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Neutrophil Activation by Monomeric Interleukin-8

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Interleukin-8 (IL-8), a pro-inflammatory protein, has been shown by nuclear magnetic resonance (NMR) and x-ray techniques to exist as a homodimer. An IL-8 analog was chemically synthesized, with the amide nitrogen of leucine-25 methylated to selectively block formation of hydrogen bonds between monomers and thereby prevent dimerization. This analog was shown to be a monomer, as assessed by analytical ultracentrifugation and NMR. Nevertheless, it was equivalent to IL-8 in assays of neutrophil activation, which indicates that the monomer is a functional form of IL-8.

Interleukin-8 is an inflammatory cytokine that promotes the accumulation and activation of neutrophil leukocytes (*1*) and has been implicated in a wide range of acute and chronic inflammatory diseases (*2*). In vitro, IL-8 induces neutrophil shape change, chemotaxis, granule release, and respiratory burst (*1*) by binding to receptors of the seven transmembrane segment class (*3*). The structure of human IL-8, as solved by NMR and x-ray methods (*4, 5*), shows a noncovalent homodimeric topology (Fig. 1). The dimer interface is formed by a β strand from each monomer and by part of the α helices that lie across the β sheet formed on dimerization. The principal interactions stabilizing the dimer are the six H bonds involving Leu^{25}, Val^{27}, and Glu^{29}. The NH_2-terminal residues Glu^4, Leu^5, and Arg^6 are essential for neutrophil activation in vitro (*6, 7*), although other structural features are also important (*6*). However, the COOH-terminal α helix was not essential for activity, which suggests that the groove between the helices is not a critical binding site.

Many growth factors are di- or trimeric, although the functional conformation may not correspond to the observed form, as structure determinations are done at higher concentrations than those in the biologically effective range. Nevertheless, receptor aggregation is a requirement for the signaling of some receptors (*8*), and cross-linking mediated by the ligand has been demonstrated in some instances (*9*).

Our aim was to obtain a monomeric form of IL-8. Conventional amino acid replacement results in changes in the chemical nature of the amino acid side chains but does not necessarily affect the dimeric structure. Our approach was to selectively disrupt the secondary structure that stabilizes the dimer interface by modification of the peptide backbone. This could be achieved by site-directed mutagenesis or chemical synthesis (*10*). Because IL-8 can be readily synthesized chemically (*11*), this method was used to obtain an analog in which the amide of Leu^{25}, which is normally involved in H bonding across the dimer interface, is methylated ($NH \rightarrow NCH_3$) (*12*). This analog (L25NMe) induced neutrophil chemotaxis (Fig. 2A) and the release of elastase (Fig. 2B), as well as the respiratory burst and the mobilization of cytosolic free calcium (*13*), and competed with labeled IL-8 for binding to the IL-8 receptors (Fig. 2C). In all the assays, the activities of L25NMe and IL-8 were similar. The sedimentation equilibrium profiles (Fig. 3) of the L25NMe analog and of IL-8 corresponded to those expected for a monomer and a dimer, respectively (*14*). No evidence for higher order aggregates was found for either form.

In NMR studies, relatively large (>0.3

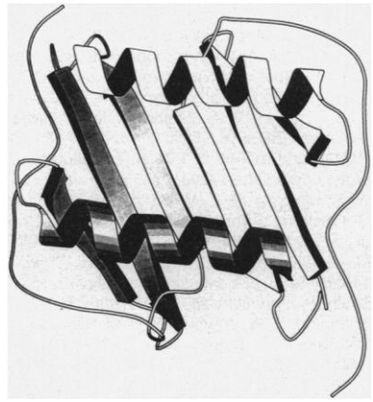

Fig. 1. A schematic diagram of IL-8 generated with the program MOLSCRIPT (*19*) which used the coordinates of the NMR structure (*4*).

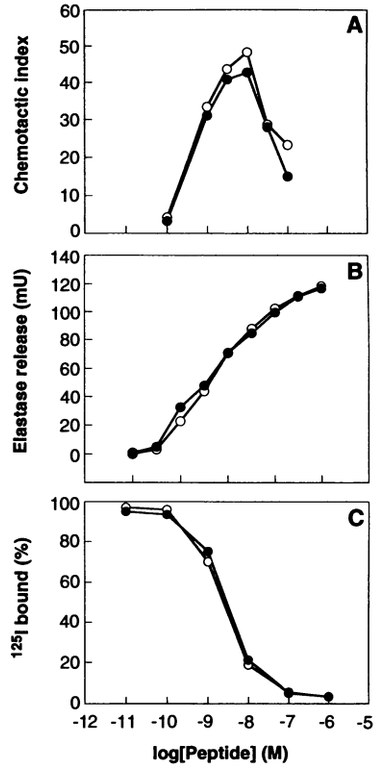

Fig. 2. Biological activities of the L25NMe analog (●) and IL-8 (○) on human neutrophils as assessed by (**A**) chemotaxis, (**B**) elastase release, and (**C**) receptor binding. The chemotactic index is the ratio of neutrophils that migrated in the presence or absence of chemoattractant and elastase release is expressed as milliunits (mU) of enzyme activity (*20*). Displacement of ^{125}I-labeled IL-8 by unlabeled IL-8 or L25NMe was determined as described (*21*). All measurements were done in duplicate at various molar concentrations (M) of peptide. All the assays were done at least three times, with different neutrophil preparations. In all cases, concentrations were calculated from the measured weight of the lyophilized material and the molecular weight of the monomer.

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parts per million) CαH chemical shift differences between IL-8 and the L25NMe analog are observed only for residues that constitute the dimer interface: the residues of the β sheet (26 to 28) and the COOH-terminus (66 to 70) (*13*). The CαH chemical shifts have been shown to directly correlate with secondary structure (*15*); therefore, these results indicate that the secondary structures of the L25NMe analog and of IL-8 are similar, except for residues at the dimer interface. For IL-8, approximately 20 nuclear Overhauser effects (NOEs) were observed across the dimer interface (*4*), which indicates spatial proximity of protons between the two subunits (Fig. 4A). For the L25NMe analog, NOEs that could be attributed to dimer formation were not present (Fig. 4B). The fingerprint region of the correlated spectroscopy (COSY) spectrum of IL-8 and the L25NMe analog in ^2H_2O is shown in Fig. 5. In the case of IL-8, a number of cross peaks are seen, which result from slowly exchanging amide protons. Three cross peaks (Leu[25], Val[27], and Glu[29]) are from the amide protons involved in H bonding across the dimer interface (*4*). However, with the L25NMe analog, the cross peaks corresponding to the dimer interface are not observed and the number of slowly exchanging protons is considerably reduced. Together, the NMR data clearly demonstrate that the L25NMe analog is a monomer and that the structural changes are largely restricted to the region corresponding to the dimer interface.

Although the L25NMe analog is shown here to be monomeric, whether the IL-8 dimer observed at high concentrations dissociates at physiological concentrations is uncertain. Gel filtration experiments suggested that in plasma, IL-8 could exist as a monomer (*16*). Our sedimentation equilibrium studies showed that the dissociation constant of dimerization (K_d) for IL-8 is less than 60 μM. Denaturation experiments showed that IL-8 unfolds by means of an intermediate, which is most likely the monomer, but did not provide the K_d (*17*). Platelet factor 4 and low-affinity platelet factor 4, both members of the IL-8 family, form multiple aggregation states in solution, with the monomer favored when physiological conditions are approximated (*18*). Thus, proteins of the IL-8 family in general form multimers at higher concentrations, but the monomer may constitute the active species.

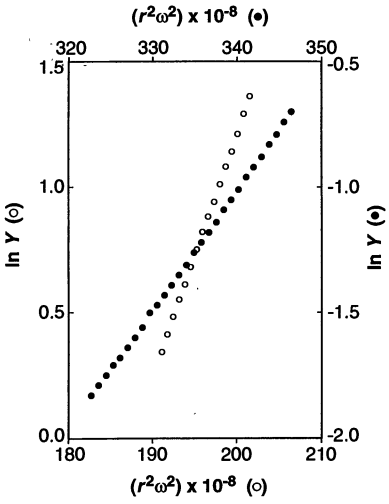

Fig. 3. Molecular weight determination of IL-8 and L25NMe by analytical ultracentrifugation. Plots of ln *Y* versus $r^2\omega^2$ are shown, where *Y* is the concentration in fringe displacement units, *r* is the distance from the axis of rotation, and ω is the angular velocity. The buffer used was 100 mM sodium acetate (pH 6.0). Molecular weights were calculated from the slope of the line (*14*). The L25NMe analog (●) was run at an initial concentration of 2.66 mg/ml at 26,000 rpm; IL-8 (○) was run at an initial concentration of 0.48 mg/ml at 20,000 rpm. The apparent molecular weights of L25NMe (7900) and IL-8 (16,200) approximate those of the monomer and dimer, respectively.

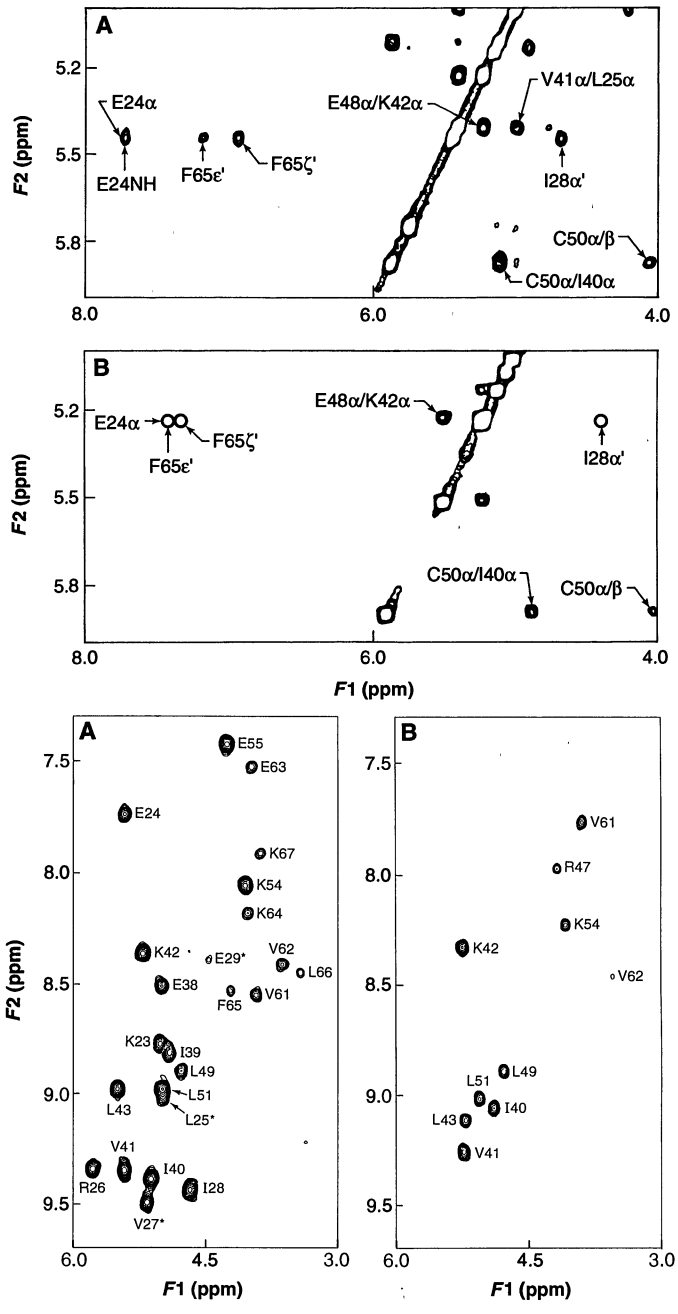

Fig. 4 (top). NMR nuclear Overhauser and exchange spectroscopy (NOESY) spectra of (**A**) IL-8 and (**B**) the L25NMe analog. The complete chemical shifts of the L25NMe and the IL-8 assignments were made by use of NOESY, double-quantum filtered correlation spectroscopy (DQFCOSY), and total correlation spectroscopy (TOCSY) experiments (*13*) that used standard pulse sequences (*22*) on a Varian Unity 600 spectrometer (Varian Instruments, Palo Alto, California). Shown are portions of the ^1H 600 MHz, two-dimensional NOESY spectra (mixing time, 150 ms) at 40°C in a ^2H_2O buffer containing 20 mM sodium acetate (pH ~4.5). *F*1 and *F*2 are the first and second frequency axes, respectively. For IL-8, NOEs across the dimer interface from E24CαH to I28CαH and F65CεH and F65CζH are seen. These cross peaks were not seen for the L25NMe analog; the circles indicate the positions, according to the assignments, where the peaks would have been observed (*23*). **Fig. 5 (bottom).** Proton exchange studies of the L25NMe analog. A profile of the slowly exchanging protons as seen in the fingerprint region (NH to CαH) of the ^1H 600-MHz, two-dimensional COSY spectra at 25°C in a ^2H_2O buffer containing 20 mM sodium acetate (pH ~4.5) for (**A**) IL-8 and (**B**) the L25NMe analog. The spectra were taken within 12 and 3 hours of dissolving the samples of IL-8 and L25NMe, respectively. The amide protons (L25, V27, and E29) (*23*) across the dimer interface are indicated by an asterisk. For L25NMe, many cross peaks seen in IL-8 are missing, including those at the dimer interface.

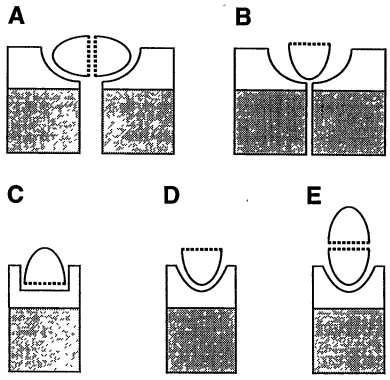

Fig. 6. Possible models for the interaction of IL-8 monomers and dimers with the receptor. In models A and B, the dimeric and monomeric forms, respectively, of IL-8 bind to a two-chain receptor. In model C, a monomer binds to a single-chain receptor through the region corresponding to the dimer interface. In model D, a monomeric IL-8 binds to a single-chain receptor, and in model E, a single-chain receptor binds the dimeric IL-8, but only one of the subunits participates in binding.

The results with the L25NMe analog demonstrate that a monomeric form of IL-8 is sufficient for receptor binding and full in vitro biological activity, and allow us to discuss models for the productive interaction of IL-8 with its receptor (Fig. 6). Model A, where dimeric IL-8 is required to cross-link two receptor molecules, is ruled out by the monomeric analog. In model C, IL-8 binds as a monomer; however, the interactions that normally occur at the dimer interface now facilitate receptor binding. This is unlikely, as the L25NMe modification does not permit the necessary H bonding. Model E, in which dimeric IL-8 binds to a single chain receptor, is not ruled out by the data; but in terms of energy used, dimerization at biologically relevant concentrations may not be favored. Model B, where the monomeric ligand binds two receptor chains at two distinct sites, is similar to the situation observed for growth hormone (9). However, receptor clustering is not generally considered a requirement for signal transduction by the seven transmembrane segment receptors. Therefore, model D, where monomeric IL-8 binds to a single chain receptor, seems the most likely mechanism of interaction.

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12. Interleukin-8 and the analog were synthesized as shortened forms (residues 4 to 72). The IL-8, 4 to 72 form has slightly higher potency than the IL-8, 1 to 72 form (*6*). When analyzed by ion spray mass spectrometry, the average molecular weights of the Leu^{25} *N*-methyl analog (L25NMe) and of IL-8, 4 to 72 were 8109.3 ± 1 and 8094.3 ± 1.3, respectively. The difference in molecular weight corresponds to a CH_2 group, which reflects the chemical difference in the two molecules.
13. T. Geiser, K. Rajarathnam, B. Sykes, I. Clark-Lewis, unpublished data.
14. Molecular weights were determined by low-speed sedimentation equilibrium analytical centrifugation with Raleigh interference optics (Beckman Instruments, Palo Alto, CA). The molecular weights were calculated with the equation

$$\frac{2RT}{(1-\bar{\nu}\rho)}\,\frac{d(\ln Y)}{d(r^2\omega^2)}$$

where R is the gas constant, T is the temperature in kelvin, $\bar{\nu}$ is the partial specific volume, ρ is the density, Y is the concentration in fringe displacement units, and $d(\ln Y)/d(r^2\omega^2)$ is the slope from the plots in Fig. 3.
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23. Abbreviations for the amino acid residues are: C, Cys; E, Glu; F, Phe; I, Ile; K, Lys; L, Leu; R, Arg; and V, Val.

24. Supported by grants from PENCE, the Medical Research Council (MRC) of Canada, and the Swiss National Science Foundation. I.C.-L. is supported by an MRC Scholarship. The authors acknowledge P. Owen, P. Borowski, and L. Vo for assistance in protein synthesis, purification, and characterization; L. Hicks for sedimentation equilibrium; K. Oikawa for the fluorescence and CD studies; and G. McQuaid for the maintenance of the NMR spectrometers. M. Loetscher performed the binding assays. C. Davidson-Hall assisted with manuscript preparation.

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Requirement of Human Renal Water Channel Aquaporin-2 for Vasopressin-Dependent Concentration of Urine

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Concentration of urine in mammals is regulated by the antidiuretic hormone vasopressin. Binding of vasopressin to its V2 receptor leads to the insertion of water channels in apical membranes of principal cells in collecting ducts. In nephrogenic diabetes insipidus (NDI), the kidney fails to concentrate urine in response to vasopressin. A male patient with an autosomal recessive form of NDI was found to be a compound heterozygote for two mutations in the gene encoding aquaporin-2, a water channel. Functional expression studies in *Xenopus* oocytes revealed that each mutation resulted in nonfunctional water channel proteins. Thus, aquaporin-2 is essential for vasopressin-dependent concentration of urine.

The cloning of water-selective channels has begun to reveal the molecular basis of renal water transport. The water-selective channel, aquaporin-1 or CHIP28, is responsible for the constitutively high water permeability in the proximal tubule and the descending thin limb of the loop of Henle (*1*). In mammals, other mechanisms are needed to