^{1}H NMR evidence that Glu-38 interacts with the N-terminal functional domain in interleukin-8

Krishnakumar Rajarathnam[a], Ian Clark-Lewis[b], Beatrice Dewald[c], Marco Baggiolini[c], Brian D. Sykes[a,*]

[a] *Protein Engineering Network of Centres of Excellence (PENCE) and the Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2S2, Canada*
[b] *PENCE, the Biomedical Research Centre, the Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC V6T 1Z3 Canada*
[c] *Theodor-Kocher Institute, University of Bern, P.O. Box 99, CH-3000 Bern, Switzerland*

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Abstract In order to assess the importance of the buried Glu-38 observed in the structure of interleukin-8, an analog in which Glu-38 was replaced with Ala (E38A analog) was investigated by ^{1}H NMR spectroscopy and neutrophil activation. Detailed analysis of the NMR NOESY data showed that the solution structure of the E38A analog is essentially the same as that for the native protein. Also, the neutrophil elastase activity of the E38A analog was similar to that of the native protein. However, the Gln-8 and Cys-9 amide proton chemical shifts, which are significantly downfield-shifted in the native protein, exhibit more 'normal' values. This observation indicates that in the native protein, Glu-38 side-chain carboxylate interacts with Gln-8 and Cys-9 amide protons. Although the N-terminal residues are critical for function, this interaction is not essential for neutrophil activation.

Key words: Interleukin-8; Chemokine; Structure-function; NMR; H-bonding interaction

1. Introduction

Interleukin-8 (IL-8) belongs to a family of proteins called chemokines (chemoattractant cytokines) that are actively involved in recruitment of leukocytes to sites of inflammation [1,2]. They have four conserved cysteines and are classified into CXC chemokines or CC chemokines on the basis of whether the first two cysteines are separated by one amino acid (C–X–C) or are adjacent (C–C). The two subfamilies are also functionally distinct: whereas the CXC chemokines activate neutrophils, CC chemokines activate monocytes, lymphocytes and eosinophils. IL-8 remains the best characterized chemokine and its structure–function relationship has been the focus of a number of studies [3–9].

The structure of IL-8, determined by NMR and X-ray methods, showed it to be a homodimer [10,11]. A monomeric IL-8 analog was shown to be fully active, suggesting that dimerization is not essential for functional activation [7]. The monomer consists of a series of turns in the N-terminus followed by three β strands and a C-terminal α-helix. The four cysteines are involved in disulfide bond formation (Cys-7 and Cys-34; Cys-9 and Cys-50) and have been shown to be essential for tertiary structure and function. Functional studies have shown that the most critical residues for activity are the flexible N-terminal residues Glu-4, Leu-5, Arg-6 ('ELR' motif) [3,5]. In addition, residues 31–34, which form a contiguous surface with the 'ELR' motif, and residues 10–22, which are also solvent exposed, were also shown to be essential [4]. Structural studies of a number of analogs combined with the above findings suggest that the core of the protein acts as a scaffold from which the 'ELR' motif and the other surface residues are suspended in a way which facilitates receptor binding and neutrophil activation [6].

Glu-38 is highly conserved among all human and other mammalian CXC chemokines (Fig. 1). The NMR and X-ray structures of native dimeric IL-8 [10,11], NMR structure of an active monomeric IL-8 [12], two solution structures of MGSA [13,14], and X-ray structure of PF-4 [15] are known to date. In all of the structures, Glu-38 (Gln in PF-4) was observed to be buried and pointed in a direction towards the N-terminal residues (Fig. 2). In both IL-8 and MGSA NMR structures, Gln-8 amide proton was significantly downfield-shifted, indicating that they are involved in an H-bonding interaction and we suspected that the interaction could involve the Glu-38 carboxylate side chain. Buried charged residues are unusual and if present are generally stabilized by electrostatic or H-bonding interactions [16]. We were interested in finding out if Glu-38 in IL-8 is involved in such an interaction and also whether this interaction plays a functional role. In order to address these questions, we substituted Glu-38 → Ala (E38A) and have studied this analog by NMR methods and have addressed the functional response by assaying elastase release from the neutrophils.

2. Materials and methods

IL-8 E38A analog was chemically synthesized, purified and characterized as described previously [17]. The elastase release activity was determined with freshly isolated neutrophils as described previously [18].

^{1}H NMR spectra were acquired on a Varian Unity 600 spectrometer at 30°C and 40°C as discussed in detail previously [12]. The protein concentration was ~2 mM in 20 mM sodium acetate aqueous buffer, pH 5.2. The NOESY (150 ms) [19], TOCSY (55 ms) [20] and DQF-COSY [21] were collected for resonance assignments and quadrature detection along the t_1 dimension was achieved using the States method [22]. The solvent was suppressed using presaturation and for NOESY and DQF-COSY experiments, the loss of intensity of the cross peaks near H_2O signal was minimized using the SCUBA se-

*Corresponding author. Fax: (1) (403) 492-1473.

Abbreviations: IL-8, interleukin-8; NAP-2, neutrophil-activating peptide 2; MGSA, melanoma growth-stimulatory activity; PF-4, platelet factor 4; NMR, nuclear magnetic resonance; DQF-COSY, double quantum-filtered correlated spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlated spectroscopy; DSS, 2,2-dimethyl-2-silapentane-5-sulfonic acid

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quence [23]. All spectra were referenced to the internal DSS at 0.00 ppm.

3. Results and discussion

Assignment of the chemical shifts of the E38A analog was accomplished using standard 2-dimensional NMR methods. Scalar connectivities were identified from the TOCSY and DQF-COSY spectra and through space connectivities were identified from the NOESY spectra. The assignments were fairly straight forward as the chemical shifts of most of the protons were similar to the native protein. The downfield region of the 1-dimensional spectra in H_2O of the native and the E38A analog is shown in Fig. 3. The two downfield-shifted signals were previously assigned to Gln-8 and Lys-20 amide protons in the native protein and it is seen that the peak from Gln-8 is missing in the E38A analog indicating it has shifted more upfield. Chemical shifts of the $C^{\alpha}H$ protons are sensitive to secondary and tertiary structure, whereas the chemical shifts of the amide protons in addition to the secondary and tertiary structure, are also sensitive to parameters such as H-bonding and exchange with the bulk solvent [24]. The differences in the chemical shifts of the amide and the $C^{\alpha}H$ protons between the native and the E38A analog are shown in Fig. 4. Chemical shifts of the $C^{\alpha}H$ protons are essentially similar between the two proteins except for small differences ($\sim$0.1 ppm) at the site of substitution and for residues at the N-terminus, suggesting that the structures are largely similar. However, in the case of amide protons, very large chemical shift differences between the two proteins are seen for Gln-8 ($\sim$3 ppm) and Cys-9 ($\sim$1 ppm). Detailed analysis of the NOESY data indicated that the tertiary structure of the two proteins are largely similar. Hence, the chemical shift differences of the Gln-8 and Cys-9 amide protons can be directly attributed to the Glu $\rightarrow$ Ala substitution and that the Gln-8 and Cys-9 amide protons are involved in an H-bonding interaction with Glu-38 carboxylate oxygens in the native protein.

On the basis of the native NMR structure, the downfield shift of Gln-8 was attributed to H-bonding with the His-33 side chain $N^{\varepsilon 2}$ [10]. However, we had shown earlier that in the H33A analog, Gln-8 and Cys-9 have similar chemical shifts as the native protein [6]. In the X-ray structure, both the carboxylate oxygens of Glu-38 are optimally oriented for H-bonding to the Gln-8 and Cys-9 amide protons [11]. In the native NMR [10] and in the monomeric NMR structures [12], Glu-38 was found to be disordered but nevertheless buried and oriented towards the N-terminal region. The present NMR data of the E38A analog provide evidence that Glu-38 side-chain carboxylate interacts with Gln-8 backbone amide proton. The large change in chemical shift ($>$3 ppm) also suggests that that Glu-38 adopts a unique conformation similar to what is seen in the X-ray structure and it is likely that the side-chain orientation in the NMR structures were not fixed due to lack of distance and/or dihedral constraints.

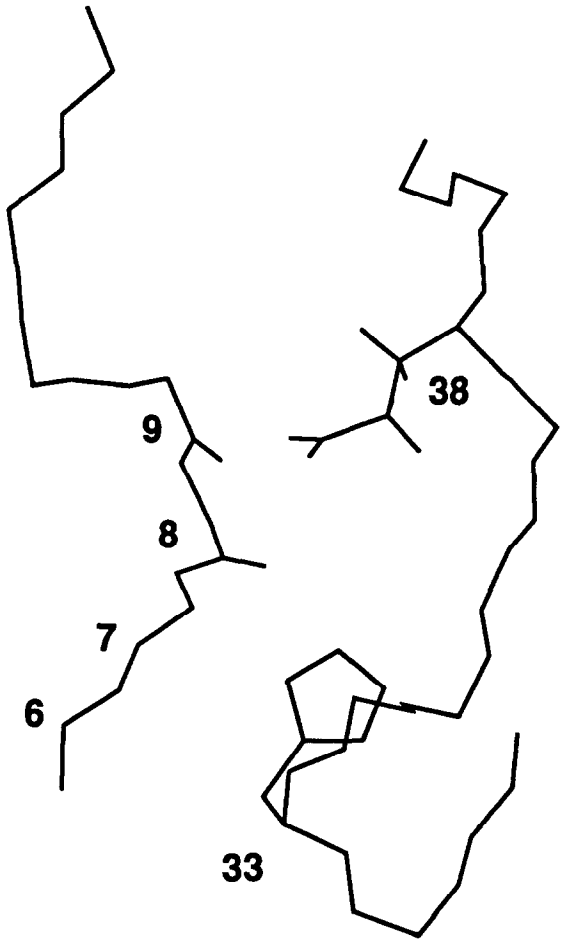

Fig. 2. Schematic presentation of the N-terminal region and the orientation of Glu-38 side chain in the IL-8 structure.

Sequences of human and other mammalian α-chemokines

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                      5    10   15   20   25   30   35   40
hIL-8                 ~ELRCQCIKTYSKPFHPKFIKELRVIESGPHCANTEIIVKLSD~
hMGSA                 ~ELRCQCLQTLQ-GIHPKNIQSVNVKSPGPHCAQTEVIATLKN~
hNAP-2                ~ELRCMCIKTTS-GIHPKNIQSLEVIGKGTHCNQVEVIATLKD~
hENA-78               ~ELRCVCLQTTQ-GVHPKMISNLQVFAIGPQCSKVEVVASLKN~
hIP-10                ~TVRCTCISISNQPVNPRSLEKLEIIPASQFCPRVEIIATMKK~
hPF-4                 ~DLQCLCVKTTS-QVRPRHITSLEVIKAGPHCPTAQLIATLKN~

guinea pig IL-8       ~ELRCQCIKIHTTPFHPKFIKELKVIESGPRCANSEIIVKLSD~
rabbit IL-8           ~ELRCQCIKTHSTPFHPKFIKELRVIESGPHCANSEIIVKLVD~
sheep IL-8            ~ELRCQCIKTHSTPFHPKFIKELRVIESGPHCENSEIIVKLTN~
pig IL-8              ~ELRCQCINTHSTPFHPKFIKELRVIESGPHCENSEIIVKLVN~
dog IL-8              ~ELRCQCIKTHSTPFHPKYIKELRVIDSGPHCENSEIIVKLFN~
rat MGSA              ~ELRCQCLQTVA-GIHFKNIQSLKVMPPGPHCTQTEVIATLKN~
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Fig. 1. A comparison of the amino acid sequences of the N-terminal region of human and other mammalian α-chemokines.

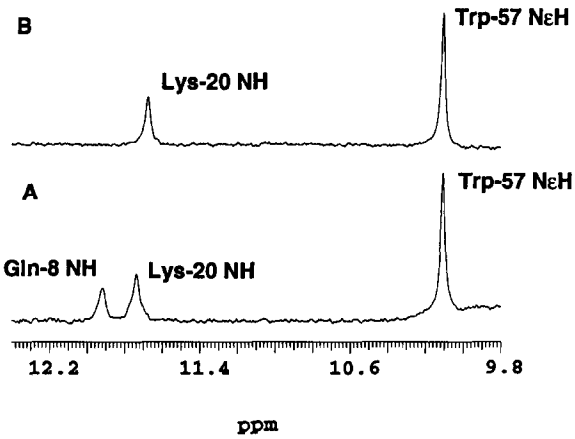

Fig. 3. Downfield region of the 600 MHz ^{1}H NMR spectra at 40°C of the (A) native IL-8 and (B) E38A analog.

Similar substantial downfield shifts for the Gln-9[1] (~11.2 ppm) and moderate downfield shifts for the Cys-10[1] (~9.5 ppm) amide protons are observed in MGSA [13,14] and the rat CINC/Gro [25] suggesting that H-bonding interaction exists between Gln-9, Cys-10 amide protons and Glu-39[1] sidechain carboxylate in these proteins. This was confirmed to be the case as the Gln-9 amide shift was upfield-shifted in a MGSA mutant in which Glu-39 was substituted with Ala. Glu-39 was found to be disordered but oriented towards the N-terminal domain in the MGSA NMR structures [13,14] but the chemical shifts of the Gln-9 and Cys-10 amide protons suggest that Glu-39 adopts a unique conformation in MGSA structure.

Two receptors (IL-8R1 and IL-8R2) have been identified for neutrophils, of which IL-8R2 (also called B-type) binds IL-8, NAP-2 and MGSA with equal affinity and the IL-8R1 (also called A-type) binds IL-8 with high affinity and NAP-2 and MGSA with low affinity [26–28]. The Duffy antigen receptor for chemokines (DARC) is less specific and binds members of both CXC and CC chemokines with medium affinity [29,30]. The activity of the E38A analog was assessed by measuring the elastase release from neutrophils (Fig. 5). It was observed that the activity is essentially the same as that seen for the native protein, suggesting that the loss of the H-bonding interaction between Glu-38 and the functionally important N-terminal residues Gln-8 and Cys-9 is not critical for receptor binding. Interestingly, mutation of Glu-39 to Ala in MGSA showed a 9-fold reduction in binding to neutrophils (data not shown). The data for the MGSA mutant is consistent with a previous study which showed a 12-fold reduction for IL-8R2 receptor and neutrophil activity and a 64-fold reduction in binding to the DARC receptor [31]. This observation suggests that this H-bonding interaction plays a role in MGSA for maximal interaction with the receptor.

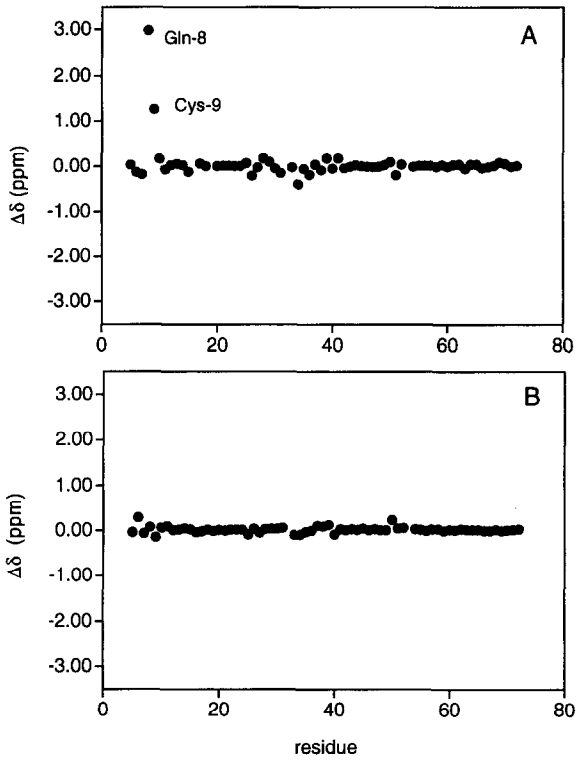

Fig. 4. A plot of the difference in chemical shifts between IL-8 (4–72) and the E38A (4–72) substitution analog for the (A) amide and (B) $C^{\alpha}H$ protons. A positive difference implies a larger chemical shift in the native protein. Both proteins were synthesized in the 4–72 version as the IL-8 (4–72) analog was shown to be as potent as the native (1–72) analog in receptor binding and functional activation [4].

[1]The residues Gln-9, Cys-10 and Glu-39 in MGSA and rat CINC/Gro correspond to residues Gln-8, Cys-9 and Glu-38 in interleukin-8 (Fig. 1).

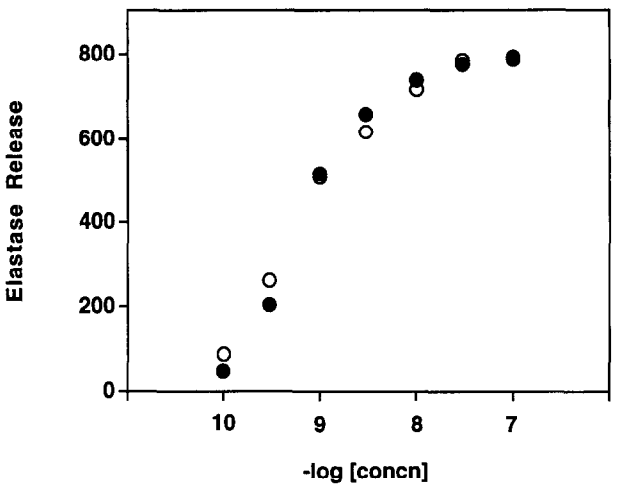

Fig. 5. Neutrophil elastase release activity of native IL-8 (○) and E38A mutant (●). Data are representative of three assays using different neutrophil preparations.

It is believed that buried charged residues which are conserved play a structural and/or a functional role [16]. In the case of IL-8, it retains substantial secondary structure in 9M urea and at temperature of 85°C indicating that it is largely stabilized by the two disulfide bonds and the hydrophobic core. Hence it is unlikely that the H-bonding interaction between the Glu-38 and Gln-8 is critical for structural stability. Indeed, thermal denaturation of the E38A analog showed a similar profile as observed for the native protein. The functional data suggets that this H-bonding interaction plays a differential role between IL-8 and MGSA. We speculate that in IL-8, this interaction is completely unrelated to activation of the IL-8 receptor, but may play a role in other events, such as an aspect of Il-8 function that we have not yet examined or in the initiation and kinetics of protein folding.

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References

[1] Baggiolini, M., Dewald, B. and Moser, B. (1994) Adv. Immunol. 55, 97–179.
[2] Miller, M.D. and Krangel, M.S. (1992) Crit. Rev. Immunol. 12, 17–46.
[3] Clark-Lewis, I., Schumacher, C., Baggiolini, M. and Moser, B. (1991) J. Biol. Chem. 266, 23128–23134.
[4] Clark-Lewis, I., Dewald, B., Loetscher, M., Moser, B. and Baggiolini, M. (1994) J. Biol. Chem. 269, 16075–16081.
[5] Hébert, C.A., Vitangcol, R.V. and Baker, J.B. (1991) J. Biol. Chem. 266, 18989–18994.

[6] Rajarathnam, K., Clark-Lewis, I. and Sykes, B.D. (1994) Biochemistry 33, 6623–6630.
[7] Rajarathnam, K., Sykes, B.D., Kay, C.M., Dewald, B., Geiser, T., Baggiolini, M. and Clark-Lewis, I. (1994) Science 264, 90–92.
[8] Schraufstätter, I.U., Ma, M., Zenaida, G.O., Barritt, D.S. and Cochrane, C.G. (1995) J. Biol. Chem. 270, 10428–10431.
[9] Moser, B., Dewald, B., Barella, L., Schumacher, C., Baggiolini, M. and Clark-Lewis, I. (1993) J. Biol. Chem. 268, 7125–7128.
[10] Clore, G.M., Appella, E., Yamada, M., Matsushima, K. and Gronenborn, A.M. (1990) Biochemistry 29, 1689–1696.
[11] 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. and Wlodawer, A. (1991) Proc. Natl. Acad. Sci. USA 88, 502–506.
[12] Rajarathnam, K., Clark-Lewis, I. and Sykes, B.D. (1995) Biochemistry 34, 12983–12990.
[13] Kim, K.-S., Clark-Lewis, I. and Sykes, B.D. (1994) J. Biol. Chem. 269, 32909–32915.
[14] Fairbrother, W.J., Reilly, D., Colby, T.J., Hesselgesser, J. and Horuk, R. (1994) J. Mol. Biol. 242, 252–270.
[15] Zhang, X., Chen, L., Bancroft, D.P., Lai, C.K. and Maione, T.E. (1994) Biochemistry 33, 8361–8366.
[16] Schueler, O. and Margalit (1995) J. Mol. Biol. 248, 125–135.
[17] Clark-Lewis, I., Moser, B., Walz, A., Baggiolini, M., Scott, G.J. and Aebersold, R. (1991a) Biochemistry 30, 3128–3135.
[18] Peveri, P., Walz, A., Dewald, B. and Baggiolini, M. (1988) J. Exp. Med. 167, 1547–1559.
[19] Jeener, J., Meier, B.H., Bachmann, P. and Ernst, R.R. (1979) J. Chem. Phys. 71, 4546–4553.
[20] Davis, D.G. and Bax, A. (1985) J. Am. Chem. Soc. 107, 2821–2822.
[21] Rance, M., Sørensen, O.W., Bodenhausen, G., Wagner, G., Ernst, R.R. and Wüthrich, K. (1983) Biochem. Biophys. Res. Commun. 117, 479–485.
[22] States, D.J., Haberkorn, R.A. and Reuben, D.J. (1982) J. Magn. Reson. 48, 286–292.
[23] Brown, S.C., Weber, P.L. and Mueller, L. (1988) J. Magn. Reson. 77, 166–169.
[24] Wishart, D.S., Sykes, B.D. and Richards, F.M. (1991) J. Mol. Biol. 222, 311–333.
[25] Hanzawa, H., Haruyama, H., Watanabe, K. and Tsurufuji, S. (1994) FEBS Lett. 354, 207–212.
[26] 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. and Beckmann, M.P. (1993) J. Biol. Chem. 268, 7283–7289.
[27] LaRosa, G.J., Thomas, K.M., Kaufmann, M.E., Mark, R., White, M., Taylor, L., Gray, G., Witt, D. and Navarro, J. (1992) J. Biol. Chem. 267, 25402–25406.
[28] Schumacher, C., Clark-Lewis, I., Baggiolini, M. and Moser, B. (1992) Proc. Natl. Acad. Sci. USA 89, 10542–10546.
[29] Horuk, R., Chitnis, C.E., Darbonne, W.C., Colby, T.J., Rybicki, A., Hadley, T.J. and Miller, L.H. (1993) Science 261, 1182–1184.
[30] Hadley, T.J., Lu, Z-h., Wasniowska, K., Martin, A.W., Pelper, S.C., Hesselgesser, J. and Horuk, R. (1994) J. Clin. Invest. 94, 985–991.
[31] Hesselgesser, J., Chitnis, C.E., Miller, L.H., Yansura, D.G., Simmons, L.C., Fairbrother, W.J., Kotts, C., Wirth, C., Gillece-Castro, B.L. and Horuk, R. (1995) J. Biol. Chem. 270, 11472–11476.