C3- and C3/C5-Epimerases Required for the Biosynthesis of the Capsular Polysaccharides from Campylobacter jejuni

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ABSTRACT: Campylobacter jejuni is a human pathogen and one of the leading causes of food poisoning in Europe and the United States. The outside of the bacterium is coated with a capsular polysaccharide that assists in the evasion of the host immune system. Many of the serotyped strains of C. jejuni contain a 6-deoxy-heptose moiety that is biosynthesized from GDP-D-glycero-D-manno-heptose by the successive actions of a 4,6-dehydratase, a C3/C5-epimerase, and a C4-reductase. We identified 18 different C3/C5-epimerases that could be clustered together into three groups at a sequence identity of >89%. Four of the enzymes from the largest cluster (from serotypes HS:3, HS:10, HS:23/36, and HS:41) were shown to only catalyze the epimerization at C3. Three enzymes from the second largest cluster (HS:2, HS:15, and HS:42) were shown to catalyze the epimerization at C3 and C5. Enzymes from the third cluster were not characterized. The three-dimensional structures of the epimerases from serotypes HS:3, HS:23/36, HS:15, and HS:41 were determined to resolutions of 1.5−1.9 Å. The overall subunit architecture places these enzymes into the diverse “cupin” superfamily. Within X-ray coordinate error, the immediate regions surrounding the active sites are identical, suggesting that factors extending farther out may influence product outcome. The X-ray crystal structures are consistent with His-67 and Tyr-134 acting as general acid/base catalysts for the epimerization of C3 and/or C5. Two amino acid changes (A76V/C136L) were enough to convert the C3-epimerase from serotype HS:3 to one that could now catalyze the epimerization at both C3 and C5.

INTRODUCTION

Campylobacter jejuni is a Gram-negative pathogenic bacterium commonly found in chickens and cattle and is the leading cause of food poisoning in North America and Europe.1,2 C. jejuni has shown high adaptability, antibiotic resistance, and an ability to evade the host immune response.3 Like many enteric bacteria, the various strains of C. jejuni produce different carbohydrate-based lipooligosaccharides (LOS) and capsular polysaccharides (CPS). These polysaccharides are essential for the structural integrity and maintenance of the bacterial cell wall.3,4 Deletion of the polysaccharide synthesis genes drastically decreases the pathogenicity of C. jejuni.5 Thus, the enzymes responsible for the biosynthesis of these polysaccharides are potential therapeutic targets.6

The capsular polysaccharides from C. jejuni are composed of repeating units of monosaccharides attached to one another via glycosidic bonds. Perhaps the most well-characterized example comes from C. jejuni NCTC 11168 (serotype HS:2). This CPS consists of a repeating series of D-ribose, N-acetyl-D-galactosamine, D-glucuronate, and D-glycero-D-gluco-heptose sugars, as shown in Scheme 1.6 There are at least 12 known CPS structures identified to date, and nine of these contain either a heptose or a 6-deoxy-heptose moiety.7 A total of 10 different heptose variations have been structurally characterized within the known serotypes of C. jejuni (Scheme 2), and it is currently thought that all of the heptose variations originate from GDP-D-glycero-α-D-manno-heptose.8−10

Of the 10 heptose sugars shown in Scheme 2, the biosynthetic pathways for only two of them have been...
functionally characterized. The Creuzenet laboratory has shown that GDP-6-deoxy-α-D-altro-heptose from the HS:23/36 serotype is synthesized from GDP-D-glycero-α-D-manno-heptose via the combined action of a 4,6-dehydratase, a C3-epimerase, and a C4-reductase. We have demonstrated that GDP-D-glycero-β-L-gluco-heptose from the HS:2 serotype can be synthesized via the combined activities of a C4-dehydrogenase, a C3/C5-epimerase, and a C4-reductase. These two pathways are summarized in Scheme 3. At C3 and C5, there are four possible stereocchemical combinations, and all four have been identified in the 10 heptoses examined to date. It is apparent that an epimerase is responsible for the racemization of the stereochemistry at C3 and/or C5 after oxidation of C4, but it is not clear as to whether the ultimate stereochemistry of the heptose is dictated by the substrate profile for the C4-reductase.

Here, we have purified and functionally characterized seven different C3/C5-epimerases from various strains of C. jejuni. Three of them were found to epimerize C3 and C5, while four of them were found to epimerize only C3. The three-dimensional structures for four of these enzymes were determined to high resolution. Two mutations to the C3-only group of epimerases conferred the ability to epimerize both C3 and C5.

■ MATERIALS AND METHODS

Materials. Lysogeny broth (LB) medium, isopropyl-β-D-thiogalactopyranoside (IPTG), and NADPH were purchased from Research Products International. The protease inhibitor cocktail, lysozyme, kanamycin, imidazole, Tris, and HEPES were obtained from Sigma-Aldrich. Ammonium bicarbonate, 2-mercaptoethanol, KCl, and MgCl₂ were acquired from Sigma-Aldrich. DNase I was purchased from Roche. HisTrap columns and Vivaspin 20 spin filters were obtained from Cytiva. The 3 kDa Nanosep spin filters were purchased from Pall Corporation (Port Washington, NY). Deuterium oxide was acquired from Cambridge Isotope Laboratories Inc. Oligonucleotide primers were bought from Integrated DNA Technologies Inc. Polymerase chain reaction (PCR) reagents, pfu polymerase buffers, Escherichia coli XL1 Blue, and BL21 (DE3) strains were purchased from New England Biolabs. The kit for the isolation of DNA was obtained from Qiagen.

Equipment. Ultraviolet spectra were collected on a SpectraMax 340 (Molecular Devices) ultraviolet-visible plate reader using 96-well Greiner plates. ¹H NMR spectra were recorded on a Bruker Avance III 400 MHz system equipped with a broadband probe and sample changer. Mass spectrometry data were collected on a Thermo Scientific Q Exactive Focus system run in negative ion mode.

Sequence Similarity Network Analysis of Epimerases. The FASTA protein sequence for Cj1430 from C. jejuni NCTC 11168 (serotype HS:2) was used as the initial BLAST (Basic Local Alignment Search Tool) query in the EFI-EST database (Enzyme Function Initiative-Enzyme Similarity Tool, https://ef.igb.illinois.edu/ef-est/). The sequence similarity networks (SSN) were generated by submitting the FASTA

sequences to the EFI-EST webtool. All network layouts were created and visualized using Cytoscape 3.8.2.17

Homologues to Cj1430 from C. jejuni NCTC 11168. The DNA for the expression of the genes homologous to Cj1430 from C. jejuni NCTC 11168 were chemically synthesized and codon-optimized from either Twist Biosciences or GenScript. These genes included those from serotypes HS:2 (UniProt entry: Q0P8I6), HS:3 (UniProt entry: F2X701), and HS:15 (UniProt entry: F2X7A6). The DNA was inserted between the Ndel and XhoI restriction sites of a pET-28a (+) expression vector. The gene constructs encode for the expression of an N-terminal His$_6$-affinity tag, and the complete amino acid sequences of the seven proteins purified for this investigation are shown in Figure S1.

Protein Expression and Purification. The wild-type epimerases from the HS:2, HS:3, HS:10, HS:15, HS:23/36, HS:41, and HS:42 serotypes, in addition to the variants made from the HS:3 and HS:15 serotypes, were purified according to the procedure reported previously.13 Similarly, the wild-type C4-reductases from serotypes HS:2, HS:3, and HS:15 were expressed and purified according to a published procedure.19 E. coli BL21(DE3) competent cells were transformed by the appropriate plasmids. Single colonies were inoculated in 50 mL of LB medium (20 g/L yeast extract, 35 g/L tryptone, 5 g/L sodium chloride, pH 7.0) supplemented with 50 μg/mL kanamycin and grown at 37 °C with shaking. The starter cultures were used to inoculate 1 L of LB medium, grown at 37 °C with shaking to an OD$_{600}$ of ~0.8. Expression was induced by the addition of IPTG to a final concentration of 1.0 mM. The cultures were subsequently incubated for 18 h at 15 °C, with shaking at 140 rpm. The cells were harvested by centrifugation at 7000g for 10 min at 4 °C, frozen in liquid N$_2$, and stored at −80 °C.

Purification of the seven epimerases was conducted at 22 °C. In a typical purification, ~5 g of frozen cell paste were resuspended in 50 mL buffer A (50 mM HEPES, pH 7.5, 250 mM KCl, 5.0 mM imidazole) supplemented with 0.1 mg/mL lysozyme, 0.05 mg/mL protease inhibitor cocktail powder, 40 U/mL DNase I, and 10 mM MgCl$_2$. The suspended cells were lysed by sonication (Branson 450 Sonifier), and the supernatant solution was collected after centrifugation at 10,000g for 30 min. The supernatant solution was loaded onto a prepacked 5-mL HisTrap column and eluted with a linear gradient of buffer B (50 mM HEPES, pH 7.5, 250 mM KCl, 500 mM imidazole). Fractions containing the desired protein, as identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were combined and concentrated in a 20-mL spin filter with a 10 kDa molecular weight cutoff. The imidazole was removed from the protein by dialysis using buffer C (50 mM HEPES, pH 7.5, 250 mM KCl). The protein was concentrated to 5–10 mg/mL, aliquoted, frozen in liquid N$_2$, and stored at −80 °C. Typical yields of 20–30 mg were obtained from 1 L of culture.

Determination of Protein Concentration. Concentrations of the proteins were determined spectrophotometrically using computationally derived molar absorption coefficients at 280 nm.18 The values of ε$_{280}$ (M$^{-1}$ cm$^{-1}$) used for the epimerases from serotypes HS:2, HS:3, HS:10, HS:15, HS:23/36, HS:41, and HS:42 are 41,370, 34,380, 35,870, 39,880, 34,380, 34,380, and 39,880, respectively.
Mutagenesis of the Epimerase from Serotype HS:3. Mutations were made to the C3-epimerase from serotype HS:3 in an attempt to alter the reaction specificity to match that of the C3/C5-epimerase from HS:2. This was done by changing the conserved residues found at specific locations within the HS:3 serotype for those found in the epimerase from the HS:2 serotype. All site-directed amino acid changes were constructed using the QuickChange mutagenesis kit from Qiagen. Three single-site variants (A76V, C136L, and E128K), two double-site variations (A76V/C136L and E128K/T129E), and one triple-site substitution (A76V/A122S/C136L) of the epimerase from serotype HS:3 were prepared. For the A76V, C136L, and E128K single-site variants, the gene for the wild-type epimerase from HS:3 was used as the template. For the A76V/C136L and E128K/T129E variants, the genes from A76V and E128K, respectively, were used as the initial template. For the A76V/A122S/C136L modification, the gene for A76V/C136L was used as the template. Polymerase chain reactions (PCR) were conducted to amplify the genes using a PTC-200 Peltier thermal cycler (MJ Research, Waltham, MA). Mutations were confirmed by DNA sequencing (Eton Bioscience Inc.).

Mutation of His67 and Tyr134 from the Epimerases from Serotypes HS:3 and HS:15. Mutation of the two putative general acid/base catalysts in the active site of the epimerases from the HS:3 and HS:15 serotypes was completed. Two single-site variants (H67N and Y134F) of each epimerase were prepared. The DNA for the H67N and Y134F of the epimerase from HS:3 and HS:15, respectively, were chemically synthesized and codon-optimized by GenScript. The DNA was inserted between the NdeI and XhoI restriction sites of a pET-28a (+) expression vector. The genes enable the expression of an N-terminal His6-terminally His6-tag, and the complete amino acid sequences of the eight variants are provided in Figure S3.

Determination of Kinetic Constants. The kinetic constants for the reaction catalyzed by the various epimerases were determined using a coupled enzyme assay with the appropriate C4-reductase by monitoring the oxidation of NADPH to NADP+ at 340 nm. GDP-6-deoxy-4-keto-α-L-lyxo-heptose (2) was obtained by incubation of 4.0 mM GDP-6-deoxy-4-keto-α-L-lyxo-heptose (2) with 5.0 μM GDP-6-deoxy-4-keto-α-L-lyxo-heptose 4,6-dehydratase from C. jejuni 81–176 (HS:23/36) in buffer D at 20 °C. The dehydratase was removed using a 3 kDa molecular weight cutoff spin filter. For the determination of the kinetic constants, the concentration of GDP-6-deoxy-4-keto-α-L-lyxo-heptose (2) was varied between 10 μM and 2.5 mM. The assays were carried out in a total reaction volume of 250 μL with 100 nM epimerase, 20 μM C4-reductase, and 300 μM NADPH in buffer D at 25 °C. The apparent values of κcat and kcat/Km were determined by fitting the initial velocity data to eq 1 using SigmaPlot 11.0, where ν is the initial velocity of the reaction, Ei is the enzyme concentration, S is the substrate concentration, κcat is the turnover number, and Km is the Michaelis constant.

\[
ν/E_i = κ_{cat}[S]/(K_m + [S])
\]

Crystallographic Analyses. Crystals of the tag-free enzyme at 40 mg/mL and 5.0 mM GDP were grown at room temperature from 10 to 12% poly(ethylene glycol) 8000, 0.75 M tetramethylammonium chloride, and 100 mM homopiperazine-1,4-bis(2-ethanesulfonic acid) at pH 5.0. For X-ray data collection, the crystals were transferred to a cryo-protectant solution composed of 20% poly(ethylene glycol) 8000, 1.0 M tetramethylammonium chloride, 300 mM NaCl, 5.0 mM GDP, 10% ethylene glycol, and 100 mM homopiperazine-1,4-bis(2-ethanesulfonic acid) at pH 5.0.

Epimerase from Serotype HS:3. Crystals of the tag-free enzyme at 24 mg/mL and 5.0 mM GDP were grown at room temperature from 18 to 20% poly(ethylene glycol) 8000, 0.75 M tetramethylammonium chloride, and 100 mM homopiperazine-1,4-bis(2-ethanesulfonic acid) at pH 5.0. For X-ray data collection, the crystals were transferred to a cryo-protectant solution composed of 28% poly(ethylene glycol) 3350, 250 mM NaCl, 250 mM KCl, 5.0 mM GDP, 18% ethylene glycol, and 100 mM 3-[4-(2-hydroxyethyl)-piperazin-1-yl]propane-1-sulfonic acid at pH 8.0.

Epimerase from Serotype HS:15. Crystals of the tag-free enzyme at 24 mg/mL and 5.0 mM GDP were grown at room temperature from 26 to 28% poly(ethylene glycol) 8000 and 100 mM 3-morpholinopropane-1-sulfonic acid at pH 7.0. For X-ray data collection, the crystals were transferred to a cryo-protectant solution composed of 31% poly(ethylene glycol) 8000, 300 mM NaCl, 5.0 mM GDP, 5% ethylene glycol, and 100 mM 3-morpholinopropane-1-sulfonic acid at pH 7.0.
Table 1. X-ray Data Collection and Model Refinement Statistics

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**a**R_{free} = (Σ|F_o| - |F_i|) / Σ|F_o|) × 100. **b**Statistics for the highest resolution bin. **c**R-factor = (Σ|F_o| - |F_i|) / Σ|F_o|) × 100, where F_o is the observed structure-factor amplitude and F_i is the calculated structure-factor amplitude. **d**Distribution of Ramachandran angles according to PROCHECK.  

Ethylene glycol, and 100 mM 3-morpholinopropane-1-sulfonic acid at pH 7.0.

X-ray data were collected at 100 K utilizing a BRUKER D8-VENTURE sealed tube system equipped with Helios optics and a PHOTON II detector. These X-ray data were processed with SAINT and scaled with SADABS (Bruker AXS). All structures were solved via molecular replacement with the software package PHASER and using the structure of Cj1430 from C. jejuni NCTC 11168 (HS:2) (PDB entry 7M14) as the search probe. 15,19 The models were refined by iterative cycles of model building in COOT 20,21 and refinement with REFMAC. 22 X-ray data collection and refinement statistics are provided in Table 1.

### RESULTS AND DISCUSSION

**Bioinformatic Analysis of the C3/C5-Epimerases.** To better understand the sequence diversity within the C3/C5-epimerases responsible for the synthesis of the stereocchemical modifications to the heptose moieties of the CPS from C. jejuni, a bioinformatic analysis was conducted. The amino acid sequence of the epimerase from C. jejuni NCTC 11168 (serotype HS:2) was used as the initial BLAST (Basic Local Alignment Search Tool) query in the EFI-EST database for the closest 1000 sequences. The sequence similarity network (SSN) was constructed at a sequence identity of 60% and is presented in Figure 1a. The epimerase from serotype HS:2 is shown in yellow, and the epimerases from the other serotyped strains of C. jejuni are shown in green. To gain deeper insight into the sequence differences for the specific epimerases from serotyped strains of C. jejuni, we retrieved the complete FASTA protein sequences of the epimerases for the 18 serotyped strains of C. jejuni from UniProt and compiled them into a local custom database. The SSN shows that at a cutoff of 89%, the epimerases form three separate groups (Figure 1b). The largest group contains 10 sequences and includes those from serotypes HS:2, HS:3, HS:10, HS:15, HS:23/36, HS:41, and HS:42. The second most populated group contains the epimerases from HS:2 and HS:15. The third group from the HS:5, HS:11, and HS:45 serotypes are likely required for the biosynthesis of 3,6-dideoxy heptoses. A sequence identity matrix for all 18 enzymes is provided in Table S1.

**Isolation and Characterization of Seven Epimerases.** We purified the epimerases from seven different strains of C. jejuni including those from serotypes HS:2, HS:3, HS:10, HS:15, HS:23/36, HS:41, and HS:42. The epimerases were produced in E. coli BL21(DE3) with a 21-residue His₉-containing affinity tag appended to the N-terminus. The
proteins were isolated using immobilized metal ion affinity chromatography.

Isolation of GDP-6-deoxy-4-keto-α-D-lyxo-heptose (2). The enzyme-catalyzed formation of GDP-6-deoxy-4-keto-α-D-lyxo-heptose (2) involves the transient oxidation/dehydration/reduction of GDP-α-glycero-α-D-manno-heptose (1). To prepare sufficient quantities of the appropriate substrate for the various epimerases, GDP-α-glycero-α-D-manno-heptose (1) was incubated with GMH dehydratase from serotype HS:23/36 to enzymatically synthesize GDP-6-deoxy-4-keto-α-D-lyxo-heptose (2) in high yield (~95%).
The sole reaction product is GDP-6-deoxy-4-keto-

Figure 3. ¹H NMR 400 MHz spectra of product formed from GDP-6-deoxy-4-keto-α-D-lyxo-heptose (2) by the addition of C3-epimerases from serotype HS:3, HS:10, HS:23/36, or HS:41. The sole reaction product is GDP-6-deoxy-4-keto-α-D-arabino-heptose (3) formed by the catalytic activity of epimerases from serotypes HS:3 (A), HS:10 (B), HS:23/36 (C), or HS:41 (D). In these experiments, 4.0 μM of the epimerase was incubated with 4.0 mM of compound 2 for 30 min prior to the acquisition of the NMR spectrum of the products.

¹H NMR spectrum of (1) is presented in Figure S5a, and the isolated dehydrated product (2) is presented in Figure S5b.

Reactions Catalyzed by the Epimerases from Various Strains of C. jejuni. The reaction profiles for the C3/C5-epimerases from seven different strains of C. jejuni (serotypes HS:2, HS:3, HS:10, HS:15, HS:23/36, and HS:42) were determined using the enzymatically prepared GDP-6-deoxy-4-keto-α-D-lyxo-heptose (2) as the substrate. When 2 was incubated with the epimerase from serotypes HS:2, HS:15, or HS:42, three new resonances were detected between 4.5 and 6.0 ppm in the region expected for the anomeric hydrogen of C1 (Figure 2). These results clearly indicate that each of the two sets of epimerases from serotypes HS:3, HS:10, HS:23/36, or HS:41, HS:2, HS:15, HS:23/36, HS:41, and HS:42, three new resonances were detected between 4.5 and 6.0 ppm in the region expected for the anomeric hydrogen of C1 (Figure 2). These results clearly indicate that each of the two sets of epimerases from sequence identities to one another of >89% in the SSN (Figure 1b and Table S1) have distinct product outcomes.

The likely reaction products that can be formed by any one of the seven epimerases tested are highlighted in Scheme 4. The sole epimerization at C3 or C5 will generate GDP-6-deoxy-4-keto-α-D-arabino-heptose (3) or GDP-6-deoxy-4-keto-β-L-ribo-heptose (4), respectively, whereas the double epimerization at C3 and C5 will form GDP-6-deoxy-4-keto-β-L-xylo-heptose (5). It has been previously shown that the doublet of doublets at 5.37 ppm originates from the C3-isomerized product 3 and that the C5-isomerized product 4 resonates at 5.31 ppm. The C3/C5-isomerized product 4 resonates at 5.02 ppm. The likely reaction products that can be formed by any one of the seven epimerases tested are highlighted in Scheme 4. The sole epimerization at C3 or C5 will generate GDP-6-deoxy-4-keto-α-D-arabino-heptose (3) or GDP-6-deoxy-4-keto-β-L-ribo-heptose (4), respectively, whereas the double epimerization at C3 and C5 will form GDP-6-deoxy-4-keto-β-L-xylo-heptose (5). It has been previously shown that the doublet of doublets at 5.37 ppm originates from the C3-isomerized product 3 and that the C5-isomerized product 4 resonates at 5.31 ppm. The C3/C5-isomerized product 4 resonates at 5.02 ppm.

Determination of Equilibrium Constants. The equilibrium constants from the product ratios for [3]/[2], [4]/[2], [5]/[3], and [5]/[4] are 0.22, 0.44, 3.6, and 1.8, respectively.

Relative Reaction Rates. The reaction for the epimerization of C3 and C5 was followed by ¹H NMR spectroscopy as a function of time. The substrate, GDP-6-deoxy-4-keto-α-D-lyxo-heptose (2), was incubated with 40 nM of the Cj1430 epimerase from C. jejuni NCTC 11168 (HS:2), and the time course for the formation of the reaction products 3, 4, and 5 was monitored over a period of 450 min at pH 7.5 (Figures 4 and 5). The relative concentrations of 2, 3, 4, and 5 were determined by integration of the NMR signals for the hydrogen at C1. The relative concentration of substrate 2 decreases as a function of time and eventually reaches a plateau. At the earliest reaction times, the relative concentration of 3, the C3-epimerized product, exceeded that of 4, the C5-epimerized product. At later reaction times, the absolute concentration of 3 decreased relative to the other reaction products 4 and 5. These results suggest that the epimerization of C3 is initially faster than the epimerization of C5.

Catalytic Activity of the Epimerases from Serotypes HS:3 and HS:15. The catalytic activities of two epimerases were determined using a coupled enzyme assay with the appropriate C4-reductase by monitoring the oxidation of NADPH as a function of time. To obtain the catalytic activity of the epimerases from serotypes HS:3 and HS:15, the C4-reductases from serotypes HS:3 (locus_tag HS3.14) and HS:15 (locus_tag HS15.12) were used. The assays were carried out at pH 7.5 and 25 °C. The kinetic constants were determined using GDP-6-deoxy-4-keto-α-D-lyxo-heptose (2) as the initial substrate for either of the two epimerases. The kinetic constants for the epimerase from serotype HS:3 are $k_{cat} = 5.8 \pm 0.2 \text{s}^{-1}$, $K_m = 440 \pm 35 \text{μM}$, and $k_{cat}/K_m = 13,200 \pm 800 \text{M}^{-1} \text{s}^{-1}$. For the epimerase from serotype HS:15, the kinetic constants are $k_{cat} = 2.6 \pm 0.1 \text{s}^{-1}$, $K_m = 180 \pm 17 \text{μM}$, and $k_{cat}/K_m = 14,400 \pm 1000 \text{M}^{-1} \text{s}^{-1}$. These kinetic constants are similar to that reported for the epimerase from serotype HS:2 ($k_{cat} = 3.5 \pm 0.2 \text{s}^{-1}$, $K_m = 136 \pm 16 \text{μM}$, and $k_{cat}/K_m = 25,700 \pm 3400 \text{M}^{-1} \text{s}^{-1}$) using GDP-β-glycero-4-keto-α-D-lyxo-heptose as substrate.
Mutation of Active Site Residues. The three-dimensional structures of the C3- and C3/C5-epimerases reported here are all superimposable with one another (shown below), but the reaction outcomes are different. An amino acid sequence alignment for the seven epimerases evaluated for this investigation is provided in Figure 6. Even though the four epimerases that catalyze changes in stereochemistry at both C3 and C5, there are regions in both sets of enzymes that are fully conserved in the other group. In addition to these differences, there are five residues near the two critical active site residues (His-67 and Tyr-134) that are differentially conserved between these two groups of enzymes. These residues include Ala-76, Ala-122, Glu-128, Ser/Thr-129, and Cys-136 for the C3-epimerases and Val-76, Ser-122, Lys-128, Glu-129, and Leu-136 for the C3/C5-epimerases (Figure 6).

We investigated the influence of these differentially conserved residues from the HS:3 serotype in an attempt to convert a C3-epimerase into one that catalyzes epimerization at both C3 and C5. The A76V, C136L, E128K/T129E, A76V/C136L, A76V/A122S/C136L, A39Y/V40L/D41L/L44V/N46D/L48I/I51K/H57N/K59H/H60F, and H100W/R102K/N106S/Q107Y/D108K/K111Q/I112L/V115L/H117P/G118N/F119M epimerase variants made of the wild-type enzyme from serotype HS:3 were assayed for their ability to catalyze the epimerization of GDP-6-deoxy-4-keto-α-D-lyxo-heptose (2). Epimerization activity at both C3 and C5 was observed only for the A76V/C136L and A76V/A122S/C136L variants after incubation with GDP-6-deoxy-4-keto-α-D-lyxo-heptose (2) for 15 h at 20 °C (pD 7.5) (Figures 7 and S6).

**Catalytic Activity of the Mutated Epimerases from Serotype HS:3.** The catalytic activities of the two mutant epimerases from HS:3 that possess C3/C5-epimerase activity were determined using a coupled enzyme assay using the C4-reductase from HS:2 by monitoring the oxidation of NADPH as a function of time. The assays were carried out at pH 7.5 and 25 °C. The kinetic constants were determined using GDP-6-deoxy-4-keto-α-D-lyxo-heptose (2) as the initial substrate for either of the two mutant epimerases. The kinetic constants for the formation of the C3/C5-epimerized product are $k_{cat} = 0.032 ± 0.001 \text{ s}^{-1}$, $K_m = 156 ± 12 \mu M$, and $k_{cat}/K_m = 205 ± 12 \text{ M}^{-1} \text{ s}^{-1}$ for the A76V/C136L variant using the C4-reductase from serotype HS:2. The $k_{cat}$, $K_m$, and $k_{cat}/K_m$ for the formation of the C3-epimerized product is $2.4 ± 0.2 \text{ s}^{-1}$, $156 ± 12 \mu M$, and $15,400 ± 1750 \text{ M}^{-1} \text{ s}^{-1}$, respectively, using the C4-reductase from HS:3 as the coupling enzyme. For the A76V/A122S/C136L variant, the kinetic constants for isomerization at C3/C5 are $k_{cat} = 0.052 ± 0.001 \text{ s}^{-1}$, $K_m = 125 ± 12 \mu M$, and $k_{cat}/K_m = 419 ± 62 \text{ M}^{-1} \text{ s}^{-1}$ using the C4-reductase from serotype HS:2. The $k_{cat}$, $K_m$, and $k_{cat}/K_m$ for the isomerization at C3 are $2.5 ± 0.2 \text{ s}^{-1}$, $125 ± 12 \mu M$, and $20,000 ± 2500 \text{ M}^{-1} \text{ s}^{-1}$, respectively, using the C4-reductase from serotype HS:3. It is clear that the two mutants are able to catalyze the additional isomerization at C5 but the rate, relative to that for the isomerization at C3, is significantly slower.

**Crystal Structures of Epimerases from C. jejuni.** The crystals of the C-3-epimerase from C. jejuni serotype HS:3 in complex with GDP belonged to the space group P2$_1_2_1_2$. With a dimer in the asymmetric unit. The structural model was refined to an overall R-factor of 19.3% at 1.5 Å resolution. Each subunit of the dimer consists of 13 $\alpha$-carbons for the two subunits in the asymmetric unit superimpose with a root-mean-square deviation of 0.3 Å.

As can be seen in Figure 8a, the first four N-terminal amino acid residues and the $\beta$-hairpin motif formed by Asn-22 to Thr-36 from one subunit reach over to the second subunit to form a binding platform for the GDP ligand. As a consequence, the active site is shared between the two subunits. A stereo view surrounding the ligand (labeled B in the X-ray coordinate file) is displayed in Figure 8b. The guanine ring is positioned into the active site by the side chains of Asn-22, Thr-33, and Lys-54 and a water molecule (subunit A). The ribose hydroxyls lie within 3.2 Å of the backbone amide and carbonyl oxygen of Met-1 of subunit A and a water molecule. The pyrophosphoryl group is surrounded by six waters and the guanidinium groups
of Arg-28 (subunit A) and Arg-64 (subunit B). The positions of the catalytic residues, His-67 and Tyr-134 (subunit B), are shown in Figure 8b. Note that Ile-66 adopts the cis peptide conformation. Also included in Figure 8b are the locations of Ala-76 and Cys-136, which were changed to valine and leucine, respectively, in this investigation.

Crystals of the C-3 epimerase from \textit{C. jejuni} serotype HS:23/36 in complex with GDP belonged to the space \textit{P2}_1 with a dimer in the asymmetric unit. The model was refined to an \textit{R}-factor of 16.7\% at 1.55 Å resolution. The \(\alpha\)-carbons for the two C-3 epimerases from serotypes HS:3 and HS:23/36 superimpose with a root-mean-square deviation of 0.4 Å (subunits B), and the regions surrounding the ligands are identical within experimental error.

The crystals of the C3/C5-epimerase from \textit{C. jejuni} serotype HS:15 in complex with GDP belonged to the space \textit{P2}_1 with four dimers in the asymmetric unit. The model was refined to an \textit{R}-factor of 19.1\% at 1.9 Å resolution. The crystals of the C3/C5-epimerase from \textit{C. jejuni} serotype HS:42 in complex with GDP belonged to the space \textit{P4}_1212 with a dimer in the asymmetric unit. The model was refined to an \textit{R}-factor of 20.7\% at 1.9 Å resolution. The \(\alpha\)-carbons for these two C3/C5-epimerases superimpose with an approximate root-mean-square deviation of 0.4 Å. Importantly, within X-ray coordinate error, the immediate active site architectures for the four enzymes structurally interrogated in this investigation are identical including the cis peptides preceding the catalytic histidines.

**Mechanism of Action.** Two different clusters of C3- and C5-epimerases have been characterized from the various serotypes of \textit{C. jejuni}. The larger cluster is restricted to the epimerization of C3 of the common substrate GDP-6-deoxy-4-keto-\(\alpha\)-D-lyxo-heptose (2), while the smaller cluster efficiently epimerizes both C3 and C5. A third cluster that is apparently involved in the biosynthesis of 3,6-dideoxy heptose products has not thus far been characterized. It is apparent from the three-dimensional structures of the C3 and C3/C5-epimerases that these enzymes use a common set of residues that function as the general acid/base groups for the abstraction and donation of protons to C3 and/or C5 during the epimerization of these chiral centers. Unfortunately, we were unable to obtain a high-resolution X-ray structure of either epimerase subgroup in the presence of a complete substrate/product. However, the previously determined three-dimensional structures of the homologous enzymes RmlC by the Naismith laboratory\(^{26}\) and

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ChmJ by the Holden laboratory strongly support the proposal that His-67 is utilized as the base to abstract a proton from either C3 or C5 of the bound substrate and that Tyr-134 is used as the general acid to reprotonate the carbanionic intermediate.

To test this proposal, we mutated His67 and Tyr134 from a C3-epimerase (from serotype HS:3) and a C3/C5-epimerase (from serotype HS:15) to an asparagine and phenylalanine, respectively. The four variants were purified to homogeneity and subsequently tested for catalytic activity using GDP-6-deoxy-4-keto-\(\alpha\)-D-lyxo-heptose (2) as the substrate at a fixed concentration.

**Figure 6.** Multiple sequence alignment for the epimerases from serotypes HS:2, HS:3, HS:10, HS:15, HS:23, HS:12, and HS:42. The residues highlighted in yellow illustrate those that are conserved in the C3-epimerases while another amino acid is conserved (shaded gray) in the C3/C5-epimerases. Two active site general acid/base groups (histidine and tyrosine) are highlighted in blue.

**Figure 7.** \(^1\)H NMR 400 MHz spectra of products formed from GDP-6-deoxy-4-keto-\(\alpha\)-D-lyxo-heptose (2) by the addition of wild-type and variant epimerases from serotype HS:3. Reaction products GDP-6-deoxy-4-keto-\(\alpha\)-D-arabino-heptose (3), GDP-6-deoxy-4-keto-\(\beta\)-L-ribo-heptose (4), and GDP-6-deoxy-4-keto-\(\beta\)-L-xylo-heptose (5) formed by the catalytic activity of epimerases from HS:2 (A), HS:3 (B), A76V/C136L HS:3, (C) and A76V/A122S/C136L HS:3 (D). The epimerases (4 \(\mu\)M) were incubated with (2) for 15 h at 20 °C (pD 7.5).
concentration of 1.0 mM. None of the four variants were able to catalyze the isomerization of C3 of this substrate with a rate constant greater than 0.003 s$^{-1}$. Under these conditions, the wild-type C3-epimerase from serotype HS:3 has a rate constant of 5.4 s$^{-1}$ and the C3/C5-epimerase from serotype HS:15 catalyzes the epimerization of C3 with a rate constant of 3.0 s$^{-1}$. Thus, the four variants are reduced in catalytic activity by a factor of more than 3 orders of magnitude. The Creuzenet laboratory has previously shown that mutation of H67 and Y134 in the C3/C5- and C3-epimerases from serotypes HS:2 and HS:23/36, respectively, also showed significant reductions in catalytic activity, but the specific rate constants were not quantified. In the proposed catalytic reaction mechanism for the C3- and C3/C5-epimerases, His67 abstracts a proton from C3 and the negative charge is delocalized to the carbonyl group at C4. In the second step, the process is reversed and C3 is reprotoxized on the opposite face by Tyr134. It is also clear that for the C3/C5-epimerases, the process can subsequently be repeated at C5 for the ultimate isomerization at both C3 and C5. The chemical mechanism is depicted in Scheme 5.

What is not so clear is what specifically separates the C3-epimerases from the C3/C5-epimerases. Based on the identification of differentially conserved residues within the C3- and C3/C5-epimerases, we have successfully changed Cys-136 and Ala-76, found exclusively in the C3-epimerases, to Leu-136 and Val-76, found only in the C3/C5-epimerases, and demonstrated that a C3-epimerase could be transformed into one that now catalyzes the epimerization at both C3 and C5. These two residues are indeed close to the two active site general acid/base groups, and thus it is quite probable that the two residues found within the C3-epimerases restrict the subtle conformational changes needed to access both C3 and C5. In the GDP-bound structures, the thiol group of Cys-136 is 4.8 Å from the phenolic oxygen of Tyr-134. Electrostatic interactions between these two residues may restrict the mobility of Tyr-134 and thus render this residue unable to function properly for the epimerization of C5.

With the C3/C5-epimerases, it is also quite apparent that the mono-epimerized products are released from the active site and that eventually an equilibrium mixture of four species is formed in solution. This means that the ultimate determinant of the final product formed is the C4-reductase. For example, in the HS:15 serotype, the heptose product is 6-deoxy-L-gulo-heptose. This product is formed via the epimerization at only C5, and thus the C4-reductase must exclusively reduce the C5-epimerized intermediate (4). Perhaps this precludes the need for a C5-only epimerase.

![Figure 8. Structure of the C-3 epimerase from C. jejuni serotype HS:3. Shown in panel (a) is a ribbon drawing of the dimer as observed in the asymmetric unit. Those secondary structural elements involved in domain swapping are highlighted in green. The bound ligands (GDP) are displayed in sphere representations. The arrow indicates the 2-fold rotational axis of the dimer. A closeup view of the active site, in stereo, is provided in panel (b). Those side chains colored in green belong to subunit A, whereas those displayed in blue are contributed by subunit B. The dashed lines indicate possible interactions between the protein and the ligand within 3.2 Å. Water molecules are drawn as red spheres.](https://doi.org/10.1021/acs.biochem.2c00364)

**Scheme 5. Proposed Mechanism of Action**

In C. jejuni, the 6-deoxy-heptoses found within the capsular polysaccharides are made from GDP-D-glycero-D-manno-heptose via the combined actions of a 4,6-dehydratase, C3/C5-epimerase, and a C4-reductase. Here, we have demonstrated that there are two major classes of epimerases in C. jejuni; those that catalyze the epimerization at C3 and those...
that catalyze the epimerization at both C3 and C5. Three-dimensional structures of both classes were determined to high resolution, and the active sites were shown to be nearly identical, reflecting greater than 76% sequence identity between the two epimerase classes. The mutation of two residues in the epimerase from serotype HS:5 was sufficient to convert an enzyme that catalyzes the epimerization at C3-only into one that could epimerize both C3 and C5. Two residues, His-67 and Tyr-134, are positioned to function as the general acid/base catalysts for the epimerization of C3 and C5. The structural constraints that limit the epimerization at a single chiral center are not clear.

**ASSOCIATED CONTENT**

**Supporting Information**
The Supporting Information is available free of charge at https://pubs.acs.org/10.1021/acs.biochem.2c00364.

Amino acid sequences of the purified proteins; enzyme modifications; sequence identity matrix; and NMR spectra of carbohydrate substrates and products (PDF)

**Accession Codes**
Epimerases from serotypes HS:2 (UniProt entry: Q0PP84), HS:3 (UniProt entry: F2X702), HS:10 (UniProt entry: F2X784), HS:15 (UniProt entry: A0A3Z9HSX9), HS:23/36 (UniProt entry: Q6EF58), HS:41 (UniProt entry: QSM6T7), and HS:42 (UniProt entry: F2X7E5).
C4-reductases from serotypes HS:2 (UniProt entry: Q0PP86), HS:3 (UniProt entry: F2X701), and HS:15 (UniProt entry: F2X7A6).

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