THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 34, pp. 24332–24339, August 23, 2013

A Novel Bacterial Enzyme with D-Glucuronyl C5-epimerase Activity*

Received for publication, April 11, 2013, and in revised form, June 27, 2013 Published, JBC Papers in Press, July 3, 2013, DOI 10.1074/jbc.M113.476440

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Background: D-Glucuronyl C5-epimerase activity is essential in glycosaminoglycan biosynthesis.
Results: This is the first demonstration of a bacterial enzyme having D-glucuronyl C5-epimerase activity.
Conclusion: This novel enzyme, probably involved in bacterial capsular polysaccharide biosynthesis, shows catalytic properties similar to murine heparan sulfate D-glucuronyl C5-epimerase.
Significance: Increased insight into bacterial cell wall biosynthesis and a potential biotechnological tool applicable in chemo-enzymatic synthesis of industrially relevant heparin-like glycosaminoglycans are provided.

Glycosaminoglycans are biologically active polysaccharides that are found ubiquitously in the animal kingdom. The biosynthesis of these complex polysaccharides involves complicated reactions that turn the simple glycosaminoglycan backbone into highly heterogeneous structures. One of the modification reactions is the epimerization of D-glucuronic acid to its C5-epimer L-iduronic acid, which is essential for the function of heparan sulfate. Although L-iduronic acid residues have been shown to exist in polysaccharides of some prokaryotes, there has been no experimental evidence for the existence of a prokaryotic D-glucuronyl C5-epimerase. This work for the first time reports on the identification of a bacterial enzyme with D-glucuronyl C5-epimerase activity. A gene of the marine bacterium *Bermanella marisrubri* sp. RED65 encodes a protein (RED65_08024) of 448 amino acids that has an overall 37% homology to the human D-glucuronic acid C5-epimerase. Alignment of this peptide with the human and mouse sequences revealed a 60% similarity at the carboxyl terminus. The recombinant protein expressed in *Escherichia coli* showed epimerization activity toward substrates generated from heparin and the *E. coli* K5 capsular polysaccharide, thereby providing the first evidence for bacterial D-glucuronyl C5-epimerase activity. These findings may eventually be used for modification of mammalian glycosaminoglycans.

Glycosaminoglycans (GAGs)[4] are a group of carbohydrate polymers that are typically composed of disaccharides of an amino sugar and hexuronic acids. GAGs are widely spread in the animal kingdom, having important biological functions. For some time GAGs were believed to exist exclusively in animals; however, today there is an increasing number of examples of GAGs of prokaryotic origin. These prokaryotic GAGs are less complex and lack some modifications typically seen in animal GAGs. For example, heparan sulfate is a ubiquitously expressed GAG in animals, having essential biological functions (1). Similar to the capsular polysaccharide of the bacterium *Escherichia coli* K5, this complicated animal GAG is initially synthesized as a simple polymer composed of repeating disaccharides of D-glucuronic acid (GlcUA) and *N*-acetyl-D-glucosamine (GlcNAc). This relatively simple polymer is subsequently adjusted by a series of modification reactions, producing distinct structures of the polysaccharide in different tissues (2). One critical modification is the conversion of GlcUA to its C5-epimer, L-iduronic acid (IdoUA), catalyzed by D-glucuronyl C5-epimerase. It is demonstrated that this modification is crucial for animal development (3). The IdoUA residues in the polymers lead to increased flexibility of the GAG chains (4), facilitating binding properties for specific polysaccharide-protein interactions (5). Although IdoUA occasionally is found in prokaryotic GAGs (6–9), the enzyme responsible for this C5-epimerization reaction has not been identified in a prokaryote.

In a previous study, we performed an *in silico* screen on available prokaryotic genomes for putative D-glucuronyl C5-epimerases which revealed multiple candidate genes in both archaea and bacteria (10). In this work we report on the identification, cloning, expression, and characterization of a C5-epimerase from the marine bacterium *Bermanella marisrubri* sp. RED65.

EXPERIMENTAL PROCEDURES

L-Iduronic acid standard was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Capsular polysaccharide of *E. coli* K5 (K5 polysaccharide) was ordered from Iduron (Manchester, UK). The ^{3}H-labeled substrates derived from K5 and heparin were prepared as described before (11). All other chemicals were bought from Sigma-Aldrich.

* This project was carried out in close collaboration with MSD Oss (formerly NV Organon/Schering Plough) and was supported by The Netherlands Ministry of Economic Affairs and the B-Basic partner organizations through B-Basic, a public-private NWO-Advanced Chemical Technologies for Sustainability program and Swedish Research Council Grant K2012-67X-21128-04-4.
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[4] The abbreviations used are: GAG, glycosaminoglycan; GlcUA, D-glucuronic acid; IdoUA, L-iduronic acid; NDSB, nondetergent sulfobetaine; PAD, pulse amperometric detection; TCEP, Tris(2-carboxyethyl)phosphine.

Restriction enzymes, *Pfu* DNA polymerase, and T4 DNA ligase were from Invitrogen and New England Biolabs.

Gene Cloning, Expression, and Purification—A synthetic gene was designed based on hypothetical protein RED65_08024, codon optimized for the codon usage bias of *E. coli*. The gene was cloned in vector pRSF-1b (Novagen) using restriction sites KpnI and BamHI, fusing the gene in-frame with a polyhistidine tag at the nonconserved amino terminus to prevent interference on the activity (12). The resulting vector, named pWUR537, was used to transform *E. coli* strains DH5α and Rosetta (DE3) containing pRARE (helper plasmid coding for rare tRNAs).

An overnight culture of *E. coli* Rosetta (DE3) containing pWUR537 was used to inoculate (1% v/v) a shake flask with 1 liter of Luria Bertani medium supplemented with 50 μg/ml kanamycin, 50 μg of chloramphenicol, and 1 mM $MgSO_4$. At A_{600} = 0.5, gene expression was induced by adding 0.05 mM isopropyl-1-thio-β-D-galactopyranoside. The culture was incubated overnight at 30 °C. Cells were harvested by centrifugation (5,000 × *g* for 15 min) and stored at −20 °C until further use. Pelleted *E. coli* cells were resuspended in 20 mM Tris-HCl buffer (pH 7.5) containing 200 mM NaCl (buffer A) followed by sonication (intermittent cooling on ice). The cell debris was removed by a centrifugation step (16,000 × *g* for 10 min). The resulting cell lysate was filtered (0.45 μm) and was used directly for activity measurements.

Protein purification was achieved by applying the cell lysate on a Talon cobalt affinity column (Clontech). After washing with 2 column volumes of buffer A and 1 column volume of buffer A containing 10 mM imidazole, RED-C5-epimerase was eluted with 1 column volume of buffer A containing 10–250 mM imidazole in a linear gradient. RED-C5-epimerase-containing fractions were pooled and applied onto a HiPrep desalting column to remove imidazole. Enzyme presence and purity were checked by SDS-PAGE analysis. A broad range protein marker (Bio-Rad) was used to estimate the molecular mass. The presence of RED-C5-epimerase was confirmed by trypsin digestion LC-MS analysis as described previously (13).

The oligomeric state of RED-C5-epimerase was determined by size exclusion chromatography on a Superdex 200 HR10/10 column (24 ml) (Amersham Biosciences) equilibrated in 20 mM Tris-HCl (pH 7.5) containing 200 mM NaCl. Approximately 0.1 mg of purified RED-C5-epimerase was loaded onto the column, using a flow rate of 0.5 ml/min. Markers used for calibration were blue dextran 2000 (>2,000 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa).

Detection of C5-epimerase Activity—Cell lysate (10 ml of culture lysed in 200 μl of lysis buffer) of the bacterial clone expressing RED-C5-epimerase was mixed with tritium-labeled substrates derived from *O*-desulfated heparin (5,000 cpm; specific radioactivity 10×10^6 cpm/mg polysaccharide) or *N*-deacetylated/sulfated K5 capsular polysaccharide (3,000 cpm; specific radioactivity 1×10^6 cpm/mg polysaccharide). The enzyme/substrate mixture was incubated at 30 °C overnight. Subsequently, the reaction mixtures were analyzed using a biphasic liquid scintillation procedure to measure tritium release as described previously (14, 15). Murine D-glucuronyl C5-epimerase constructs (full-length and a truncated form) fused to maltose-binding protein were used as positive and negative control, respectively.

HPLC-PAD Analysis—For detection of the formation of IdoUA, an HPLC-PAD (pulse amperometric detection) analysis using a Dionex system was used. Cell lysate or purified RED-C5-epimerase was mixed with *N*-acetyl-de-*O*-sulfated heparin (sodium salt; Sigma-Aldrich). The mixture was incubated at 30 °C overnight. Subsequently, protein was inactivated by heating at 100 °C and removed from the reaction mixture by centrifugation. The reaction mixture was hydrolyzed (90 °C for 4 h) by addition of 1 volume of hydrochloric acid to an end concentration of 1 M. Hydrochloric acid was removed by evaporation in a SpeedVac, and hydrolyzed samples were redissolved in Millipore water. Final traces of hydrochloric acid were removed by titration with sodium hydroxide. Samples were analyzed for their hexuronic acid content by anion exchange chromatography on a Dionex system equipped with a CarboPac-100 column. The used effluent was 45 mM sodium hydroxide, with an increasing gradient of sodium acetate (0–960 mM).

RESULTS

Protein Sequence Analysis—A BLAST search (16) with the human heparan sulfate D-glucuronyl C5-epimerase protein sequence as query confirmed the presence of highly homologous proteins in different mammalian species (17). Although homologous sequences were found in genomes of both bacteria and archaea as well, most of these genes identified have a relatively low homology (10). However, among this group of prokaryotic sequences, the candidate C5-epimerase protein sequence (RED-C5-epimerase) of the marine bacterium *B. marisrubri* sp. RED65 (18) showed significantly higher homology to animal heparan sulfate D-glucuronyl C5-epimerase than any other prokaryotic sequence. Based on its sequence, RED-C5-epimerase is placed within PFAM family C5-epim_C (PF0662), representing the carboxyl-terminal domain of heparan sulfate D-glucuronyl C5-epimerase (19). The gene coding for RED-C5-epimerase (RED65_08024) contains 1,347 base pairs, resulting in a protein of 448 amino acids with a theoretical molecular mass of 51,914 Da.

We constructed a multiple sequence alignment (T-Coffee server (20)), to illustrate the homology between RED-C5-epimerase and the animal heparan sulfate D-glucuronyl C5-epimerases (Fig. 1). The multiple sequence alignment was visualized using GeneDoc version 2.6.[5] RED-C5-epimerase has 23% identity and 37% similarity with the human heparan sulfate D-glucuronyl C5-epimerase, mostly based on high similarity at the carboxyl terminus (41% identity, 60% similarity). In contrast with the animal proteins, RED-C5-epimerase has no predicted transmembrane helix at the amino terminus. The overall secondary structure is predicted (PSIPRED Server (22)) to be similar to the animal heparan sulfate D-glucuronyl C5-epimerase (Fig. 1).

Neighborhood Analysis—To have a prediction on the physiological role of RED-C5-epimerase, we performed a gene

[5] K. B. Nicholas and H. B. Nicholas, unpublished data.

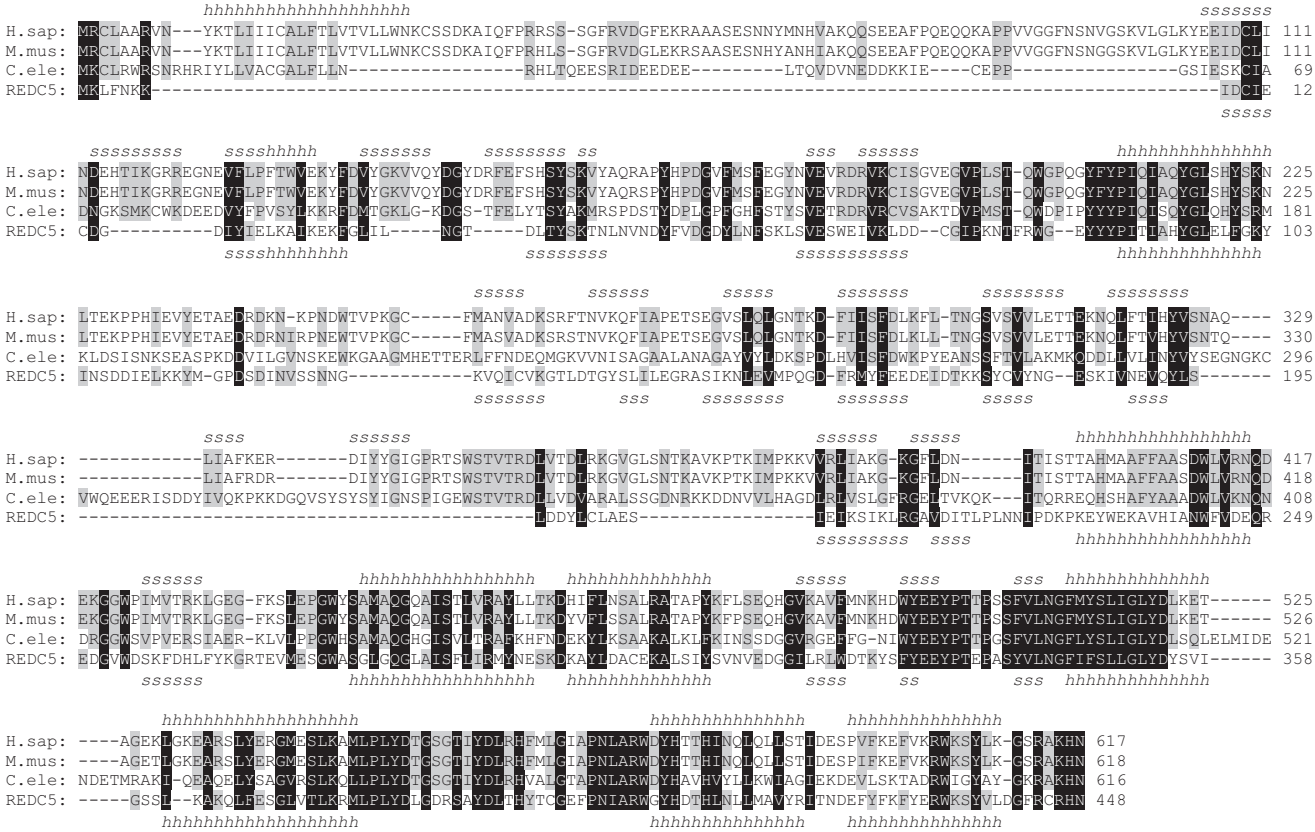

FIGURE 1. **Multiple sequence alignment of RED-C5-epimerase.** Hypothetical protein RED_08024 from *B. marisrubri* sp. Red65 (REDC5) is compared with characterized animal heparan sulfate D-glucuronyl C5-epimerases (*H. sap*, *Homo sapiens*; *M. mus*, *Mus musculus*; *C. ele*, *C. elegans*). Secondary structure predictions are indicated for human and *B. marisrubri* (*h* indicates predicted α-helices; *s* indicates predicted β-strands).

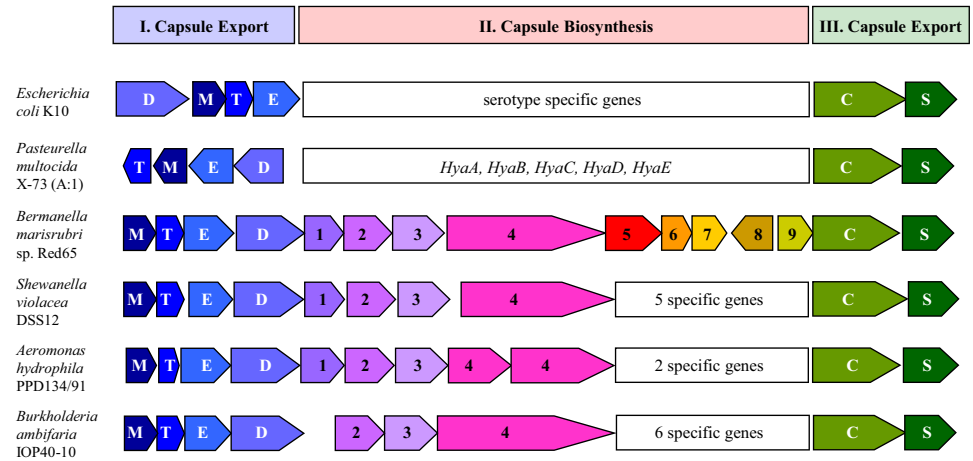

FIGURE 2. **Genomic context of Red_08024 (in *red*) compared with other bacterial capsular polysaccharide biosynthesis gene clusters (23, 24, 27).** The flanking genes in *blue* (region 1; *M*, *kpsM*; *T*, *kpsT*; *E*, *kpsE*; and *D*, *kpsD*) and *green* (region 3; *C*, *kpsC*; and *S*, *kpsS*) are likely to be involved in polysaccharide export. The genes in between (region 2) are likely to be involved in serotype-specific capsular polysaccharide biosynthesis. The conserved genes are predicted to be a: glycosyl transferase (ORF1, *dark purple*), UDP-*N*-acetyl glucosamine-2-epimerase (ORF2, *purple*), UDP-*N*-acetyl-D-mannosaminuronic acid dehydrogenase (ORF3, *violet*), and glycosyltransferase (ORF4, *magenta*). The D-glucuronyl C5-epimerase (ORF5, *red*) is unique to *B. marisrubri* sp. RED65. The other region 2 genes in *B. marisrubri* are predicted to be (conserved) hypothetical proteins (ORF6, ORF7), glycosyl transferase (ORF8), and 2-dehydro-3-deoxyphosphooctonate aldolase (ORF9).

neighborhood analysis. The genomic context of RED65_08024 comprises genes involved in the biosynthesis of capsular polysaccharides. In bacteria these genes typically are organized in gene clusters. Studies on the organization of similar clusters in *E. coli* (23) or *Pasteurella multocida* (24), show that these clusters consist of three functional regions. Regions 1 and 3 are well conserved, and the encoded proteins are involved in capsular polysaccharide export, whereas proteins encoded by region 2 are involved in the biosynthesis of serotype-specific polysaccharides and therefore are highly variable (25). RED65_08024 and another eight ORFs are flanked by these capsular polysaccharide export regions 1 and 3 (Fig. 2).

We compared the genomic context of RED-C5-epimerase with the organization of similar genes in other bacteria. We

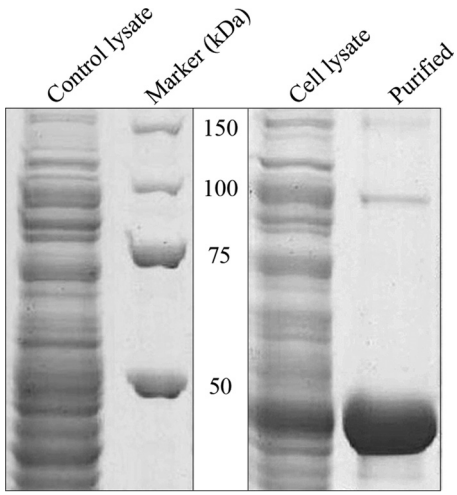

FIGURE 3. **Recombinant RED-C5-epimerase production in *E. coli*.** RED-C5-epimerase presence is shown in cell lysate and after purification. The control lysate is made from *E. coli* not harboring the recombinant RED-C5-epimerase-containing vector pWUR537. The full-length presence of the protein has been confirmed by trypsin digestion LC-MS analysis.

observed high resemblance with the capsular gene clusters of the aquatic bacteria *Shewanella violacea* DSS12 (26), *Aeromonas hydrophila* PPD134/91 (27), and *Burkholderia ambifaria* IOP40-10. Not only do they all possess the regions 1 and 3 genes involved in capsular polysaccharide transport, they also have several genes in common of the type-specific region 2. However, the presence of a C5-epimerase appears to be restricted to *B. marisrubri* sp. RED65. Most likely this candidate C5-epimerase is involved in a species- or type-specific sugar modification, which is expected for a bacterial D-glucuronyl C5-epimerase, as IdoUA presence in prokaryotes seems to be a species- or type-specific constituent (10).

Characterization of the Recombinant Protein of RED-C5-epimerase—The RED-C5-epimerase gene was successfully expressed in *E. coli*, resulting in high amounts (~20 mg/liter cell culture) of protein (Fig. 3). Based on SDS-PAGE analysis, the protein size was estimated slightly smaller than the theoretical size of 52 kDa. However, protein truncation can be excluded, as trypsin digestion LC-MS analysis did confirm the presence of both termini. We could purify the protein using the amino-terminal polyhistidine tag; however, we observed a tendency for protein aggregation.

To determine the oligomeric state of the protein, we performed size exclusion chromatography on a Superdex-200 HR 10/10 column (Fig. 4). In freshly prepared protein samples, RED-C5-epimerase is present in a dimeric/tetrameric form, in approximately a 2:1 ratio, and appears to be in a dynamic equilibrium. Upon storage, RED-C5-epimerase has the tendency to form larger soluble aggregates. Nondetergent sulfobetaines (NDSBs) are known to efficiently prevent protein aggregation (28). We tested the effect of NDSB201 (up to 1 M) but did not observe less aggregation at 4 °C, although NDSB201 did prevent precipitation of the protein upon freezing. Addition of reducing agents such as Tris(2-carboxyethyl)phosphine (TCEP, 2 mM) prevented aggregation, maintaining the initial dimeric/tetrameric oligomeric state. High levels of reducing agent (*e.g.* 50 mM β-mercaptoethanol) even partly reversed

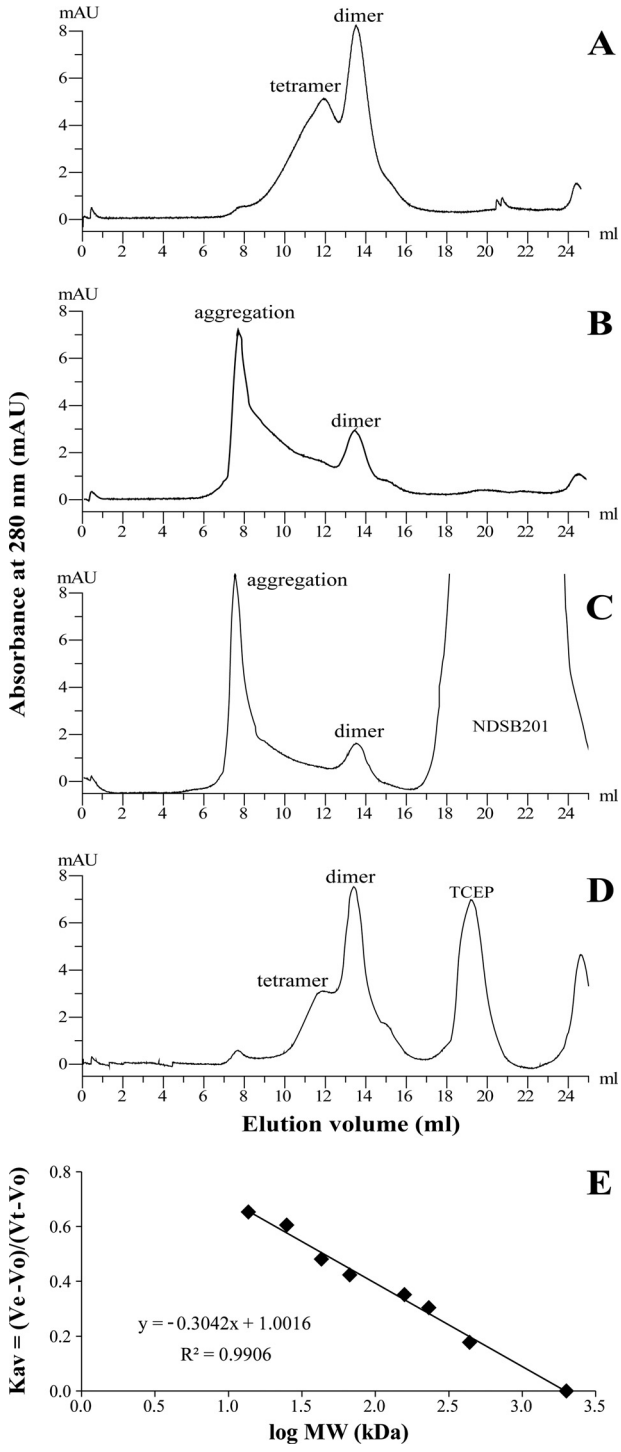

FIGURE 4. **Size exclusion chromatography to determine the oligomeric state of RED-C5-epimerase.** *A*, in freshly prepared samples RED-C5-epimerase is present in a dimeric/tetrameric form occurring in a 2:1 ratio. *B*, in time the protein has the tendency to form larger aggregates. *C*, despite the fact that the nondetergent sulfobetaines NDSB201 does prevent precipitation upon freezing, it has no obvious effect in prevention of aggregation. *D*, addition of the reducing agents TCEP does help to prevent aggregation, maintaining the initial oligomeric state. *E*, calibration curve is shown.

aggregation. This effect suggests that the formation of intramolecular and intermolecular disulfide bonds between any of the nine cysteine residues in the RED-C5-epimerase peptide causes the observed aggregation.

TABLE 1
Activity measurement of D-glucuronyl C5-epimerase-mediated tritium release

Substrate	Enzyme	Buffer	^3H release
			cpm
Heparin[a]	RED-C5-epimerase (5 μl)	Tris with TCEP	25
Heparin	RED-C5-epimerase (10 μl)	Tris with TCEP	15
Heparin	RED-C5-epimerase (50 μl)	Tris with TCEP	15
Heparin	RED-C5-epimerase (5 μl)	Tris with TCEP + Tween 20	58
Heparin	RED-C5-epimerase (10 μl)	Tris with TCEP + Tween 20	116
Heparin	RED-C5-epimerase (50 μl)	Tris with TCEP + Tween 20	200
Heparin	Mouse C5-epimerase (5 μl)	HEPES	1200
Heparin	Mouse C5-epimerase (10 μl)	HEPES	1540
Heparin	Mouse C5-epimerase (50 μl)	HEPES	2054
K5 polysaccharide[b]	RED-C5-epimerase (5 μl)	Tris with TCEP	32
K5 polysaccharide	RED-C5-epimerase (10 μl)	Tris with TCEP	84
K5 polysaccharide	RED-C5-epimerase (50 μl)	Tris with TCEP	117
K5 polysaccharide	RED-C5-epimerase (5 μl)	Tris with TCEP + Tween 20	25
K5 polysaccharide	RED-C5-epimerase (10 μl)	Tris with TCEP + Tween 20	32
K5 polysaccharide	RED-C5-epimerase (50 μl)	Tris with TCEP + Tween 20	181
K5 polysaccharide	Mouse C5-epimerase (50 μl)	HEPES	305
K5 polysaccharide	Mouse C5-epimerase (50 μl)	HEPES	320

[a] Desulfated and re-*N*-sulfated heparin were incubated with purified murine C5-epimerase in the presence of ^3H_2O. Tritium was introduced into both IdoUA and GlcUA residues as described in Ref. 21.
[b] *E. coli* K5 was cultured in the presence of C5-[^{3}H]glucose to introduce ^{3}H-labeled GlcUA. The purified capsular polysaccharide was deacetylated and *N*-sulfated.

Enzyme Activity Analyses—To test for C5-epimerase activity, cell lysate containing recombinant RED-C5-epimerase was incubated with ^{3}H-labeled substrates derived from modified heparin and K5 polysaccharide (11), by the biphasic separation method (14). For both substrates we detected significant D-glucuronyl C5-epimerase activity, although at a substantially lower level compared with the murine C5-epimerase (Table 1). Notably, we were unable to detect activity in the absence of TCEP in the incubation buffer. Also, the addition of the detergent Tween 20 has a positive effect on the enzymatic activity (Table 1). Incubations were done at 30 °C, as 37 °C resulted in inactivation of the enzyme.

To confirm the formation of GlcUA *versus* IdoUA residues in polysaccharides, we analyzed the products of de-*O*-sulfated heparin after incubation with the recombinant RED-C5-epimerase. We performed chemical hydrolysis with HCl, resulting in partial hydrolysis of heparin thereby liberating monomeric hexuronic acids. Only a small fraction can be recovered as monomeric sugar; most sugars remain unreleased in oligosaccharides. Tight control of hydrolysis time, temperature, and acid concentration is crucial to prevent substantial formation of side products and to prevent differential release of GlcUA and IdoUA, due to differences in acid lability of their glycosidic bonds (29). Therefore, a control sample was included in each run to validate the hydrolysis conditions. Monomeric GlcUA can be separated from IdoUA by high performance anion exchange chromatography (30, 31). We optimized the protocol for analysis of the hydrolyzed reaction mixture on a Dionex system, monitoring hexuronic acids with an electrochemical detector using PAD (Fig. 5*A*).

Using an anion exchange column (CarboPac PA-100) we were able to separate GlcUA and IdoUA. An important note is to keep salt levels low in the samples, as the retention time of IdoUA decreases at high salt concentrations, resulting in poor separation of both hexuronic acids. Using this HPLC-PAD method we tested C5-epimerase activity of the RED-C5-epimerase on various available D-glucuronyl-containing GAGs and GAG precursors, including chondroitin, hyaluronan, UDP-GlcUA, *N*-sulfated K5 polysaccharide, and de-*O*-sulfated heparin. Most of the samples did not give any significant changes in the hexuronic acid content after incubation with RED-C5-epimerase, except for the de-*O*-sulfated heparin. Incubation of this substrate with both crude cell lysate or purified enzyme resulted in the change of the proportion of GlcUA *versus* IdoUA residues. In heparin the ratio between both hexuronic acids typically is 35% GlcUA and 65% IdoUA. This was also visible in the control samples. However, overnight incubations of de-*O*-sulfated heparin with RED-C5-epimerase resulted in a

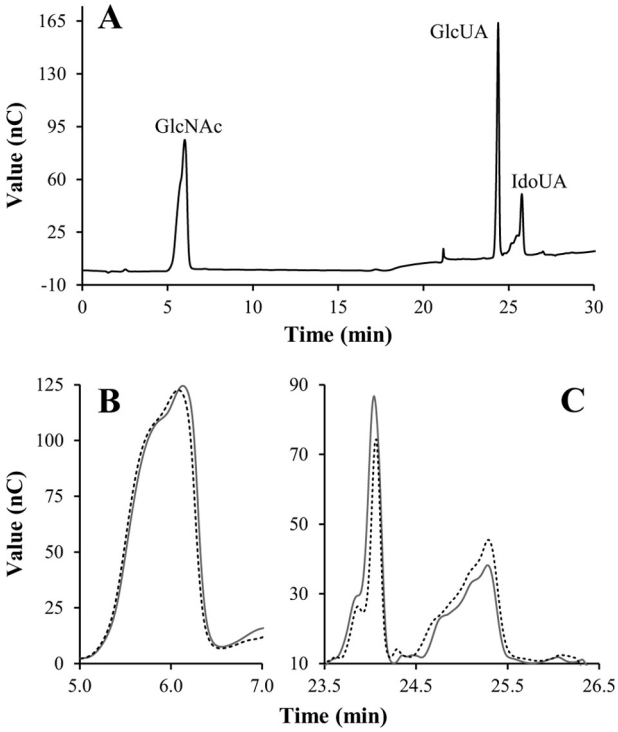

FIGURE 5. **Separation of GlcNAc, GlcUA, and IdoUA using HPLC-PAD.** Separation of standards (*A*), GlcNAc (*B*), and GlcUA (*C*) *versus* IdoUA recovery is shown upon hydrolyses of (de-*O*-sulfated) heparin. *Black/dashed line*, control sample; *gray/solid line*, sample incubated with RED-C5-epimerase.

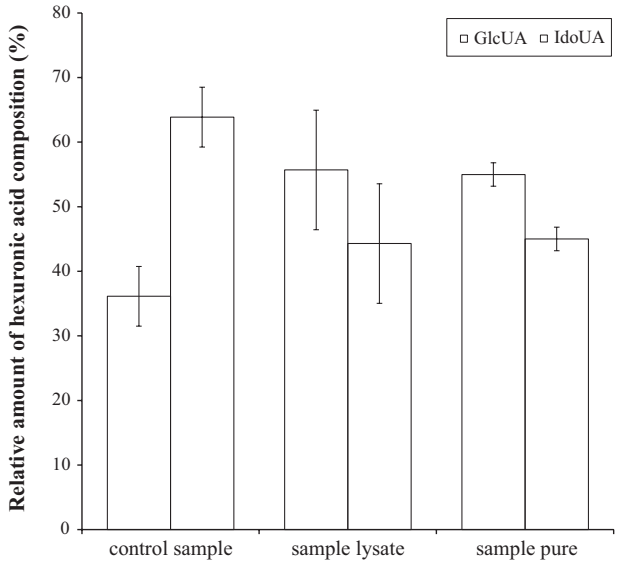

FIGURE 6. **Hexuronic acid ratio in heparin after treatment with RED-C5-epimerase.** Both incubation with cell lysate and pure protein shows partial epimerization of IdoUA (*white bars*) into GlcUA (*gray bars*), whereas the control sample shows the initial ratio, indicating no epimerization.

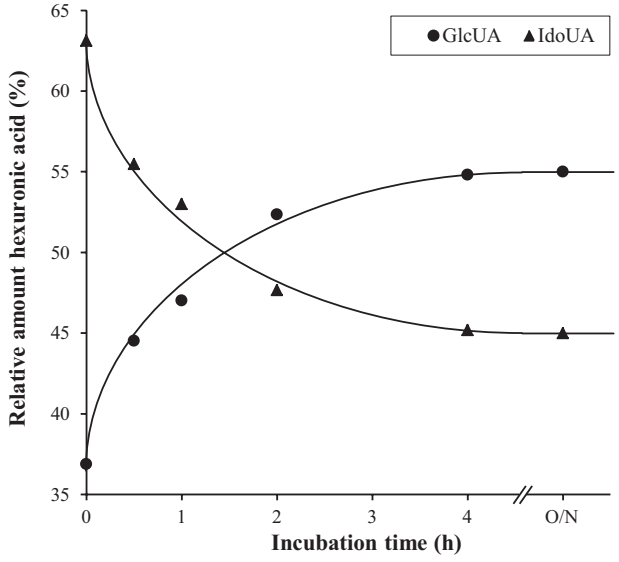

FIGURE 7. **Time series analysis of the hexuronic acid content of heparin as a result of RED-C5 activity.** Incubations showed a gradually decrease in the conversion rate of the hexuronic acids until an equilibrium was reached with a ratio of approximately 55% GlcUA and 45% IdoUA.

shift in the ratio, where IdoUA levels dropped to 45% and GlcUA increased to 55%, whereas GlcNAc levels remained unaffected (Fig. 5*B* and Fig. 6). Control samples were treated identically but were incubated with cell lysate lacking the RED-C5-epimerase.

Using the same HPLC method, a time series experiment was performed to monitor the enzyme activity over time. Incubations showed a gradually decrease in the conversion rate of the hexuronic acids until an equilibrium was reached with a ratio of approximately 55% GlcUA and 45% IdoUA (Fig. 7). Extending the reaction time did not result in a higher net conversion of the hexuronic acids, neither did the addition of higher or additional amounts of enzyme.

DISCUSSION

IdoUA is a common and essential sugar residue in several animal GAGs; however, it has been shown that IdoUA is not only typical for eukaryotic GAGs, but occasionally also can be found as a constituent in serotype-specific prokaryotic cell wall polysaccharides (6–10). From the animal GAG biosynthesis we know that IdoUA is synthesized via a C5-epimerization of GlcUA (review, see Ref. 32). In prokaryotes IdoUA is likely to be synthesized in a similar way, which implies the involvement of a prokaryotic D-glucuronyl C5-epimerase. We selected a hypothetical gene from the marine bacterium *B. marisrubri* sp. RED65 which has a relative high homology to animal heparan sulfate D-glucuronyl C5-epimerases. This similarity is mostly based on the conserved carboxyl-terminal domain of the protein, but also based on a very similar predicted secondary structure. Phylogenetic analysis (10) indicates that prokaryotic C5-epimerase sequences do not cluster to the clade of animal heparan sulfate D-glucuronyl C5-epimerases, making any (recent) horizontal gene transfer from animal to prokaryote unlikely. The presence of this enzyme in "higher animals" (*e.g.* humans) as well as in "lower animals" (*e.g. Caenorhabditis elegans*) may imply an evolutionary scenario with an initial development of this enzyme in bacteria or archaea with a horizontal gene transfer event to a common ancestor of animals, after which molecular complexity (amino-terminal domain) increased. We cloned and expressed the gene coding for the candidate C5-epimerase in *E. coli* and tested gene product RED-C5-epimerase for heparan sulfate D-glucuronyl C5-epimerase activity. We were able to detect significant levels of tritium release from ^{3}H-labeled substrates derived from modified heparin and K5 polysaccharides, indicating C5-epimerase activity. This enzymatic conversion of hexuronic acids in de-*O*-sulfated heparin was confirmed using an HPLC-PAD method (Figs. 6 and 7).

An epimerization reaction typically ends up in an equilibrium between the two specific epimers. For animal heparan sulfate D-glucuronyl C5-epimerases it has been postulated that the epimerization equilibrium is slightly toward GlcUA over IdoUA (11). Thus, it is tempting to speculate that RED-C5-epimerase has similar catalytic properties and results in similar conversion ratios as the animal heparan sulfate D-glucuronyl C5-epimerase. The data show that an equilibrium of GlcUA and IdoUA in de-*O*-sulfated heparin was reached upon incubation with RED-C5-epimerase, in a pattern similar to the product of murine heparan sulfate D-glucuronyl C5-epimerase (11). This equilibrium state is clearly shown in Fig. 7. No further conversion was observed after prolonged incubation or after addition of more enzyme. The observed GlcUA conversion must be an effect of the RED-C5-epimerase, as control samples did not show a shift in the hexuronic acid ratio. We have been unable to prove significant IdoUA levels for the RED-C5-epimerase/K5 polysaccharide incubations using HPLC-PAD analysis, although the enzyme did release tritium from ^{3}H-labeled modified K5 polysaccharide. Likely, the K5 polysaccharide is a poor substrate, which is also the case for the murine heparan sulfate C5-epimerase (Table 1). Whether this is due to a differ-

ential *N*-sulfation pattern between the K5 polysaccharide and de-*O*-sulfated heparin should be further verified.

As far as we know, this is the first time that experimental evidence is provided on a prokaryotic enzyme with heparan sulfate D-glucuronyl C5-epimerase activity. Obviously, the bacterial enzyme has distinct features compared with the murine enzyme. Apart from the substantial lower activity toward the substrates tested, the bacterial enzyme functions in a different buffer system requiring the reducing agent TCEP as additive. TCEP prevents the formation of aggregates which results in inactivation of the enzyme; apparently only the dimeric/tetrameric enzyme fraction is active. The dimeric and tetrameric fractions appear to be in a dynamic equilibrium, and therefore we were unable to conclude which of the two fractions is active. Based on the effect of TCEP, we conclude that the observed protein aggregation is most likely caused by inappropriate disulfide bond formation. Compared with the murine heparan sulfate D-glucuronyl C5-epimerase (6 cysteines; of which 3 are in the signal peptide), the RED-C5-epimerase is relatively cysteine-rich (9 cysteines, no signal peptide), which increases the potential to form intradisulfide bonds. This aggregation may have led to depletion of the competent enzyme molecules, resulting low enzymatic activity. The inappropriate formation of disulfide bonds should be a key consideration for potential exploitation of RED-C5-epimerase for potential applications. Considering that the activity of the RED-C5-epimerase is relatively low, it may indicate that the physiological substrate of this enzyme in the bacterium could differ from heparin and heparan sulfate. Analysis of the *B. marisrubri* sp. RED65 cell wall polysaccharides could be a first step in providing more insight in both the physiological role and substrate specificity of RED-C5-epimerase. Moreover, the identification of novel bacterial C5-epimerases not only increases our insight into bacterial cell wall polysaccharide biosynthesis but eventually may provide potential new biotechnological tools applicable in the controlled chemo-enzymatic synthesis of industrially relevant IdoUA-containing GAGs like heparin, heparan sulfate, or variants.

Acknowledgments—We thank Henk Schols and Willy van den Berg (Wageningen University, The Netherlands) for help in initiating the HPLC-PAD analysis and Bram Snijders (London Research Institute, Cancer Research UK, United Kingdom) for the trypsin digest LC-MS analysis.

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