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# Pattern and Temporal Sequence of Sulfation of CCR5 N-Terminal Peptides by Tyrosylprotein Sulfotransferase-2: An Assessment of the Effects of N-Terminal Residues[†]

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ABSTRACT: CC chemokine receptor 5 (CCR5) is the receptor for several inflammatory chemokines and is a coreceptor for HIV-1. Posttranslational sulfation of tyrosines in the N-terminal regions of chemokine receptors has been shown to be important in the binding affinity for chemokine ligands. In addition, sulfation of CCR5 is crucial for mediating interactions with HIV-1 envelope protein gp120. The major sulfation pathway for peptides derived from the N-terminal domains of CCR5 and CCR8 and variations of the peptides were determined by in vitro enzymatic sulfation by tyrosylprotein sulfotranferase-2 (TPST-2), subsequent separation of products by RP-HPLC, and mass spectrometry analysis. It was found that the patterns of sulfation and the rates of sulfation for CCR5 and CCR8 depend on the number of amino acids N-terminal of Tyr-3. Results herein address previous seemingly contradictory studies and delineate the temporal sulfation of N-terminal chemokine receptor peptides.

Chemokines and their corresponding receptors direct the migration of leukocytes in response to pro-inflammatory signals or for homeostatic purposes (*1*). The chemokine receptors are G protein-coupled receptors composed of a short extracellular N-terminal domain followed by seven transmembrane domains and a cytoplasmic C-terminal tail. The N-terminal extracellular domains of the chemokine receptors are critical for interactions with their ligands. This domain has been shown to be modified by posttranslational tyrosine sulfation in several chemokine receptors, including human CC chemokine receptor 5 (CCR5)[1] and mouse CC chemokine receptor 8 (CCR8) (*2*−*7*).

CCR5 is the receptor for chemokines CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, and CCL8/MCP-2 (*8*, *9*), and human CCR5 is also a coreceptor for HIV-1 (*10*−*14*). Previous studies have clearly demonstrated that tyrosine sulfation of the N-terminal domain of CCR5 contributes to the binding affinity of CCL3/MIP-1α, CCL4/MIP-1β, and HIV-1 gp120−CD4 complexes as assessed by site-directed mutagenesis and by chlorate inhibition of sulfation (*6*). The region spanning amino acids 2−18 of CCR5 (CCR5 2−18, DYQVSSPIYDINYYTSE) is thought to contain all the residues in this domain that directly contribute to interactions with HIV-1 gp120−CD4 and has four tyrosine residues that can be potentially sulfated (Tyr-3, -10, -14, and -15, underlined) (*6*, *15*−*17*). In the original report by Farzan et al., a series of four CCR5 constructs in which three of four N-terminal tyrosines were changed to phenylalanine (YFFF, FYFF, FFYF, and FFFY, amino acids at positions 3, 10, 14, and 15, respectively, are represented by each letter) were expressed. Of these four CCR5 variants, the YFFF variant incorporated [$^{35}$S]sulfate most efficiently (*6*). On the basis of analyses of additional CCR5 variants, Farzan et al. concluded that Tyr-3 and at least one additional tyrosine residue in the N-terminal domain of CCR5 could be sulfated.

Subsequently, Seibert et al. analyzed the pattern and time course of sulfation of the CCR5 2−18 peptide by purified recombinant TPST-1 and TPST-2 (*18*). They reported that sulfation of CCR5 2−18 occurred in a nonrandom, sequential manner in which Tyr-14 or Tyr-15 was sulfated first, followed by Tyr-10, and finally Tyr-3 (*18*). Thus, an inconsistency exists in that while Tyr-3 can be sulfated efficiently in the CCR5 YFFF variant in cells, Tyr-3 was the last tyrosine in the CCR5 2−18 peptide to be sulfated in a purified system.

One explanation for this apparent inconsistency is that the studies by Farzan examined sulfation of the full-length receptor in which the N-terminal methionine was presumably present while Seibert et al. examined sulfation of a CCR5 peptide substrate lacking the native N-terminal methionine. Therefore, this study involves the re-examination of the pattern of sulfation of CCR5 and CCR8 N-terminal peptides. Specifically, the order of sulfation for CCR5 1−18 and CCR5 2−18 peptides by recombinant TPST-2 was compared using a novel subtractive strategy to determine the sites of sulfation (*19*). For CCR5 2−18, Tyr-14 and Tyr-15 were sulfated first, followed by Tyr-10, with Tyr-3 sulfated last. This largely

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[1]Abbreviations: TPST, tyrosylprotein sulfotransferase; CCR5, CC chemokine receptor 5; CCR8, CC chemokine receptor 8; HIV-1, human immunodeficiency virus type 1; CCL3, -4, -5, and -8, CC chemokine family ligands 3, 4, 5, and 8, respectively; CID, collision-induced dissociation; ESI, electrospray ionization; RP-HPLC, reverse phase high-performance liquid chromatography.

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confirms the observations of Seibert et al. However, the pattern of sulfation of CCR5 1−18 was radically different. In the case of CCR5 1−18, Tyr-3 was sulfated first, followed by Tyr-14 or Tyr-15, and finally Tyr-10. In addition, the overall rate of sulfation was higher for CCR5 1−18 than for CCR5 2−18. The same overall effect of the N-terminal methionine on the order and time course of sulfation was also observed for peptides modeled on the N-terminus of CCR8, another tyrosine-sulfated chemokine receptor which shares the same three N-terminal residues (MDY) with CCR5. Moreover, addition of an acetyl group to the N-terminus of CCR8 2−17 alters the major pathway and rate of sulfation compared to those of the nonacetylated form. These data suggest that the role of Tyr-3 is perhaps more important than previously believed and that additional studies may be required to fully understand the function of this residue in interactions with the HIV-1 gp120−CD4 complex.

## EXPERIMENTAL PROCEDURES

*Materials.* HPLC grade acetonitrile, glycerol, ammonium acetate crystalline, and imidazole were purchased from Thermo Fisher (Pittsburgh, PA). The buffers *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) and 3-*N*-morpholinopropanesulfonic acid (MOPS) were purchased from Sigma (Milwaukee, WI). Approximately 80% pure adenosine 3′-phosphate 5′-phosphosulfate (PAPS) was purchased from Calbiochem (San Diego, CA). Recombinant human TPST-2 was expressed and purified as previously described (*20*). The human CCR5 peptides MDYQVSSPIYDINYYTSE-$NH_2$ (CCR5 1−18) and DYQVSSPIYDINYYTSE-$NH_2$ (CCR5 2−18) were synthesized by Ezbiolab (Westfield, IN). The mouse CCR8 peptides MDYTMEPNVTMTDYYPD-$NH_2$ (CCR8 1−17) and DYTMEPNVTMTDYYPD-$NH_2$ (CCR8 2−17) were synthesized by Biomatik (Wilmington, DE). The altered mouse CCR8 peptide ADYTMEPNVTMTDYYPD-$NH_2$ (CCR8 1−17 M1A) was synthesized by Chi Scientific (Maynard, MD). N-Terminally acetylated peptides were prepared using sulfosuccinimidyl acetate (S-NHSAc) (Pierce, Rockford, IL). The lyophilzed peptides were dissolved into 100 mM sodium phosphate and 0.15 M NaCl (pH 7.2). A 25-fold molar excess of freshly prepared S-NHSAc in ultrapure water was added and incubated for 1 h at room temperature.

*In Vitro Sulfation of CCR5 and CCR8 N-Terminal Peptides.* Recombinant human TPST-2 was used to enzymatically sulfate the chemokine receptor peptides from CCR5 and CCR8. CCR5 1−18 or 2−18 at 15 μM was incubated with 500 μM PAPS in 100 μL of 20 mM MOPS, 100 mM NaCl, and 10% glycerol (pH 7.5). For sulfation of CCR5 peptides, 3 μM TPST-2 was added, and for sulfation of CCR8, 1.5 μM TPST-2 was added; the reactions were allowed to proceed for 8 h at 30 °C. The concentration of PAPS and the reaction temperature were adjusted from previously described conditions to allow for more efficient sulfate incorporation. The enzyme concentration used for sulfation of CCR5 peptides is the same as that reported previously (*19*). However, the amount of enzyme required for sulfation of CCR8 was half of the reported concentration. This may be due to differences in the enzyme's preference for substrate or due to the greater number of N-terminal tyrosine residues in CCR5.

The reactions catalyzed by TPST-2 were monitored at several time points (0, 0.5, 1, 2, 4, and 8 h). At each time point, 50 μL aliquots were analyzed by RP-HPLC. The percentage of each species was calculated by integration of the chromatographic peaks and division by the sum of integrations for all species observed.

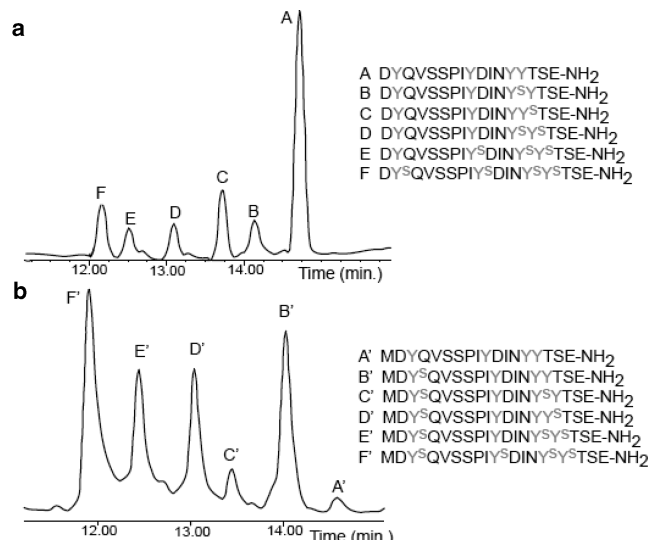



FIGURE 1: RP-HPLC analysis of (a) CCR5 2−18 and (b) CCR5 1−18 peptides generated by in vitro enzymatic sulfation by TPST-2. The reaction of CCR5 2−18 (15 μM) or CCR5 1−18 (15 μM) with TPST-2 (3 μM) was conducted for 8 h in the presence of 500 μM PAPS. The identities of the peaks were later determined by site-of-sulfation analysis.

*RP-HPLC of Peptides.* The enzymatically sulfated peptides were analyzed by RP-HPLC, using a Zorbax C8 column (4.6 mm × 150 mm, 5 μm particle size) (Agilent, Palo Alto, CA). Liquid chromatographic separation and peptide detection were performed on a Waters HPLC system with MassLynx version 4.0. The Waters 600E multisolvent delivery system consisted of a Waters Delta 600 pump, a Waters 2487 UV detector, and a Waters 600 controller. RP-HPLC was performed using a flow rate of 1 mL/min with a linear gradient from 5 to 100% solvent B over 30 min. Solvent A was 20 mM $NH_4OAc$ (pH 6.8), and solvent B was 20 mM $NH_4OAc$ (pH 6.8) in an 80:20 acetonitrile/$H_2O$ mixture. Chromatograms were monitored by UV absorbance at 215 nm. Fractions were collected and analyzed by mass spectrometry. Fractions that were found to correspond to tyrosine-sulfated peptides were lyophilized and used for site-of-sulfation determination.

*Derivatization of Tyrosine Residues Using Sulfosuccinimidyl Acetate.* The methodology for the determination of sites of sulfation has been reported previously (*19*). Briefly, lyophilized peptides were reconstituted in 100 μL of 200 mM HEPES (pH 7.0) and 3 mM imidazole. A fresh stock solution of S-NHSAc was prepared in DMSO. S-NHSAc was added to the peptide solution to a final concentration of 30 mM and incubated at 4 °C overnight. After incubation, the solution was desalted using Oasis SPE HLB cartridges (Waters, Milford, MA) preconditioned with 1 mL of methanol followed by 1 mL of 100 mM $NH_4OAc$. After the sample had been loaded, the cartridge was washed with 3 mL of 100 mM $NH_4OAc$ and the peptide was eluted with 200 μL of methanol.

*Electrospray Ionization Mass Spectrometry.* Mass spectra were recorded on a LTQ linear ion trap mass spectrometer with an electrospray ionizaion (ESI) source (Thermo Electron, San Jose, CA). The data acquisition software used was Xcalibur, version 2.0. Samples were introduced by direct infusion at a rate of 5 μL/min. Spectra were obtained using a spray voltage of 3.6 kV and a capillary temperature of 200 °C and collected in the negative ion mode. Tandem mass spectra (MS/MS) were obtained using a spray voltage of 4.5 kV and a capillary temperature of 175 °C in the positive ion mode. For the tandem mass spectrometry

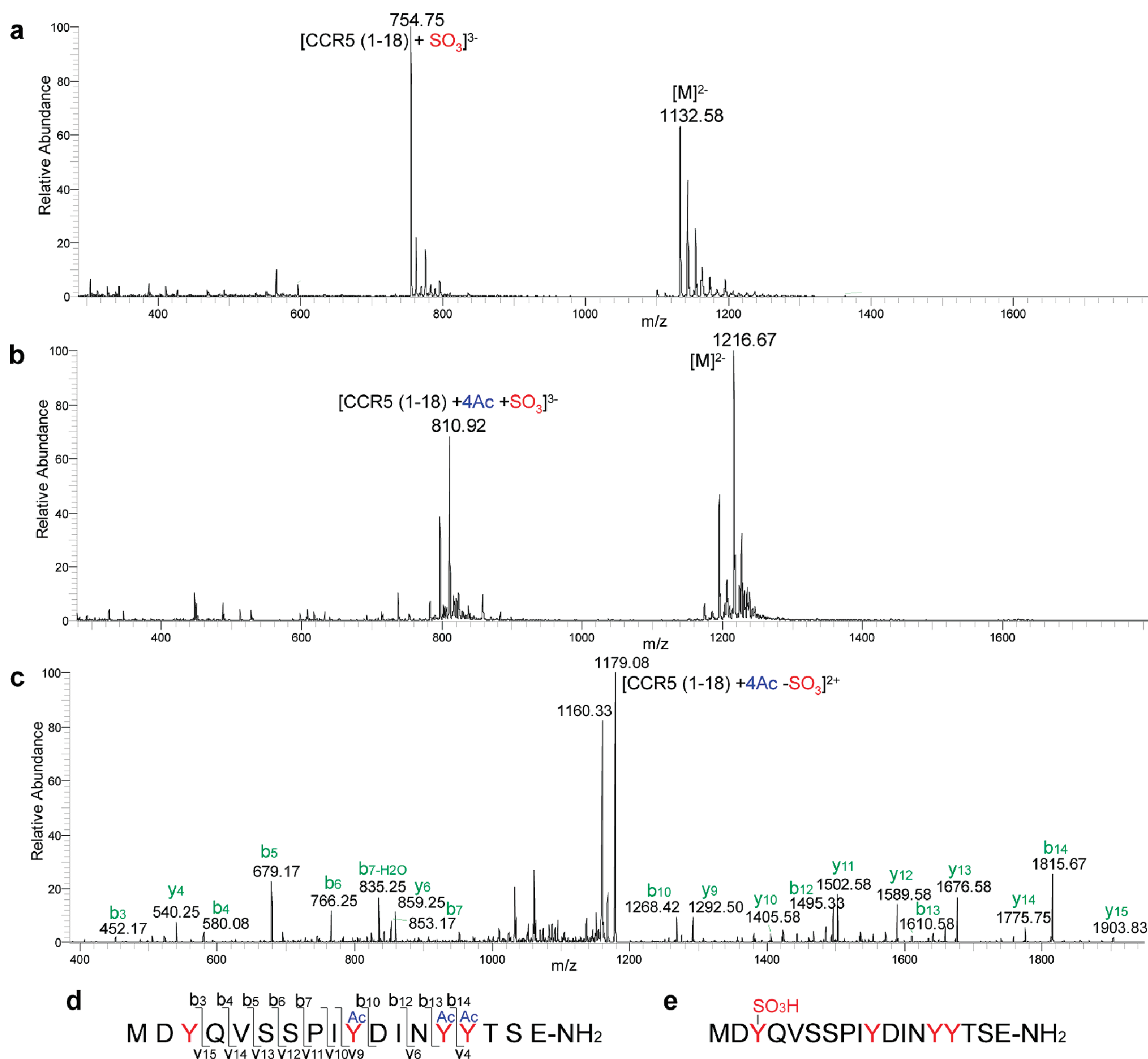



FIGURE 2: Determination of the site of sulfation on the monosulfated CCR5 1−18 peptide. Shown are (a) a mass spectrum of monosulfated CCR5 1−18 from peak B′ in Figure 1b, (b) a mass spectrum of monosulfated CCR5 1−18 after reaction with S-NHSAc, and (c) a MS/MS spectrum of the acetylated, monosulfated CCR5 1−18 peptide. Note the ion corresponding to $m/z$ 494.16, which is Y3 acetylated, is not observed. (d) MS/MS fragmentation scheme of CCR5 1−18. From the MS/MS spectrum, we determined that Tyr-10, Tyr-14, and Tyr-15 were acetylated. (e) Structure of monosulfated CCR5 1−18. Since Tyr-3 was not acetylated, it was inferred that Tyr-3 was initially sulfated.

experiments, selection of the precursor ion was achieved using an isolation width of 3 Da, and the ion was activated at 18% normalized collision energy using helium as the collision gas. Each mass spectrum obtained consists of an average of 40 scans.

## RESULTS

*Pattern of Sulfation for CCR5 and CCR8 by TPST-2.* To examine the effect of the N-terminal methionine on sulfation of the CCR5 N-terminus, the patterns of sulfation for CCR5 1−18 and CCR5 2−18 by TPST-2 were compared. In vitro enzymatic sulfation of the peptides by recombinant TPST-2 generated a mixture of sulfated peptides with the addition of one to four sulfate groups. These differentially sulfated peptides were subsequently separated by RP-HPLC (Figure 1) and analyzed using negative ion electrospray ionization mass spectrometry. The peptides were subsequently examined using a subtractive approach to determine the sites of tyrosine O-sulfation as described by Yu et al. (*19*). Each sulfated peptide species was chemically modified with S-NHSAc, which acetylates both tyrosyl hydroxyl and primary amino groups. Since tyrosine O-sulfation is labile when analyzed by mass spectrometry in the positive ion mode and during CID (*21*, *22*), the sites of sulfation were determined by a subtractive methodology (Figure 2 and Figure S1 of the Supporting Information). Collision-induced dissociation of the enzymatically sulfated and chemically acetylated peptide allows for assignment of tyrosine O-sulfation sites since any tyrosine in the peptide that is not acetylated must have been sulfated prior to CID.

For the pattern of sulfation of CCR5 2−18, Tyr-14 and Tyr-15 were found to be sulfated first, followed by Tyr-10, with Tyr-3 sulfated last (Figure 3 and Figures S2 and S3 of the Supporting Information). These results are in agreement with the previously published pattern of sulfation for CCR5 2−18 (*18*). In contrast, the sulfation pattern of CCR5 1−18 by TPST-2 is radically different. In the case of CCR5 1−18, Tyr-3 is sulfated first, followed by Tyr-14 or Tyr-15, and finally Tyr-10 (Figure 3). Thus, the presence of the native N-terminal methionine residue changes the pattern of sulfation for CCR5 N-terminal peptides.

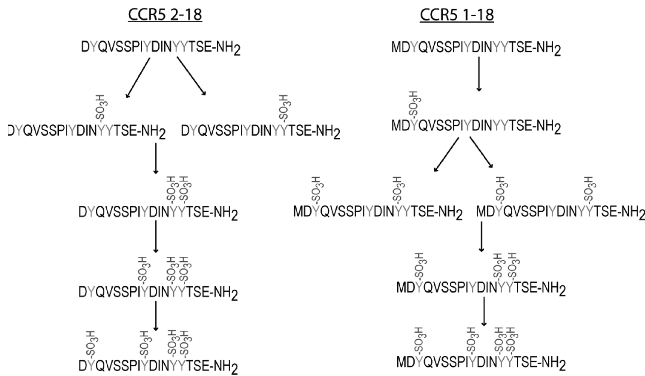



FIGURE 3: Major pathway for sulfation of CCR5 2−18 and CCR5 1−18 by TPST-2. The pathway for sulfation of CCR5 1−18 was derived from site-of-sulfation data shown in Figure 2 and Figure S1.

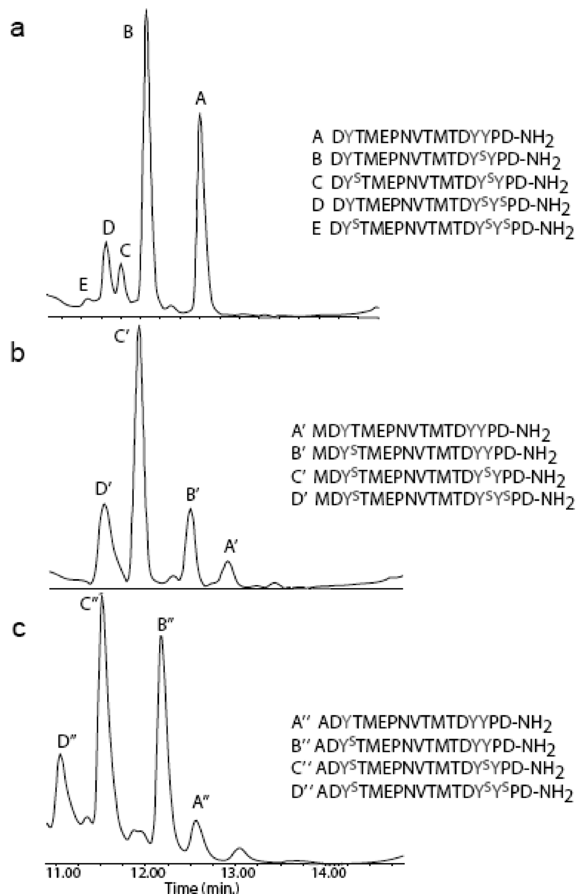
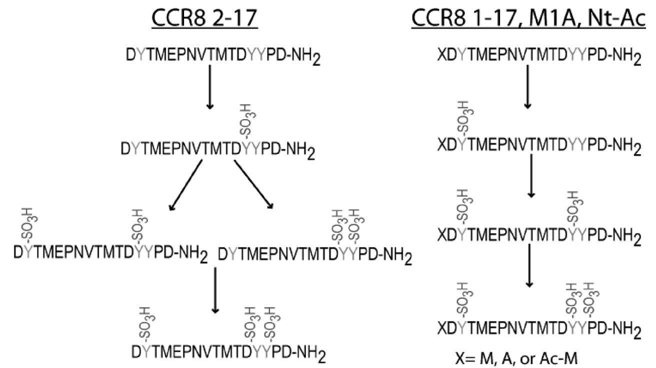



FIGURE 4: RP-HPLC analysis of (a) CCR8 2−17, (b) CCR8 1−17, and (c) CCR8 1−17 M1A peptides generated by in vitro enzymatic sulfation by TPST-2. The reaction of CCR8 2−17 (15 $\mu$M), CCR8 1−17 (15 $\mu$M), and CCR8 1−17 M1A (15 $\mu$M) with TPST-2 (1.5 $\mu$M) was conducted for 8 h in the presence of 500 $\mu$M PAPS. The chromatogram for the reaction of CCR8 1−17 with TPST-2 is shown after 4 h.

To investigate if the presence or absence of Met-1 had a similar effect on another chemokine receptor, the sulfation patterns of CCR8 1−17 and CCR8 2−17 were analyzed (Figure 4). CCR8 was chosen because the first three residues of CCR8, MDY, are identical to those in CCR5. It was determined that sulfation of CCR8 2−17 by TPST-2 occurs with the following pattern: Tyr-14 sulfated first, followed by Tyr-3 and Tyr-15 (Figure 5). The sulfation pattern for CCR8 1−17, however, differs in that Tyr-3 is again sulfated first, followed by Tyr-14, with Tyr-15 last. The



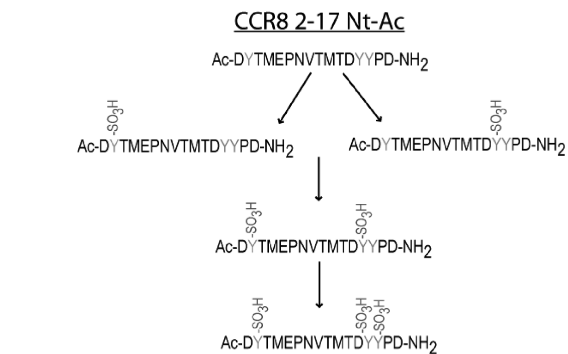




FIGURE 5: Major pathway for sulfation of CCR8 2−17, CCR8 1−17, CCR8 1−17 M1A, and CCR8 2−17 N-terminally acetylated (Nt-Ac) by TPST-2.

patterns of sulfation for CCR8 1−17 and CCR8 2−17 are consistent with those observed for CCR5 1−18 and CCR5 2−18. These data indicate that Tyr-3 is preferentially sulfated in CCR5 and CCR8 when the N-terminal methionine is present.

To determine if other N-terminal amino acids would also alter the order of sulfation by TPST-2, CCR8 1−17 M1A and CCR8 1−17 were analyzed and compared (Figure 5). The pattern of sulfation for CCR8 1−17 M1A was identical to that for CCR8 1−17 in which Tyr-3 is sulfated first, followed by Tyr-14, with Tyr-15 last (Figure 5). The preferential sulfation of Tyr-3 does not appear to be contingent on the identity of the residue two amino acids prior (position −2) to Tyr-3, but more so on the mere presence of an amino acid at that position. We were not able to conduct a comparable analysis of CCR5 M1A because of the inability of custom peptide synthesis companies to synthesize a pure and soluble form of CCR5 1−18 M1A.

In an attempt to further understand the basis of the different sulfation patterns for the CCR8 peptides, CCR8 1−17 and CCR8 2−17 variants were acetylated at the N-terminus (Nt-Ac) and tested. The pattern of sulfation for CCR8 1−17 Nt-Ac was identical to that of CCR8 1−17 (Figure 5). Thus, acetylation of the N-terminus of CCR8 1−17 has no effect on the order of sulfation. The pattern of sulfation for CCR8 2−17 Nt-Ac, however, is unique and follows the pattern of Tyr-3 or Tyr-14 being sulfated first and Tyr-15 being sulfated last (Figure 5). Interestingly, the pattern of sulfation of CCR8 2−17 Nt-Ac appears to be a hybrid between that of CCR8 2−17 and that of CCR8 1−17 in which both peptide species with Tyr-3 or Tyr-14 sulfated first were observed. The addition of an acetyl group to the N-terminus of CCR8 2−17 clearly affects its major pathway of sulfation.

*Time Course of CCR5 and CCR8 Sulfation by TPST-2.* To determine if the rates of sulfate incorporation for CCR5 and CCR8 peptides are affected by the presence of Met-1, the time

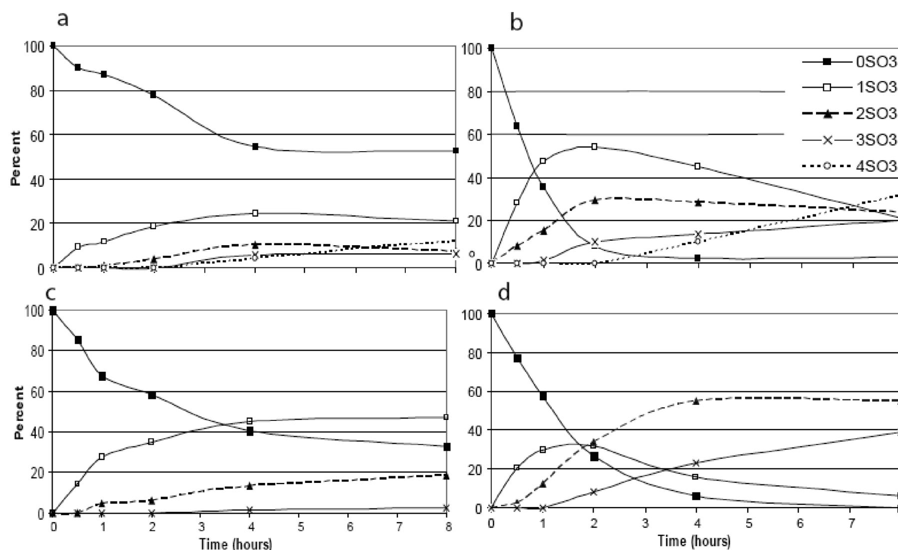



FIGURE 6: Time course of in vitro sulfation by TPST-2 for (a) CCR5 2−18, (b) CCR5 1−18, (c) CCR8 2−17, and (d) CCR8 1−17. At each time point, aliquots were taken and the reaction was quenched. The reaction was monitored by RP-HPLC. The relative amounts of each species were calculated by integrating the peak areas.

courses of CCR5 1−18 and CCR5 2−18 sulfation were compared under identical conditions. The same comparison was made for CCR8 1−17 and CCR8 2−17. At each time point, an aliquot was taken and analyzed by RP-HPLC. The percentage of each unsulfated and sulfated species at each time point was calculated by integration of the chromatographic peaks and divided by the sum of integrations for all species observed.

The sulfation time course plots reveal that unsulfated CCR5 1−18 was quickly depleted (Figure 6 a,b). In contrast, the rate of unsulfated CCR5 2−18 depletion was less dramatic, and the rate of sulfated product formation was much slower. After 8 h, the percentage of tetrasulfated CCR5 1−18 produced was almost 3-fold greater than the percentage of tetrasulfated CCR5 2−18. The percentage of trisulfated CCR5 1−18 was also approximately 3-fold greater than that of the trisulfated CCR5 2−18. The percentage of unmodified CCR5 1−18 remaining after the 8 h reaction was 15-fold less than that of unmodified CCR5 2−18. These data show that CCR5 1−18 is clearly the preferred substrate of TPST-2. Experiments aimed at determining the kinetic constants for the substrates with and without the N-terminal methionine are currently underway.

Similar results were obtained when the time courses for sulfation of CCR8 1−17 and sulfation of CCR8 2−17 by TPST-2 were examined (Figure 6 c,d). The unsulfated CCR8 1−17 peptide was completely depleted over the course of the reaction as sulfated products appeared. In contrast, a relatively large percentage of the unmodified CCR8 2−17 peptide remained after 8 h. The percentage of disulfated CCR8 1−17 produced was more than twice that of disulfated CCR5 2−18. Also at 8 h, the amount of trisulfated CCR8 1−17 produced was 10 times greater than that of trisulfated CCR8 2−17. These data demonstrate that the peptides with the N-terminal methionine were more efficiently sulfated by TPST-2 than the peptides lacking Met-1. Moreover, the time course plot of CCR8 1−17 M1A showed rates of sulfation comparable to that of CCR8 1−17 (Figure 6 and Figure S4a of the Supporting Information).

The rates of sulfation of CCR8 1−17 Nt-Ac were similar to that of CCR8 1−17 (Figures 6 and S4b). However, the degree of sulfate incorporation for CCR8 2−17 Nt-Ac (Figures 6 and S4c) was much greater than that for CCR8 2−17 and comparable to that for CCR8 1−17. Addition of an acetyl group clearly has a large impact on the rate of sulfation of the CCR8 peptide.

## DISCUSSION

To address the apparent discrepancy surrounding the pattern of sulfation, it was shown that peptides CCR5 1−18 and CCR5 2−18 differ in the order of sulfation. The presence of Met-1 resulted in a change in the order of sulfation in which Tyr-3 becomes the preferred TPST-2 substrate. These results are in agreement with the two observations that Tyr-3 can be sulfated efficiently in the full-length CCR5 YFFF variant in HeLa cells, whereas Tyr-3 is the last tyrosine in CCR5 2−18 to be sulfated.

A proposed consensus feature for tyrosine sulfation is the presence of acidic residues on the N-terminal side of the target tyrosine (*23*−*25*). Substitution of an acidic residue in the position directly N-terminal of the tyrosine substrate has been shown to lead to an increase in $K_m$ values (*25*). As an aspartic acid precedes Tyr-3 in CCR5, this particular tyrosine is expected to be a good substrate for sulfation. Similarly, analysis of the consensus sequence of naturally sulfated peptides and proteins reveals that acidic amino acids are commonly found at positions −5 to −1 of the tyrosine residue, particularly at position −1 (*26*, *27*). The observation that Tyr-3 is sulfated last in CCR5 2−18 provoked investigation.

Results also indicate that Met-1 can be replaced with alanine without consequence for the pattern of sulfation or the rate of sulfate incorporation. Hence, the presence but not identity of the residue at the −2 position appears to be crucial. Perhaps TPST-2 may require at least two amino acids N-terminal of its target substrate to efficiently catalyze tyrosine sulfation. This proposed explanation is supported by previous studies showing that serial elimination of amino acids N-terminal of a target tyrosine results in multifold increases in $K_m$ values (*24*, *25*). Determination of the order of sulfation for CCR8 2−17 Nt-Ac provides a rationale behind the proposed substrate requirement of two amino acids preceding the target tyrosine. Acetylation of the N-terminus affects the pattern of sulfation of CCR8 2−17 but not CCR8 1−17. A possible explanation for this observation is that the positive charge on the amine group of the aspartic acid forms a

salt bridge and affects the substrate's ability to be recognized by TPST-2, whereas acetylation will remove the positive charge from the amine, allowing efficient sulfation. Sulfation appears to be affected by charge distribution surrounding the sulfation site, particularly in position −1. This conclusion is consistent with previous studies demonstrating that a basic, positively charged residue in the position directly N-terminal to the tyrosine hinders sulfation (*23*). Since some monosulfated CCR8 2−17 Nt-Ac at Tyr-14 is still observed, the positive charge on the amine group of aspartic acid does not completely account for the differences in the order of sulfation of CCR8 2−17 and CCR8 1−17. An additional possible explanation is a minimal requirement of two amino acids prior to the target tyrosine for sufficient interactions with TPST-2 and/or PAPS. Given that the identity of the amino acid at position −2 does not seem to be crucial, it is plausible that these interactions with the enzyme involve the peptide backbone.

The rates of sulfation for the various N-terminal CCR5 and CCR8 peptides were examined in an effort to improve our understanding of the importance of preferential sulfation of Tyr-3. The rate of initial monosulfation of CCR5 1−18 at Tyr-3 is faster than the rates of initial monosulfation of CCR5 2−18 at Tyr-14 or Tyr-15. It may be that the role of Tyr-3 sulfation is to promote stoichiometric sulfation of the other tyrosine residues within the peptide. There is precedence for the increase in the affinity of TPST for peptide variants in which one site is already tyrosine O-sulfated. For example, Niehrs et al. determined the apparent $K_m$ of synthetic peptides with a varying number of potential sulfation sites and found that the $K_m$ decreased exponentially with the number of potential tyrosine sulfation sites (*24*). Another study showed that the $K_m$ of a nonsulfated CCR8 peptide is 5-fold greater than that of the monosulfated variant (*28*). In the case of CCR5 1−18, the efficient sulfation of one site (Tyr-3) may improve the kinetics for sulfation of other target sites (Tyr-14 or Tyr-15). The same effect was found in the rate of sulfation incorporation for CCR8 1−17 and CCR8 2−17.

Several studies have documented the importance of sulfation of Tyr-10 and Tyr-14 for binding of CCR5 chemokine ligands and HIV-1 gp120 using a variety of techniques, including mutagenesis of the tyrosine residues, synthetic peptide binding, NMR techniques, and isothermal calorimetry (*16*, *17*, *29*−*33*). However, the potential importance of Tyr-3 is open to question. Alanine mutagenesis of CCR5 showed that substitutions of tyrosine at position 3 (Y3A) or of aspartic acid at position 2 (D2A) resulted in inhibition of binding to the gp120−CD4 complex from several strains of HIV-1 (*17*, *29*). Conversely, another study used a panel of sulfated CCR5 N-terminal peptides to bind the gp120−CD4 complex and showed that sulfation of Tyr-3 does not appear to significantly contribute to binding (*30*). The observed reduction in the level of binding of Y3A or D2A CCR5 mutants to the gp120−CD4 complex may be the result of the predicted lower stoichiometric sulfation of Tyr-10 and Tyr-14 in those mutants. Nevertheless, it is also possible that sulfation at Tyr-3 is directly involved in interactions with the gp120−CD4 complex. Recent work reported the structural basis of CXCL12/SDF-α recognition of the three potential CXCR4 N-terminal sulfotyrosines, and future parallel work on CCR5 may provide evidence of a physiological role for Tyr-3 (*34*, *35*). Given the data presented in this study, and those cited by others, it appears that the role of Tyr-3 is likely to be more important than previously estimated. We, however, cannot ascertain that the pattern of sulfation of synthetic peptides by soluble TPST recapitulates the pattern of sulfation of the native chemokine receptors by full-length TPST in an intact cell. Nevertheless, our findings should prompt a reassessment of the potential role of sulfation of CCR5 Tyr-3 in its interaction with gp120 and with its chemokine ligands.

In summary, by using various CCR5 and CCR8 N-terminal peptides, we showed that the order of sulfation can vary significantly for the two peptides that differ only by the presence or absence of one amino acid at position −2 of Tyr-3. The CCR5 and CCR8 peptides that include Met-1 or the substitution with Ala-1 are better substrates for sulfation by TPST-2 than peptides lacking Met-1 or Ala-1.

## SUPPORTING INFORMATION AVAILABLE

Figures S1−S4, MS/MS spectra of di- and trisulfated CCR5 1−18, MS/MS spectra of monosulfated CCR5 2−18, and a summary of sulfation rates for variations of the CCR8 N-terminal peptide. This material is available free of charge via the Internet at http://pubs.acs.org.

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