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Distinct Differences in Structural States of Conserved Histidines in Two Related Proteins: NMR Studies of the Chemokines CXCL1 and CXCL8 in the Free Form and Macromolecular Complexes

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Abstract

Hydrogen-bonding and ionic interactions play fundamental roles in macromolecular recognition and function. In contrast to lysines and arginines, how histidines mediate these interactions is less well-understood due to the unique properties of its side chain imidazole that include an aromatic ring with two titratable nitrogens, a pK_a that can vary significantly, and the ability to exist in three distinct forms: protonated imidazolium and two tautomeric neutral ($N^{\delta 1}$ and $N^{\epsilon 2}$) states. Here, we characterized the structural features of histidines in the chemokines CXCL8 and CXCL1 in the free, GAG heparin-bound, and CXCR2 receptor N-terminal domain-bound states using solution NMR spectroscopy. CXCL8 and CXCL1 share two conserved histidines, one in the N-loop and the other in the 30s loop. In CXCL8, both histidines exist in the $N^{\epsilon 2}$ tautomeric state in the free, GAG-bound, and receptor-bound forms. On the other hand, in unliganded CXCL1, each of the two histidines exists in two states, as the neutral $N^{\epsilon 2}$ tautomer and charged imidazolium. Further, both histidines exclusively exist as the imidazolium in the GAG-bound and as the $N^{\epsilon 2}$ tautomer in the receptor-bound forms. The N-loop histidine alone in both chemokines is involved in direct GAG and receptor interactions, indicating the role of the 30s loop varies between the chemokines. Our observation that the structural features of conserved histidines and their functional role in two related proteins can be quite different is novel. We further propose that directly probing the imidazole structural features is essential to fully appreciate the molecular basis of histidine function.

Abstract

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Author Contributions

K.R. and K.M.S. designed the research and analyzed the data. K.M.S. performed the experiments. K.R. wrote the manuscript. Both authors reviewed the results and approved the final version of the manuscript.

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H-bonding and ionic interactions mediate macromolecular interactions and are fundamental to all physiological phenomena.^{1–3} The basic amino acids lysine, arginine, and histidine are major drivers of H-bonding and electrostatic interactions. The side chain structures and properties such as pK_a of these amino acids are quite different, indicating that how the side chain properties dictate different aspects of the binding, such as affinity and specificity, will be different. Whereas the lysine amine and arginine guanidinium groups are at the end of a long aliphatic chain and their pK_a is >10 , the imidazole side chain is cyclic with a pK_a that can vary significantly. Whereas the pK_a of a histidine in model peptides is ~ 6 , pK_a in proteins can vary between <3 and >10 , indicating that the pK_a is highly dependent on the local structure.^{4–10} Further, the imidazole ring can exist in three forms due to two titratable nitrogens: protonated imidazolium and two tautomeric neutral ($N^{\delta 1}$ and $N^{\epsilon 2}$) states^{11–14} (Figure 1). Accordingly, the role of histidines and the molecular basis of how histidine imidazole interacts with its cognate partner within the free protein and in complex formation can vary.

Humans express ~ 50 chemokines, which play a crucial role in directing immune and nonimmune cells in health and disease.¹⁵ It is well-established that chemokine function is intimately coupled to binding to two classes of macro-molecules: glycosaminoglycans (GAGs) that are sulfated linear polysaccharides and receptors that belong to the GPCR (G protein-coupled receptor) class.^{16–19} GAGs are expressed by most cell types and are also an integral component of the extracellular matrix.^{20,21} GAG interactions play a crucial role in regulating chemokine concentration gradients and receptor activity, which together orchestrate cellular trafficking.^{22,23} Receptor signaling triggers ultrastructural changes that are essential for directed cell migration.^{24,25} The chemokines CXCL1 and CXCL8, members of a subset of seven chemokines that function as agonists for the CXCR2 receptor, orchestrate neutrophil recruitment in response to infection and injury.^{26,27} Sequences reveal two conserved histidines, one located in the N-terminal loop and the second in the 30s loop

(Figure 2A). Binding studies have shown that receptor and GAG binding involves only the N-loop histidine and that the 30s loop histidine is remote from the binding interface and cannot be directly involved in binding either to the GAG or the receptor.^{28–32} At this time, little is known regarding the structural features of His or how the imidazole side chain engages its cognate partners in GAG-binding proteins.

In principle, crystal structures of chemokine complexes should provide insights into which and how histidine imidazole side chain nitrogens engage their cognate partners in the GAG and the receptor. Structures are not available, and even if they were, it is quite challenging to unambiguously identify which of the three forms exists in the bound structure. Most crystal structures lack the resolution to detect protons or to be able to distinguish between nitrogens and carbons. Moreover, H-bonding interactions observed in the crystal structure may not hold in the solution phase due to differences in side chain conformation and constraints introduced by crystal packing. On the other hand, NMR chemical shifts and coupling constants of the imidazole ring nitrogens and protons have been shown to be sensitive probes for identifying the ionic and tautomeric states. Indeed, imidazole chemical shifts have been shown to be quite useful for describing how histidine side chain properties impact protein folding, structure, and function.^{4–10}

In this study, we determined the ionic and tautomeric states of histidines in the free and bound forms of CXCL1 and CXCL8 using NMR spectroscopy. Our data indicate that the roles of histidines are quite different between these related proteins. Both histidines in CXCL8 exist in the neutral N^{e2} tautomeric state in the free and bound forms. On binding GAG and the receptor, chemical shifts of N-loop histidine alone are perturbed, indicating 30s loop histidine plays no role in binding. In contrast, both histidines in CXCL1 show two distinct sets of peaks characteristic of the charged imidazolium and neutral N^{e2} tautomer. In the receptor-bound form, both histidines exist in the N^{e2} tautomeric state, whereas both exist exclusively in the imidazolium state in the GAG-bound form. These observations are remarkable, indicating selective binding of one or the other conformer to different binding partners and that the remote histidine adopts the same conformational state as the histidine within the binding interface. We conclude that the structural features and function of histidines can be quite complex, that their roles can vary within a protein and between two closely related proteins, and that directly probing the imidazole side chain as described here is essential to understand the molecular basis of histidine function.

MATERIAL AND METHODS

Expression and Purification of CXCL1 and CXCL8.

The recombinant chemokines were expressed in *E. coli* strain BL21(DE3), and ¹⁵N-labeled protein was produced by growing cells in minimal medium containing ¹⁵NH₄Cl as the sole nitrogen source.³¹ Transformed *E. coli* BL21(DE3) cells were grown in minimal medium containing ampicillin to an A₆₀₀ of 0.8 and induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 8 h at 37 °C. Purity and molecular weight of the proteins were confirmed using analytical high-pressure liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS), respectively. The heparin octasaccharide (dp8) was purchased from Iduron (United Kingdom). The main

disaccharide unit in dp8 is IdoA,2S-GlcNS,6S (~75%) and shows some variation in sulfation.

NMR Experiments.

NMR spectra were recorded on Bruker 750 and 800 MHz NMR spectrometers at 25 °C. The spectra of the CXCL1-dp8 complex were also collected at 40 °C. ^1H - ^{15}N HMQC spectra of the free chemokine and dp8-bound and CXCR2 N-terminal domain-bound complexes were acquired with a 22 ms delay for selective observation of the long-range ^1H - ^{15}N coupling constant of histidines ($2J_{\text{N-H}} = 6\text{--}12$ Hz). The chemical shifts of side chain imidazole nitrogen and proton chemical shifts were assigned as described.⁵ A total of 2048 complex points with a spectral width of 12 019 Hz was acquired in the proton dimension, and a total of 256 complex points was acquired in the indirectly detected ^{15}N dimension. The ^{15}N spectral width was 120 ppm centered at 215 ppm. The heparin dp8-bound and CXCR2 N-terminal domain-bound complexes were prepared by mixing 200 μM CXCL8 or CXCL1 with heparin dp8 or CXCR2 N-domain in 1:4 and 1:3 molar ratios, respectively, in 50 mM sodium phosphate, pH 6.0 buffer. The pK_a were determined using backbone amide and imidazole side chain chemical shifts of CXCL8 and CXCL1 that were obtained from ^1H - ^{15}N HSQC and ^1H - ^{15}N HMQC spectra collected at pH between 2.5 and 8.5.

RESULTS AND DISCUSSION

Histidine imidazole nitrogen-attached protons are generally not detected by NMR experiments because of their rapid exchange with the bulk water. However, the nonexchangeable $\text{H}^{\delta 2}$ and $\text{H}^{\epsilon 1}$ protons (attached to $\text{C}^{\delta 2}$ and $\text{C}^{\epsilon 1}$, respectively) can be detected via two- or three-bond couplings to the nitrogens in the heteronuclear correlation experiments.^{12,13} Cross-peak intensities reflect the magnitude of the coupling constants, as $^2\text{J}_{\text{NH}}$ coupling compared to the three-bond $^3\text{J}_{\text{N}\delta 1\text{--H}\delta 2}$ coupling results in stronger signals, and the latter peak is either weak or often not observed in either the protonated or neutral states (Figure 1). The cross-peak pattern in the HMQC spectrum allows assignment of the ionic and tautomeric states and the imidazole nitrogen and proton ($\text{N}^{\delta 1}$, $\text{N}^{\epsilon 2}$, $\text{H}^{\delta 2}$, and $\text{H}^{\epsilon 1}$) chemical shifts. The chemical shifts of ring nitrogens and protons have been reported using model peptides. In the protonated state, $\text{N}^{\epsilon 2}$ and $\text{N}^{\delta 1}$ have chemical shifts of ~173 and 176 ppm. $\text{N}^{\epsilon 2}$ and $\text{N}^{\delta 1}$ have chemical shifts of ~164 and 249 ppm in the $\text{N}^{\epsilon 2}\text{H}$ tautomer, and chemical shifts of 249 and 164 ppm in the $\text{N}^{\delta 1}\text{H}$ tautomer. Intermediate values are indicative of fast exchange between the neutral and charged forms. Experimental and theoretical studies have shown $\text{N}^{\epsilon 2}\text{H}$ tautomer is favored by ~5-fold over the $\text{N}^{\delta 1}\text{H}$ tautomer.¹¹

Previous NMR studies have characterized histidines in several classes of proteins, from enzymes and glycosidases to those that bind metals and mediate immunity.^{4–10} These studies were mostly focused on characterizing active site histidines and have shown that chemical shifts are quite sensitive and are composites that reflect their location whether on the surface or buried, their structural environment, and extent of polar, H-bonding, and packing interactions. However, very little is known regarding how histidines mediate macromolecular interactions, and in particular, binding to sulfated polysaccharides such as GAGs. Unlike active site histidines that are buried in the structure, surface histidines mediate

macromolecular interactions. In this study, we characterized the structural features of two conserved histidines in two related proteins that interact with GAGs and GPCRs.

Role of Histidines in CXCL8 Interactions.

The chemical shifts of His18 and His33 imidazole ^{15}N and ^1H resonances in CXCL8 were assigned from the ^1H - ^{15}N HMQC spectrum and previously reported proton chemical shifts.³³ The chemical shifts and the cross-peak patterns indicated that both His18 and His33 exist in the neutral $\text{N}^{\text{e}2}\text{H}$ tautomeric state at pH 6.0 (Figure 3). The pK_{a} of the histidines was determined from pH titrations from multiple ring nitrogens and protons, and the data for the side chain protons are shown (Figure 3B). The values of 3.8 for His18 and 4.5 for His33 indicate that the local environment of both histidines is distinctly different compared to a solvent exposed histidine. Low pK_{a} values of <3 for imidazole nitrogens have been observed in several proteins, and structures and computational studies reveal that several factors such as ASA, proximity of charged residues, and local hydrophobic/electronic environment together determine pK_{a} in the folded protein.^{4,6,39} Both NMR and X-ray structures show that the His18 imidazole ring is tightly packed against the Lys20 side chain and that imidazole $\text{N}^{\delta 1}$ functions as an H-bond acceptor and forms an H-bond with Lys20 amide proton^{34–36} (Figure 2B). Further, the Lys20 backbone amide is significantly downfield shifted in the free protein, and its chemical shift changes by as much as 3.9 ppm on reducing the pH, further confirming that the His18 $\text{N}^{\delta 1}$ is H-bonded to the Lys20 backbone amide (Figure 2C). In the case of His33, in both NMR and X-ray structures, the accessible surface area (ASA) of His33 is $\sim 20\%$, indicating most of the His33 side chain is buried in the folded structure. The crystal structure indicates that the imidazole $\text{N}^{\text{e}2}\text{H}$ functions as an H-bond donor to the carbonyl of Glu29.³⁵ However, NMR structure shows that the His33 $\text{N}^{\text{e}2}$ functions as a H-bond acceptor and that it forms an H-bond with the Gln8 backbone amide proton.³⁴ Our data are inconsistent with the NMR structure, and mutational studies have also shown that it is not His33 but the Glu38 side chain carboxylate that forms an H-bond with the Gln8 backbone amide.^{37,38} Our data are more in agreement with the X-ray structure, but we cannot say with certainty that the low pK_{a} is due to H-bonding with a backbone carbonyl, as the 30s loop residues are conformationally flexible.

We characterized the binding of heparin dp8 to CXCL8 using ^1H - ^{15}N HMQC spectra (Figure 4). At pH 6.0, both histidines exist in the neutral $\text{N}^{\text{e}2}\text{H}$ tautomeric state in the dp8-bound form. In the dp8-bound form, $\text{N}^{\delta 1}$ resonances appear upfield and $\text{N}^{\text{e}2}$ resonances downfield for both histidines. The His18 $\text{N}^{\delta 1}$ chemical shift moves upfield by 32 ppm and $\text{N}^{\text{e}2}$ resonance moves downfield by 1.7 ppm. $\text{H}^{\delta 2}$ and $\text{H}^{\text{e}1}$ chemical shifts are downfield shifted by 0.12 ± 0.02 ppm. The chemical shifts of $\text{N}^{\delta 1}$ and $\text{H}^{\text{e}1}$ are comparable to those observed in the HMQC spectrum of the free protein measured at pH 4.5, indicating that His18 is more positively charged in the bound form. The backbone chemical shift of Lys20 broadens out on heparin binding, suggesting weakening of the H-bond and dynamic equilibrium between the free and H-bonded forms. The altered imidazole chemical shift profile, retention of intramolecular H-bonding to Lys20 (though weaker), and the tautomeric state of His18 in the bound form collectively indicate that $\text{N}^{\text{e}2}\text{H}$ must be engaged in electrostatic/H-bonding interactions with the heparin sulfate/carboxylate and that His18 retains the same orientation and packing interactions in the dp8-bound complex as in the

free protein. The His33 shifts were essentially unaltered, indicating that it plays no role in the binding and that its immediate electronic environment is unaltered in the dp8-bound complex.

The spectrum of CXCL8 bound to the CXCR2 N-terminal domain peptide at pH 6.0 shows that both histidines exist in the N^{e2} tautomeric state (Figure 4). His18 N^{δ1} and N^{e2} shifts move upfield and downfield by 7.0 and 0.7 ppm, respectively. H^{δ2} and H^{e1} shifts are downfield shifted by 0.07 ± 0.02 ppm. The His33 imidazole nitrogen and proton resonances were essentially unaltered, indicating that the N-loop histidine alone mediates receptor interactions.

Role of Histidines in CXCL1 Interactions.

The chemical shifts of CXCL1 His19 and His34 imidazole ¹⁵N and ¹H resonances were assigned from the HMQC spectra and previously available chemical shifts.^{39,40} The chemical shifts and the cross-peak patterns and intensities show two distinct sets of peaks for each of the two histidines, indicating that both histidines exist in two states, as the neutral N^{e2} and charged imidazolium. Two distinct sets of peaks for each of the two histidines indicate that they are in slow exchange in the NMR time scale (Figure 5). The HMQC spectrum at 25 °C was essentially similar to the spectrum at 40 °C (not shown), and spectra as a function of pH indicated a gradual shift in the populations with the imidazolium dominating at a lower pH (Figure 6). The chemical shifts of the N^{e2} tautomeric state of His34 are also quite unusual.

The pK_a of histidines determined from pH titrations indicate a value of ~5.2 for His19 and ~5.8 for His34 (Figure 6). Similar to CXCL8, the CXCL1 solution structures show that the His19 imidazole ring is packed against the lysine side chain. NOESY data also show a number of NOEs between His19 and Lys21 (data not shown). The Lys21 backbone amide is downfield shifted at pH 7.0 and moves upfield by 1.5 ppm at pH 3.0, suggesting it is H-bonded to the His19 N^{δ1} at pH 7.0 (Figure 7). However, for His34, the solution structures reveal different orientations for the imidazole side chain that can be attributed to the conformational flexibility of the 30s loop residues.^{40,41} Similar to CXCL8, its ASA is ~20%, and it is also interesting that the pK_a of His34 is comparable to that of a solvent exposed histidine and does not provide any clue regarding its structural states. Collectively, these observations indicate that local dynamics, correlated motions of the N-terminal and 30s loop residues mediated by the disulfide, and/or side chain structural properties must be responsible for the distinct histidine imidazole conformers in the folded protein.

We characterized the binding of heparin dp8 to CXCL1 using ¹H-¹⁵N HMQC spectra (Figure 8B). The cross-peak patterns and intensities in the dp8-bound form indicate that both histidines exist exclusively in the imidazolium state. The imidazolium state of His19 in the bound form suggests that interactions with heparin sulfates/carboxylates are more likely electrostatic in nature. In the bound HSQC spectrum, the chemical shift of the Lys21 backbone amide also moves upfield by 1.5 ppm to a value similar to what is observed in the pH 3.0 spectrum, indicating the Lys21 backbone amide is no longer H-bonded to His19 N^{δ1} in the heparin-bound complex. Differences in His34 spectral characteristics indicate an altered local electronic environment in the bound form though it is not involved in direct

heparin binding. Our previous studies have shown that the binding geometry of heparin in CXCL1 involves two nonoverlapping domains, and in particular, N-terminal arginine, which is proximal to the 30s loop, constitutes one of the binding domains.²⁷ Therefore, it is possible that differences in heparin geometries between CXCL1 and CXCL8 determine both the local electronic environment and 30s loop histidine imidazole structural properties.

We also characterized binding of the CXCR2 N-terminal domain peptide to CXCL1. The bound spectrum at pH 6.0 shows that both histidines exist in the N^{e2} tautomeric state unlike the imidazolium state in the heparin-bound form (Figure 8C). Considering the 30s loop histidine is not involved in direct receptor interactions, a preferred conformer in the receptor-bound state also indicates that the 30s loop residues are involved in indirect coupled binding interactions.

CONCLUSIONS

In summary, our data collectively indicate that the role of histidines in CXCL8 and CXCL1 is quite complex and that the side chain structural properties allow an array of interactions that provide exquisite control in regulating receptor and GAG interactions. Though a role for histidines has been inferred in GAG binding, this study provides the first experimental evidence of how imidazole side chain engages GAG heparin and also shows that the nature of interactions can be quite different between two related proteins. Further, our observations that one histidine conformer is preferred in the heparin-bound form and the other in the receptor-bound form and that a histidine located in the remote site adopts the same tautomeric state as the binding interface histidine in both complexes are novel. Most studies reported in the literature have focused on active site histidines that are buried and are involved in catalysis or binding cofactors. These studies have shown that the imidazole side chain is quite sensitive to its environment and can function as both an H-bond donor and acceptor with both backbone and side chain atoms and with water molecules. Our studies indicate that the structural features of histidines involved in macromolecular interactions can also be quite complex and that their interactions in closely related proteins can be different. We conclude that exploiting the sensitivity of NMR chemical shifts of imidazole side chain is essential to fully appreciate the molecular basis of histidine function.

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ABBREVIATIONS

CXCL8	CXC chemokine ligand 8
CXCL1	CXC chemokine ligand 1

NMR	nuclear magnetic resonance
HMQC	heteronuclear multiple quantum coherence

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Figure 1. A schematic of the neutral $\text{N}^{\epsilon 2}$, neutral $\text{N}^{\delta 1}$, and charged imidazolium states of the histidine side chain and the expected ^1H - ^{15}N HMQC spectrum of each species.

Figure 2.

(A) Sequences of CXCL1 and CXCL8. (B) Ribbon representation of CXCL8 with histidine residues highlighted in red. (C) Schematic showing H-bonding interaction between the His18 side chain and the Lys20 backbone amide proton. (D) NMR evidence for CXCL8 Lys20 backbone amide H-bonding. Overlay of ^1H - ^{15}N HSQC spectra of CXCL8 at pH 7.5 (black) and pH 3.0 (red) shows a significant chemical shift change as a function of pH.

Figure 3.
(A) ^1H - ^{15}N HMQC spectrum of CXCL8 at pH 6.0 showing that both His18 and His33 exist as $\text{N}^{\epsilon 2}$ tautomers. (B and C) pH titration curves of $^{15}\text{N}^{\delta 1}$ chemical shifts of CXCL8 His18 and His33.

Figure 4. ^1H - ^{15}N HMQC spectra of CXCL8 at pH 6.0 in the (A) free, (B) dp8-bound, and (C) CXCR2 receptor N-terminal domain-bound forms. (D) Overlay of ^1H - ^{15}N HMQC spectra of the free (black) and dp8-bound (red) forms. (E) Overlay of ^1H - ^{15}N HMQC spectra of the free (black) and CXCR2 receptor N-terminal domain-bound (green) forms. Prominent chemical shift changes on binding are indicated by an arrow.

Figure 5.
 ^1H - ^{15}N HMQC spectra of CXCL1 at pH 6.0 showing both His19 and His34 exist in two distinct states. Neutral and charged conformers are shown in red and blue, respectively. Expanded imidazolium region is shown in the right panel. The two conformers are distinguished by the ' symbol.

Figure 6. ^1H - ^{15}N HMQC spectra of free CXCL1 as a function of pH at 40 °C. Changes in $\text{N}^{\delta 1}$ chemical shifts of His19 and His34 in the pH titration curve are shown in green and red, respectively.

Figure 7. NMR evidence for H-bonding of CXCL1 Lys21 backbone amide. Overlay of ^1H - ^{15}N HSQC spectra of CXCL1 at pH 7.5 (black) and pH 3.0 (red) shows a significant chemical shift change for Lys21 as a function of pH.

Figure 8. ^1H - ^{15}N HMQC spectra of CXCL1 at pH 6.0 in the free (A), dp8-bound (B), and receptor-bound (C) forms. His19 and His34 cross-peak patterns are indicated for both neutral (red) and charged (blue) states.