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Analysis of internal motions of interleukin-13 variant associated with severe bronchial asthma using ^{15}N NMR relaxation measurements

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Abstract

The single nucleotide polymorphism interleukin-13 (IL-13) R110Q is associated with severe bronchial asthma because its lower affinity leads to the augmentation of local IL-13 concentration, resulting in an increase in the signal transduction via IL-13R. Since the mutation site does not directly bind to IL-13Rα2, we carried out NMR relaxation analyses of the wild-type IL-13 and IL-13-R110Q in order to examine whether the R110Q mutation affects the internal motions in IL-13 molecules. The results showed that the internal motion in the micro- to millisecond time scale on helix D, which is suggested to be important for the interaction between IL-13 and IL-13Rα2, is increased in IL-13-R110Q compared with that in the wild-type IL-13. It therefore appears that the difference in the internal motions on helix D between the wild-type IL-13 and IL-13-R110Q may be involved in their affinity differences with IL-13Rα2.

Keywords: Interleukin-13; Interleukin-13 receptor; Internal motion; NMR

Interleukin-13 (IL-13) is a critical mediator of allergic inflammation. IL-13 is an immunoregulatory cytokine secreted predominantly by activated Th2 cells [1–3]. IL-13 shares approximately 25% homology with IL-4, and they have in fact many overlapping activities. However, IL-13 does not always share all functional characteristics with IL-4 [4,5]. In particular, IL-13 is now thought to be an especially critical factor in asthma, which is characterized by airway hyperreactivity, mucus overproduction, and chronic eosinophilic inflammation [6,7]. IL-13 is therefore an obvious target for pharmacologic intervention in the treatment of allergic disease such as bronchial asthma.

There are two types of IL-13 receptor (IL-13R). One type is composed of an IL-4Rα chain and an IL-13Rα1 chain [8,9]. These units play an important role in the binding of IL-13 and IL-4, respectively. IL-13 binds to IL-13Rα1 at low affinity in the absence of IL-4Rα, whereas in the presence of IL-4Rα, the affinity between them becomes much higher. Another type of IL-13R has been reported to include IL-13Rα2 [10]. IL-13Rα2 binds IL-13 with greater avidity than IL-13Rα1, even in the absence of IL-4Rα [11]. However, IL-13Rα2 cannot transduce the IL-13 signal due to the lack of intracellular signaling motifs. It has therefore been assumed that IL-13Rα2 acts as a decoy receptor [12].

Abbreviations: IL-13, interleukin-13; HSQC, heteronuclear single quantum coherence; R1, longitudinal relaxation rate; R2, transverse relaxation time; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; TOCSY, total correlation spectroscopy; τ_m, rotational correlation time; S^2, spatial restriction of the ^{15}N–^{1}H bond vectors, Rex, chemical exchange in line width.

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In genetic studies, single nucleotide polymorphisms (SNPs) have been found in IL-13. Such SNPs are closely associated with serum IgE levels in patients with asthma and atopic dermatitis in various age and ethnic groups [13–17]. In particular, it has been reported that IL-13-R110Q, in which arginine-110 is substituted with glutamine, correlates with high IgE levels and atopic dermatitis [14,15]. Arima et al. have demonstrated that the R110Q mutation does not affect the binding activity of IL-13R composed of IL-13Rα1 and IL-4Rα, whereas the affinity of IL-13-R110Q with IL-13Rα2 is slightly lower than that of the wild-type IL-13 (K_d of IL-13Rα2–IL-13 complex for the wild-type IL-13, 54.7 ± 1.6 pM, K_d of IL-13Rα2–IL-13-R110Q complex for IL-13-R110Q, 83.7 ± 3.1 pM) [18]. It has been suggested that the lower affinity between IL-13-R110Q and IL-13Rα2 leads to its lesser clearance by IL-13Rα2, resulting in an augmentation of local IL-13 concentrations and a subsequent increase in signal transduction via IL-13R [18]. The three-dimensional structures of IL-13Rα1 and IL-13Rα2 have not yet been solved. From mutation analysis of IL-13, it has been found that R110 in IL-13 is not directly involved in the binding to IL-13R or IL-13Rα2 [19]. Thus, so far, it is not clear why the R110Q mutation affects binding activity.

By NMR relaxation analysis, Zidek et al. showed that the internal motion of the backbone of the mouse major urinary binding protein I, not in the interaction region, increased upon binding its ligand [20]. These results suggested that the change of internal motions in a protein affect to the binding affinity. Under the circumstances, we have paid close attention to the internal motions of IL-13 in the association between IL-13 and IL-13Rα2. As is well known, NMR relaxation analysis provides valuable information regarding protein interactions by extracting dynamic parameters at atomic resolution [21]. Therefore, in order to examine whether the R110Q mutation affects internal motions in the IL-13 molecule, we carried out NMR relaxation analyses of the wild-type IL-13 and IL-13-R110Q. Our results did show such a difference at the region where IL-13 is suggested to bind to IL-13Rα2.

Materials and methods

Expression and purification of denatured IL-13. The expression vector pET22b containing the gene of human IL-13 fused with $(His)_6$-tag and an enterokinase (EK) cleavage site at N-terminal was introduced into *Escherichia coli* BL21(DE3) (Novagen, Madison, WI). The transformant cells were grown in M9 minimal medium containing 1g/l of $^{15}NH_4Cl$ as the sole nitrogen source at 37 °C. The culture was allowed to grow until an OD_{600} of 0.6 is reached and the expression of IL-13 was incubated by addition of 1 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) and incubation for 3 h. The cells were harvested by centrifugation for 10 min at 8000 rpm. The pellets were suspended in 20 mM sodium phosphate buffer, pH 7.0, and sonicated 20 times for 30 s in a cool ice bath. IL-13 was expressed in the form of inclusion bodies from *E.coli*. After centrifugation for 10 min at 12,000 rpm, the inclusion bodies were re-suspended in 10 ml of 20 mM sodium phosphate buffer, pH 7.0, containing deoxyribonuclease I (20 μg/ml) and ribonuclease A (20 μg/ml), and incubated for 1 h at 40 °C to decompose nucleic acids. The mixture was centrifuged for 30 min at 7000 rpm. The precipitates were collected and dissolved at 5 mg/ml in 50 mM Tris–HCl buffer, pH 8.0, containing 6 M guanidine–HCl. The solutions containing denatured fusion proteins were adsorbed to Ni–nitrilotriacetic acid (NTA) column (3.0×1.0 cm) and eluted with 50 mM sodium phosphate buffer, pH 4.5, containing 6 M guanidine–HCl.

Refolding of IL-13. The solution containing denatured IL-13 was dialyzed against 0.1 M Tris–HCl buffer, pH 8.5, containing 8 M urea and then prepared at the protein concentration of 0.2 mg/ml by the same buffer. Mercaptoethanol (final concentration of 4 mM) was added in the solution for the reduction. After the incubation at 40 °C for 1.5 h, the reduced solution was slowly diluted 4-fold into 0.1 M Tris–HCl buffer, pH 8, containing 5 mM cystamine, 5 mM cysteine, 3 M urea and 30% glycerol and stirred for 3 days at 4 °C.

After the precipitant was removed by centrifugation, the supernatant was mixed 1:1 with Ni–NTA resins equilibrated in 0.1 M Tris–HCl buffer, pH 8, containing 3 M urea and 30% glycerol and then gently shook for overnight at 4 °C. The protein–resin complex was poured into an empty column (1.0×3.0 cm). After washing with 20 mM sodium phosphate buffer, pH 6.1, containing 10% glycerol, IL-13 was eluted with 20 mM sodium phosphate buffer, pH 6.1, containing 10% glycerol and 250 mM imidazole. The fractions containing IL-13 were collected, and dialyzed against 20 mM sodium phosphate buffer, pH 6.1, containing 10% glycerol. And then, His-tag was processed with 2 U of enterokinase toward 3 mg protein by incubation for 14 h at 20 °C. Each reaction mixture was applied to cation exchange column (1.0×3.0 cm, CM-Toyopearl 650 M, Tosho, Tokyo), and the wild-type IL-13 was eluted with 20 mM sodium phosphate buffer, pH 6.1, containing 1 M NaCl.

NMR measurements. The NMR sample was prepared to contain 0.3 mM protein in 25 mM Na_2HPO_4, 50 mM NaCl buffer, pH 6.1, in 90% H_2O/10% D_2O. NMR experiments were performed at 30 °C on a Varian Inova 600 MHz spectrometer equipped with a triple-resonance, pulse-field gradient probe with an actively shielded z gradient and a gradient amplifier unit. Sequential assignment of main-chain ^1H and ^{15}N resonances of the wild-type IL-13 and IL-13- R110Q were made on the basis of ^1H–^{15}N HSQC, ^{15}N-edited NOESY-HSQC [22], and ^{15}N-edited TOCSY-HSQC [23] spectra. Processing and analysis of NMR data and peak-picking were performed on NMRPipe/NMRDraw package [24]. The ^{15}N longitudinal relaxation rate ($R1$), transverse relaxation rate ($R2$), and ^1H–^{15}N heteronuclear nuclear Overhauser enhancement (NOE) were recorded for the wild-type IL-13 and IL-13-R110Q, respectively. Pulse sequences for the measurement of the ^1H–^{15}N NOE values, and the $R1$ and $R2$ relaxation times have been described [25,26]. The $R1$ values were measured using spectra recorded with seven different delay times; $T = 32.94$, 92.94, 172.94, 332.94, 652.94, 1292.94, and 1812.94 ms. The $R2$ values were measured using spectra recorded with seven different delay times; $T = 4$, 42, 82, 122, 202, 282, and 342 ms. The $R1$ and $R2$ values were obtained by fitting the intensities of the peak heights in each spectrum to a single exponential curve using a non-linear least-squares fitting program, CURVEFIT, available from the web site of Palmer's group (http://cpmcnet.columbia.edu./dept/gsas/biochem/labs/palmer/software.html). Errors in R1 and R2 were estimated by Monte Carlo analysis using CURVEFIT. ^1H–^{15}N steady-state NOE values were obtained by recording spectra with and without ^1H saturation for 3.0 s, and by calculating the ratios of the intensities of the peak heights. The errors in NOE were estimated by use of the root-mean-square value of the background noise [26].

Relaxation data analysis. The overall rotational correlation times of wild-type IL-13 and IL-13-R110Q were calculated from the $R2/R1$ ratios using a program, R2R1_TM, available from the web site of Palmer's group (http://cpmcnet.columbia.edu./dept/gsas/biochem/labs/palmer/software.html). The values of S^2, τ_e, Rex and S_f^2 were calculated with a program, Modelfree 4.0 [27], available from the same site, by applying the isotropic diffusion model with the obtained overall rotational correlation time.

Results and discussion

Expression, folding, and purification of IL-13 and IL-13 mutant

Wild-type IL-13 and IL-13-R110Q, which has a fused His-tag and enterokinase cleavage sequence at the N-terminal of IL-13, were overexpressed in *E. coli* as inclusion bodies. Purifications of the inclusion bodies were performed using a Ni–NTA column. From 1 l of culture, approximately 30 mg of the denatured IL-13s was obtained. Next, we attempt to fold the denatured IL-13s by a batch procedure on Ni–NTA resins after dilution of denaturant, as described in Material and methods. The yields for the folding of His-tag IL-13s were approximately 20%. Furthermore, His-tag was processed by enterokinase. In order to confirm whether the final production of folded IL-13 possesses the correct conformation, the HSQC spectra were measured for wild-type IL-13 (Fig. 1). The spectrum was similar to those reported previously [28]. Similarly, we also confirmed that the refolded IL-13-R110Q formed a correct conformation (data not shown). Eisenmesser et al. have reported for the development of a method to produce human IL-13. Namely, IL-13 gene fused to maltose-binding protein was inserted into the expression vector with a cleavage site for the tobacco etch virus protease. Coexpression of the fusion protein and the protease led to high levels of IL-13 production [28]. In the present study, we developed a convenient method to obtain the human IL-13.

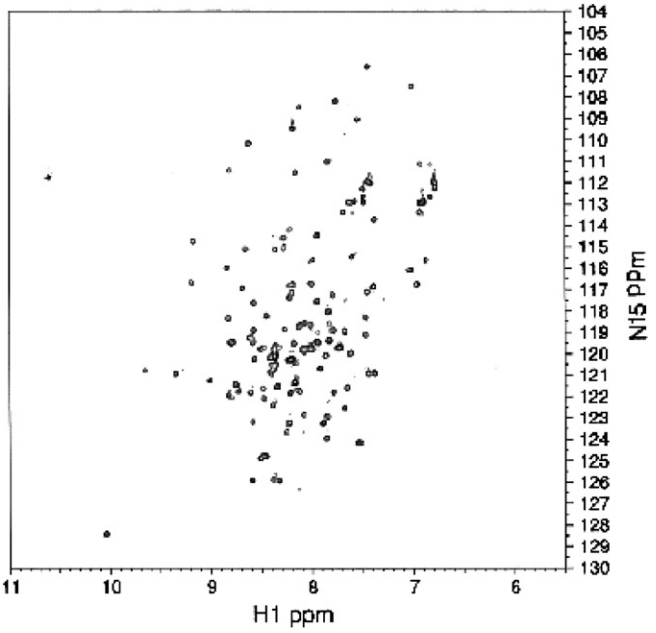

Fig. 1. ^{1}H–^{15}N HSQC spectrum of the wild-type IL-13. ^{1}H–^{15}N HSQC spectrum of 0.3 mM IL-13 in H_2O/D_2O (90%/10%, v/v), 25 mM Na_2HPO_4, pH 6.1, 50 mM NaCl at 30 °C is shown.

Relaxation analysis of wild-type IL-13 and IL-13-R110Q

In the ^{1}H–^{15}N chemical shift assignments, the backbone amide ^{1}H and ^{15}N resonance of the IL-13s were assigned based on ^{1}H–^{15}N HSQC, ^{15}N-edited NOESY-HSQC, and ^{15}N-edited TOCSY-HSQC spectra on the basis of the published assignments of IL-13 [22,23]. There are 105 amino acid residues in IL-13, excluding the N-terminal and three Pro residues. The resonances of the wild-type IL-13 and IL-13-R110Q were observed for 75 and 74 HN–N correlation peaks out of 105, respectively. The obtained $R1$, $R2$, and ^{1}H–^{15}N NOE values are shown in Fig. 2. For the isotropic motion, the rotational correlation time (τ_m) was calculated from $R2/R1$ values of residues. The calculated τ_m was 6.984 and 7.022 ns for the wild-type IL-13 and IL-13-R110Q, respectively. Then, under the application of the obtained τ_m value, $R1$, $R2$ and ^{1}H–^{15}N NOE were fit with five models consisting of the following subsets of the extended model-free parameters: (1) S^2; (2) S^2 and τ_e; (3) S^2 and Rex; (4) S^2, τ_e and Rex; and (5) S_f^2, S_s^2 and τ_e, where S^2 is the square of the generalized order parameter, τ_e is the effective correlation time, Rex is the chemical exchange term, and S_f^2 is the square of the order parameter for the internal motion on the fast time scale. Model-free analysis, a widely accepted method for evaluating the internal motions of a molecule, combines a generalized order parameter, such as a measure of the spatial restriction of the ^{15}N–^{1}H bond vectors (S^2), which contributes to small amplitude motions on the pico- to nanosecond time scale, and the chemical exchange in line width (Rex), which is a microenvironment change on the micro- to millisecond time scale. The order parameters and Rex values for IL-13 are shown in Fig. 3. For most residues in the wild-type IL-13, the order parameters were in the range of 0.8–1.0, indicating that internal motions on the pico- to nanosecond time scale are largely restricted. In contrast, lower-order parameters were observed in loop regions such as the AB loop (N22, K24, A25, L28, L38, T39), BC loop (V53, S54), and CD loop (H72, K73, A76, G77, Q78), reflecting increased motion in these regions. In the comparison of the order parameters between the wild-type IL-13 and IL-13-R110Q, the values in overall molecules were similar, whereas those in the region of helix D of IL-13-R110Q were slightly lower than those in that of the wild-type. On the other hand, the behaviors of Rex terms were different between the wild-type IL-13 and IL-13-R110Q. The appearances of Rex terms show the internal motions in the micro- to millisecond time scale. In particular, observed Rex terms in the region of helix D of IL-13-R110Q were increased compared with those of the wild-type, indicating increased motion in the region of helix D. It has been reported that such internal motions on the micro- to millisecond time scale are involved in the molecular recognition process [29]. In the solution structure of IL-13 cited from Eisenmesser et al. [30], because the side chain Nζ1-H1 atom of Arg110 is close to the side chain Oε1 atom of Glu108, which is located at C terminal of helix D, it is likely that

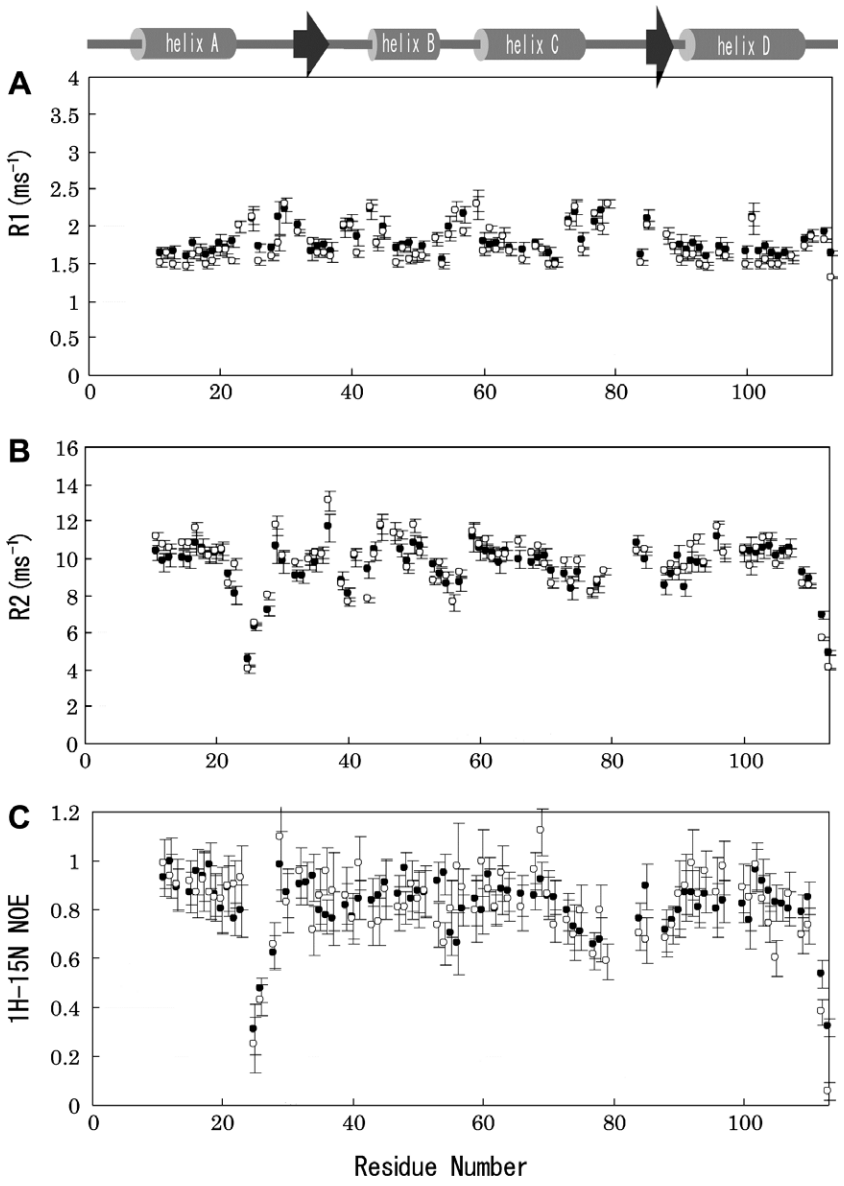

Fig. 2. ^{15}N $R1$ (A), $R2$ (B) and ^{1}H–^{15}N NOE (C) values of the wild-type IL-13 (closed circle) and IL-13-R110Q (open circles) at pH 6.1 and 30 °C.

these side chains interact with each other via hydrogen bond (Fig. 4). Therefore, the disappearance of the hydrogen bond in IL-13-R110Q might affect internal motion in the region of helix D.

Relationship between internal motions and the binding affinity of IL-13 with IL-13Rα2

It has previously been reported [19] that helix D in IL-13 may be important to the interaction between IL-13 and IL-13Rα2. Namely, the alanine-scanning mutagenesis has revealed that Lys103, Lys104, and Arg107 of helix D in IL-13 are responsible for interacting with IL-13Rα2, and Lys103 particularly represents a hot spot for the interaction with IL-13Rα2 [19]. Moreover, it has been shown that Ala92 of helix D affects the interaction with IL-13Rα2, although Ala92 does not affect the interaction with IL4/13R [19]. Since internal motions on the micro- to millisecond time scale have been reported to be involved in the molecular recognition process [29], it is important to discuss the Rex terms. In our results, the regions where Rex terms are exclusively observed in IL-13-R110Q in a comparison of Rex terms between the wild-type IL-13 and IL-13-R110Q are shown in red on the IL-13 structure cited in Eisenmesser et al. [30] (Fig. 4). In particular, differences in Rex terms were observed in the regions of helix D. These regions contain Lys103 and Ala92, which are responsible for interacting with IL-13Rα2, as shown in Fig. 4. It has been reported that the change of internal motions in a protein affect to the binding affinity [20]. Therefore, the increase in internal motion in these regions in IL-13-R110Q might cause an increase in the entropic penalty derived from binding events, resulting in lower

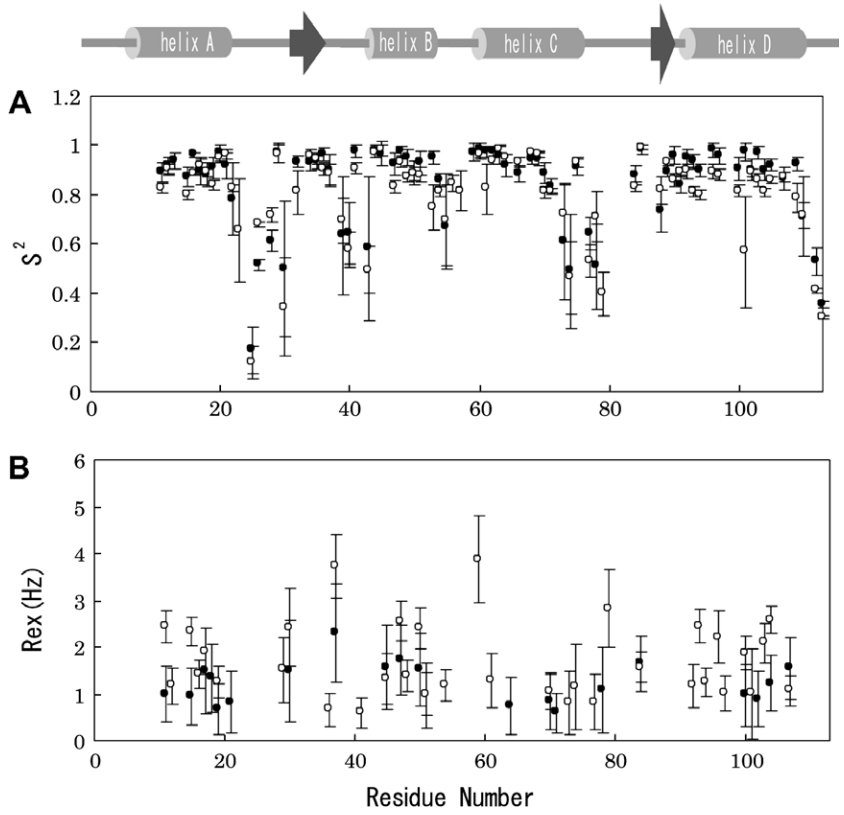

Fig. 3. Order parameters and Rex values of IL-13s. (A) Order parameters of the wild-type IL-13 (closed circle) and IL-13-R110Q (open circle). (B) Rex values of the wild-type IL-13 (closed circle) and IL-13- R110Q (open circle).

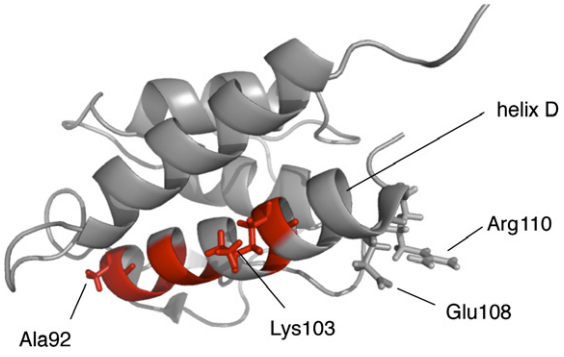

Fig. 4. The three-dimensional structure of IL-13 cited from Eisenmesser et al. [30]. The regions where Rex terms are exclusively observed in IL-13-R110Q compared with those in the wild-type IL-13 are colored in red. The side chains of Ala92, Lys103, Glu108, and R110 are shown as a stick model. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

affinity between IL-13-R110Q and IL-13Rα2. As such, it appears that the difference in internal motions on helix D between the wild-type IL-13 and IL-13-R110Q may be involved in their difference in affinity.

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