^{1}H NMR Studies of Interleukin 8 Analogs: Characterization of the Domains Essential for Function†

Krishnakumar Rajarathnam,‡ Ian Clark-Lewis,§ and Brian D. Sykes*,‡

Protein Engineering Network of Centres of Excellence and the Department of Biochemistry, 713 Heritage Building, University of Alberta, Edmonton, Alberta, Canada T6G 2S2, and Protein Engineering Network of Centres of Excellence and the Department of Biochemistry, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3

Received December 17, 1993; Revised Manuscript Received March 22, 1994®

ABSTRACT: ^{1}H NMR studies were carried out on interleukin 8 (IL-8) analogs in order to probe the structural features that are essential for receptor binding and function. The analogs studied were the chemically synthesized IL-8 (1–72), a series of N-terminally truncated derivatives (4–72, 5–72, and 6–72), and derivatives with single amino acid substitutions (I10A, R6K, and H33A). Previous functional studies have shown that the N-terminal residues, especially the residues at positions 4–6, and the β turn containing Cys-34,which is disulfide linked to Cys-7, are important for receptor affinity and functional activity [Clark-Lewis, I., Schumacher, C., Baggiolini, M., & Moser, B. (1991) *J. Biol. Chem. 266*, 23128–23134; Clark-Lewis, I., Dewald, B., Loetscher, M., Moser, B., & Baggiolini, M. (1994) *J. Biol. Chem.* (in press)]. The 6–72 and R6K analogs also showed properties of an antagonist. Analysis of the ^{1}H NMR parameters such as chemical shifts, amide proton chemical shift temperature coefficients, and NOESY data indicates that the core structure is the same for all these proteins. Small differences were observed in some of the NMR properties for some of the residues in the N-terminal region and the turn containing Cys-34. Detailed analysis suggests that there is no correlation between these differences and observed function. Thus functional differences between the N-terminal analogs are a direct consequence of changes in receptor binding due to substitutions/deletions in the N-terminal sequence and not due to structural changes elsewhere. NMR data for the H33A analog, which had the same activity as native IL-8, showed that the turn is essentially the same as in the native IL-8 and other analogs. Our data suggests that the protein core acts as a scaffold from which the N-terminal residues and the turn containing Cys-34 are suspended in the correct orientation to bind the receptor and elicit function.

Interleukin 8 (IL-8)[1] is a member of a growing family of related proinflammatory cytokines called chemokines. The IL-8 subfamily includes such proteins as platelet factor 4 (PF-4), melanoma growth stimulatory activity (MGSA), and neutrophil-activating protein 2 (NAP-2) and is characterized by the sequence C-X-C near the N-terminus (Oppenheim et al., 1991; Miller & Krangel, 1992; Baggiolini & Clark-Lewis, 1992). IL-8 is a chemoattractant and activator of neutrophils. It is produced by a variety of cell types, such as monocytes, fibroblasts, keratinocytes, endothelial cells, and it is induced by factors such as tumor necrosis factor (TNF), interleukin 1 (IL-1), and lipopolysaccharides (LPS). IL-8 induces migration of neutrophils to the site of inflammation and has been implicated in a wide range of acute and chronic inflammatory diseases including psoriasis and rheumatoid arthritis (Seitz et al., 1991; Koch et al., 1992; Antialla et al., 1992).

Studies using deletion/substitution analogs of IL-8 and chimeric constructs of IL-8 and IP-10 (γ-interferon-induced protein) have provided a wealth of information regarding structure–function relationships in IL-8. Studies with truncation analogs showed that the C-terminus helix is important but not essential, whereas a few residues near the N-terminus (Glu-4, Leu-5, and Arg-6) are essential for binding and function (Clark-Lewis et al., 1991b). The 4–72 analog showed the same binding as, and a 3-fold higher elastase release activity than, the native protein (1–72). The 5–72 analog had a 3-fold lower binding and a ~90-fold lower activity, whereas the 6–72 analog had a ~120-fold lower binding and no activity (Table 1). The highly differential response clearly indicates the importance of the N-terminal residues. Modification of the residues Glu-4, Leu-5, and Arg-6 also resulted in poor binding and activity, the effect being most dramatic for Arg-6 (Hébert et al., 1991; Moser et al., 1993). Even a conservative Arg → Lys substitution resulted in complete loss of activity (Table 1). The 6–72 and R6K analogs showed properties of an antagonist as they blocked IL-8 activity by competitively binding to the receptor (Moser et al., 1993). Residues Glu-4, Leu-5, and Arg-6 ("ELR" motif) are conserved in related proteins such as MGSA/Gro-α, Gro-β, Gro-γ, and NAP-2 (Schumacher et al., 1992). However, the tripeptide ELR and other peptides having the ELR motif were inactive, indicating that additional features are involved in binding and function. Recent studies using substitution analogs and chimeric constructs of IL-8/IP-10 have shown that, in addition to the ELR residues, residues 10–23 and the turn 30–35 are also important for binding and function (Clark-Lewis et al., 1993b). Steric bulk of Ile at position 10 was shown to be important, as the I10A analog had 30-fold less binding and

† This work is supported by grants from the Protein Engineering Network of Centres of Excellence.

* To whom correspondence should be addressed. Telephone: 403 492 5460: FAX 403 492 1473.

‡ University of Alberta.

§ University of British Columbia.

® Abstract published in *Advance ACS Abstracts,* April 15, 1994.

[1] Abbreviations: IL-8, interleukin 8; NAP-2, neutrophil-activating peptide 2; MGSA, melanoma growth stimulatory activity; PF-4, platelet factor 4; IP-10, γ-interferon-induced protein; NMR, nuclear magnetic resonance; DQFCOSY, double quantum filtered correlated spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlated spectroscopy; rmsd, root mean square deviation; DSS, sodium 2,2-dimethyl-2-sila-5-pentanesulfonate.

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Table 1: Binding and Elastase Release Activity of IL-8 Analogs

	residue												
	1	2	3	4	5	6	7	8	9	10	33	relative activity[a]	relative binding[b]
IL-8 (1–72)	Ser	Ala	Lys	Glu	Leu	Arg	Cys	Gln	Cys	Ile	His	1[c]	1[c]
IL-8 (4–72)				Glu	Leu	Arg	Cys	Gln	Cys	Ile	His	0.37[c]	1[c]
IL-8 (5–72)					Leu	Arg	Cys	Gln	Cys	Ile	His	83[c]	3[c]
IL-8 (6–72)						Arg	Cys	Gln	Cys	Ile	His	none[c]	124[c]
IL-8 (R6K)				Glu	Leu	Lys	Cys	Gln	Cys	Ile	His	none[e]	880[f]
IL-8 (I10A)				Glu	Leu	Arg	Cys	Gln	Cys	Ala	His	22[g]	29[g]
IL-8 (H33A)				Glu	Leu	Arg	Cys	Gln	Cys	Ile	Ala	1.2[g]	1[g]

[a] Elastase release activity (Moser et al., 1993); relative activity is calculated by dividing the activity of the analog by the activity of the native IL-8. A larger relative activity corresponds to a less active analog. [b] K_d was measured by competitive binding assay using radiolabeled IL8 (1–72); relative binding is calculated by dividing the K_d of the analog by the K_d of the native IL-8 (I-72). [c] From Clark-Lewis et al. (1991). [d] The analogs were synthesized in the 4–72 forms and therefore compared to IL-8 (4–72) in determining the relative activity and binding. [e] From Moser et al. (1993). [f] B. Moser and I. Clark-Lewis, unpublished results. [g] Clark-Lewis et al. (1994).

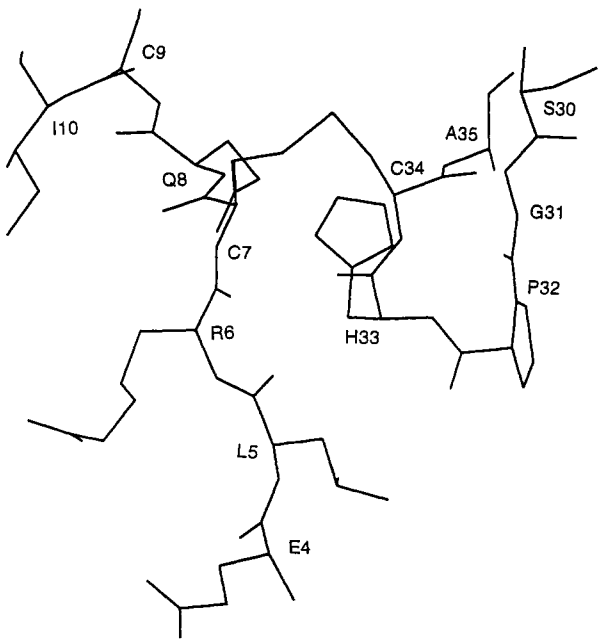

FIGURE 1: Schematic presentation of the N-terminus and the loop containing Cys-34 of IL-8 from the NMR structure (Clore et al., 1990).

22-fold less activity[2] [Table 1; see Clark-Lewis et al. (1994)]. The turn 30–35 contains Cys-34 which is disulfide linked to Cys-7 in the N-terminus. Residues Gly-31, Pro-32, and Cys-34 are conserved and are expected to play a structural role. Residue His-33 is also conserved in all the related proteins and is thought to play a role in modulating function. However, the analog H33A and other substitutions showed essentially the same binding and activity as the native protein (Table 1).

The three-dimensional structure of IL-8 is known from both NMR and X-ray studies (Clore et al., 1989, 1990; Clore & Gronenborn, 1991; Baldwin et al., 1991). It has two disulfide bonds which are conserved in all related proteins and are absolutely essential for function. The protein exists as a dimer in solution, and the core of the structure consists of three β strands arranged in a Greek key motif and a C-terminal α helix. The first and the second β strand are connected by a turn (residues 30–35) which is linked to the N-terminus by a disulfide bridge between Cys-7 and Cys-34. This turn and the N-terminus present a contiguous surface projecting from the edge of the β sheet into solution (Figure 1). Although both NMR and X-ray techniques show largely similar structures, some differences are evident. NMR results show that the functionally critical N-terminal residues (4–6) are flexible, whereas the X-ray structure shows these residues to be ordered largely due to a salt bridge between Glu-4 and Lys-23′ of the other monomer in the dimer. The main difference between the well-defined parts of the protein was in the loop containing residues 30–35. In the NMR structure, His-33 $N^{\epsilon 2}$ accepts a H-bond from the backbone of Gln-8 NH, whereas in the crystal structure, $N^{\epsilon 2}H$ donates a H-bond to the backbone of the carbonyl group of Glu-29. Also in the NMR structure, the distance between the helices is 14.8 Å, whereas in the X-ray structure the distance is 11.1 Å. It was proposed that IL-8 is capable of undergoing a conformational change, and the NMR and X-ray structures correspond to the open (active) and closed (inactive) forms, respectively; hence the differences observed may have functional relevance (Clore & Gronenborn, 1991).

Of the different analogs tested, modifications involving the N-terminal residues provide the greatest interest, as they not only were sensitive to deletions and modifications but generated antagonists which could have pharmacological importance. Given the wide ranging functional effects of the modifications close to the N-terminal region, it was essential to determine the structural effects of the deletions/substitutions in order to correlate structure and function. More specifically, we wish to address whether the effects are direct or due to structural changes elsewhere in the protein. Here we describe detailed NMR studies of the chemically synthesized IL-8 (1–72) and the 4–72, 5–72, 6–72, R6K, H33A, and I10A analogs.

MATERIALS AND METHODS

Protein Synthesis and Purification. All IL-8 analogs were chemically synthesized, purified, and characterized as discussed in detail previously (Clark-Lewis et al., 1991a).

NMR Methods. ^1H NMR experiments were performed on a Varian Unity 600 spectrometer using spectral windows of 10 000 Hz in H_2O and 8000 Hz in ^2H_2O at 40 °C. All the NMR samples were in 20 mM acetate, pH ~5.2 ± 0.2, and the protein concentrations were ~2mM. The 2D NMR spectra were collected over 4096 points along t_2 and 256–320 increments along t_1, and the quadrature detection was achieved as described by States et al. (1982). The solvent was presaturated for 2 s, and for some of the experiments, the loss of intensity of cross peaks near the H_2O signal was minimized using the SCUBA sequence (Brown et al., 1988). Resonances were assigned from DQFCOSY(Rance et al., 1983) and TOCSY (mix, 55 ms) experiments (Davis & Bax, 1985); NOESY (Jeener et al., 1979) spectra were collected at 80- and 150-ms mixing times for structure determinations. The amide proton chemical shift temperature coefficients were calculated by collecting NOESY spectra at 20, 30, and 40 °C. The NOESY and TOCSY spectra were processed using a Gaussian function with resolution enhancement or a shifted sine bell function; DQFCOSY spectra were processed using

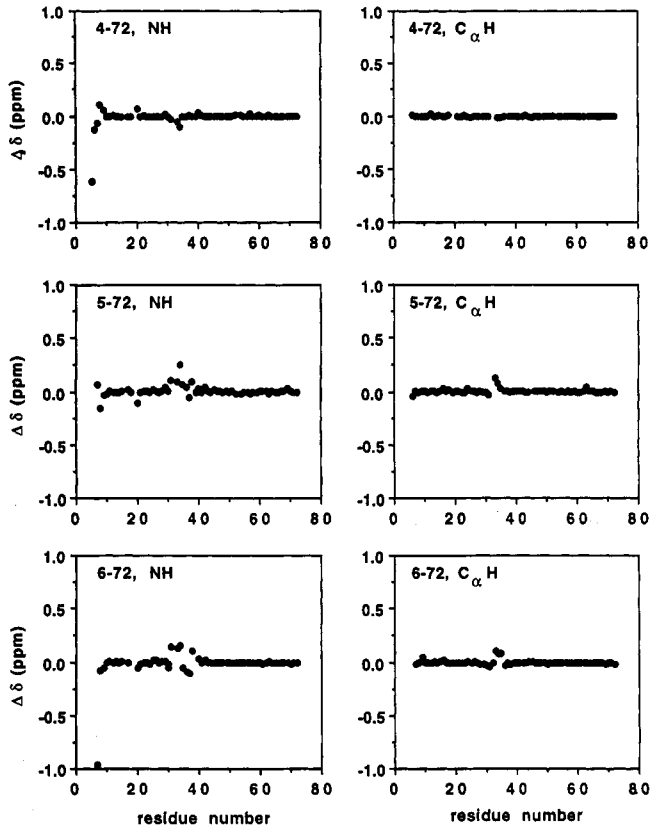

FIGURE 2: Plots of the difference in chemical shifts between native IL-8 (1–72) and the deletion analogs 4–72, 5–72, and 6–72 for the amide and C_α protons.

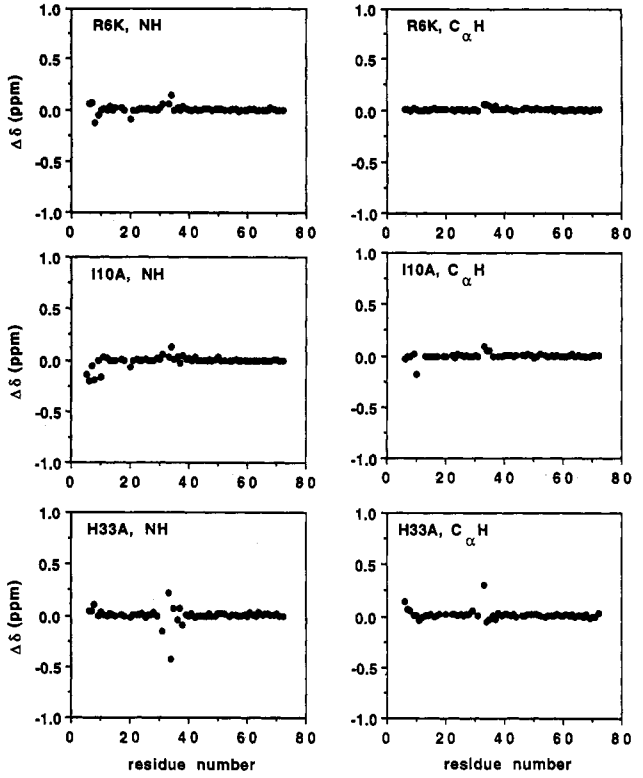

FIGURE 3: Plots of the difference in chemical shifts between IL-8 (4–72) and the substitution analogs H33A, I10A, and R6K for the amide and C_α protons. The substitution analogs were synthesized in the 4–72 version, as the IL-8 (4–72) analog was shown to be as potent as the native (1–72) analog in receptor binding and functional activation (Clark-Lewis et al., 1991b).

a sine bell function, and in all cases, the spectra were zero-filled to 4K × 4K points in both dimensions. All spectra are referenced to the externally added DSS.

RESULTS

Chemical Shift Analysis

The ^{1}H NMR chemical shifts of the proton resonances in all of the analogs were assigned in a fairly straightforward manner, as the NMR spectra were largely similar to that of the native IL-8 (1–72) (given in supplementary material). The chemical shift differences of the amide and C_α protons between the native IL-8 (1–72) and the different truncation and substitution analogs are discussed here in detail to see whether they can serve as sensitive probes for structural changes in the protein (Figures 2 and 3). It is observed that the chemical shifts of most of the protons, especially of the amino acids in the β sheet and the α helix region, are the same, implying similar secondary and tertiary structure. For all of the analogs, small differences in chemical shifts are observed only for residues in the N-terminus and the turn region (30–35) which is disulfide linked to the N-terminus (Figure 1). Some changes in chemical shifts are to be expected due to small perturbations in the local structure. Since large differences in function are observed (Table 1), it is important to address whether the changes in function are a direct consequence of the deletions/substitutions, are due to structural changes in the protein, or are from a combination of both.

Deletion Analogs. Figure 2 shows the difference in chemical shifts of the C_α and the amide protons of the deletion analogs 4–72, 5–72, and 6–72. For the 4–72 analog, which has binding similar to, but 3-fold higher activity than, the native 1–72 analog (Clark-Lewis et al., 1991b) (Table 1), only the His-33 C_α proton chemical shift showed some chemical shift difference (0.05 ppm), and all other protons were within experimental error (+0.02 ppm). Small differences (≤0.1 ppm) are observed for the amide protons for only two residues, Cys-34 and His-33. Cys-34 is disulfide linked to Cys-7 in the N-terminus, and the changes in chemical shifts of His-33 and Cys-34, though small, could be the result of structural changes in the N-terminus transmitted by the disulfide bond. For the 5–72 analog, which had 3-fold less binding and ~90-fold less activity, the C_α proton chemical shifts showed larger differences (~0.1 ppm) for residues His-33 and Cys-34. The differences in amide chemical shifts (≤0.2 ppm) for the 5–72 analog were higher than those for the 4–72 analog (Figure 2). Interestingly for the 6–72 analog, which had ~120-fold less binding and no activity, the changes in chemical shift of both the C_α proton and the amide protons were of the same order and direction as those of the 5–72 analog (Figure 2).

The amide proton chemical shifts in addition to structural differences are also sensitive to dynamic parameters such as differences in the pH and H-bonding (see Discussion). When amide chemical shifts of the native IL-8 and all three of the truncation analogs are considered together, larger chemical shift differences are observed for 5–72 than for 6–72 for some protons (Cys-34, Ala-35). For the 4–72 analog, chemical shift differences of some of the protons are negative (His-33, Cys-34) (more downfield shifted in the 4–72 analog), whereas for the 5–72 and the 6–72 analogs, the chemical shift differences for the same protons are positive (more upfield shifted in the 5–72 and 6–72 analogs). The NMR data was also collected for the 6–72 analog at pH ~4.6, and it was observed that the amide shift of Cys-34 was smaller by as much as 0.15 ppm, and that for His-33, by 0.08 ppm. These

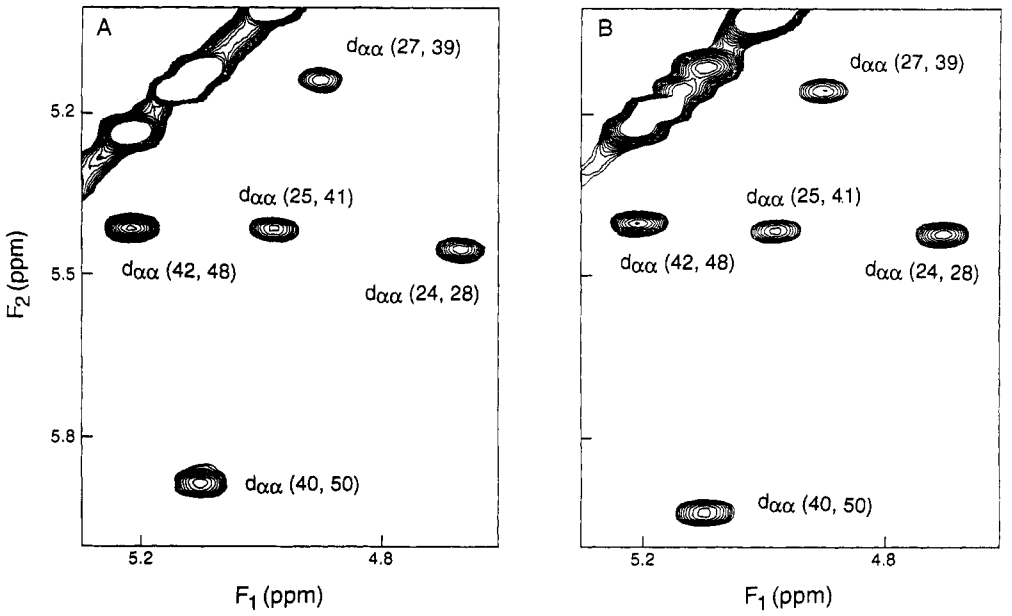

FIGURE 4: 600-MHz 2D NOESY ^{1}H NMR spectra of (A) the native IL-8 (1–72) and (B) the 6–72 analog at 40 °C showing long-range $d_{\alpha\alpha}$ NOE cross peaks that are characteristic of the β sheet structure. $d_{\alpha\alpha}$ (24, 28) corresponds to a NOE across the dimer interface.

shifts were closer to those of the native protein at pH 5.2. This suggests that part of the difference in the amide shifts is due to differential exchange rates of these protons, besides being due to small differences in the pH of the NMR samples.

Substitution Analogs. Figure 3 shows the difference in chemical shifts of the C_α and amide protons of the substitution analogs R6K, I10A, and H33A. The R6K analog, though Arg → Lys is a conservative substitution, had ~900-fold less binding and was completely inactive (Moser et al., 1993) (Table 1). The C_α proton shifts are largely the same as those in the native protein[2] with a small difference (~0.1 ppm) observed for the Cys-34 amide proton. In the case of the I10A analog, the substitution involves a site farther removed from the N-terminus, as there are two intervening cysteines at positions 7 and 9. Functional studies showed that the steric bulk at position 10 is important, as this analog had ~30-fold less binding and 22-fold less activity (Clark-Lewis et al., 1994) (Table 1). The largest C_α proton chemical shift difference is for Ala-10 (~0.2 ppm) and can be directly attributed to the substitution,[2] and small chemical shift differences of the same order as those seen for the R6K analog (0.04 ppm) are seen for the Cys-34 and His-33 protons (Figure 3). Once again the largest chemical shift difference for the amide protons is for Cys-34, and differences in chemical shift are also observed for the N-terminal residues which are of the same order as observed in other analogs. In the case of the H33A analog, the substitution involves a residue which is directly in the turn. His-33 was thought to be crucial as a structural restraint, as the imidazole side chain was found to interact with the Gln-8 backbone NH in the native NMR structure. However, the H33A analog had binding and activity similar to those of the native protein (Clark-Lewis et al., 1994) (Table 1). The largest C_α proton chemical shift difference is for Ala-33 (0.3 ppm),[2] and smaller differences (~0.1 ppm) are also observed for residues Arg-6 and Cys-7 (Figure 3). However, much larger shift differences are observed for the amide protons. The largest chemical shift difference is for Cys-34 (~0.5 ppm), and differences of more than 0.1 ppm are also observed for two other residues in the turn, Gly-31 and Ala-33.

NOESY Analysis

In order to determine the nature of the structural changes in more detail, the NOESY spectra, which give through-space information, were analyzed. The same long-range NOEs which define the tertiary structure in the native protein are seen in all of the analogs. Some characteristic $d_{\alpha\alpha}$ NOEs between the β strands, Glu-24/Ile-49, Leu-25/Val-41, Val-27/Ile-39, Ile-40/Cys-50, and Lys-42/Glu-48, are shown Figure 4. The sequential NOEs which characterize the α helix are shown in Figure 5. The α helix orientation is found to be the same, as indicated by connectivities seen between Pro-19 and Val-61 and between Phe-65 and residues at the dimer interface, Leu-25, Val-27, and Glu-29 (not shown). In the native protein, NOEs observed for the residues in the turn (30–35) are either intraresidue or sequential in nature, and the only long-range NOEs are those which define the turn, namely, those between residues Ser-30/Thr-37 and Gly-31/Cys-34. In the case of 4–72 and 5–72 analogs, the NOE pattern was the same as seen for the native protein, as shown by the NOEs to the Cys-34 amide proton in Figure 6. Though most of the NOEs were observed for the 6–72 analog, some of them were weaker compared to the native protein (Figure 6). For the substitution analogs R6K and I10A, the NOE pattern for residues in the turn was similar to that of the native protein, whereas larger differences were observed for the H33A analog (Figure 6). The NOESY data reiterate that there is little correlation between the change in NMR properties and the observed function.

Amide Temperature Coefficient Analysis

The observation that the chemical shift changes are larger for the amide than for the C_α proton suggests that there may be differences in the dynamics as reflected by changes in H-bonding and/or exchange rates (Wishart et al., 1991; Pardi et al., 1983). Temperature coefficients of the exchangeable amide protons have been reported to be good indicators of the

[2] If the random coil chemical shifts (Wishart et al., 1991) of the substituted residues are considered (Arg → Lys, Ile → Ala, and His → Ala), the difference in the C_α proton chemical shift for position 6 in the R6K analog increases from 0.01 to 0.08 ppm, the difference for position 10 in I10A decreases from 0.18 to 0.08 ppm, and the difference for position 33 in H33A mutant decreases from 0.3 to 0.1 ppm.

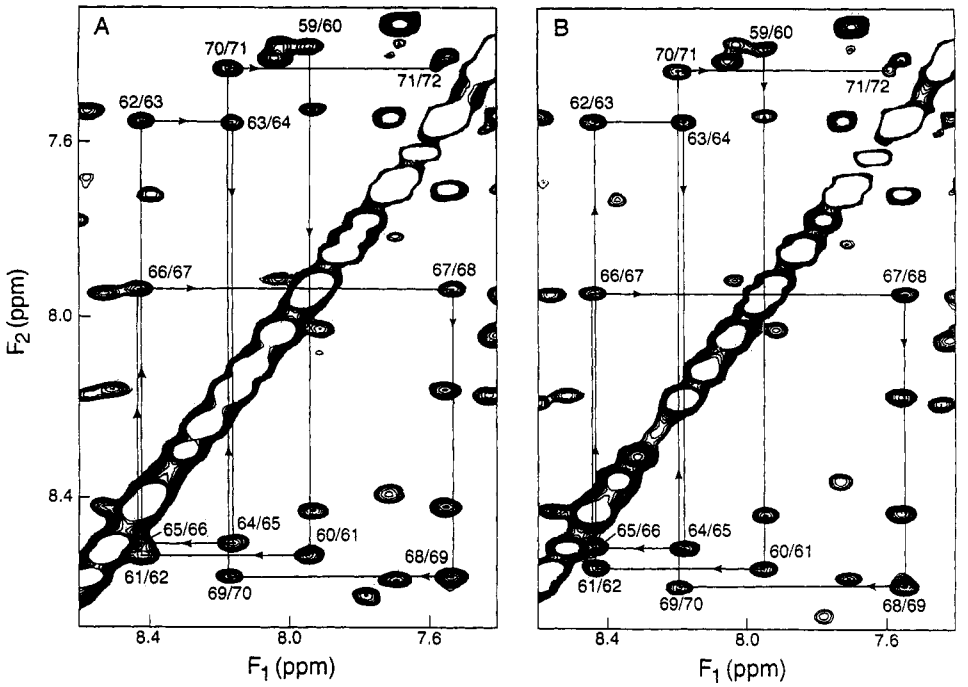

FIGURE 5: 600-MHz 2D NOESY ^{1}H NMR spectra of (A) the native IL-8 (1–72) and (B) the 6–72 analog at 40 °C showing d_{NN} (i, i + 1) NOE cross peaks that are characteristic of an α helix.

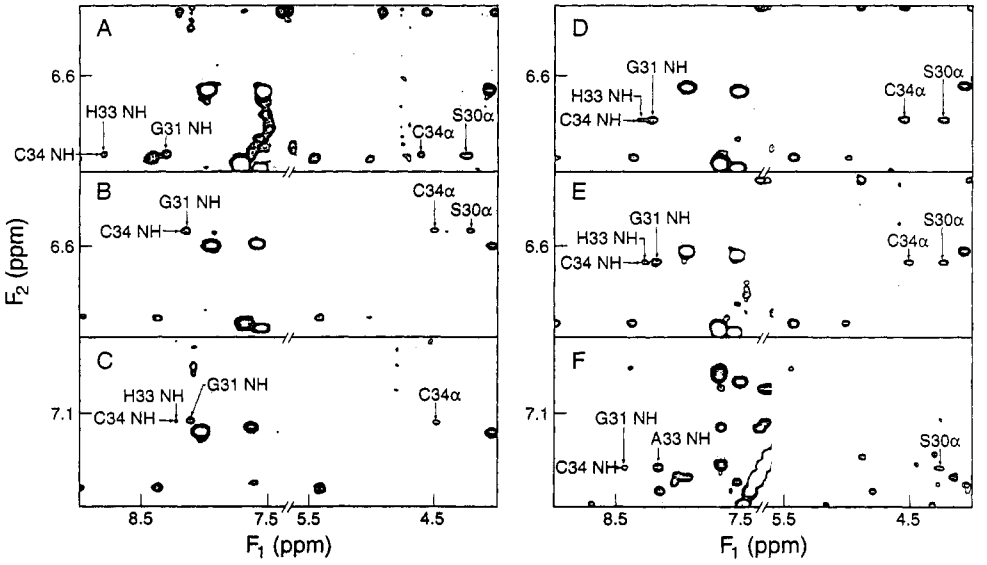

FIGURE 6: Sections of the NOESY NMR spectra showing the NOEs from the Cys-34 amide proton in the deletion analogs (A) 4–72, (B) 5–72, and (C) 6–72 and the substitution analogs (D) R6K, (E) I10A, and (F) H33A. The NMR spectrum of the 1–72 analog is similar to that of the 4–72 analog and is not shown.

dynamic state of the protein (Heinz et al., 1992). Temperature coefficients were measured for the 6–72 analog, an analog which was inactive and showed relatively large differences in chemical shifts and NOEs, and the native IL-8. It is observed that the temperature coefficients are the same for most of the protons within experimental error (Figure 7). In general, small temperature coefficients (≤5 ppb/K) are indicative of H-bonding. Some differences are observed for the temperature coefficients in the turn region and the N-terminus. In the 6–72 analog, the temperature coefficients for Gly-31, His-33, Cys-34, Ala-35, Cys-7, Gln-8, and Cys-9 are actually lower than in the native analog, suggesting that these residues are less mobile in the 6–72 analog.

DISCUSSION

Functional Implications of the Observed NMR Properties. The amide and the C_α proton, being the backbone protons, are sensitive to changes in structure and dynamics of the protein (de Dios et al., 1993; Wishart et al., 1991; Pardi et al., 1983; Clayden & Williams, 1982). In general, chemical shifts of the C_α proton are affected by secondary structural features such as backbone ψ and ϕ angles and tertiary structural features such as ring current contributions from aromatic side chains (Wishart et al., 1991; Clayden & Williams, 1982). The amide chemical shifts are also extremely sensitive to changes in dynamic aspects of the structure, such as H-bonding and solvent exposure, in addition to changes in the secondary and tertiary structure (de Dios et al., 1993; Wishart et al., 1991; Pardi et al., 1983).

The synthetic IL-8 has been shown to have the same functional properties as genetically expressed IL-8 (Clark-Lewis et al., 1991b). In order to ascertain that they are structurally the same, we have completely assigned the

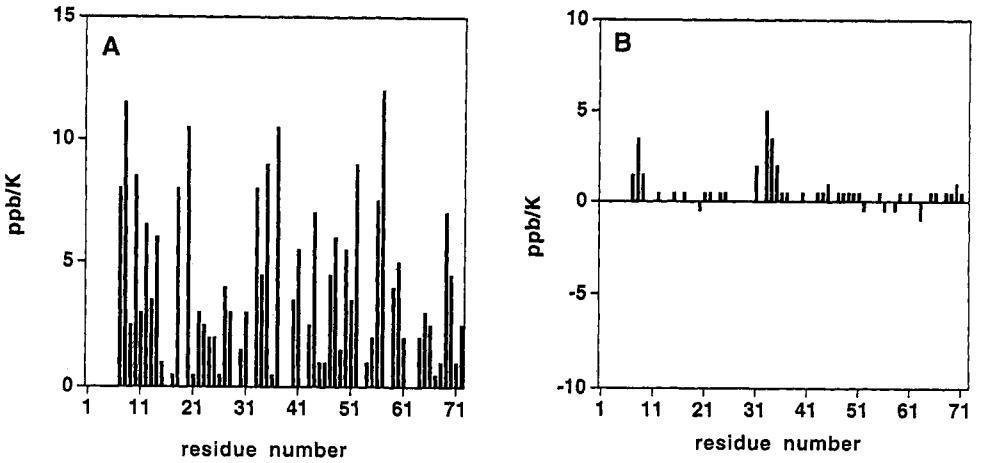

FIGURE 7: Comparison of the temperature coefficients of the ^1H NMR chemical shifts of the amide resonances of the native IL-8and the 6–72 analog: (A) The amide proton temperature coefficients of the native IL-8 and (B) the difference in temperature coefficients of the amide protons between the 6–72 and the native analog. A positive difference implies that the temperature coefficient was lower in the analog.

chemical shifts of the synthetic 1–72 analog using standard 2D ^1H NMR techniques. The chemical shifts of the chemically synthesized 1–72 analog were essentially the same as those reported for the recombinant IL-8 (1–72) (shown in the supplementary material). This further demonstrates that the synthetic approach is a viable alternative to recombinant techniques for engineering IL-8 analogs.

To correlate the structural changes with function, we have completely assigned all of the amide and C_α protons of the deletion (4–72, 5–72, and 6–72) and substitution analogs (R6K, I10A, and H33A). In the first series of analogs, the 5–72 and 6–72 analogs showed similar small perturbations in NMR chemical shifts, although there were large functional differences between them. In the second set of analogs, the H33A analog showed the largest perturbation in chemical shifts but was completely active. Clearly there is no correlation between the extent of change in chemical shifts and the extent of change in function. Detailed analysis of the NOESY spectra showed that the NOE pattern is essentially the same in all of the different analogs except for small differences in the 30–35 turn region. Similarly, the amide proton chemical shift temperature coefficients were essentially the same but for small changes in the turn region. Whereas the changes in the NOESY data for the 6–72 analog suggested that these residues are more mobile (Figure 6), the temperature coefficient data suggested that these residues are less mobile (Figure 7). The above observations suggest that the extent of the change in the NOESY and the temperature coefficient data is minimal and indicate structural changes which are within the experimental error of the methodologies. This also suggests that of the different NMR parameters used as criteria for structural changes, the chemical shifts are the most sensitive.

Though both 6–72 and R6K analogs show properties of antagonists, 6–72 analog is found to be the more potent of the two (Moser et al., 1993). However, the NMR properties of the R6K analog are indistinguishable from those of the 4–72 analog, indicating that the structure of the R6K analog is identical to that of the 4–72 analog. This suggests that the side chain of Arg is critical to receptor binding. The I10A analog showed 22-fold less activity (Clark-Lewis et al., 1994) (Table 1). The NMR properties of the I10A analog were studied in order to determine whether the functional changes are due to reduced steric bulk at position 10 or to structural changes elsewhere. The chemical shift and NOESY data showed that the Ile → Ala substitution does not result in any structural changes and that the structure is essentially the same as that of the native protein. The data for the 4–72, 5–72, and 6–72 truncation analogs and the I10A and R6K substitution analogs clearly argue that it is the specificity of the amino acid side chains (steric bulk, charge distribution, etc.) of the N-terminal residues which is important for the interaction of IL-8 with the receptor.

His-33 is conserved in all of the neutrophil-activating IL-8-related proteins. In the native IL-8 NMR structure, the His-33 imidazole side chain N_ϵ is found to be H-bonded to the Gln-8 NH, and more importantly, it is close to the functionally essential N-terminal ELR residues and the 7–34 disulfide bond. However, substitution of His-33 with other amino acids, such as Glu, Gln, and Ala, had no affect on activity.[2] NMR studies of the H33A analog showed that removal of the His-33 side chain resulted in changes that are more dynamic than structural and that were confined to the turn and the N-terminus as seen in all other analogs. Though H33A showed the largest differences in chemical shifts, this analog was as active as the native protein (Clark-Lewis et al., 1994) (Table 1), indicating that there is no correlation between the observed differences in the NMR properties and function.

Cys-34 which is part of a turn, and Cys-7 are involved in disulfide bond formation, which is shown to be essential for the integrity of the tertiary structure (Clark-Lewis et al., 1993b). Though it is likely that the turn is involved in receptor binding, our data does not directly reflect that this is the case. Though both NMR and X-ray structures show that the turn containing Cys-34 is well defined in the individual structures (Clore & Gronenborn, 1991), the largest difference in terms of rmsd (>2 Å) was observed for this part of the protein. Recent studies of the backbone dynamics by ^{15}N relaxation measurements showed that the N-terminal residues Glu-4, Leu-5, and Arg-6 have significant degrees of internal motion consistent with the earlier ^1H NMR data (Grasberger et al., 1993; Clore et al., 1990). Residues Cys-7, Cys-34, Ala-35, and Asn-36 also showed a large degree of mobility, and interestingly residues Ser-30, Gly-31, and His-33 showed mobility parameters similar to those of the residues in the core of the protein (Grasberger et al., 1993). This and our NMR data suggest an intrinsic flexibility/mobility of the residues in the turn and the N-terminus and also that they can undergo concerted motion through the disulfide linkage (Figure 1). The NMR data of the analogs also suggest that the differences observed between the NMR and X-ray structures of the native IL-8 are of no functional relevance and are a consequence of the methods used.

Implications for Receptor Binding. Two receptors for IL-8 have been identified and characterized in neutrophils, and they belong to the superfamily of seven transmembrane domain containing proteins that bind to G-proteins (Holmes et al., 1991; Murphy & Tiffany, 1991; Moser et al., 1991). Recent studies have identified the N-terminal region and the extracellular loop 3 on the receptor as part of the binding domain (Hébert et al., 1993). It was observed that the same receptors bind MGSA and NAP-2 (both have the ELR sequence) but with differential selectivity (Gayle et al., 1993; Lee et al., 1992). Interestingly, receptors which uniquely bind MGSA but not IL-8 have been reported (Horuk et al., 1993; Unemori et al., 1993). A related chemokine, PF-4, which lacks the ELR sequence, does not bind to the IL-8 receptor, whereas a synthetic analog, "ELR-PF4", binds to the IL-8 receptor (Clark-Lewis et al., 1993a). However, the ELR analog of a related chemokine, γIP-10, and the unmodified γIP-10 failed to bind to the IL-8 receptor (Dewald et al., 1992; Clark-Lewis et al., 1993b). The above observations suggest that, in addition to the ELR sequence, additional features differentiate between related proteins in a selective manner at the receptor-binding level. This specificity may arise from the tertiary structure of the chemokines or from the structure of the receptor or both. In the case of chemokines, it is likely that this selectivity may come from the turn containing Cys-34 which forms a contiguous surface with the N-terminus or from the region corresponding to the residues 10–23.[2] This in part may explain the presence of what seem to be a number of proteins whose functions are similar: the structural/functional differences at a molecular level in these domains fine-tune the biochemical responses at the site of inflammation.

When the structural and functional data of the IL-8 analogs and related proteins are considered together, a molecular basis for receptor binding emerges. The NMR data suggest that the core of IL-8 acts as a scaffold from which the N-terminal residues (4–6) and the disulfide-linked turn are suspended in a way which facilitates receptor binding and triggers neutrophil activation. The observation that the 6–72 analog has a higher binding constant and was a better antagonist than the R6K analog though the R6K analog was structurally the same as the native protein suggests that structural features such as side-chain steric bulk and local positive charge distribution are critical to initiate binding. The structural and functional data from the H33A analog and other His-33 analogs suggest that the turn containing Cys-34 is important for disulfide formation with the N-terminus and hence for the tertiary structure. Though it is possible that this turn and the disulfide bond may play a direct role in receptor binding, further studies with multiple substitutions in the turn and the N-terminus should provide further insight into the relative contribution of these residues to binding and function. Structural data of related proteins such as MGSA, NAP-2, and IP-10 also should aid in understanding the mechanism of interaction for IL-8 and its family of proteins.

ACKNOWLEDGMENT

We express our sincere thanks to Dr. Wishart for his insightful suggestions; to Gerry Quaid for the upkeep of the spectrometers; and to Philip Owen, Peter Borowski, and Luan Vo for the protein synthesis.

SUPPLEMENTARY MATERIAL AVAILABLE

Two tables of the complete chemical shifts of the native IL-8 (1–72) and the 6–72 analog and two tables of the chemical shifts of C_α and the amide protons for the native IL-8 and the deletion (4–72, 5–72, and 6–72) and substitution analogs (H33A, I10A, and R6K) (8 pages). Ordering information is given on any current masthead page.

REFERENCES

Antilla, H. S. I., Rietamo, S., Erkko, P., Ceska, M., Moser, B., & Baggiolini, M. (1992) *J. Invest. Dermatol. 98*, 96–101.
Baggiolini, M., & Clark-Lewis, I. (1992) *FEBS Lett. 307*, 97–101.
Baldwin, E. T., Weber, I. T., Charles, R., Xuan, J.-C., Appella, E., Yamada, M., Matsushima, K., Edwards, B. F. P., Clore, G. M., Gronenborn, A. M., & Wlodawer, A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 502–506.
Brown, S. C., Weber, P. L., & Mueller, L. (1988) *J. Magn. Reson. 77*, 166–169.
Clark-Lewis, I., Moser, B., Walz, A., Baggiolini, M., Scott, G. J., & Aebersold, R. (1991a) *Biochemistry 30*, 3128–3135.
Clark-Lewis, I., Schumacher, C., Baggiolini, M., & Moser, B. (1991b) *J. Biol. Chem. 266*, 23128–23134.
Clark-Lewis, I., Dewald, B., Geiser, T., Moser, B., & Baggiolini, M. (1993a) *Proc. Natl. Acad. Sci. U.S.A. 90*, 3574–3577.
Clark-Lewis, I., Dewald, B., Moser, B., & Baggiolini, M. (1993b) in *Peptides, Chemistry and Biology: Proceedings of the 13th American Peptide Symposium* (Hodges, R. S, & Smith, J. A., Eds.), ESCOM, Leiden, The Netherlands (in press).
Clark-Lewis, I., Dewald, B., Loetscher, M., Moser, B., & Baggiolini, M. (1994) *J. Biol. Chem. 269*, (in press).
Clayden, N. J., & Williams, R. J. P. (1982) *J. Magn. Reson. 49*, 383–396.
Clore, G. M., & Gronenborn, A. M. (1991) *J. Mol. Biol. 217*, 611–620.
Clore, G. M., Appella, E., Yamada, M., Matsushima, K., & Gronenborn, A. M. (1989) *J. Biol. Chem. 264*, 18907–18911.
Clore, G. M., Appella, E., Yamada, M., Matsushima, K., & Gronenborn, A. M. (1990) *Biochemistry 29*, 1689–1696.
Davis, D. G., & Bax, A. (1985) *J. Am. Chem. Soc. 107*, 2821–2822.
de Dios, A. C., Pearson, J. G., & Oldfield, E. (1993) *Science 260*, 1491–1496.
Dewald, B., Moser, B., Barella, L., Schumacher, C., Baggiolini, M., & Clark-Lewis, I. (1992) *Immunol. Lett. 32*, 81–84.
Gayle, R. B., Sleath, P. R., Srinivason, S., Birks, C. W., Weerawarna, K. S., Cerretti, D. P., Kozlosky, C. J., Nelson, N., Bos, T. V., & Beckmann, M. P. (1993) *J. Biol. Chem. 268*, 7283–7289.
Grasberger, B. L., Gronenborn, A. M., & Clore, G. M. (1993) *J. Mol. Biol. 230*, 364–372.
Hébert, C. A., Vitangcol, R. V., & Baker, J. B. (1991) *J. Biol. Chem. 266*, 18989–18994.
Hébert, C. A., Chuntharapai, A., Smith, M., Colby, T., Kim, J., & Horuk, R. (1993) *J. Biol. Chem. 268*, 18549–18553.
Heinz, D. W., Hyberts, S. G., Peng, J. W., Priestle, J. P., Wagner, G., & Grutter, M. G. (1992) *Biochemistry 31*, 8755–8766.
Holmes, W. E., Lee, J., Kuang, W.-J., Rice, G. C., & Wood, W. I. (1991) *Science 253*, 1278–1280.
Horuk, R., Yansura, D. G., Reilly, D., Spencer, S., Bourell, J., Henzel, W., Rice, G., & Unemori, E. (1993) *J. Biol. Chem. 268*, 541–546.
Jeener, J., Meier, B. H., Bachmann, P., & Ernst, R. R. (1979) *J. Chem. Phys. 71*, 4546–4553.
Koch, A. E., Polverini, P. J., Kunkel, S. L., Harlow, L. A., DiPietro, L. A., Elner, V. M., Elner, S. G., & Strieter, R. M. (1992) *Science 258*, 1798–1801.
Lee, J., Horuk, R., Rice, G. C., Bennett, G. L., Camerato, T., & Wood, W. I. (1992) *J. Biol. Chem. 267*, 16283–16287.
Miller, M. D., & Krangel, M. S. (1992) *Crit. Rev. Immunol. 12*, 17–46.
Moser, B., Schumacher, C., von Tscharner, V., Clark-Lewis, I., & Baggiolini, M. (1991) *J. Biol. Chem. 266*, 10666–10671.
Moser, B., Dewald, B., Barella, L., Schumacher, C., Baggiolini, M., & Clark-Lewis, I. (1993) *J. Biol. Chem. 268*, 7125–7128.

Murphy, P. M., & Tiffany, H. L. (1991) *Science 253*, 1280–1283.

Oppenheim, J. J., Zachariae, C. O. C., Mukaida, N., & Matsushima, K. (1991) *Annu. Rev. Immunol. 9*, 617–648.

Pardi, A., Wagner, G., & Wüthrich, K. (1983) *Eur. J. Biochem. 137*, 445–454.

Rance, M., Sørensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 117*, 479–485.

Schumacher, C., Clark-Lewis, I., Baggiolini, M., & Moser, B. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10542–10546.

Seitz, M., Dewald, B., Gerber, N., & Baggiolini, M. (1991) *J. Clin. Invest. 87*, 463–469.

States, D. J., Haberkorn, R. A., & Reuben, D. J. (1982) *J. Magn. Reson. 48*, 286–292.

Unemori, E. N., Amento, E. P., Bauer, E. A., & Horuk, R. (1993) *J. Biol. Chem. 268*, 1338–1342.

Webb, L. M. C., Ehrengruber, M. U., Clark-Lewis, I., Baggiolini, M., & Rot, A. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7158–7172.

Wishart, D. S., Sykes, B. D., & Richards, F. M. (1991) *J. Mol. Biol. 222*, 311–333.