Solution Structure of Eotaxin, a Chemokine That Selectively Recruits Eosinophils in Allergic Inflammation*

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The solution structure of the CCR3-specific chemokine, eotaxin, has been determined by NMR spectroscopy. The quaternary structure of eotaxin was investigated by ultracentrifugation and NMR, and it was found to be in equilibrium between monomer and dimer under a wide range of conditions. At pH 5.0 and low ionic strength, eotaxin was found to be predominantly a monomer. The three-dimensional structure of the eotaxin monomer solved at pH 5.0 revealed that it has a typical chemokine fold, which includes a 3-stranded β-sheet and an overlying α-helix. Except for the N-terminal residues (residues 1–8), the core of the protein is well defined. The eotaxin structure is compared with the chemokines regulated upon activation, normal T-cell expressed and secreted (RANTES) and monocyte chemoattractant protein-1 (MCP-1); eotaxin binds only CC chemokine receptor CCR3, whereas RANTES binds many receptors including CCR3, and MCP-1 binds a distinct receptor, CCR2. The RMSD of the eotaxin ensemble of structures with the RANTES average minimized monomeric subunit is 5.52 ± 0.87 Å over all backbone atoms and 1.14 ± 0.09 Å over backbone atoms of residues 11–28 and 34–65. The most important difference between the structures is in the N-terminal residues that are unstructured in eotaxin but structured in RANTES and MCP-1. Several residues in the loop region of RANTES show similar packing in eotaxin (residues 11–17). As the N-terminal and loop regions have been shown to be critical for receptor binding and signaling, this structure will be useful for determining the basis for CCR3 selectivity of the eotaxin.

Eotaxin (1, 2) is a member of the chemoattractant cytokine (chemokine) family of proteins that mediates the migration and accumulation of eosinophils at sites of parasitic infection and allergic response (3–5). The chemokines play a pivotal role in the control of leukocyte chemotaxis and represent attractive targets for the blockade of inflammatory disease progression. Eotaxin belongs to the CC chemokine family which is distinguished by the presence of two adjacent cysteines close to the N-terminus (6). The CC chemokines activate a wide range of leukocytes including eosinophils, basophils, monocytes, dendritic cells, and T lymphocytes. Chemokine activities are mediated through the binding and activation of seven transmembrane helix G-coupled receptors (6), and eight distinct functional CC chemokine receptors have been identified (6–9). Their broad leukocyte distribution coupled with a lack of CC chemokine specificity means there is generally considerable redundancy among the CC chemokine family. However, the cloning and characterization of eotaxin and the related protein eotaxin-2 (10) and its receptor CCR3 (11) has revealed its unique and most important feature, a high selectivity for eosinophils (1, 2). Eotaxin has been implicated as the predominant chemokine in the recruitment of eosinophils and the eosinophil-mediated tissue damage associated with asthma. Thus eotaxin is a possible target for anti-asthmatic drugs.

The three-dimensional structures of RANTES and MCP-3 have been solved (12–14). Both of these proteins bind CCR3 among other receptors, so there is presently no structure for a chemokine that is selective in its binding to CCR3. In this study we have determined the solution structure of eotaxin by multinuclear NMR and compared it with the structure of other CC chemokines. Implications of the structure for its specific binding to CCR3 and the possible design of structure-aided protein therapeutics for asthma and other eosinophil-mediated diseases are discussed.

MATERIALS AND METHODS

Chemical Synthesis of Eotaxin—Eotaxin (Fig. 1) was synthesized by stepwise solid phase methods using t-butyloxycarbonyl protection chemistry. After hydrogen fluoride deprotection, the polypeptide was folded and purified by reverse-phase HPLC as described previously (15). HPLC of the resulting product revealed a major peak and a significant minor peak that could not be resolved by re-chromatography. The major peak was resolved by ion-exchange chromatography on a sulfonic acid silica column, followed by reverse-phase HPLC. [15N]Val, [15N]Leu, [15N]Phe, [15N]Ala, [15N]Ser, and [15N]Gly were incorporated as the isotopically labeled t-butyloxycarbonyl derivative (Cambridge Isotope Laboratories, Andover, MA). Purity of the products was assessed by ion-exchange HPLC and mass spectrometry. The measured mass of each of the final proteins, as determined by electrospray mass spectrometry, was consistent with the average mass calculated from the atomic composition.

1 The abbreviations used are: CCR3, CC chemokine receptor 3; CXCR, CXC chemokine receptor; NMR, nuclear magnetic resonance; RANTES, regulated upon activation, normal T-cell expressed and secreted; MCP, monocyte chemoattractant protein; DQF-COSY, double-quantum-filtered correlated spectroscopy; TOCSY, total correlation spectroscopy; NOESY, two-dimensional nuclear Overhauser enhancement spectroscopy; ROESY, two-dimensional rotating frame nuclear Overhauser spectroscopy; NOE, nuclear Overhauser enhancement; HSQC, heteronuclear single quantum coherence; HMOC, heteronuclear multiple quantum coherence; HPLC, high pressure liquid chromatography; RMSD, root mean square deviation.

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DNA Expression—Recombinant human eotaxin was prepared from the eotaxin gene subcloned into an expression vector pET15b (Novagen) in Escherichia coli strain BL21(DE3). Cells were harvested after 3 h of induction in M9 minimal medium supplemented with 1 g of NH4Cl and 10 g of glucose/liter at 37 °C. For each hydrogen bond, two distance restraints were used (JHCD 1.8–2.4, rHCD 1.8–3.5) and were added after initial examination of ensembles of structures. The refinement rounds were performed with simulated annealing using 6000 steps of high temperature dynamics (30 ps) and 4000 cooling steps (20 ps). A set of 32 final structures was selected from the 40 refined structures on the basis of lowest total energy and agreement with the experimental data. Analysis of the (ϕ, ψ) backbone torsion angles and quality of the structures was made using the program PROCHECK (20).

RESULTS AND DISCUSSION

Eotaxin Is in a Monomer-Dimer Equilibrium—Analytical ultracentrifugation sedimentation equilibrium studies were performed to determine the association state of eotaxin under a variety of experimental conditions (Table I). The calculated molecular mass of eotaxin by sedimentation equilibrium in 100 mM NaCl and 50 mM NaPi, at pH 7.0 was 9.8 kDa at 35 °C, 10.4 kDa at 30 °C, and 10.9 kDa at 25 °C. The observed mass distribution between 9 and 11 kDa indicates that eotaxin shows a monomer-dimer equilibrium. At 35 °C, the data could be fitted to a monomer-dimer equilibrium with a dissociation constant (Kd) of 1.3 mm (Table I). However, the dimerization was much weaker at pH 5.0, 20 °C, where the calculated molecular mass was 8.4 kDa in 100 mM NaCl and 50 mM NaPi, close to the calculated molecular weight for the monomer (Mx = 8365).

The monomer-dimer equilibrium was also investigated using NMR spectroscopy. A series of one-dimensional 1H NMR experiments was acquired as a function of eotaxin concentration at 30 °C, pH 5.0, in 20 mM NaAc buffer and are shown in Fig. 2A. The downfield signal at 10.25 ppm assigned to Trp-57 NeH is seen to change as the concentration is increased, with the emergence of a second broader line at 10.35 ppm corresponding to the dimer species. Chemical exchange peaks between the monomer-dimer resonances of Trp-57 NeH were clearly visible on TOCSY and NOESY spectra, confirming the existence of slow exchange (on the NMR time scale) of nuclei between monomer and dimer environments. Cross-peaks were observed in 600 MHz TOCSY spectra (mixing time 55 ms) between the Trp NeH resonances with a chemical shift difference (Δδ) = 0.1 ppm, indicating that the rate of exchange (kex) of monomer-dimer must be in the range 20 s−1 ≤ kex ≤ 360 s−1. The monomer and dimer concentrations in each state were calculated based on estimates of the mole fractions given the constant total area under the Trp-57 NeH resonances. If xM ↔ xM2, where M is monomer, x is the stoichiometric coefficient, and M2 the associated state, x can be estimated from Equation 1.

$$\ln[M] = x\ln[M] + \ln K$$  \(\text{(Eq. 1)}\)

A plot of [M2] versus ln[M] at pH 5.0 gives a value of x = 1.9, showing that two monomers are associating to form dimers, in agreement with the sedimentation data.

The monomer-dimer equilibrium was also investigated by collecting one-dimensional spectra in D2O at a protein concentration of 1.2 mM and under several temperature and pH conditions. The resonance lines corresponding to the CaH proton of Tyr-26 in the monomer (5.76 ppm) and dimer (5.80 ppm) species were well resolved, and their relative peak areas were
used to monitor the mole fractions of monomer and dimer. Fig. 2, B and C, shows the temperature dependence at two values of pH (5.0 and 6.8, respectively) showing that dimerization is pH-dependent, and the dimer population increases at higher pH values. \( K_d \) values are summarized in Table I. The effect of ionic strength is more severe with a 4–10-fold reduction of the \( K_d \) upon addition of 20 mM sodium phosphate. The dependence of dimer association as a function of pH indicates that electrostatic interactions may play a role in stabilizing the dimer structure, or alternatively, pH-induced conformational changes may disfavor formation of the dimer species at lower pH.

Both the sedimentation and NMR data indicated that eotaxin is predominantly monomeric at pH 5.0, and low ionic strength buffers also favor this form. The NMR assignments of eotaxin were carried out under conditions where eotaxin is predominantly in the monomeric state (30 or 40 °C, pH 5.0 in 20 mM NaAc and at concentrations between 1 and 2 mM). In addition, when recombinant \(^{15}\)N-labeled eotaxin became available, it showed no tendency to dimerize, presumably due to steric interference involving the GSHM N-terminal extension at the CC chemokine dimer interface.

Solution Structure of Monomeric Eotaxin—Resonance assignments were achieved using standard two- and three-dimensional sequential assignment techniques and a complete list of chemical shifts has been deposited in the BioMagResBank (code 4155). Detailed analysis of the NOEY spectra of eotaxin at 1–2 mM, pH 5.0 and 30 °C, revealed that neither residues at the N-terminus nor the residues at the first \( \beta \)-strand showed NOEs which could be attributed to intermonomer contacts. At 3 mM eotaxin concentration no new NOEs were observed, but several peaks were identified and assigned as exchange peaks between the monomer and a dimer. Interestingly, the CaH resonance of the N-terminal residue Val-5 has a “random coil” value of 4.46 ppm in the monomer (21), but it shows an exchange peak to a \( \beta \)-strand type chemical shift (4.93 ppm) in the dimer. This was the only direct structural information we could obtain on the dimeric form of eotaxin which suggests a dimeric structure typical of other CC chemokines. Accordingly, all the assigned NOEY cross-peaks could be accounted for using a monomeric model for eotaxin.

The solution structure of eotaxin was determined from NMR data using the simulated annealing protocol within the program X-PLOR (18). In the final round of calculations, 735 NOE-derived distance restraints and 74 dihedral restraints were used corresponding to 13 restraints per residue over the well ordered regions of the protein. The distribution of NOEs is shown in Fig. 3A. Forty structures were calculated of which eight structures were discarded due to either residual NOE violations and/or poor covalent geometry for some residues. The final ensemble of structures is of high quality with no NOE violations >0.3 Å or dihedral angle violations >0.5°, good covalent geometry as judged by small deviations from ideality, and approximately 99% of the well ordered \( \phi/\psi \) angles are in allowed regions of the Ramachandran plot (Table II).

| Technique | State | \( \text{pH} \) | Temperature (°C) | \( K_d \) (\( \mu \text{M} \)) | Buffer |
|-----------|-------|---------------|-----------------|-----------------|--------|
| NMR\(^a\) | M/D   | 5.0           | 10              | 1600            | 20 mM NaAc |
| NMR       | M/D   | 5.0           | 20              | 5400            | 20 mM NaAc |
| NMR       | M/D   | 5.0           | 30              | 8500            | 20 mM NaAc |
| NMR       | M/D   | 5.0           | 40              | 18,000          | 20 mM NaAc |
| Sed. Equilm.\(^b\) | M      | 5.0           | 20              | 100 mM NaCl, 50 mM NaPi |
| Sed. Equilm. | M/D   | 6.8           | 10              | 600             | 20 mM NaAc |
| Sed. Equilm. | M/D   | 6.8           | 20              | 800             | 20 mM NaAc |
| Sed. Equilm. | M/D   | 6.8           | 30              | 1000            | 20 mM NaAc |
| Sed. Equilm. | M/D   | 6.8           | 40              | 1600            | 20 mM NaAc |
| Sed. Equilm. | M/D   | 7.0           | 10              | 60              | 20 mM NaAc, 20 mM NaPi |
| Sed. Equilm. | M/D   | 7.0           | 20              | 130             | 20 mM NaAc, 20 mM NaPi |
| Sed. Equilm. | M/D   | 7.0           | 30              | 220             | 20 mM NaAc, 20 mM NaPi |
| Sed. Equilm. | M/D   | 7.0           | 40              | 370             | 20 mM NaAc, 20 mM NaPi |
| Sed. Equilm. | M/D   | 7.0           | 25              | 240 ± 50        | 100 mM NaCl, 50 mM NaPi |
| Sed. Equilm. | M/D   | 7.0           | 31              | 290 ± 10        | 100 mM NaCl, 50 mM NaPi |
| Sed. Equilm. | M/D   | 7.0           | 35              | 1300 ± 300      | 100 mM NaCl, 50 mM NaPi |

\(^a\)\( K_d \) values calculated for the 10 and 20 °C NMR data are likely to be less accurate than 30 and 40 °C due to overlap of the Tyr-26 monomer peak and Asp-48 dimer peak at lower temperatures, leading to an overestimation of the \( K_d \).

\(^b\) Sed. Equilm., sedimentation equilibrium. All ultracentrifugation data were collected in solutions containing 100 mM NaCl and 50 mM NaPi, \( K_d \) values are quoted as the average value determined at rotor speeds of 26,000 and 30,000 rpm.

\(^c\) Under these conditions only the monomer species was observed, and no \( K_d \) was calculated.
NOE or energetic penalty.

The eotaxin monomer has a chemokine-like fold consisting of three anti-parallel \( \beta \)-strands with an overlying \( \alpha \)-helix (Fig. 4C). The program Procheck was used to identify regions of secondary structure following the Kabsch-Sander definitions. Following the first eight residues which contain no defined structure, the first disulfide is undefined, whereas the second adopts a left-handed spiral. The loop (N-loop, residues 11–19) contains a bend at residue 16 and leads into a 3/10 helix (residues 19–22). Strand \( \beta 1 \) contains a \( \beta \)-bulge at residue Glu-24 and extends from residues Ser-25 to Ile-29. Residues Thr-30 to Lys-37 connect strand \( \beta 1 \) to strand \( \beta 2 \) and contain a structured type I/III turn (30-s turn) over residues Ser-31 to Cys-34. This region is characterized by only a few sequential NOE restraints, no long range NOEs, and rapid solvent exchange (Ser-31, Gly-32, and Lys-33-amide protons show exchange peaks with water in NOESY-HSQC spectra). The region from Thr-30 to Lys-37 shows defined local structure, but the lack of tertiary interactions leads to an apparent segmental motion of this entire region. Strand \( \beta 2 \) extends from Ala-38 to Lys-43 leading into a type I turn (40-s turn; residues 44–47), and the third strand \( \beta 3 \) runs from Asp-48 to Ala-51. A type III turn (50-s turn) (residues 52–55) connects strand \( \beta 3 \) to the \( \alpha \)-helix (residues 57–68), which packs onto the three-stranded \( \beta \)-sheet. Beyond Lys-68 (Ser-69 has an ordered \( \phi \) angle (\( S(\phi) = 0.97 \)) but disordered \( \psi \) (\( S(\psi) = 0.75 \))), the structure is not defined which is due to the lack of tertiary interactions at the C-terminal part of the structure.

The exchange rate of the individual amide protons with deuterons in the bulk solvent provides a good assessment of the stability of the secondary and tertiary structure of the protein. The initial one-dimensional spectrum (pH 5.0, 30 °C, 20 mM sodium acetate buffer, pH 5.0) recorded after 10 min shows 17 well resolved and identifiable resonances corresponding to the core \( \beta \)-sheet regions and the \( \alpha \)-helix (data not shown). In the dimer structure of the chemokine RANTES, approximately 25 protons were observed after 2 h at 35 °C with several highly protected amides in the core \( \beta \)-sheet structure persistent after 60 h (12). However, amide exchange experiments of monomeric versus dimeric interleukin-8 have shown that the amide protons exchange more quickly throughout the structure in the monomeric versus the dimeric form (23).

Comparison of Chemokine Tertiary Structures—Several CC chemokine structures have been solved by NMR and x-ray crystallography including RANTES (12, 13), MCP-3 (14), MIP-1\( \beta \) (24), and MCP-1 (25, 26). Comparisons will be restricted to MCP-1 and RANTES. RANTES binds CCR3 but also other receptors, whereas MCP-1 shows very high sequence homology to eotaxin but binds a distinct receptor CCR2. At the monomeric level, the core of the CC chemokine structures shows similar secondary structure consisting of three anti-parallel strands and an overlying \( \alpha \)-helix. A superposition of the monomeric subunits of MCP-1, RANTES, and eotaxin over these regions is shown in Fig. 5A. The greatest difference is seen in the N-terminal region of the proteins, which adopts a well ordered \( \beta \)-strand type structure in RANTES and MCP-1 but is disordered in eotaxin. The MCP-3 monomer structure also has a disordered NH\( _2 \)-terminal region (14). Eotaxin has inherent flexibility in this region which appears to be a common and important feature of CC chemokines in the monomeric form. The core of the structures superimpose very closely with the exception of the 30-s loop where the three proteins show considerable differences. In part this is due to the different packing against the N-terminal region, which also shows large variability between the monomer and dimer structures. In RANTES and MCP-1, the structured N-terminal regions are similar leading to the formation of comparable “end on end” dimers, whereas the equivalent residues are disordered in eotaxin (Fig. 5B). The N-terminal region is attached to the 30-s
TABLE II

Structural statistics and atomic root mean square (r.m.s.) differences for 32 calculated eotaxin structures and minimized average structure

|                      | (Eotaxin)            | Eotaxin_min       |
|----------------------|----------------------|-------------------|
| All (735)            | 0.015 ± 0.002        | 0.012             |
| Restraints (Å) ^a    |                      |                   |
| Short (1 < |j – i| ≤ 5) (74)    | 0.018 ± 0.002     | 0.017             |
| Sequential (|j – i| = 1) (209)    | 0.016 ± 0.004     | 0.013             |
| Long (|j – i| > 5) (130)     | 0.001 ± 0.002     | 0.007             |
| Intra-residue (256)  | 0.016 ± 0.003        | 0.012             |
| Dihedral restraints  | 0.148 ± 0.048        | 0.103             |
| Long (|R^2 averaged) (40) | 0.012 ± 0.001     | 0.011             |
| H bonds (28)         | 0.006 ± 0.003        | 0.007             |
| Energies (kcal mol^-1) |                   |                   |
| E_NOE                | 8.38 ± 2.160         | 5.39              |
| E_DIHE               | 0.11 ± 0.06          | 0.018             |
| E_REPEL              | 26.47 ± 2.42         | 23.1              |
| Deviations from idealized geometry ^c |               |                   |
| Bonds (Å)            | 0.0300 ± 0.0001      | 0.003             |
| Angles (degree)      | 0.52 ± 0.01          | 0.52              |
| Improper (degree)    | 0.35 ± 0.01          | 0.34              |
| Backbone atoms (9–68)| 0.55 ± 0.11          | 0.39              |
| Backbone atoms (9–30,35–68) | 0.47 ± 0.08     | 0.37              |
| Heavy atoms (9–68)   | 1.05 ± 0.10          | 0.72              |
| Heavy atoms (9–30,35–68) | 1.00 ± 0.07     | 0.72              |
| Buried heavy atoms (9–68) | 0.55 ± 0.06     | 0.42              |
| Core region          | 80.10 ± 3.0          | 77.8              |
| Additionally allowed regions | 19.20 ± 3.2 | 22.2             |
| Generously allowed regions | 0.7 ± 1.0 | 0          |
| Forbidden regions    | 0 ± 0                | 0                 |

(RMSDs from experimental ^a)

^a The r.m.s. deviation of the experimental restraints is calculated with respect to the upper and lower limits of the input restraints for 32 structures (Eotaxin) and minimized average structure (Eotaxin_min).

^b The values for E_NOE and E_DIHE are calculated from a square well potential with a force constant of 50 kcal mol^-1 Å^2 and 200 kcal mol^-1 rad^-2. E_REPEL is calculated with a force constant of 4 kcal mol^-1 Å^-4, and the final van der Waals radii were set to 0.80 times the value used in the CHARMM force field.

^c The values for bonds, angles, and impropers show the deviation from ideal values based on perfect stereochemistry.

^d Root mean square deviations from the average structure. The average structure was obtained by averaging the coordinates of the individual structures.

^e As determined by the program PROCHECK (20).
loop via the first disulfide (residues 9–34), a link that allows the 30-s loop and N terminus to undergo an apparent concerted motion. This motion leads to the apparent differences in the “average” monomer and dimer structures. The C-terminal helices in eotaxin, MCP-1, and MCP-3 are longer than RANTES and are unstructured over the last six residues. Examination reveals that the packing of the hydrophobic core in eotaxin and MCP-1 is very highly conserved. Many residues that form the core are conserved, and similar $\chi_1$ rotamers for residues Val-39, Ile-40, Phe-41, Thr-43, Ile-49, Cys-50, Trp-57, Val-58, Ser-61, and Tyr-64 in eotaxin are observed in MCP-1.

The orientation of the helix with respect to the strands is defined by the packing of hydrophobic residues of the helix and the strands including Leu-65 with Leu-26 and Phe-41 in strands 1 and 2. Interestingly, a polar residue Ser-61 is also buried and shows contacts with residues Leu-20 and Leu-23 in the $\alpha_{10}$ helix and Phe-41. The hydroxyl proton of Ser-61 is observable at 4.95 ppm, and a clear NOE is observed to the $C_e^3$H of Trp-57 confirming that the hydroxyl is well buried and possibly H-bonded to the carbonyl oxygen of Trp-57 (Fig. 6A). The hydroxyl proton and side chain protons show similar chemical shifts in MCP-1 where Ser-61 is conserved and also buried. In both eotaxin and MCP-1, Ser-61 replaces a buried hydrophobe found in other CC chemokine structures. For example, RANTES has a buried tyrosine at position 61 (Fig. 6B). To compensate and maintain adequate packing of the core, eotaxin has a buried tyrosine at position 64, whereas other CC chemokines have a charged or polar group (e.g., Ser-64 in RANTES).

**Fig. 4. Ensemble and average minimized structure of eotaxin.** A, superimposition of the 32 simulated annealing structures of eotaxin on the average structure. The average structure was generated by averaging the coordinates of the 32 final eotaxin structures. The structures were overlaid on the average structure using just the backbone N, Ca, and C atoms of residues 9–68. B, in an orientation that optimizes clarity, the heavy atoms of well ordered side chains are labeled and superimposed on the average structure. The N and C termini are omitted for clarity. C, in the same orientation, a schematic diagram showing the restrained minimized average structure of eotaxin created with the program MOLSCRIPT (38). In this representation the helix is represented as a flat ribbon and the sheet with a broad arrow. The loops and turns are represented by a slim ribbon.
Between eotaxin and MCP-1 there is also similarity in the local packing between conserved residues Ile-29 and Ser-31 and between Ser-31 and Cys-34 which predominantly defines the average structure excluding the N terminus and disulfides (residues 1–10) and the 30-s turn (residues 31–34) superimposed on MCP-1 (residues 13–32 and 37–69) with an RMSD of 1.29 Å. RANTES contains one less residue between the first β-strand and the 30-s turn so eotaxin (residues 11–28 and 34–65) was superimposed on the minimized average monomeric subunit of RANTES (residues 12–29 and 34–65) giving an RMSD of 1.19 Å. 

Fig. 5. A, comparison of the tertiary structures of eotaxin, RANTES, and MCP-1. The minimized mean structure of one monomeric unit of both RANTES (blue, protein data base accession code 1RTO) and MCP-1 (yellow, protein data base accession code 1DOM) was used for overlaying on eotaxin (red). Backbone heavy atoms of the eotaxin minimized average structure excluding the N terminus and disulfides (residues 1–10) and the 30-s turn (residues 31–34) superimposed on MCP-1 (residues 13–32 and 37–69) with an RMSD of 1.29 Å. RANTES contains one less residue between the first β-strand and the 30-s turn so eotaxin (residues 11–28 and 34–65) was superimposed on the minimized average monomeric subunit of RANTES (residues 12–29 and 34–65) giving an RMSD of 1.19 Å. B, comparison of ensembles of structures between RANTES dimer and eotaxin monomer.

Fig. 6. Comparison of the tertiary structures of eotaxin and RANTES over the monomer units. Ribbon diagrams of eotaxin (A) and RANTES (B) are displayed. Side chains of residues constituting the bulk of the hydrophobic core are displayed in yellow with their corresponding molecular surfaces. The substitution of Tyr-61 in RANTES for Ser-61 in eotaxin is highlighted by shading these residues white. This figure was prepared using the program RIBBONS (39).
conformation of the 30-s turn. Following Cys-34, the hydrophobic portion of the Gln-36 side chain packs against the aromatic ring of Phe-11, and this interaction is also clearly seen in RANTES between Phe-12 and Asn-36 (Fig. 7). The aromatic rings are displaced by approximately 1.0 Å between eotaxin and RANTES which appear to be a result of the concerted motion of the entire 30-s loop and the disordered N terminus (via the first disulfide). The side chain χ₁ of Gln-36 in eotaxin is disordered (S = 0.45), and the χ₂ of Asn-36 appears disordered in RANTES, so differences in the orientation of this group are difficult to quantify. Nevertheless the two phenylalanines are solvent-exposed and show packing to a similar region of the 30-s turn.

Structure/Function of Eotaxin—Eotaxin is in equilibrium between monomer and dimer at near physiological pH, and the calculated $K_d$ indicates that at functional concentrations (0.1 to 100 nM) eotaxin is present as a monomer. RANTES ($K_d$ 35 μM) and MIP-1β ($K_d$ 0.25 μM) are dimeric at pH 2.5, but they associate into heavily aggregated species above approximately pH 3.5 (12, 24). Conversely, 1-309 (27) and MCP-3, pH 6.5 (14), are monomeric. The other major family, the CXC chemokines, also show a variety of association states (28). Several dimerize through an alternative interface along the 1st β-strand (e.g., interleukin-8, melanoma growth stimulating activity (MGSA)); others form tetramers (e.g., NAP-2 and PF-4), whereas SDF-1 is monomeric (29). In addition, recent studies have shown that the CC chemokine MCP-1 is able to form both CXC and CC type dimer interfaces (26). There is a broad spectrum of physical behavior among the chemokine family, but no direct correlation between association properties and CCR- or CXCR-mediated function has been demonstrated.

Structure/function studies have been reported for MCP-1, RANTES, and MCP-3 (30–33), and it has been shown through truncation mutants of MCP-1, RANTES, and MCP-3 that the entire region of the N terminus preceding the first cysteine is critical for receptor binding and activation (31). In addition, alanine scanning mutagenesis of the N terminus and N-loop of RANTES has highlighted several residues important for CCR3 activation within the scaffold of the protein (34). Within the N-loop, the mutations Phe-12 to Ala and Arg-17 to Ala caused significant loss of CCR3 activation. Interestingly, the packing of Phe-11 is very similar in RANTES and eotaxin (Fig. 7), and the solvent exposure and orientation of this group may also be important for CCR3 binding in eotaxin. Similarly in eotaxin, Arg-16 is conserved, solvent-exposed, and oriented similarly to Arg-17 in RANTES suggesting a possible role for this residue in CCR3 activation. From structural and functional studies with CC and CXC chemokines, we have proposed the following scaffold hypothesis of chemokine receptor binding: A flexible N-terminal region is supported by the core of the protein and presented to the receptor in a fashion that optimizes binding and activation (29, 30, 35). This is proposed to occur through a two-step mechanism where the N-loop makes an important initial contact with the receptor followed by activation of the receptor via the N-terminal residues. The initial contact may constitute a “recognition step” responsible for receptor subtype specificity. Interestingly elements of the N-loop appear to be structurally conserved between RANTES and eotaxin, and they may provide the necessary interactions for CCR3 selectivity.

The primary sequence of the flexible N-terminal region of chemokines in itself is not always informative. Although the “ELR” motif defines chemokines that activate neutrophils via CXCR1 and CXCR2, there is no corresponding consensus sequence for the CC chemokines that is associated with CCR activation. For example, eotaxin and eotaxin-2 bind CCR3 specifically, whereas RANTES and MCP-3,4 bind CCR3 and other receptors. Some inherent flexibility may therefore be crucial whereby the N termini may adopt conformations that can equally satisfy essential ligand receptor interactions on the same receptor, despite differing primary sequences. This structural study shows that the N terminus of eotaxin is flexible in the monomeric form, and this may extend to other CC chemokine monomeric species, despite their properties in the dimeric state. We suggest that the monomeric form of eotaxin, like other chemokines, is the active species, and the flexible, exposed N terminus is critical in allowing it to adopt an orientation upon receptor binding that will trigger receptor activation.

The involvement of eotaxin in the highly selective recruit-
ment of eosinophils and eosinophil-mediated tissue damage associated with asthma suggests that eotaxin receptor binding is a possible target for anti-asthmatic drugs. Increased in vivo expression of eotaxin has also been detected in the bronchial mucosa of asthmatic patients and correlated with infiltration of eosinophils (36). Understanding the mechanisms through which eosinophils are selectively recruited is of fundamental importance and will aid the treatment of these diseases, perhaps through the blockage of eosinophil-endothelial adhesion or chemotaxis. Future studies based on the three-dimensional structure of eotaxin will address the molecular basis for CCR3 or chemotaxis.

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