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Regulation of Chemokine Recognition by Site-Specific Tyrosine Sulfation of Receptor Peptides

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SUMMARY

Sulfation of tyrosine is a common posttranslational modification of secreted proteins that influences numerous physiological and pathological processes. Studies of tyrosine sulfation have been hindered by the difficulty of introducing sulfate groups at specific positions of peptides and proteins. Here we report a general strategy for synthesis of peptides containing sulfotyrosine at one or more specific position(s). The approach provides a substantial improvement in both yield and convenience over existing methods. Using synthetic sulfopeptides derived from the chemokine receptor CCR3, we demonstrate that sulfation enhances affinity for the chemokine eotaxin by ~7-fold or more than 28-fold, depending on which of two adjacent tyrosine residues is sulfated. The synthetic methodology will substantially enhance efforts to understand the functional and structural consequences of protein tyrosine sulfation.

INTRODUCTION

Tyrosine sulfation is an increasingly recognized posttranslational modification of secreted and integral membrane proteins, playing important roles in blood coagulation, leukocyte adhesion and trafficking, hormonal regulation, retroviral and parasitic infection, and the humoral immune response (Kehoe and Bertozzi, 2000; Seibert and Sakmar, 2008). Leukocyte trafficking and blood cell infection by both HIV-1 and the malarial parasite *Plasmodium vivax* are mediated by sulfated tyrosine residues in the amino-terminal extracellular regions of chemokine receptors (Farzan et al., 1999, 2000, 2002; Choe et al., 2005; Preobrazhensky et al., 2000; Liu et al., 2008). Remarkably, HIV-1 inhibitory antibodies incorporate sulfotyrosine residues in their complementarity-determining regions, thereby mimicking recognition of HIV surface glycoprotein gp120 by chemokine receptors (Dorfman et al., 2006; Huang et al., 2007). Similarly, proteins and peptides containing tyrosine-sulfated regions of chemokine receptors can inhibit HIV infection (Cormier et al., 2000; Farzan et al., 2002), *P. vivax* infection (Choe et al., 2005), and/or chemokine binding (Choe et al., 2005; Fong et al., 2002). Consequently, there is considerable interest in determining the roles played by specific sulfotyrosine residues in recognition of chemokines and pathogen proteins.

The presence of sulfotyrosine in expressed proteins can be readily demonstrated by radioactive sulfate-labeling and by functional characterization under conditions that promote or inhibit enzymatic sulfation or promote sulfate hydrolysis. However, identification of the specific residues that are sulfated and elucidation of their functional roles is more challenging. One common approach is site-directed mutagenesis (typically Tyr→Phe mutations) of the putatively sulfated residues (Farzan et al., 1999; Choe et al., 2005; Fong et al., 2002), but these experiments report on the roles of the mutated residues rather than directly on the roles of the sulfate moieties themselves. In favorable cases, it is possible to introduce sulfate groups site-specifically by in vitro enzymatic catalysis (Veldkamp et al., 2006). However, enzymatic sulfation frequently yields a complex mixture of products that may be difficult to separate (Seibert et al., 2002). In order to study the functions of specific sulfate groups, it is therefore advantageous to complement the mutational and enzymatic approaches by using synthetic peptides containing sulfate groups on specific tyrosine residues.

To date, the synthesis of tyrosine-sulfated peptides, particularly those incorporating more than one sulfotyrosine residue, has been a considerable challenge. Available synthetic methods may be classified according to two major approaches: the chemical sulfation of peptides *after* they have been synthesized, or the incorporation of tyrosine sulfate monoesters *during* peptide synthesis (Seibert and Sakmar, 2008). However, both of these approaches have major limitations that have prevented them from being broadly useful. The chemical sulfation of tyrosine residues *after* peptide synthesis suffers from the low specificity of sulfation reagents, the need for special protecting group strategies, and the difficulty of achieving resin cleavage and side chain deprotection without degradation of the acid-labile tyrosine sulfate monoesters. Furthermore, the chemical sulfation of multiple tyrosine residues, especially in larger peptides, can suffer from incomplete reactions with sulfating reagents. Thus, chemical sulfation of peptides is generally time-consuming, low-yielding, and limited in scope. A more general approach is the incorporation of tyrosine sulfate monoesters, such as FmocTyr(SO_3Na)OH, *during* solid-phase peptide synthesis (Penke and Nyerges, 1991; Kitagawa

et al., 2001). Unfortunately, coupling of FmocTyr(SO_3Na)OH and elongation of the resulting peptide can be sluggish (Leppanen et al., 2000), and attempts to incorporate multiple sulfotyrosine residues into a peptide have been plagued by poor resin swelling and the need for extended coupling times (Young and Kiessling, 2002). Furthermore, this approach also suffers from incomplete resin cleavage and side-chain deprotection as well as some hydrolysis of sulfotyrosine during TFA treatment, thus frequently resulting in low overall yields of the desired peptides.

In this article, we propose a broadly applicable method for the synthesis of tyrosine-sulfated peptides. By synthesis of previously studied peptides, we demonstrate the advantages of this new method over existing approaches. In addition, by applying the new method to peptides derived from the N-terminal region of a chemokine receptor, we demonstrate that chemokine recognition is sensitively dependent on both the presence and the specific position of sulfotyrosine residues.

RESULTS AND DISCUSSION

Peptide Synthesis Strategy

The major difficulty of incorporating sulfotyrosine derivatives during peptide synthesis is the instability of the tyrosine sulfate monoester, especially under acidic conditions. We have recently described the development of acid-stable alkyl protecting groups for sulfate monoesters (Simpson and Widlanski, 2006). In the work presented here, we utilized the neopentyl protecting group for sulfate monoesters in the high-yielding synthesis of tyrosine-sulfated peptides. The general strategy involves the incorporation of a neopentyl-protected sulfate monoester of tyrosine into a growing peptide chain (Figure 1). Resin cleavage and deprotection of the completed peptide with TFA can be achieved with minimal degradation of the protected sulfate monoesters and the neopentyl group can be removed under mild conditions to reveal a sulfotyrosine residue.

Synthesis of the Protected Sulfotyrosine Monomer

For the production of tyrosine-sulfated peptides by this strategy, the requisite Fmoc-protected sulfotyrosine monomer was synthesized in high overall yield (Figure 2). A protected tyrosine derivative was treated with neopentyl chlorosulfate (NPCS) to produce sulfate monoester **1** in 94% yield. Treatment of this compound with 90% aqueous TFA removed Boc and *t*-butyl groups, and an Fmoc protecting group was installed by stirring the resulting compound with *N*-(9-fluorenylmethoxycarbonyloxy) succinimide in dioxane and 10% aqueous sodium carbonate. Purification by column chromatography gave compound **2** as a white solid in 70% yield for the two combined steps. Benzyloxycarbonyl (Cbz) and benzyl protecting groups were not used in this synthesis as previously reported (Simpson and Widlanski, 2006) because the removal of these groups under hydrogenolysis conditions (Pd/C and H_2) results in partial reduction (~10%) of the neopentyl-protected sulfate monoester of tyrosine to phenylalanine. The reduction product cannot be easily separated from the desired protected sulfate monoester.

Synthesis of Sulfotyrosine-Containing Peptides

We first tested the proposed synthesis strategy by preparing two tyrosine-sulfated peptides that had been previously synthesized

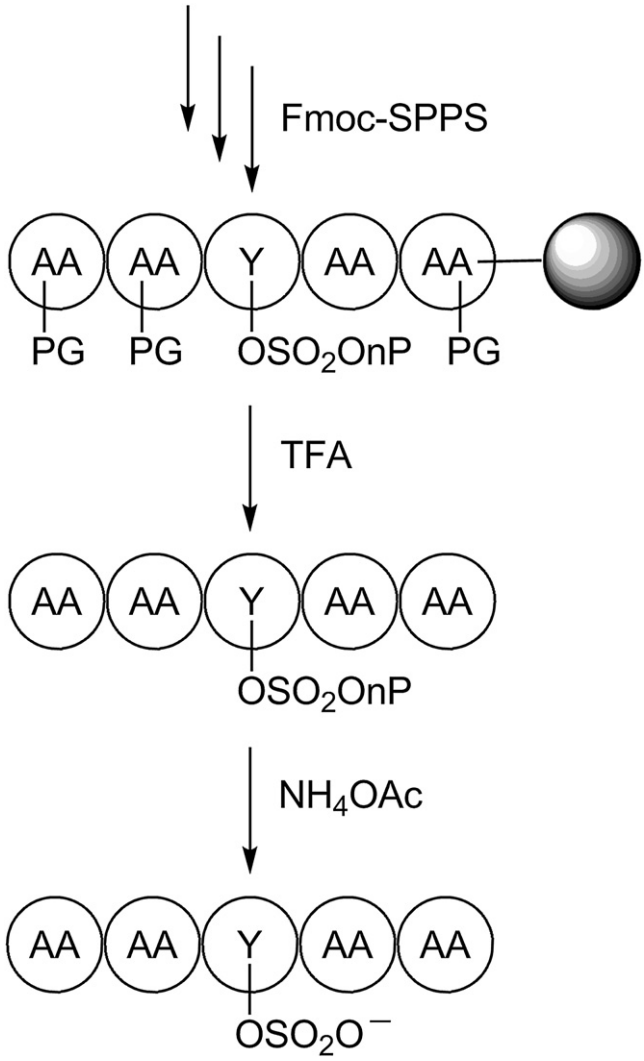

Figure 1. General Strategy for the Chemical Synthesis of Tyrosine-Sulfated Peptides

SPPS represents solid-phase peptide synthesis; PG, side-chain protecting group; nP, neopentyl.

by other methods: peptide **4**, corresponding to residues 5–12 of the cell adhesion molecule P-selectin glycoprotein ligand-1 (PSGL-1), and peptide **6**, the human digestive hormone cholecystokinin (CCK)-12 (Figure 3). Standard diisopropylcarbodiimide-hydroxybenzotriazole protocols were used in the manual or automated coupling of all Fmoc amino acids and the coupling of tyrosine derivative **2** required neither special conditions nor extended reaction times. Deblocking of Fmoc groups after each coupling was accomplished by treatment with 20% piperidine. Although standard resins should be useful in this strategy, the highly acid-sensitive 2-chlorotrityl resin was utilized in the synthesis of peptide acids and the Sieber amide resin in the synthesis of a peptide amide. These resins allow for quantitative cleavage and convenient isolation of fully protected peptides with 1%–3% TFA in dichloromethane. Following detachment of the protected peptides, side-chain deprotection was achieved by treatment with 95% TFA and scavengers at room

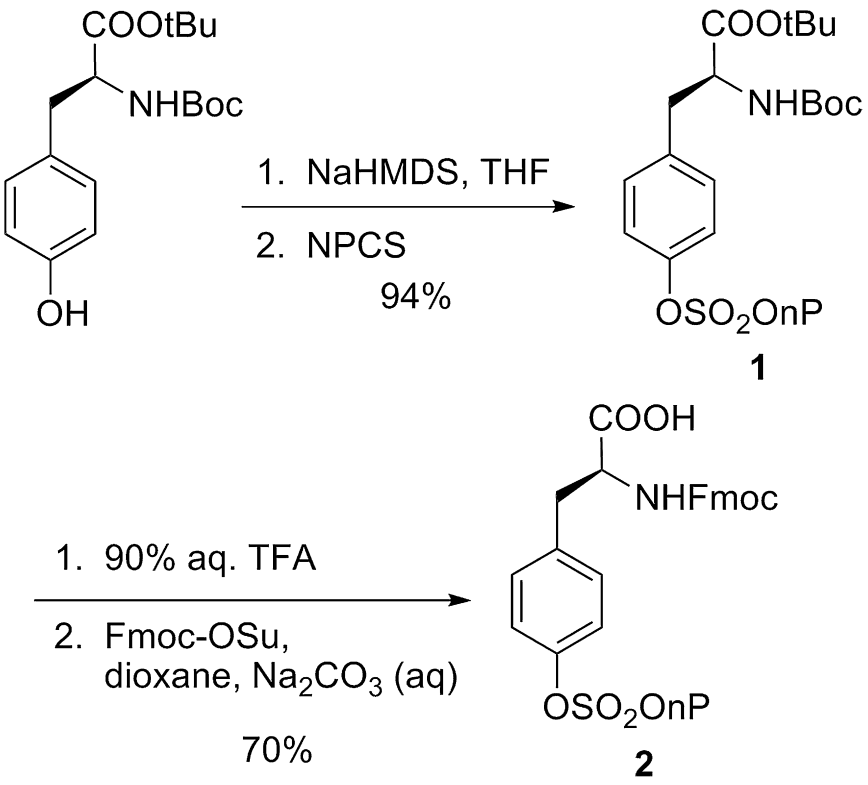

Figure 2. Synthesis of the Protected Sulfotyrosine Monomer
NPCS represents neopentyl chlorosulfate; nP, neopentyl.

temperature. The neopentyl-protected sulfate monoester remained intact during these steps, with little or no degradation.

Neopentyl groups can be removed by treatment with unhindered nucleophiles in a hot polar aprotic solvent. However, an unanticipated result led to the discovery of milder and more convenient conditions for deblocking. When the neopentyl-protected PSGL-1 peptide **3** was dissolved in aqueous ammonium acetate, loss of the neopentyl group and preservation of the resulting sulfate monoester was observed. Treatment with aqueous sodium acetate produced a similar result, whereas treatment with aqueous ammonium chloride resulted in precipitation of the peptide. Although neopentyl protecting groups are exceedingly stable in organic solvents, they appear to be susceptible to acid-catalyzed solvolysis in polar protic solvents. Indeed, acetolysis of neopentyl sulfonates proceeds via an assisted S_N1 reaction with methyl participation and rearrangement (Ando et al., 1976). Thus, crude neopentyl-protected sulfotyrosine peptides **3** and **5** (Figure 3) were dissolved in a minimum amount of dimethylsulfoxide, diluted with 1-2 M ammonium acetate and placed in a 37°C water bath for 6–12 hr. The resulting sulfate monoesters are stable in ammonium acetate buffer (pH 7) and were loaded directly onto a reversed-phase high-performance liquid chromatography (RP-HPLC) column for purification.

Using these procedures, the monosulfated PSGL-1-derived peptide **4** was produced in 98% overall yield based on resin loading. In contrast, a reported synthesis of the *N*-acetylated form of this peptide was accomplished in 5% overall yield using chemical sulfation with sulfur trioxide/dimethyl formamide complex (Young and Kiessling, 2002); *N*-terminal acetylation was required to avoid the formation of a sulfamate upon treatment with sulfur trioxide. Similarly, a peptide amide, CCK-12 sulfate **6**, was produced in 85% overall yield based on resin loading. This peptide has previously been synthesized via the incorporation of FmocTyr(SO_3Na)OH during solid-phase synthesis (Kitagawa et al., 2001). Apparently, the reported yield of 26% from the protected-peptide resin does not include the yield of coupling steps. Thus, for both peptides **4** and **6**, the approach used in the present work represents a substantial improvement over existing methods for the synthesis of sulfotyrosine-containing peptides.

Peptides Derived from Chemokine Receptor CCR3

We have used this peptide synthesis strategy to explore the role of tyrosine sulfation in recognition of the chemokine eotaxin by its receptor CCR3. CCR3 is expressed primarily on eosinophils, basophils, and Th2 cells, and activation of CCR3 by eotaxin regulates the recruitment of these cell types in allergic responses and parasitic infections (Jose et al., 1994; Rankin et al., 2000; Yawalkar et al., 1999). In addition, CCR3 can support HIV infection in vitro, although its function in HIV pathology is not well established (Choe et al., 1996; He et al., 1997). The amino-terminal extracellular region of CCR3 is sulfated when expressed in HeLa cells as a fusion to the Fc domain of immunoglobulin IgG1 (Farzan et al., 1999). This region contains two consecutive tyrosine residues (Tyr-16 and Tyr-17) sequentially adjacent to acidic residues, a common feature of tyrosine sulfation sites (Figure 3). Based on comparisons of sequence context with known sulfated and nonsulfated tyrosines, Tyr-17 is strongly predicted to be sulfated, and Tyr-16 is considered more likely than not to be sulfated (Liu et al., 2008). A 35-residue nonsulfated peptide corresponding to the complete N-terminal region (i.e., preceding the first transmembrane helix) binds to eotaxin with an equilibrium dissociation constant (K_d) of 80 ± 38 μM (Ye et al., 2000). Nuclear magnetic resonance (NMR) experiments indicated that the central region of this peptide is involved in the interaction with eotaxin and a truncated, nonsulfated peptide (residues 8–23) binds to eotaxin with a dissociation constant of about 136 ± 23 μM, only slightly weaker than the full-length *N*-terminal peptide (Ye et al., 2000). Considering that this shorter peptide encompasses the two putative tyrosine sulfation sites, we hypothesized that sulfation of this peptide at one or both positions would substantially enhance the affinity of chemokine binding. To test this hypothesis, we synthesized four 16-mer peptides (Figure 3) corresponding to residues 8–23 of CCR3 with sulfation on each of the tyrosine residues (peptides Su16

Peptide	Sequence	Yield
3	YEYLDYDF	
4	YEYLDYDF	98%
5	ISDRDYMGWMDF-$CONH_2$	
6	ISDRDYMGWMDF-$CONH_2$	85%

Peptide	Sequence	Yield
7 (Su0)	VETFGTTSY$_{16}$Y$_{17}$DDVGLL	55%
8	VETFGTTSY$_{16}$Y$_{17}$DDVGLL	
9 (Su16)	VETFGTTSY$_{16}$Y$_{17}$DDVGLL	68%
10	VETFGTTSY$_{16}$Y$_{17}$DDVGLL	
11 (Su17)	VETFGTTSY$_{16}$Y$_{17}$DDVGLL	72%
12	VETFGTTSY$_{16}$Y$_{17}$DDVGLL	
13 (Su1617)	VETFGTTSY$_{16}$Y$_{17}$DDVGLL	65%

Figure 3. Sequences and Yields of the Peptides Synthesized in this Study

Neopentyl-protected sulfotyrosine residues are highlighted in bold green, sulfotyrosine residues are highlighted in bold red. Each indicated yield is the overall percent yield of the final tyrosine-sulfated peptide based on initial resin loading.

and Su17), with sulfation on both tyrosine residues (Su1617) and without sulfation (Su0).

The nonsulfated peptide (Su0) was synthesized using the solid phase methods described above, except that double or triple couplings of the final three *N*-terminal residues (T10, E9, and V8) were required due to the slow rate of the coupling reactions at these positions. Peptide Su0 (**7**) was obtained in 55% overall yield. The three neopentyl-protected sulfotyrosine peptides **8**, **10**, and **12** were synthesized in the same manner, and the sulfotyrosine residues were deprotected by dissolving the peptides in aqueous ammonium acetate and heating to 37°C to yield the desired sulfated peptides Su16 (**9**), Su17 (**11**), and Su1617 (**13**) in 68%, 72%, and 65% overall yields, respectively. Despite the need for the final sulfate monoester deprotection step and the use of the bulky neopentyl protecting groups during the synthesis, the mono- and bis-sulfated peptides were obtained in higher yield than the corresponding nonsulfated peptide, further demonstrating the utility of the synthesis strategy.

Eotaxin Binding by CCR3-Derived Peptides

Binding of each peptide to eotaxin was monitored by collecting ^{1}H-^{15}N correlation NMR spectra of uniformly ^{15}N-enriched eotaxin in the presence of increasing concentrations of each unlabeled peptide (Figures 4A–4C). For each peptide, the observed changes in eotaxin chemical shifts were fit globally for all resonances that shifted in response to peptide binding, yielding the equilibrium dissociation constant (K_d) for that peptide (Figures 4D–4F). In agreement with our previous observations (Ye et al., 2000), Su0 bound to eotaxin with an equilibrium dissociation constant (K_d) of 140 ± 40 μM and induced concentration-dependent $^{1}H_N$ chemical shift changes of 0.01–0.04 ppm for a small number (8) of amide resonances (Figure 4G). In contrast, Su17 bound to eotaxin with a 7-fold enhanced affinity (K_d = 22 ± 4 μM) and induced considerably larger chemical shift changes of the same amide resonances as well as several additional resonances (Figure 4G). Interestingly, Su16 and Su1617 bound to eotaxin too tightly for the dissociation equilibrium constants to be determined from chemical shift changes under the conditions of the NMR experiment, indicating that the K_d values for these interactions are less than ~5 μM. Thus, the influence of tyrosine sulfation on chemokine binding affinity is not only substantial but also dependent on the specific position of the sulfate group. This result raises the possibility that regulation of sulfation pattern might be an effective mechanism for regulating the chemokine affinity and responsiveness of chemokine receptors.

The ~7-fold to more than 28-fold affinity enhancements observed for the CCR3-derived peptides compare favorably with those observed previous for sulfated peptides derived from the N-terminal regions of other chemokine receptors. Fong et al. reported that sulfation of a single Tyr residue in a peptide from receptor CX_3CR1 provided a 10-fold enhancement of binding affinity for the chemokine fractalkine (Fong et al., 2002). Similarly, Duma et al. found that a bis-sulfated peptide derived from the N-terminal region of chemokine receptor CCR5 bound stoichiometrically to the chemokine RANTES (at 200 μM concentration), indicating a K_d value lower than ~20 μM in comparison with 168 μM for the corresponding unsulfated peptide (Duma et al., 2007); the fitted K_d value for the sulfated peptide was 1.2 μM. In addition, Veldkamp et al. observed a ~3.5-fold enhancement of binding affinity for the chemokine SDF-1α upon sulfation of a single Tyr residue in an N-terminal peptide from chemokine receptor CXCR4 (Veldkamp et al., 2006) and a further enhancement of affinity upon sulfation of three Tyr residues in the same peptide (Seibert et al., 2008). Considering that chemokines are generally highly basic, it is perhaps not surprising that their interactions with receptor peptides should be enhanced by the addition of negative charges to those peptides. However, previous mutational studies of sulfated Tyr residues in chemokine receptors (Farzan et al., 2002; Fong et al., 2002; Bannert et al., 2001) have shown that interactions with chemokines are sensitive to the specific Tyr residue that has been replaced, suggesting that the position, rather than merely the presence, of sulfate is important. The present results support this conclusion by demonstrating that substantial but different enhancements of chemokine affinity result from sulfation at each of two distinct positions.

Possible Sulfotyrosine Binding Sites

Previous NMR studies (Ye et al., 2000; Skelton et al., 1999; Clubb et al., 1994; Mayer and Stone, 2000) have indicated that N-terminal peptides from chemokine receptors interact with the surface of their cognate chemokines defined by residues in the N-loop and β2-β3 hairpin structural elements (structural elements are shown in Figures 5A and 5B); we refer to this surface as the "front face" of the chemokine (the face shown in Figures 5A, 5C, and 5E). The NMR titration data presented herein

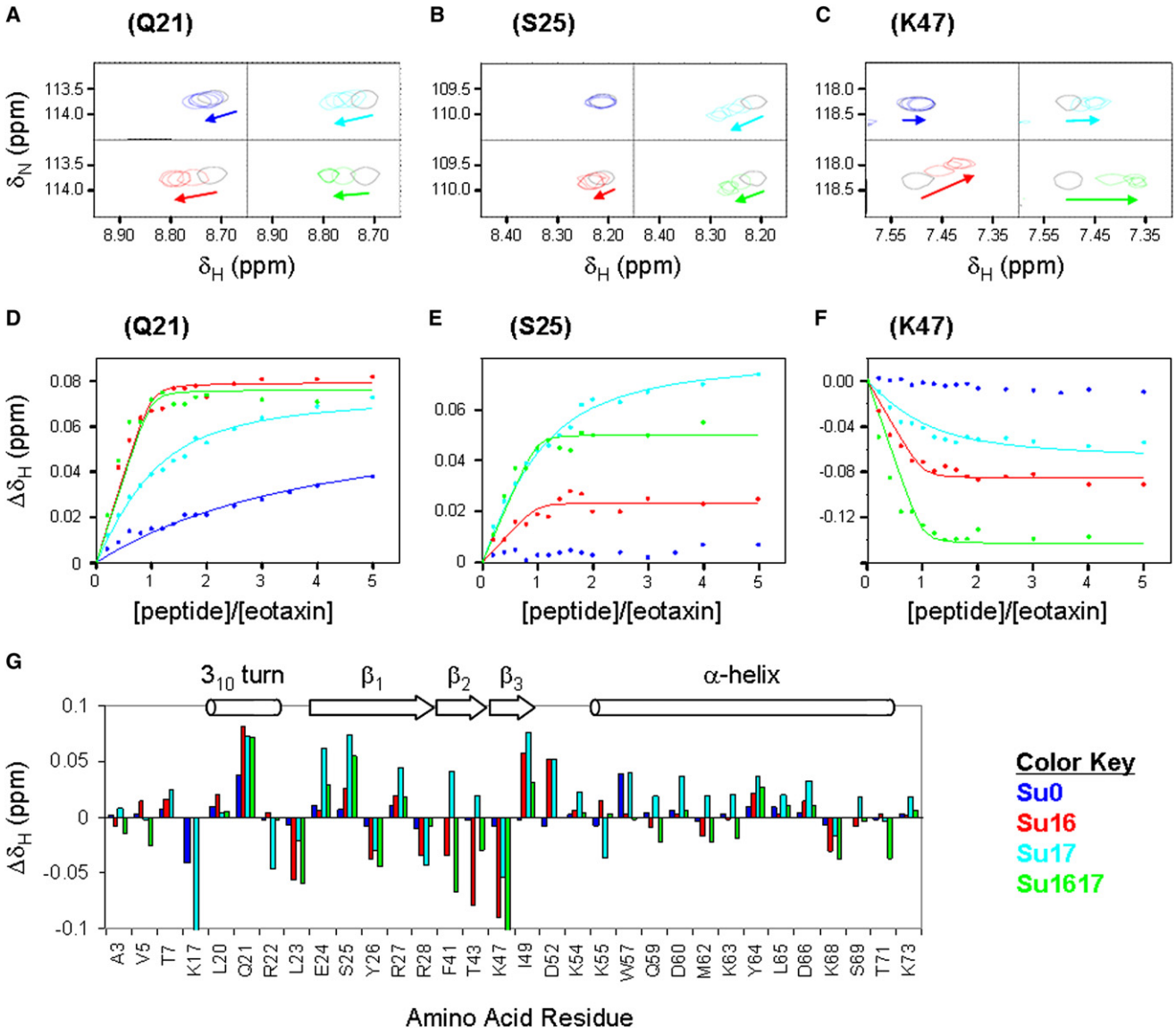

Figure 4. NMR Data for CCR3-Derived Peptides Binding to Eotaxin

(A–C) Expanded regions of the ^{1}H-^{15}N correlation (HSQC) spectrum of ^{15}N-labeled eotaxin containing the backbone amide peaks for residues Q21 (A), S25 (B), and K47 (C). Each region is shown in the absence of peptide (gray contour in each of the four panels) and in the presence of 0, 0.6, 1, 2, and 4 equivalents of Su0 (blue contours), Su16 (red contours), Su17 (cyan contours), and Su1617 (green contours) in separate panels. Arrows indicate the direction of peak movement.

(D–F) Graphs showing the change in backbone $^{1}H_N$ chemical shift for Q21 (D), S25 (E) and K47 (F) as a function of the molar ratio of added peptide to ^{15}N-labeled eotaxin. Uncertainties in $^{1}H_N$ chemical shifts are approximately 0.004 ppm. Solid lines represent the fitted binding curves obtained separately for each peptide by global analysis of all the binding data for that peptide (see text for details). Data and fitted curves are colored for each peptide as in (A–C).

(G) Bar graph showing the maximum changes in backbone $^{1}H_N$ chemical shift observed for specific residues of eotaxin upon addition of Su0 (blue), Su16 (red), Su17 (cyan), and Su1617 (green); data are shown for all residues for which significant changes in chemical shifts were observed upon binding to one or more peptide(s). Uncertainties in maximum shifts are approximately 0.004 ppm. The positions of secondary structure elements are indicated schematically at the top of the graph.

provide insights into the possible binding sites for the peptide sulfate groups on this surface of eotaxin (Crump et al., 1998). Chemical shift data were obtained for 28 of 65 backbone NH groups in eotaxin, predominantly located in the secondary structure elements (Figure 4G); other NH groups were not observed due to either resonance overlap, conformational exchange broadening, or fast exchange with solvent. In the absence of sulfation (peptide Su0), the largest chemical shift changes are observed for residues Lys-17, Gln-21, and Trp-57 (blue in Figures 5C and 5D and Figure S20), suggesting that the peptide comes into contact with the top part of the front face, possibly extending toward the α helix. Sulfation of each tyrosine residue results in additional chemical shift changes for a different (but overlapping) set of residues and the changes observed for peptide Su1617 are generally similar to those observed for one or both of the monosulfated peptides (Figure 4G).

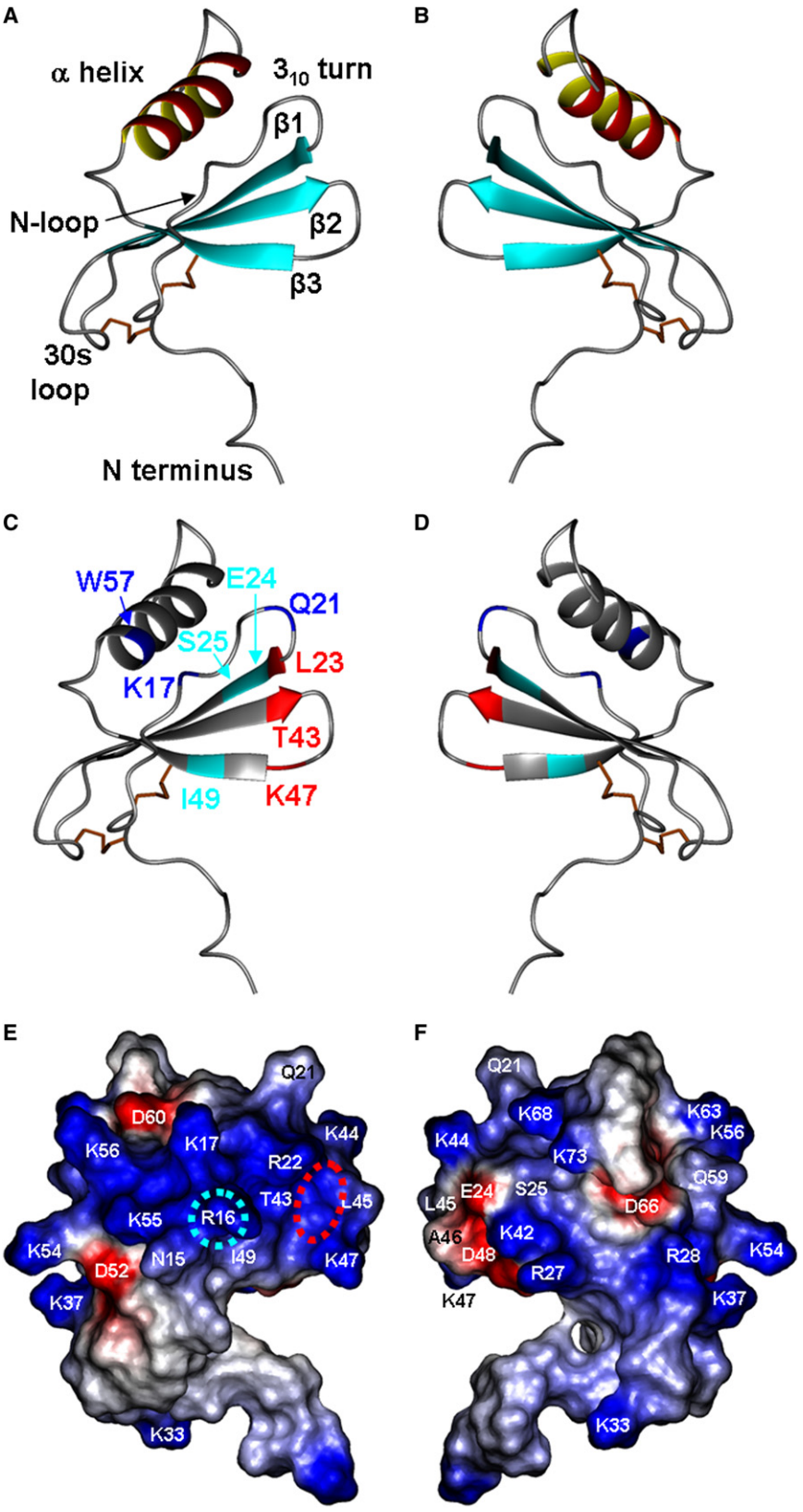

Figure 5. Structure of Eotaxin Showing the Proposed Locations of Bound Sulfotyrosine

Each panel shows front (left) and back (right) views of the structure.

(A and B) Ribbon representation with the locations of structural elements indicated.

(C and D) Ribbon representation with residues showing the largest shifts upon binding to Su0 colored blue and residues that show substantial additional shifts upon binding to Su16 and Su17 colored red and cyan, respectively.

(E and F) Surface electrostatic potential representations (blue represents positive, red is negative) showing the proposed location of the Su16 sulfate group (red circle) and a possible location of the Su17 sulfate group (cyan circle).

Sulfation of Tyr-16 causes substantial additional shifts (relative to peptide Su0) for the backbone amide resonances of Leu-23, Thr-43, and Lys-47 (red in Figures 5C, 5D, and S20). These three residues are coupled to each other through a series of cross-strand hydrogen bonds at the right-hand end of the β sheet, near the β2-β3 hairpin turn and this region of the structure includes a cluster of positively charged residues (Arg-22, Lys-44, and Lys-47), suggesting that this cluster forms the binding site for sulfotyrosine-16 of the CCR3 peptide (indicated by the red oval in Figure 5E). In previous studies of interactions between chemokines and sulfotyrosine-containing peptides, the equivalent residue to Lys-47 (Arg-47 in both SDF-1α and RANTES) has been implicated as a binding site for the sulfate group (Veldkamp et al., 2006; Duma et al., 2007). Thus, this might be a conserved sulfotyrosine recognition element on chemokine surfaces.

In contrast to the observations with Su16, sulfation of Tyr-17 causes substantial shifts (in addition to those caused by Su0) for the backbone amide resonances of Glu-24, Ser-25, and Ile-49 (cyan in Figures 5C, 5D, and S20). These three residues are located in the central region of the β sheet and their backbones are connected to each other, via Phe-41 and Lys-42, through a series of cross-strand hydrogen bonds. Thus, the chemical shift changes for these residues are likely to result from interaction of sulfotyrosine-17 with either the front or back face of the β sheet, and adjacent regions of the structure. The back face (Figure 5F) includes several positively charged residues (Arg-27, Lys-42, Lys-44, Lys-68, and Lys-73) and a substantial patch of negative potential resulting from acidic residues (Glu-24 and Asp-48). If sulfotyrosine-16 binds in the region discussed above, interaction of sulfotyrosine-17 with the back face would require that the peptide chain forms a tight turn between sulfotyrosine residues 16 and 17 and that the chain orientation is substantially different from that found previously for the complex of interleukin-8 (IL-8) with a peptidomimetic derived from its receptor CXCR1 (Skelton et al., 1999) and for the complex of SDF-1 with a CXCR4 peptide (*vide infra*) (Veldkamp et al., 2008). Alternatively, if sulfotyrosine-17 is positioned on the front face of eotaxin, it could potentially interact with the side chains of β3-strand residue Ile-49, on the floor of the hydrophobic channel, and N-loop residue Arg-16, on the chemokine surface, in the region indicated by the cyan circle in Figure 5E. Formation of these interactions would be consistent with the peptide chain being extended in an orientation similar to that found for the CXCR1/IL-8 and CXCR4/SDF-1 interactions (Skelton et al., 1999; Veldkamp et al., 2008). Recently, Veldkamp et al. have reported the structure of an obligate SDF-1 dimer bound to a triply sulfated N-terminal peptide from CXCR4 (Veldkamp et al., 2008). In this structure, sulfotyrosine-21 interacts with the site proposed for sulfotyrosine-16 of the CCR3 peptide in the current study (*vide supra*). In addition, residues 17-20 of the peptide form a turn around the β3 strand of the chemokine allowing the amino-terminal region of the peptide to extend across the dimer interface; sulfotyrosine-12 interacts with the back of the β1 strand of one monomer and sulfotyrosine-7 interacts with the α-helix of the other monomer. The mode of interaction observed for the CXCR4 peptide with SDF-1 is not possible for the CCR3 peptide binding to eotaxin because: (1) eotaxin was monomeric under the condition of our study; (2) CC chemokines dimerize in a structurally distinct manner from CXC chemokines; and (3) the spacing of sulfotyrosine residues is quite different for CCR3 versus CXCR4. Thus, it appears that recognition of the tyrosine-sulfated N-terminal regions of receptors might differ substantially between CC and CXC chemokines. Characterization of these differences will require detailed structural analysis of additional complexes.

SIGNIFICANCE

We have described a new strategy to enable the production of sulfotyrosine-containing peptides in high yield and have demonstrated the utility of the approach for obtaining insights into sulfotyrosine recognition by proteins. The synthetic strategy uses standard Fmoc solid-phase synthesis procedures and, except for the neopentyl-protected sulfotyrosine derivative, no other specially protected amino acids are required. In contrast to other methods, this strategy has been applied in automated peptide synthesis and should be scalable to the production of larger quantities without difficulty.

The synthesis strategy described herein permits the facile synthesis of differentially sulfated and multiply sulfated peptides. Using this approach, we have demonstrated that sulfation of a 16-residue peptide from the chemokine receptor CCR3 enhances the affinity of this peptide for its cognate chemokine by ~7-fold or more than 28-fold, depending on which of two adjacent tyrosine residues is sulfated. Considering that the N-terminal regions of most chemokine receptors contain multiple tyrosine residues that are known or predicted to be sulfated, it is likely that similar enhancements of affinity will be obtainable by site-specific sulfation of peptides from other chemokine receptors. More generally, the synthesis approach will enable the functional roles of sulfate groups on tyrosine residues to be determined for peptides derived from any sulfotyrosine-containing protein. Furthermore, the synthetic approach could potentially be combined with native chemical ligation methods (Dawson et al., 1994), allowing access to intact proteins with tyrosine sulfation at specific positions. Structural studies of such peptides and proteins in complex with their binding partners will provide a detailed understanding of sulfotyrosine recognition in numerous protein families. In summary, by providing ready access to site-specifically sulfated peptides, the synthesis approach described herein will overcome a major barrier to studying the biochemical function of tyrosine sulfation.

EXPERIMENTAL PROCEDURES

General Information

Resins were purchased from Novabiochem (EMD Biosciences) and Fmoc-amino acid derivatives were purchased from Anaspec, Inc. Arginine residues were protected with the 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) group, and standard *t*-butyl and Boc protecting groups were used on other amino acid side chains. Diisopropylcarbodiimide (DIPCDI), hydroxybenzotriazole (HOBt), and all other chemicals were of analytical grade and were used without further purification. Uniformly ^{15}N-labeled eotaxin was expressed and purified as described previously (Ye et al., 2000). Reactions were done in flame-dried flasks under a dry nitrogen or argon atmosphere and using

magnetic stirring. Automated peptide synthesis was performed on an Applied Biosystems 433A Peptide Synthesizer. Manual peptide synthesis was performed in Bio-Rad Poly-Prep chromatography columns. Flash column chromatography was performed with 60 Å 230–400 mesh silica gel. ^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively. Fourier transformed infrared spectra were recorded as neat liquids or as thin films (obtained by evaporation from chloroform). High-resolution mass spectra were obtained by the methods indicated.

Synthesis of Sulfotyrosine Monomer

N-(Tert-Butoxycarbonyl)-L-Tyrosine Tert-Butyl Ester Neopentyl Sulfate (1)

N-(*tert*-butoxycarbonyl)-L-tyrosine *tert*-butyl ester (Schlienger et al., 2000) (6.9 g, 20.4 mmol) was dissolved in 200 ml dry THF and the resulting solution was cooled to −75°C in a dry ice/isopropanol bath. Sodium bis(trimethylsilyl)amide (1.0 M solution in THF, 22.5 ml, 22.5 mmol, 1.1 Eq) was added dropwise to the cooled solution and stirred for 2 min. Neat neopentyl chlorosulfate (Simpson and Widlanski, 2006) (3.9 ml, 24.5 mmol, 1.2 Eq) was added quickly and the reaction mixture allowed to warm to room temperature. Upon completion (thin-layer chromatography), ethyl acetate and water were added. The organic layer was separated and washed with 5% aqueous citric acid and brine, then dried over $MgSO_4$ and concentrated in vacuo. Purification by silica gel chromatography (5→20% ethyl acetate in hexanes) gave **1** as clear viscous oil (9.39 g, 94%): ^{1}H NMR (400 MHz, MeOH-d_4) δ 7.32 (d, J = 8.4 Hz, 2H), 7.26 (d, J = 8.8 Hz, 2H), 4.25 (dd, J = 6.4, 8.4 Hz, 1H) 4.11 (s, 2H), 3.08 (dd, J = 6.4, 13.6 Hz, 1H), 2.94 (dd, J = 8.8, 13.6 Hz, 1H), 1.40 (s, 9H), 1.39 (s, 9H), 0.98 (s, 9H); ^{13}C NMR (100 MHz, MeOH-d_4) δ 172.51, 157.54, 150.42, 138.09, 131.94, 122.06, 84.42, 82.81, 80.46, 56.83, 38.09, 32.69, 28.70, 28.21, 26.24; IR (film) 2976, 1716, 1505, 1409, 1393, 1368, 1208, 1153, 965; HRMS (EI) m/z calcd for $C_{23}H_{38}NO_8S$ ($M+H^+$) 488.2313, found 488.2299.

N-(9-Fluorenylmethoxycarbonyl) L-Tyrosine Neopentyl Sulfate (2)

tert-Butyl *N*-(*tert*-butoxycarbonyl)-L-tyrosine neopentyl sulfate **1** (9.30 g, 19.07 mmol) was dissolved in 120 ml trifluoroacetic acid and 6 ml water. After stirring solution for 1 hr, TFA and water were removed in vacuo. The resulting residue was dissolved in 80 ml dioxane and 160 ml 10% aqueous Na_2CO_3. *N*-(9-fluorenylmethoxycarbonyloxy)-succinimide (7.39 g, 21.0 mmol, 1.1 Eq) was added and the reaction was stirred overnight. Upon completion, the reaction mixture was acidified (pH 3) and the product was extracted several times with CH_2Cl_2. The combined extracts were dried over $MgSO_4$ and concentrated in vacuo. Purification by silica gel chromatography (2→10% methanol in CH_2Cl_2) gave **2** as a white solid (7.38 g, 70%): ^{1}H NMR (400 MHz, MeOH-d_4) δ 7.73 (d, J = 7.6 Hz, 2H), 7.56 (m, 2H), 7.37-7.21 (m, 8H), 4.43 (m, 1H), 4.30-4.19 (m, 2 H), 4.11 (t, J = 6.8 Hz, 1H), 4.04 (s, 2H), 3.23 (dd, J = 4.6, 14 Hz, 1H), 2.96 (dd, J = 9.6, 14 Hz, 1H), 0.904 (s, 9H); ^{13}C NMR (100 MHz, MeOH-d_4) δ 174.69, 158.23, 150.48, 145.14, 142.47, 138.23, 131.90, 128.73, 128.11, 126.21, 122.02, 120.88, 84.52, 67.88, 56.49, 48.23, 37.78, 32.64, 26.15; IR (film) 2961, 1690, 1575, 1504, 1406, 1206, 1150, 1049, 960, 883; HRMS (ESI) m/z calcd for $C_{29}H_{32}O_8NS$ ($M+H^+$) 554.1849, found 544.1858.

Synthesis of Tyrosine-Sulfated Peptides: Representative Example

PSGL-1-Derived Peptide 4

Automated solid-phase peptide synthesis was done on a 0.1 mM scale with a preloaded H-Phe-2-ClTrt resin (substitution = 0.60 mmol/g, 0.168 g, 0.10 mmol). Fmoc amino acids were incorporated by the DIPCDI-HOBt coupling protocol in NMP: Fmoc amino acid (10 Eq), DIPCDI (10 Eq), and HOBt (10 Eq). Amino acids were preactivated for 20 min, and resin coupling was allowed to proceed for 40 min. After each coupling, Fmoc groups were cleaved by a 3 min treatment with 20% piperidine in NMP, followed by a second treatment for 11 min. After completion of the automated synthesis, the protected peptide-resin was treated eight times with 1% TFA in CH_2Cl_2 (4 ml, 5 min each), with filtering after each treatment. The combined filtrate was concentrated in vacuo to remove CH_2Cl_2 and the resulting residue was treated with 90% TFA (5% methanol and 5% triisopropylsilane, 2.5 ml total) at room temperature for 45 min. Diethyl ether was added and the resulting precipitate was collected by centrifugation, washed once with diethyl ether, and dried under vacuum to give the crude neopentyl-protected tyrosine-sulfated peptide **3** (0.126 g). liquid chromatography mass spectrometry electrospray-ionization (LCMS-ESI) (positive) m/z: calculated for $C_{60}H_{77}N_8O_{21}S$ 1277.49 ($M+H^+$), found 1278.0.

A portion of the crude peptide (0.050 g) was weighed into a large centrifuge tube and dissolved with a minimum amount of dimethyl sulfoxide (0.5 ml). The solution was then diluted to 50 ml with 2 M NH_4OAc, capped, and placed in a 37°C water bath. After 6 hr, the fully deprotected peptide was loaded directly onto a RP-HPLC column (2.2 × 25 cm Kromasil C18) equilibrated with 0.1 M NH_4OAc. The peptide was eluted with a gradient of 0.1 M NH_4OAc and CH_3CN at a flow rate of 5 ml/min with absorbance detection at 225 nm. The solvent was removed by lyophilization and the residue was dissolved in water and again lyophilized to give **4** as a fluffy powder (0.048 g, 98% overall yield). The sample exhibited a single peak on an analytical HPLC chromatogram. LCMS-ESI (positive) m/z: calculated for $C_{55}H_{67}N_8O_{21}S$ 1207.41 ($M+H^+$), found 1208.0 and m/z: calculated for $C_{55}H_{70}N_9O_{21}S$ 1224.44 ($M+NH_4^+$), found 1225.0.

NMR Titrations of Eotaxin with Peptides

Titrations of ^{15}N-labeled eotaxin with synthetic peptides were performed using initial samples of ^{15}N-labeled eotaxin (50 μM, 600 μl) in 20 mM sodium acetate, 0.02% NaN_3, and 10% D_2O (pH 6.5). The peptide, in the same buffer, was added in several aliquots from a 1 mM stock solution to give final molar ratios ([peptide]/[protein]) of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2, 2.5, 3, 4, and 5; the final volume was 750 μl. For the initial sample and after each addition, a gradient sensitivity-enhanced ^{15}N-HSQC spectrum (Kay et al., 1992) was recorded using spectral widths of 2,100 Hz and 10,000 Hz and 128 and 2048 complex data points in the ^{15}N and ^{1}H dimensions, respectively. Spectra were recorded at 4°C on a Varian NMR Systems 600 MHz spectrometer equipped with a triple-resonance Cold Probe. Chemical shifts were referenced to external DSS.

Analysis of Binding Data

NMR data were processed using NMRPipe then visualized and analyzed using Sparky. Spectral assignments were made by comparison to the published assignments (Crump et al., 1998; Ye et al., 1999). $^{1}H_N$ chemical shift changes (Δδ) were measured in Sparky for each residue at each peptide concentration. For each peptide, the data were then fit simultaneously, using the software OriginPro 8, for all residues whose $^{1}H_N$ chemical shift changes in the final titration point were ≥ 0.02 ppm. Data were fit to the 1:1 binding equation:

$$\Delta\delta = 0.5\Delta\delta_{max}\left(\left(1+r+\frac{K_d}{Ce}\right)-\sqrt{\left(1+r+\frac{K_d}{Ce}\right)^2-4r}\right)$$

in which: $\Delta\delta_{max}$ is the maximum change in chemical shift (fit individually for each residue); K_d is the equilibrium dissociation constant (fit globally for all residues), r is the molar ratio ([peptide]/[protein]) at each titration point, and C_e is the concentration of eotaxin at each titration point.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, twenty figures, and one table and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00005-2.

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