Published in final edited form as:
Biochemistry. 2009 June 16; 48(23): 5332–5338. doi:10.1021/bi900285c.

The Pattern and Temporal Sequence of Sulfation of CCR5 N-Terminal Peptides by Tyrosylprotein Sulfotranferase-2: An Assessment of the Effects of N-Terminal Residues

Connie H. Jen[‡], **Kevin L. Moore**[¶], and **Julie A. Leary**[‡,§]

[§]Department of Chemistry, University of California, Davis, California, 95616

[‡]Section of Molecular and Cellular Biology, University of California, Davis, California, 95616

[¶]Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation and the Departments of Cell Biology and Medicine, University of Oklahoma Health Sciences Center, and the Oklahoma Center for Medical Glycobiology, Oklahoma City, Oklahoma 73104

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 have been shown to be important in the binding affinity to 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) 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)

Corresponding Author: Julie A. Leary, Briggs Hall 130, One Shields Ave., Davis, CA 95616, Telephone: (530) 752-4685, Fax: (530) 752-3085, jaleary@ucdavis.edu.

Supporting Information Available. This material includes figures S1-S4, MS/MS spectra of di- and tri-sulfated CCR5 1-18, MS/MS spectra of mono-sulfated 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

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 were expressed wherein three of four N-terminal tyrosines were changed to phenylalanine (YFFF, FYFF, FFYF, and FFFY, amino acids at positions 3, 10, 14, and 15 are represented by each letter). Of these four CCR5 variants, the YFFF variant incorporated ^{35}S-sulfate most efficiently (6). Based on 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 were sulfated first, followed by Tyr-10, and finally Tyr-3 (18). Thus, an inconsistency exists 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 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 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 compared to that of 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 non-acetylated 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-Morpholino propanesulfonic 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 Ezbolab (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, Maryland). N-terminal acetylated peptides were prepared using sulfosuccinimidyl acetate (S-NHSAc, Pierce, Rockford, IL). The lyophilzed peptides were

dissolved into 100mM sodium phosphate, 0.15M NaCl, pH 7.2. A twenty-five-fold molar excess of freshly prepared S-NHSAc in ultrapure water was added and incubated for one hour at room temperature.

In vitro sulfation of CCR5/CCR8 N-terminal peptides

Recombinant human TPST-2 was used to enzymatically sulfate the chemokine receptor peptides from CCR5 and CCR8. Fifteen micromolar of CCR5 1-18 or 2-18 was incubated with 500 μM PAPS in 100 μL of 20 mM MOPS, 100 mM NaCl, and 10% glycerol at pH 7.5. For sulfation of CCR5 peptides, 3 μM TPST-2 was added and for sulfation of CCR8 1.5 μM of TPST-2 was added, and the reactions were allowed to proceed for 8 hours 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 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 hours). 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 dividing by the sum of integrations for all species observed.

RP-HPLC of peptides

The enzymatically sulfated peptides were analyzed by RP-HPLC, using a Zorbax C8 column (4.6×150mm, 5μm particle size Agilent, Palo Alto, CA). Liquid chromatographic separation and peptide detection was performed on a Waters HPLC 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 of 5-100% solvent B over 30 minutes. The composition of solvent A was 20 mM NH_4OAc, pH 6.8, and the composition of solvent B was 20 mM NH_4OAc, pH 6.8 in 80:20 acetonitrile:H_2O. 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 1ml of methanol followed by 1 ml of 100 mM NH_4OAc. After sample loading 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 acquired 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 5μl/min. Spectra were obtained using a spray voltage of 3.6kV, 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 4.5kV and a capillary temperature of 175°C in the positive ion mode. For the tandem mass spectrometry 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 pattern of sulfation for CCR5 1-18 and CCR5 2-18 by TPST-2 was 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 sulfotyrosines. 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 site(s) of sulfation were determined by a subtractive methodology (Figure 2 and S1). 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, S2, and S3). 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.

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 is 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, and lastly Tyr-15. The 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 CCR8 1-17 in which Tyr-3 is sulfated first, followed by Tyr-14, and lastly Tyr-15 (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 is 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 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 impacted by the presence of Met-1, the time course 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 eight hours, the percentage of tetra-sulfated CCR5 1-18 produced was almost three-fold greater than the percentage of tetra-sulfated CCR5 2-18. The percentage of tri-sulfated CCR5 1-18 was also approximately three fold greater than that of the tri-sulfated CCR5 2-18. The percentage of un-modified CCR5 1-18 remaining after the eight hour reaction was fifteen-fold less than the un-modified CCR5 2-18. These data show that CCR5 1-18 is clearly the preferred substrate of TPST-2. Experiments are currently underway to obtain the kinetic constants for the substrates with and without the N-terminal methionine.

Similar results were obtained when the time course for sulfation of CCR8 1-17 and CCR8 2-17 sulfation by TPST-2 was 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 un-modified CCR8 2-17 peptide remained after eight hours. The percentage of di-sulfated CCR8 1-17 produced was more than twice that of di-sulfated CCR5 2-18. Also at eight hours, the amount of tri-sulfated CCR8 1-17 produced was ten-times greater than that of the tri-sulfated 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, S4 a).

The rates of sulfation of CCR8 1-17 Nt-Ac were similar to that of CCR8 1-17 (Figure 6 and S4b). However, sulfate incorporation for CCR8 2-17 Nt-Ac (Figure 6 and S4c) were much greater than that for CCR8 2-17 and were comparable to that of CCR8 1-17. An 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 of 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 is prior to 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 reveal that acidic amino acids are commonly found at positions -5 to -1 of the tyrosine residue, particularly in 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 to 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 multiple fold 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 mono-sulfated 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 order to understand the importance of preferential sulfation of Tyr-3. The rate of initial mono-sulfation of CCR5 1-18 at Tyr-3 is faster than the rates of initial mono-sulfation 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 non-sulfated CCR8 peptide is five fold greater than that of the mono-sulfated 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 or of aspartic acid at position 2 (Y3A and D2A) resulted in inhibition of binding to 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 gp120/CD4 and showed that sulfation of Tyr-3 does not appear to significantly contribute to binding (30). The observed reduction in binding of Y3A or D2A CCR5 mutants to gp120/CD4 may be the result of the predicted lower stiochiometric 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 gp120/CD4. Recent work reported the structural basis of CXCL12/SDF-α recognition of the three potential CXCR 4 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by National Institutes of Health (NIH) Grant HD056022 to K.L.M. and J.A.L, and by GM63581 to J.A.L

References

1. Moser B, Loetscher P. Lymphocyte traffic control by chemokines. Nat Immunol 2001;2:123–128. [PubMed: 11175804]
2. Farzan M, Babcock GJ, Vasilieva N, Wright PL, Kiprilov E, Mirzabekov T, Choe H. The role of post-translational modifications of the CXCR4 amino terminus in stromal-derived factor 1 alpha association and HIV-1 entry. J Biol Chem 2002;277:29484–29489. [PubMed: 12034737]
3. Gutierrez J, Kremer L, Zaballos A, Goya I, Martinez AC, Marquez G. Analysis of post-translational CCR8 modifications and their influence on receptor activity. J Biol Chem 2004;279:14726–14733. [PubMed: 14736884]
4. Preobrazhensky AA, Dragan S, Kawano T, Gavrilin MA, Gulina IV, Chakravarty L, Kolattukudy PE. Monocyte chemotactic protein-1 receptor CCR2B is a glycoprotein that has tyrosine sulfation in a conserved extracellular N-terminal region. J Immunol 2000;165:5295–5303. [PubMed: 11046064]
5. Colvin RA, Campanella GS, Manice LA, Luster AD. CXCR3 requires tyrosine sulfation for ligand binding and a second extracellular loop arginine residue for ligand-induced chemotaxis. Mol Cell Biol 2006;26:5838–5849. [PubMed: 16847335]
6. Farzan M, Mirzabekov T, Kolchinsky P, Wyatt R, Cayabyab M, Gerard NP, Gerard C, Sodroski J, Choe H. Tyrosine sulfation of the amino terminus of CCR5 facilitates HIV-1 entry. Cell 1999;96:667–676. [PubMed: 10089882]
7. Moore KL. The biology and enzymology of protein tyrosine O-sulfation. J Biol Chem 2003;278:24243–24246. [PubMed: 12730193]
8. Samson M, Labbe O, Mollereau C, Vassart G, Parmentier M. Molecular cloning and functional expression of a new human CC-chemokine receptor gene. Biochemistry 1996;35:3362–3367. [PubMed: 8639485]

9. Gong W, Howard OM, Turpin JA, Grimm MC, Ueda H, Gray PW, Raport CJ, Oppenheim JJ, Wang JM. Monocyte chemotactic protein-2 activates CCR5 and blocks CD4/CCR5-mediated HIV-1 entry/replication. J Biol Chem 1998;273:4289–4292. [PubMed: 9468473]
10. Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, Berger EA. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. Science 1996:1955–1958. [PubMed: 8658171]
11. Choe H, Farzan M, Sun Y, Sullivan N, Rollins B, Ponath PD, Wu L, Mackay CR, LaRosa G, Newman W, Gerard N, Gerard C, Sodroski J. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell 1996;85:1135–1148. [PubMed: 8674119]
12. Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, Davis CB, Peiper SC, Schall TJ, Littman DR, Landau NR. Identification of a major co-receptor for primary isolates of HIV-1. Nature 1996;381:661–666. [PubMed: 8649511]
13. Doranz BJ, Rucker J, Yi Y, Smyth RJ, Samson M, Peiper SC, Parmentier M, Collman RG, Doms RW. A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. Cell 1996;85:1149–1158. [PubMed: 8674120]
14. Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, Cayanan C, Maddon PJ, Koup RA, Moore JP, Paxton WA. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature 1996;381:667–673. [PubMed: 8649512]
15. Dragic T, Trkola A, Lin SW, Nagashima KA, Kajumo F, Zhao L, Olson WC, Wu L, Mackay CR, Allaway GP, Sakmar TP, Moore JP, Maddon PJ. Amino-terminal substitutions in the CCR5 coreceptor impair gp120 binding and human immunodeficiency virus type 1 entry. J of Virol 1998;72:279–285. [PubMed: 9420225]
16. Farzan M, Choe H, Vaca L, Martin K, Sun Y, Desjardins E, Ruffing N, Wu L, Wyatt R, Gerard N, Gerard C, Sodroski J. A tyrosine-rich region in the N terminus of CCR5 is important for human immunodeficiency virus type 1 entry and mediates an association between gp120 and CCR5. J of Virol 1998;72:1160–1164. [PubMed: 9445013]
17. Rabut GE, Konner JA, Kajumo F, Moore JP, Dragic T. Alanine substitutions of polar and nonpolar residues in the amino-terminal domain of CCR5 differently impair entry of macrophage- and dualtropic isolates of human immunodeficiency virus type 1. J of Virol 1998;72:3464–3468. [PubMed: 9525683]
18. Seibert C, Cadene M, Sanfiz A, Chait BT, Sakmar TP. Tyrosine sulfation of CCR5 N-terminal peptide by tyrosylprotein sulfotransferases 1 and 2 follows a discrete pattern and temporal sequence. Proc Natl Acad Sci U S A 2002;99:11031–11036. [PubMed: 12169668]
19. Yu Y, Hoffhines AJ, Moore KL, Leary JA. Determination of the sites of tyrosine O-sulfation in peptides and proteins. Nat Methods 2007;4:583–588. [PubMed: 17558413]
20. Ouyang YB, Moore KL. Molecular cloning and expression of human and mouse tyrosylprotein sulfotransferase-2 and a tyrosylprotein sulfotransferase homologue in Caenorhabditis elegans. J Biol Chem 1998;273:24770–24774. [PubMed: 9733778]
21. Salek M, Costagliola S, Lehmann WD. Protein tyrosine-O-sulfation analysis by exhaustive product ion scanning with minimum collision offset in a NanoESI Q-TOF tandem mass spectrometer. Anal Chem 2004;76:5136–5142. [PubMed: 15373453]
22. Onnerfjord P, Heathfield TF, Heinegard D. Identification of tyrosine sulfation in extracellular leucine-rich repeat proteins using mass spectrometry. J Biol Chem 2004;279:26–33. [PubMed: 14551184]
23. Bundgaard JR, Vuust J, Rehfeld JF. New consensus features for tyrosine O-sulfation determined by mutational analysis. J Biol Chem 1997;272:21700–21705. [PubMed: 9268297]
24. Niehrs C, Kraft M, Lee RW, Huttner WB. Analysis of the substrate specificity of tyrosylprotein sulfotransferase using synthetic peptides. J Biol Chem 1990;265:8525–8532. [PubMed: 2341394]
25. Lin WH, Larsen K, Hortin GL, Roth JA. Recognition of substrates by tyrosylprotein sulfotransferase. Determination of affinity by acidic amino acids near the target sites. J Biol Chem 1992;267:2876–2879. [PubMed: 1737745]
26. Hortin G, Folz R, Gordon JI, Strauss AW. Characterization of sites of tyrosine sulfation in proteins and criteria for predicting their occurrence. Biochem Biophys Res Commun 1986;141:326–333. [PubMed: 3801003]

27. Rosenquist GL, Nicholas HB Jr. Analysis of sequence requirements for protein tyrosine sulfation. Protein Sci 1993;2:215–222. [PubMed: 8443599]

28. Danan LM, Yu Z, Hoffhines AJ, Moore KL, Leary JA. Mass spectrometric kinetic analysis of human tyrosylprotein sulfotransferase-1 and -2. J Am Soc Mass Spectrom 2008;10:1459–66. [PubMed: 18672380]

29. Blanpain C, Doranz BJ, Vakili J, Rucker J, Govaerts C, Baik SS, Lorthioir O, Migeotte I, Libert F, Baleux F, Vassart G, Doms RW, Parmentier M. Multiple charged and aromatic residues in CCR5 amino-terminal domain are involved in high affinity binding of both chemokines and HIV-1 Env protein. J Biol Chem 1999;274:34719–34727. [PubMed: 10574939]

30. Cormier EG, Persuh M, Thompson DA, Lin SW, Sakmar TP, Olson WC, Dragic T. Specific interaction of CCR5 amino-terminal domain peptides containing sulfotyrosines with HIV-1 envelope glycoprotein gp120. Proc Natl Acad Sci U S A 2000;97:5762–5767. [PubMed: 10823934]

31. Huang CC, Lam SN, Acharya P, Tang M, Xiang SH, Hussan SS, Stanfield RL, Robinson J, Sodroski J, Wilson IA, Wyatt R, Bewley CA, Kwong PD. Structures of the CCR5 N terminus and of a tyrosine-sulfated antibody with HIV-1 gp120 and CD4. Science 2007:1930–1934. [PubMed: 17901336]

32. Brower ET, Schon A, Klein JC, Freire E. Binding thermodynamics of the N-terminal peptide of the CCR5 coreceptor to HIV-1 envelope glycoprotein gp120. Biochemistry 2009;48:779–785. [PubMed: 19170639]

33. Duma L, Haussinger D, Rogowski M, Lusso P, Grzesiek S. Recognition of RANTES by extracellular parts of the CCR5 receptor. J Mol Biol 2007;365:1063–1075. [PubMed: 17101151]

34. Veldkamp CT, Seibert C, Peterson FC, De la Cruz NB, Haugner JC 3rd, Basnet H, Sakmar TP, Volkman BF. Structural basis of CXCR4 sulfotyrosine recognition by the chemokine SDF-1/CXCL12. Science Signaling 2008;1:ra4. [PubMed: 18799424]

35. Seibert C, Veldkamp CT, Peterson FC, Chait BT, Volkman BF, Sakmar TP. Sequential tyrosine sulfation of CXCR4 by tyrosylprotein sulfotransferases. Biochemistry 2008;47:11251–11262. [PubMed: 18834145]

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

CID
: collision-induced-dissociation

ESI
: electrospray ionizaion

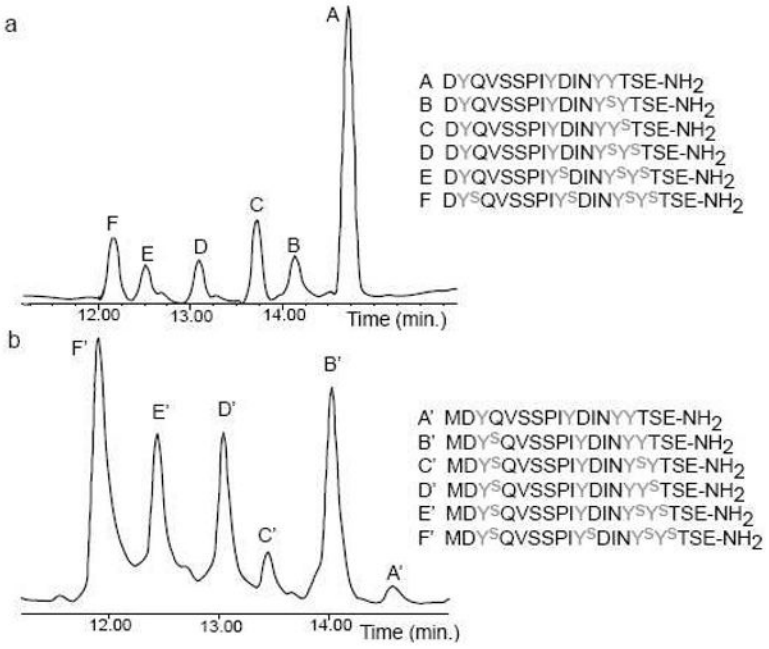

FIGURE 1.

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 carried out over 8 h in the presence of 500 μM PAPS. The identities of the peaks were later determined by site of sulfation analysis.

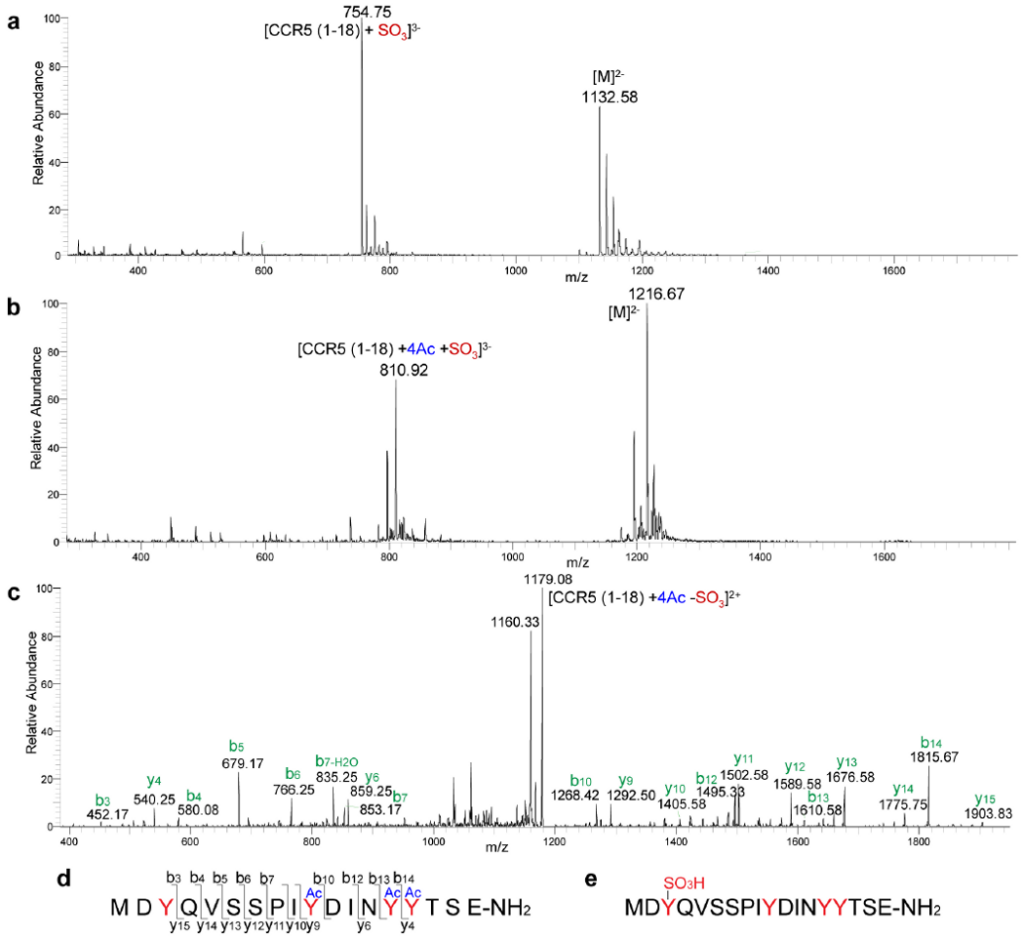

FIGURE 2.

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

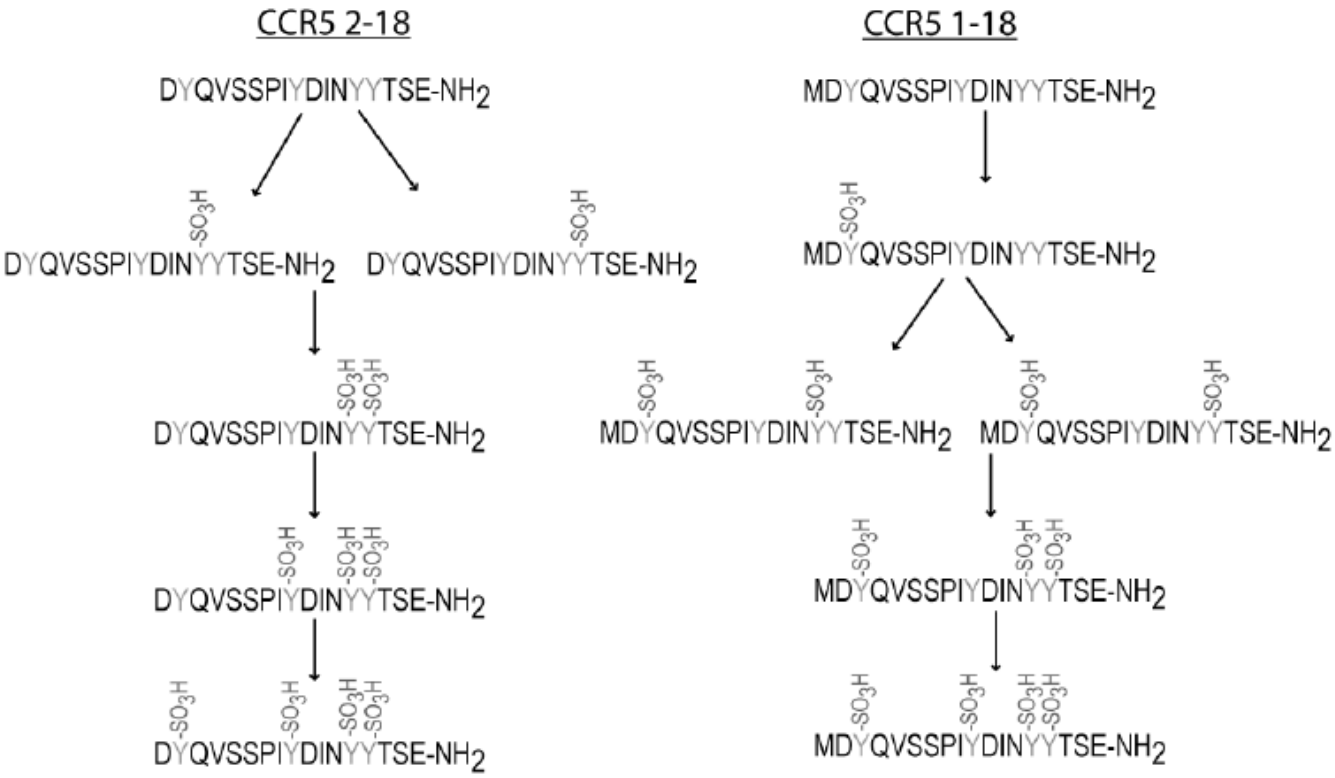

FIGURE 3.

The major pathway for sulfation of CCR5 2-18 and CCR5 1-18 by TPST-2. The pathway for sulfation CCR5 1-18 was derived from site of sulfation data shown in Figure 2 and Figure S1.

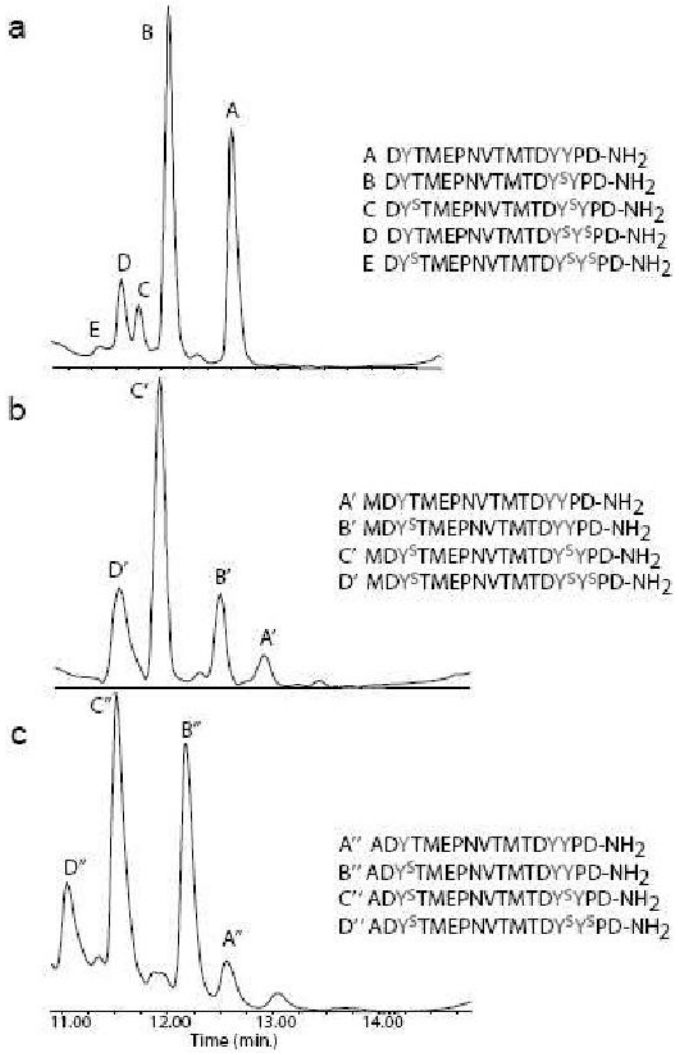

FIGURE 4.

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 μM), CCR8 1-17 (15 μM) and CCR8 1-17 M1A (15 μM) with TPST-2 (1.5 μM) was carried out over 8 hours in the presence of 500 μM PAPS. The chromatogram for the reaction of CCR8 1-17 with TPST-2 is shown after 4 h.

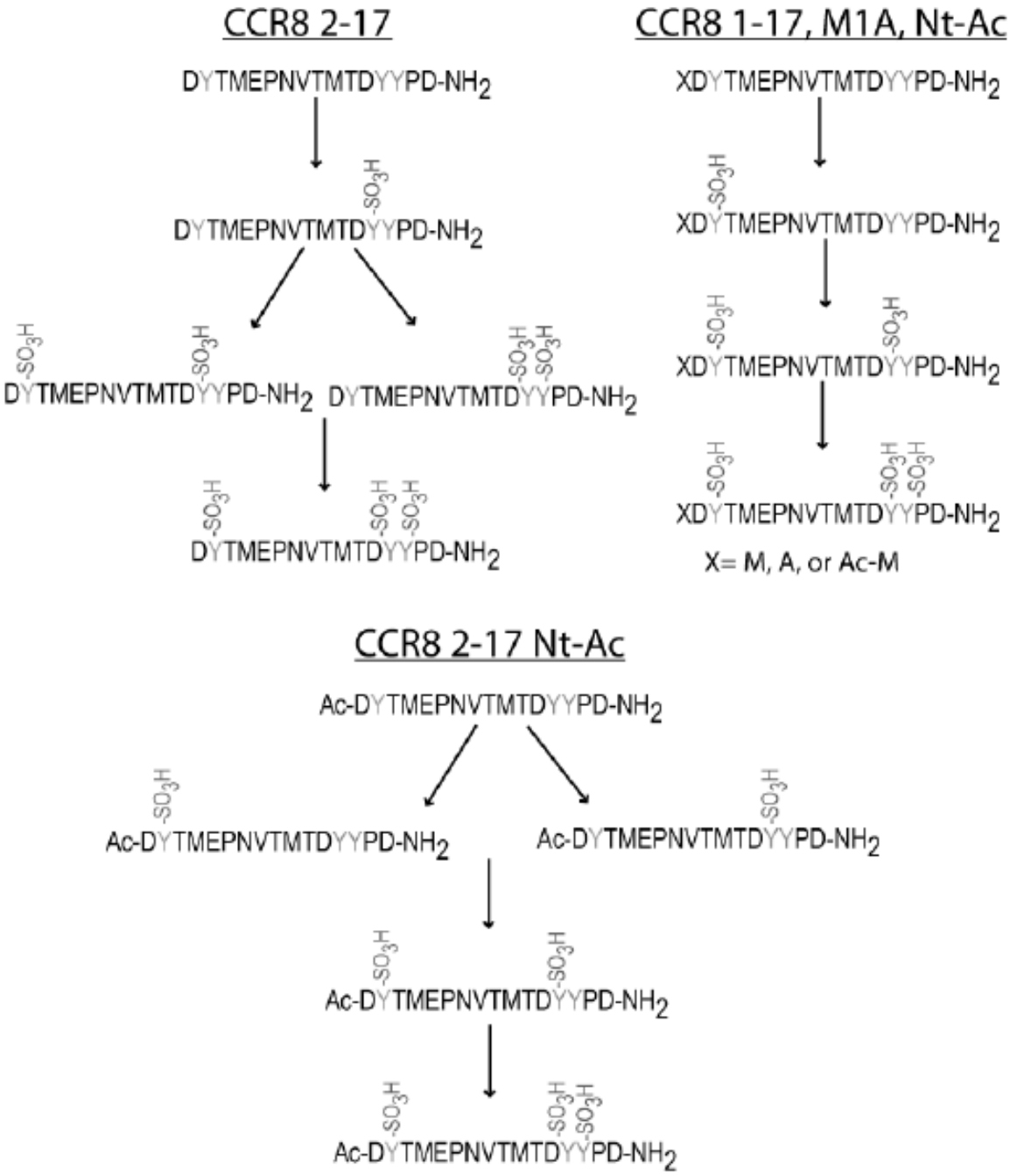

FIGURE 5.
The major pathway for sulfation of CCR8 2-17, CCR8 1-17, CCR8 1-17 M1A, and CCR8 2-17 N-term acetylated (Nt-Ac) by TPST-2.

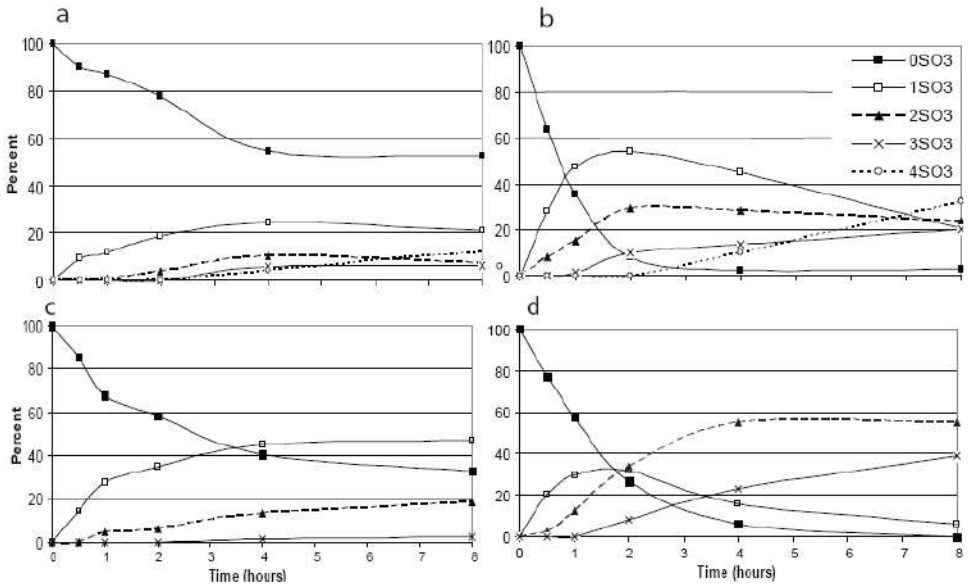

FIGURE 6.

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.