Identification of the Active Site of DS-epimerase 1 and Requirement of N-Glycosylation for Enzyme Function

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Dermatan sulfate is a highly sulfated polysaccharide and has a variety of biological functions in development and disease. Iduronic acid domains in dermatan sulfate, which are formed by the action of two DS-epimerases, have a key role in mediating these functions. We have identified the catalytic site and three putative catalytic residues in DS-epimerase 1, His-205, Tyr-261, and His-450, by tertiary structure modeling and amino acid conservation to heparinase II. These residues were systematically mutated to alanine or more conserved residues, which resulted in complete loss of epimerase activity. Based on these data and the close relationship between lyase and epimerase reactions, we propose a model where His-450 functions as a general base abstracting the C5 proton from glucuronic acid. Subsequent cleavage of the glycosidic linkage by Tyr-261 generates a 4,5-unsaturated hexuronic intermediate, which is protonated at the C5 carbon by His-205 from the side of the sugar plane opposite to the side of previous proton abstraction. Concomitant recreation of the glycosidic linkage ends the reaction, generating iduronic acid. In addition, we show that proper N-glycosylation of DS-epimerase 1 is required for enzyme activity. This study represents the first description of the structural basis for epimerization by a glycosaminoglycan epimerase.

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2 and Fig. S1.

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3 The abbreviations used are: DS, dermatan sulfate; IdoA, iduronic acid; dK4, defucosylated E. coli K4 capsular polysaccharide; MES, 4-morpholineethanesulfonic acid; DS-EpI1, DS-epimerase 1.

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EXPERIMENTAL PROCEDURES

Materials—293HEK was from ATCC, and cell culture reagents were from Invitrogen. 5-3H-Labeled defructosylated K4 capsular polysaccharide (dK4) was prepared as previously described from a K4-producing Escherichia coli strain (9). DNA Technology A/S synthesized oligonucleotides.

Modeling of DS-epimerase 1—The primary amino acid sequence was parsed into structural domains using Ginzu (10). Ginzu is an iterative method that attempts to find homologues of known structure using more sensitive but less reliable detection methods in each iteration. A PSI-BLAST search against the Protein Data Bank was followed by a fold recognition method, in this case FAS03 (11–14). If regions remained unassigned, they were parsed into domains using PFAM and then using the block structure from a multiple sequence alignment generated with PSI-BLAST against the NCBI nonredundant data base (10, 15, 16). Domains detected using PSI-BLAST and FFAS against the Protein Data Bank were annotated with the homolog of known structure, and that structure was used in subsequent homology modeling. The alignment between the query sequence and the structure was optimized using FAS03 before the homology models were generated using Rosetta (17). Several hundred models were created and visually inspected, and the alignment was manually adjusted using 2DDB to accommodate differences in loop lengths (18).

Construction of DS-epimerase 1 Wild-type and Mutant Plasmids—The open reading frame of human DS-epimerase 1 (accession number NM_001080976) was amplified from pcDNA3.1/DS-Epi1-His (8) using a nested PCR to introduce recombination sites for Gateway cloning into pDONR221 according to the manufacturer's instructions (Invitrogen). To create the C-terminal tagged expression construct for DS-epi1, pDONR221-DS-epi1 was subsequently recombined with a modified pcDNA3/CTAP vector containing a Gateway cloning cassette using an LR recombination kit (Invitrogen). Mutations were generated with the QuickChange mutagenesis kit (Stratagene), using the primers indicated in Table S2. All constructs were sequenced (MWG Biotech).

DS-epimerase 1 Expression and Purification—Expression of wild-type and mutated versions of DS-Epi1 was carried out in 293HEK cells. Confluent 25-cm² flasks were transfected with 8 μg of expression plasmid using 20 μl of Lipofectamine 2000 (Invitrogen). After 48 h of incubation, cells were lysed in 400 μl of TAP lysis buffer (50 mM Hepes (pH 8.0), 100 mM KCl, 10% glycerol, 0.1% Nonidet P-40, 2 mM EDTA, 2 mM dithiothreitol, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin)). Protein concentrations were estimated using a Bradford assay (Bio-Rad), using bovine serum albumin as a standard.

To purify recombinant proteins, 500 μg of cell lysate was bound to 10 μl of IgG-Sepharose (GE Healthcare) for 4 h at 4 °C, end over end. After binding, beads were washed once with 20 volumes of TAP lysis buffer and twice with 20 volumes of desalting buffer (20 mM MES (pH 5.5 at 37 °C), 10% glycerol, 0.5 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, protease inhibitors). The IgG gel was then resuspended in 200 μl of desalting buffer, of which 80 μl were used for determination of epimerase activity (see below). Wild-type and mutant protein expression was detected by Western blot, using 1 μg/ml of an affinity-purified anti-DS-epimerase 1 rabbit polyclonal antibody obtained by immunizing rabbits with the peptide KWSKYKHDLAAAS (Innovagen).

Tunicamycin Treatment—293HEK cells were transfected as above with pcDNA3-DS-epi1/CTAP and grown for 48 h before the addition of 1 μg/ml tunicamycin. The cells were subsequently grown for an additional 24 h prior to lysis as above.

 Determination of DS-epimerase Activity—Epimerase activity was measured as previously described (8). Briefly, cells were lysed in TAP lysis and desalted (in Fig. 6) or purified as above (in Fig. 4) before assaying. Assays were performed in a total reaction volume of 100 μl of 0.8× desalting buffer, 0.5 mg of bovine serum albumin, 2 mM MnCl₂, 0.5% Nonidet P-40, and 30,000 dpm [5-3H]dK4 (~20 μM HexA) and incubated for 18 h at 37 °C. The supernatant was subsequently distilled, and tritium release was quantified with liquid scintillation counting. Alternatively, when specified, assays using 25 μg of dermatan were performed in the same buffer composition as above in the presence of 25 mCi of tritiated water. The assay product was subsequently purified as previously described, and the incorporated tritium was quantified (9). For kinetic assays, reactions were performed as above, varying the substrate concentration from 4 to 155 μM. A time course of the reaction showed linearity up to 4 h. Kinetic parameters were calculated from the obtained initial rates of product formation (4-h assay) using a nonlinear fitting regression analysis (Hyperbolic Regression Analysis Method, available on the World Wide Web).

RESULTS AND DISCUSSION

Structure Modeling of DS-epimerase 1—DS-epimerase 1 was predicted to have three distinct domains using Ginzu (Fig. 1A). Despite low sequence similarity, the N-terminal domain showed significant structural homology to several PL5 and PL8 family lyases, such as heparinase II, hyaluronan lyase, agalactiane lyase, and chondroitinase ACI (19–21). The prediction was validated by submission of the primary sequence of DS-epimerase 1 to the Structure Prediction Metaserver (Table S1) (22). The central domain showed a significant homology only with heparinase II. The N-terminal domain (amino acids 1–390) is predicted to be an α-helical domain (SCOP superfamily, a.102.2) containing 13 α-helices shaped as an incomplete (α/α)₉ toroid. The central domain (amino acids 391–690) is a β-sheet domain, where the β-strands form a two-layered stack (predicted SCOP superfamily, b.30.5). No homolog of known structure could be detected for the C-terminal domain (amino acids 691–958), and we therefore attempted to predict the SCOP superfamily using a Rosetta de novo method, but the results were inconclusive (23). Secondary structure predictions showed that this domain contained mostly α-helices (Fig. 1A).

Since our initial prediction only generated models of the individual domains, the crystal structure of heparinase II (2FUT) was subsequently used as a template to build a full-length tertiary structure model of the N-terminal and central domain of DS-epimerase 1 (Fig. 1B). The N-terminal and central domain together form a groove where the walls are built up by α-helices 2–7 in the N-terminal domain, whereas several loops in the
central domain form the second wall. Differences between heparinase II and DS-epimerase 1 could be identified in this region, where in the latter several loops are considerably longer. The bottom of the groove is formed by a loop connecting α-helices 8 and 9 together with α-helix 11 and a loop connecting α-helices 12 and 13.

The structural homology to PL5 and PL8 family lyases strongly suggests that DS-epimerase 1 has evolved from these bacterial enzymes. In addition, the lyase and the polymer level epimerase reaction have been proposed to proceed in a similar manner (24).

Identification of the Active Site in DS-epimerase 1—In an effort to identify the active site of DS-epimerase 1 we turned to similarities with heparinase II. In the latter, the active site is located in the groove formed by the N-terminal α-helical domain together with the central domain, as seen in DS-epimerase 1 (Fig. 1B) (20). The active site of heparinase II has been suggested to consist of a histidine (His-406) or a tyrosine (Tyr-257), functioning as the general base abstracting the C5 proton from GlcA. In the second step, a tyrosine (Tyr-257) is believed to function as a general acid by donating a proton to the 4-O-glycosidic bond, resulting in cleavage of the linkage. As mentioned above, DS-epimerase 1 and heparinase II are highly diverged at the primary sequence level. However, a structure-based sequence alignment shows conservation of His-450 and Tyr-261 corresponding to the residues implied in the catalytic activity of heparinase II (Fig. 2). These residues are also conserved in DS-epimerase 2 (data not shown). Superposition of the predicted DS-epimerase 1 structure and heparinase II crystal structure (2FUT) shows that these residues are spatially conserved and well oriented in regard to the C5 proton and the glycosidic oxygen of a modeled chondroitin sulfate tetrasaccharide substrate, respectively (Fig. 3A). Based on this structural conservation, it is therefore likely that these residues also play a role in the first steps of epimerization of GlcA.

FIGURE 1. Domain structure of DS-epimerase 1. A, schematic representation of the DS-epimerase 1 domain structure, showing individual domains with SCOP predictions, as determined using Ginzu. The predicted secondary structure elements are depicted below the schematic view, where red boxes represent α-helices and blue boxes represent β-strands. N-Glycosylation sites, a predicted signal peptide (SP), and two potential C-terminal transmembrane domains (TM) are also depicted. B, schematic representation of DS-epimerase 1 showing a chondroitin sulfate tetrasaccharide located in a groove formed by the N-terminal domain (residues 1–390) (red), where each α-helix is labeled, and the central domain (residues 391–690) (blue). The chondroitin sulfate tetrasaccharide was placed in the modeled DS-epimerase 1 by manual alignment of the chondroitinase AC1 crystal structure (1HMW). N-Glycosylation sites are indicated with arrows and labeled N1–N4. The structure diagram was generated with PyMOL.

FIGURE 2. Partial structural sequence alignment of DS-epi1, heparinase II and chondroitinase AC-I. The structural sequence alignment was generated with Mammoth Multi using the homology model of DS-epi1 and the crystal structures of heparinase II (2FUT) and chondroitinase AC-I (1RW9) (29). Amino acids involved in catalysis are boxed, and secondary structure elements are indicated above (DS-epi1 and heparinase II) and below (chondroitinase AC-I) the sequences. Stars indicate residues investigated with site-directed mutagenesis. A full-length alignment can be found in Fig. S1.
A prerequisite for epimerization to occur is the presence of a general acid that replaces the abstracted C5 proton from the opposite side of the sugar plane to achieve the movement of the carboxyl group from an equatorial to an axial position. In addition, this general acid must be different from the general base abstracting the C5 proton. Based on these prerequisites, we searched for potential general acids in our modeled structure. A closer inspection of the proposed active site showed the presence of two histidines (His-203 and His-205) located at the top of α-helix 8 opposite to His-450. His-203 is pointing away from the bound substrate, whereas the position of His-205, which is pointing toward the C5 position of GlcA, fulfills the criteria of a general acid, located in such a way that it respects the axial-equatorial requirements for proton abstraction and readdition (Fig. 3A). In the reverse reaction, His-205 would instead function as a general base and His-450 as a general acid. This view is strengthened by the conservation of His-205 in heparinase II (His-202), which has the ability to cleave both GlcA and IdoA linkages (Fig. 3, A and B). A similar composite active site has also recently been suggested for chondroitinase ABC (25). Interestingly, chondroitinase ACI, which can only cleave GlcA, lacks the corresponding histidine (Fig. 3C).

Mutational Analysis of DS-epimerase 1—Candidate amino acids were mutated by site-directed mutagenesis and expressed in 293HEK cells. C-terminally tagged recombinant enzymes were subsequently purified by binding to IgG-Sepharose and assayed for epimerase activity, measured as the release of tritium from [5-3H]dK4 or as the incorporation of tritium into dermatan sulfate (dermatan).

Incubation of 5-3H-labeled dK4 with a purified wild-type version of DS-epimerase 1 resulted in substantial release of tritium (1200 dpm) from the substrate, whereas no tritium release was noted from mock lysates (Fig. 4A). Subsequently, we analyzed the effect of replacing His-450 with an alanine or asparagine. These mutants did not show any release of tritiated water from [5-3H]dK4, indicating that the C5 proton could not be abstracted (Fig. 4A). To address whether this residue is functioning as a general acid in the conversion of IdoA to GlcA, the incorporation of tritium from tritiated water into dermatan was measured. The H450A mutant did not show any incorporation of tritium into the substrate, whereas the purified wild-type protein incorporated 4200 dpm. These results are in agreement with structural findings suggesting a function of His-450 as the general base (proton abstractor) in the conversion of GlcA to IdoA and as a general acid (proton donor) in the reverse reaction (Fig. 5). It has previously been suggested that proton abstraction from GlcA is performed by a polyprotic base, such as a lysine (9). However, we could not find any lysines present in the active site in our model of DS-epimerase 1. Our structural data and the fact that the H450A/N mutants are inactive show that H450 is indeed the general base.

We then continued to investigate the role of His-203 and His-205 in catalytic activity. Mutation of His-205 into alanine...
or asparagine resulted in a complete loss of activity toward chondroitin, suggesting a role as a general acid (Fig. 4A). On the contrary, the H203A and H203N mutants still retained around 9.6 and 5.2% of the activity compared with the wild-type enzyme, indicating that His-203 is not directly involved in the catalytic mechanism. His-203 might instead be involved in positioning of the substrate within the active site (Fig. 4). The fact that no tritium release was observed from [5-3H]dK4 incubated with the H205A/N mutants and no tritiated water-derived tritium was incorporated into dermatan with the H450A mutant shows that after proton abstraction, there is no immediate exchange of the abstracted proton with the solvent. Interestingly, the absence of tritium release from [5-3H]dK4 incubated with the H205A/N mutants indicates that after proton abstraction by His-450, the intermediate can be reprotonated by the same residue.

FIGURE 5. Proposed molecular mechanism of epimerization. A schematic diagram shows the conversion of glucuronic acid to iduronic acid, initiated by C5 proton abstraction by the general base His-450 (1). Subsequent β-elimination, mediated by Tyr-261 (2), creates a 4,5-unsaturated hexuronic intermediate, which is reprotonated from the opposite side of the sugar plane by His-205 (5) with concomitant recreation of the glycosidic linkage (3, 4). The small arrows indicate the flow of electrons during the reaction.}

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The Tyr-257 is thought to be essential for heparinase II to cleave the sugar chain and is also found in a structurally equivalent position in DS-epimerase 1 (Tyr-261) (Fig. 3). This residue was therefore mutated into alanine, and the mutant was indeed shown to be inactive using both dermatan (data not shown) and chondroitin as substrates (Fig. 4A). Similar results were also obtained replacing the tyrosine with a phenylalanine. This is intriguing and suggests that during epimerization, a cleaved 4,5-unsaturated hexuronic intermediate is formed (Fig. 5). This conclusion is strengthened by the fact that a 4,5-unsaturated intermediate has been shown to exist in the C5 mannanuron epimerase reaction, which also takes place at the polymer level (26). How the glycosidic linkage is subsequently reformed in an epimerase compared with a lyase remains an open question. It is possible that the tyrosinate ion formed after cleavage is also involved in the reformation of the glycosidic linkage before reprotonation of the C5 carbon (Fig. 5). Whether this is a general mechanism for epimerases acting at the polymer level remains to be elucidated, since no structure is currently available for the heparan sulfate epimerase. A homology search using the sequence of the HS epimerase did not show any significant structural hits, suggesting that it has a currently unknown fold. This line of evidence strengthens the view that the DS-epimerases and the HS-epimerase are not evolutionarily related but does not exclude a similar reaction mechanism.

To rule out the possibility that mutation of the above residues caused any major misfolding, expression levels of the wild-type and mutant enzymes were evaluated with Western blot. When transiently overexpressed in 293HEK cells, the mutant constructs showed similar expression levels as the wild-type enzyme after purification on an IgG-Sepharose (Fig. 4B). A charged amino acid (Lys-331) located in /H9251-H9252-helix 12 outside of the active site and Tyr-256 located in the same loop as the catalytic residue Tyr-261 were also mutated into alanine to serve as general controls of mutation. These two mutants showed activity similar to that of the wild-type enzyme (Fig. 4A). Together, these data indicate that the mutations performed do not induce any major misfolding of the protein that could explain the loss of activity.
DS-epimerase 1 Active Site

A

B

C

FIGURE 6. N-glycosylation of DS-epimerase 1 is required for activity. 293HEK cell lysates overexpressing DS-epi1/CTAP (A and B) or DS-epi1/His wild type (WT) and mutants (C) were analyzed by Western blot (A and C) and by activity toward chondroitin (B). In A and B, cells were treated as indicated with tunicamycin for 24 h. A and C, Ponceau red stainings were used to estimate equal loading. The arrows indicate the shift in molecular weight. B and C, the specific activity of DS-epi1/CTAP lysate was 891 dpm/h/mg. Error bars, ± S.D. of three independent experiments.

N-Glycosylation Is Required for DS-epimerase 1 Activity—Several enzymes involved in the biosynthesis of dermatan sulfate have been shown to be dependent on N-glycosylation for proper function (27, 28). We therefore investigated the importance of N-glycosylation for enzymatic activity of DS-epimerase 1. The enzyme has four predicted N-glycosylation sites (Fig. 1), as determined by the NetNGlyc program (available on the World Wide Web) and no predicted O-glycosylation sites. To experimentally determine if DS-epimerase 1 is N-glycosylated, cells overexpressing the enzyme were treated with tunicamycin. After a 24-h incubation with 1 μg/ml tunicamycin, a second band with a shift in molecular mass of ~8 kDa could be detected (Fig. 6A). To determine if N-glycosylation is required for enzyme activity, lysates from control and tunicamycin-treated cells were compared for their ability to convert GlcA into IdoA. After tunicamycin treatment, epimerase activity was reduced by 70% compared with the control (Fig. 6B). Similar results were also obtained by purifying DS-epi1 after tunicamycin treatment (data not shown). This reduction in activity cannot simply be explained by an impaired production of the protein in the absence of N-glycosylation, since tunicamycin-treated cells showed an expression of DS-epimerase 1 equal to that of control cells (Fig. 6A). To investigate if the glycosylation status could influence detection of DS-epi1 by Western blot and hamper the interpretation of expression levels in the experiment above, lysates were treated with peptide N-glycosidase F. No difference in band intensity could be detected after digestion (data not shown). The residual activity observed is probably due to the fully glycosylated enzyme that can still be found after tunicamycin treatment (Fig. 6A). In agreement with the essential role of N-glycosylation, the full-size protein expressed in a bacterial system was inactive (data not shown).

DS-epimerase 1 Carries Four N-Glycosylation Chains—To address which predicted sites are carrying N-glycans, mutants were constructed for each site by replacing the asparagine residues with serine. The mutants were subsequently expressed in 293HEK cells and analyzed with Western blot. All four mutants showed a shift of about 2 kDa, indicating that the four predicted sites are all occupied (Fig. 6C). The mutants were expressed at the same level, indicating that no single N-glycosylation site is necessary for expression of the enzyme. This is in agreement with the tunicamycin data above. We then investigated the role of each N-glycosylation site in enzyme activity. N183S and N642S mutants had a 3.6- and 1.5-fold reduction in activity compared with the tunicamycin data above. We then investigated the role of each N-glycosylation site in enzyme activity. N183S and N642S mutants had a 3.6- and 1.5-fold reduction in $V_{max}$ values compared with that of the wild-type protein, respectively (Table 1). The N336S and N648S mutants had a strong reduction in catalytic activity (13- and 43-fold) with minor but discernable release of tritium from the substrate (Table 1). The almost complete loss of activity observed for the N336S and N648S mutants is hard to reconcile with a mechanism where the N-glycosylation chains are somehow involved in substrate binding due to their location far away from the active site (Fig. 1B). A more feasible explanation would be that the lack of large N-glycosylation chains in these regions induces local changes in structure that result in reduction of enzyme activity. This view is strengthened by kinetic analysis of the various mutants. The major effect was seen in $V_{max}$ and not $K_m$ values, indicating an aberrant enzyme function rather than impaired substrate binding (Table 1).

CONCLUSION

In summary, this study provides the first structural investigation of a glycosaminoglycan epimerase and the molecular mechanism behind epimerization of chondroitin. We conclude, through structure-based bioinformatics, that the DS-epimerases have evolved from bacterial lyases. The active
site of DS-epimerase 1 is located in a groove formed by two distinct domains of the enzyme. Based on site-directed mutagenesis and structural conservation, we propose a mechanism of epimerization where His-450 functions as the initial general base abstracting the C5 proton from GlcA. The subsequent cleavage of the glycosidic linkage by Tyr-261 generates a 4,5-unsaturated intermediate, which is protonated from the opposite side of the sugar plane by His-205 with a concomitant recreation of the glycosidic linkage by either the tyrosinate ion generated after cleavage or a second general acid, which was not identified in this study (Fig. 5). In addition, we show that proper N-glycosylation is necessary for the production of an active DS-epimerase 1.

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