of 14 ± 4 μM at 25 °C. These results demonstrated that at sub-micromolar concentrations IL-8 was a monomer.

Dissociation of IL-8 to monomers was further studied by decay fluorescence anisotropy (Badea & Brand, 1979). At 7 μM IL-8, the fluorescence anisotropy decays with a rotational correlation time of 4.7 ns (Figure 2), which is close to the predicted value for the monomer (4.2 ns) and distinct from that predicted for the dimer (8.4 ns) (Small et al., 1991).

To independently measure the dimerization equilibrium constant and also determine the thermodynamics of IL-8 dimerization, we used titration microcalorimetry (Wiseman et al., 1989). In this method, a concentrated solution of IL-8 was diluted into buffer, and the heat of dissociation to monomers was measured (Figure 3). The amount of heat absorbed upon each injection is governed by the enthalpy change of dissociation to monomers and the monomer–dimer equilibrium constant (eq 2). Analysis of the data in Figure 3 yielded a K_D of 18 ± 6 μM at 37 °C, in very good agreement with the analytical ultracentrifugation results, and a dimerization enthalpy change of −8.4 ± 0.7 kcal/mol of dimer.

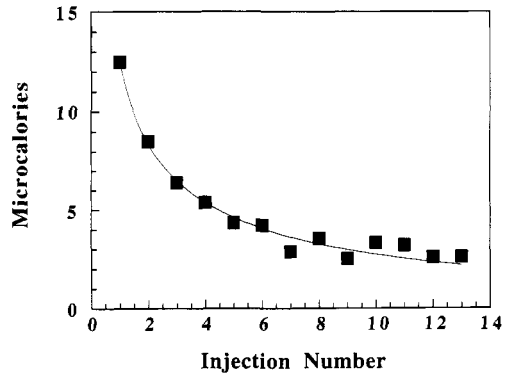

FIGURE 3: Heats of dissociation of IL-8 dimer to monomers measured by titration microcalorimetry. Consecutive 10-μL volumes of 460 μM IL-8 were diluted into 1.4 ml of 20 mM potassium phosphate, pH 7.4, and 150 mM NaCl, at 37 °C. Least-squares analysis yielded a dissociation constant of 18 ± 6 μM and a dimerization enthalpy change of $\Delta H°$ = −8.4 ± 0.7 kcal mol^{-1}.

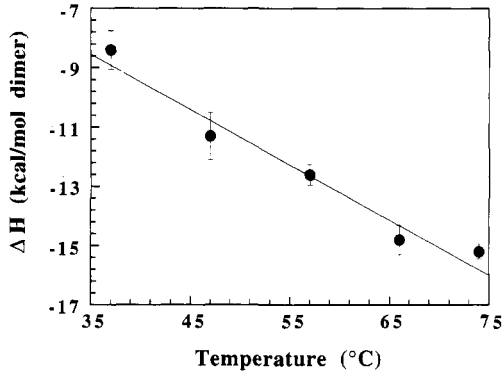

FIGURE 4: Enthalpy change for IL-8 dimerization versus temperature measured by titration microcalorimetry. The slope of the line equals the dimerization heat capacity change (Table 1).

Measurement of $\Delta H°$ versus temperature (Figure 4) gave a dimerization heat capacity change, $\Delta C°$, of −185 ± 37 cal mol^{-1} K^{-1}.

The experimentally determined thermodynamic parameters were compared to structure-based calculated predictions in order to investigate whether significant conformational changes are coupled to dissociation. The experimentally determined $\Delta H°$ and $\Delta C°$ were used to estimate the amount of polar (ΔASA_{polar}) and apolar (ΔASA_{apolar}) solvent-accessible surface area that is buried upon dimerization (Table 1), according to the empirical relationships (Murphy & Freire, 1992; Murphy et al., 1993):

$$\Delta ASA_{polar} = \Delta H°_{100}/35 \quad (3)$$

$$\Delta C° = 0.45\Delta ASA_{apolar} - 0.26\Delta ASA_{polar} \quad (4)$$

where $\Delta H°_{100}$ is the experimentally deduced dimerization enthalpy at 100 °C (−20.1 kcal/mol). Likewise, the thermodynamics for IL-8 dimerization were calculated from eqs 3 and 4 on the basis of the changes in polar and apolar accessible surface areas as determined from the NMR coordinates of the dimer (Clore et al., 1990). The results, summarized in Table 1, indicate that the calculated and experimental values for $\Delta C°$, $\Delta H°$, and ΔASA compare quite favorably. Since the structure-based calculations assume no structural rearrangement upon dissociation of dimer to monomers, the agreement with the experimental values suggests that the structure of the monomer in solution is similar to that of a subunit of the associated dimer.

Table 1: Structural and Thermodynamic Features of IL-8 Dimerization at 37 °C[a]

method	$\Delta G°$ (kcal mol^{-1})	$\Delta H°$ (kcal mol^{-1})	$\Delta S°$ [cal mol^{-1} K^{-1} (eu)]	$\Delta C°$ (cal mol^{-1} K^{-1})	ΔASA_{polar} (Å^2)	ΔASA_{apolar} (Å^2)
experiment	−6.7 ± 0.1	−8.4 ± 0.7	−5.5 ± 2.3	−185 ± 37	−570 ± 90[b]	−740 ± 100[b]
calculation	ND	−7.0 ± 2.5[c]	ND	−243 ± 26[c]	−640 ± 60[d]	−910 ± 90[d]

[a] Abbreviations: $\Delta G°$, free energy change; $\Delta H°$, enthalpy change, $\Delta C°$, heat capacity change; ΔASA_{polar} and ΔASA_{apolar}, change in polar and apolar solvent-accessible surface areas; ND, not determined. [b] Equations 3 and 4 and experimental $\Delta C°$ and $\Delta H°$. [c] Equations 3 and 4 and ΔASA values measured from IL-8 NMR structure coordinates of dimer (Clore et al., 1990). [d] Measured from IL-8 NMR structure coordinates of dimer.

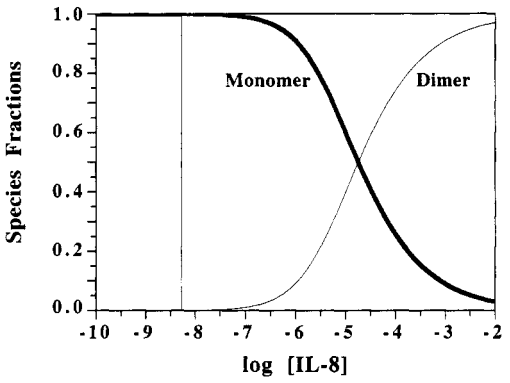

FIGURE 5: Fractions of monomer and dimer IL-8 species versus logarithm of IL-8 subunit concentration. Curves were generated with the 37 °C monomer–dimer equilibrium dissociation constant of 18 μM. The shaded area indicates the physiological concentration range (Gross et al., 1992; McElvaney et al., 1992; Rodriguez et al., 1992).

Thus, the agreement between the calculated and experimentally determined thermodynamic parameters argues for only minimal conformational changes upon dissociation of the dimer to the monomer. These conclusions are also in agreement with NMR structural analysis of a chemically modified form of IL-8 which is monomeric (Rajarathnam et al., 1994).

DISCUSSION

A K_D of 18 μM for dissociation of IL-8 dimers indicates that IL-8 is essentially a monomer at the nanomolar IL-8 concentrations sufficient for activity *in vitro* (Holmes et al., 1991) and *in vivo* (Gross et al., 1992; McElvaney et al., 1992; Rodriguez et al., 1992) (Figure 5). This argues that IL-8 binds its receptor as a monomer. This is supported by the mutagenesis evidence to date, in which there are two mutations reported at the dimer interface, K23A and N-methylated L25 (Hebert et al., 1991; Rajarathnam et al., 1994), both of which retain full biological activity even though the latter has been shown to be a monomer even at high concentrations.

Although receptor binding could induce dimerization of IL-8, the energetic cost of dimerizing IL-8 at nanomolar concentrations is several kilocalories. It is unclear whether the mechanistic benefits of dimerization would outweigh this energetic penalty. Allosteric effectors, such as heparin sulfate which increases IL-8 bioactivity *in vitro* and presumably mimics the *in vivo* interaction of IL-8 with matrix proteoglycan (Webb et al., 1993), may also affect dimerization.

The present findings may also apply to other members of the chemokine family. It was recently reported that introduction of three carboxy-terminal mutations into MIP-1α rendered it monomeric at concentrations where wild-type MIP-1α is highly self-associated, yet there was no change in biological activity (Graham et al., 1994). Furthermore, the monomer conformation of self-associated forms of chemokines appears to be conserved for IL-8, PF4, and MIP-1β (Baldwin et al., 1991; Charles et al., 1989; Clore et al., 1990; Lodi et al., 1994), despite differences in the way that IL-8 and PF4 associate compared to MIP-1β.

The tendency of chemokines to self-associate raises the question of a possible biological role of the self-associated forms. By analogy to insulin (Hua et al., 1991), which is hexameric after synthesis, it is possible that the oligomeric forms are inactive, allowing slower release of the active form after secretion and reducing autostimulation of the producer cell. Self-association may also be important for secretion, as evidenced by the failure of IL-8 mutated at the dimer interface to be secreted (Hebert et al., 1991). Production inside the cell as self-associated forms could also reduce intracellular osmotic stress at high levels of expression.

Dissociation of IL-8 to monomers also has significant implications for understanding mutagenesis data. Such data points to important roles for the amino-terminal residues E4, L5, R6, and I10 (Hebert et al., 1991) and suggests involvement of the loop between Cys 7 and Cys 34 and the carboxy-terminal α-helix (Clark-Lewis et al., 1991, 1993; Moser et al., 1993; Schraufstatter et al., 1993). Since the latter two regions form part of the dimer interface, it is possible that their exposure upon dissociation to monomer allows them to play a role in receptor binding, as in the case of insulin (Hua et al., 1991).

In conclusion, the present finding that IL-8 is monomeric at nanomolar physiological concentrations strongly suggests that the monomer is the biologically active form. Nevertheless, analysis of the thermodynamics of IL-8 dimerization suggests that only minimal structural changes are coupled to dissociation, so that the atomic coordinates of the monomer within the dimeric NMR and crystallographic structures should provide an approximate model for the molecular species that binds to the receptor. Determination of a high-resolution structure of an IL-8 monomer may provide further insight into how it and other chemokines recognize their receptors.

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