The Structural Basis for a Coordinated Reaction Catalyzed by a Bifunctional Glycosyltransferase in Chondroitin Biosynthesis"**

Research into polysaccharide chains and their roles in biology dates back to 1918 when the anti-coagulant heparin was first purified and characterized from the liver (1). Since that initial discovery, many essential roles for polysaccharides have been established. Polysaccharide chains comprise the core structure of glycosaminoglycans and are present as O- or N-glycans in proteoglycans as well as in free polymers such as chondroitin, hyaluronan, and heparin (2). Glycosaminoglycans have been credited with controlling a diverse array of biological processes such as blood coagulation, cell division, adhesion, and bacterial and viral infections (3). In addition, sulfation confers glycosaminoglycans with divergent biological functions from cell differentiation and morphogenesis (4) to fibroblast growth, nervous system, and cartilage development (5). The biosynthetic pathways of glycosaminoglycans are frequently altered in cancer cells; these alterations manifest in an array of forms, providing biological markers for the transformation process and progression of tumor cells (6).

Given their biological importance, various glycosyltransferases that are involved in the biosynthesis of glycosaminoglycans have been characterized, and their reaction mechanisms have been determined (7, 8). The majority of mammalian glycosyltransferases belong to the structural subclass of glycosyltransferases within the GT-A-fold group of enzymes and utilize different sugar molecules into chondroitin, hyaluronan or heparin/heparan chains. Although understanding the reaction mechanism of bifunctional glycosyltransferases is critical to investigating the biological functions and implications of glycosaminoglycans in diseases, it remains unknown at the present time. Here we have utilized bacterial chondroitin synthase as an enzyme model for glycosaminoglycan chain polymerase to investigate this mechanism of polymerization. Of particular interest is whether or not the two transfer reactions are coordinated in the synthesis of a glucosaminoglycan chain. And if they are coordinated, what is the mechanism?

The K4 strain of Escherichia coli-produced chondroitin synthase K4CP is one such bifunctional glycosyltransferase that catalyzes β1–3 glucuronolactone and β1–4 N-acetylgalactosaminyltransfer reactions to polymerize glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc) into a chondroitin chain [GlcA β(1–3)-GalNAc β(1–4)]n (9). K4CP consists of 686 amino acid residues, from which a truncated form was constructed by deleting the first 57 residues from the N terminus to produce K4CPΔ57. This deletion mutant fully retained the
enzyme activity of K4CP. The x-ray crystal structure of K4CPΔ57 was recently determined (10). The K4CPΔ57 structure revealed that K4CP is a single globular protein consisting of two glycosyltransferase GT-A domains that are consistent with possessing Sα2-type GalNAc and GlcA transfer reactions at the N- and C-terminal domains, respectively. The N- and C-terminal domains orient their open access sites for donor substrates in directions perpendicular to one another, and their active sites do not share the same space within the K4CP molecule. What this x-ray crystal structure revealed posed a critical question with regard to the mechanism by which K4CP catalyzes the polymerization reaction; is this a random reaction? If it is not, then how does K4CP coordinate these two active sites, which are not in the same space and are positioned perpendicularly, to propel the polymerization reaction? Conversely, the K4CP structure also revealed the intriguing structural feature of a peptide consisting of residues 58–134 that wraps around the C-terminal domain before extending back into the N-terminal domain. Here we focus on this N-terminal peptide and examine its role in the polymerization reaction as catalyzed by K4CP.

Recombinant K4CPΔ57 and its mutants were subjected to assays to determine enzyme activity and to isothermal titration calorimetry (ITC) analyses to characterize donor and acceptor substrate binding. Interaction between the 113-DWPSDL118 sequence within the N-terminal peptide with the peptide 677YTWK1682 in the C-terminal region of the K4CP molecule was characterized as the regulatory motif that determines each of the two transfer reactions as well as coordinates the polymerization reaction. We have now generated K4CP mutants that represent different states of the polymerization reaction. These states are consistent with the hypothesis that interaction between specific N- and C-terminal peptides supports an underlying mechanism that coordinates the transfer reaction to induce polymerization.

EXPERIMENTAL PROCEDURES

Materials—Thrombin and trypsin were purchased from Sigma. *Escherichia coli* BL21 (DE3) and C41 (DE3) cells were produced by Agilent Technologies (Cary, NC) and Lucigen (Middleton, WI), respectively. pGEX plasmid was obtained from GE Healthcare. Primers were generated by Invitrogen. HepES was procured from Sigma. CH polymer (a chemically desulfated derivative of CS-C from shark cartilage) was obtained from Seikagaku Corp. (Tokyo, Japan). UDP-GlcA, UDP-GalNAc, testicular hyaluronidase, and chondroitin heptasaccharide GalNAc-(GlcUA-GalNAc)3; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-[hydroxymethyl]propane-1,3-diol activated Sepharose beads, and the ECL detection system were from GE Healthcare. A semiquantitative SAX MAGNAM ion exchange column was purchased from Whatman (Clifton, NJ).

Preparation of CH6 and CH7—CH oligosaccharides were prepared from CH polymer as previously described (11). Briefly, for the preparation of even-numbered oligosaccharides such as CH6, CH polymer was digested with testicular hyaluronidase. For the preparation of odd-numbered oligosaccharides such as CH7, the hyaluronidase digests were further treated with β-glucuronidase at 37 °C. CH6 and CH7 were separated from these digests by chromatography on a Q-Sepharose ion exchange column and a Superdex 30 gel filtration column. The structures of the oligosaccharides were confirmed with MALDI-TOF Mass spectrometry (MS) spectrometer (AutoFlex, Bruker Daltonics, Bremen, Germany).

Site-directed Mutagenesis—Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene) following the protocols described in the accompanying instruction manual utilizing proper primers. The mutations were confirmed by sequencing with the Big Dye Terminator Cycle Sequencing Reaction kit (Applied Biosystems).

Purification of Recombinant Proteins—*Escherichia coli* BL21 (DE3) cells were transformed with a given pGEX plasmid in SOC medium, (Invitrogen) and the transformed cells were selected from a Luria-Bertani agar plate containing a 100 μg/ml concentration of ampicillin. Transformed cells grown in Luria-Bertani medium were inoculated into 2YT media containing 100 mg/ml ampicillin at 37 °C. When A600 of the culture reached 0.6, the temperature was set to 23.5 °C, and isopropyl-1-thio-β-D-galactopyranoside (final concentration of 0.2 mM) was added 14 h before cells were harvested. Purification of protein and confirmation of protein purity was performed as previously reported (12).

Isothermal Titration Calorimetry—Isothermal titration calorimetry measurements were carried out in HEPES buffer using an iTC200 MicroCalorimeter (GE Healthcare) at 20 °C. Substrate solutions containing UDP, UDP-GlcNAC, UDP-GalNAc, C6, and C7 at 4 mM or UDP-GlcA at 1 mM were injected into a reaction cell containing ~100–200 μM protein. Thirty injections of 7 μl at 120 s intervals were performed. Data acquisition and analysis were performed by the MicroCal Origin software package. Data analysis was performed by generating a binding isotherm and best fit using the following fitting parameters: N (number of sites), ΔH (cal/mol), ΔS (cal/mol/deg), and K (binding constant in M−1) and the standard Levenberg-Marquardt methods (13). After data analysis, K (m−1) was then converted to KD (μM).

Partial Proteolysis—One microgram of protein in HEPES buffer was incubated with 50, 5, or 0.5 ng of trypsin for 30 min at room temperature. Digestion was halted by adding 1 μl of 100 mM phenylmethylsulfonyl fluoride (Active Motif) and then boiling for 1 min. Samples were then loaded onto a NuPage 4–12% Bis-Tris gel (Invitrogen) with 6 μl of NuPage 4X LDS sample buffer (Invitrogen) and subjected to electrophoresis. Gel was then stained with Coomassie Brilliant Blue G 250 (Fluka).

Mass Spectroscopy—Two major bands stained with the Colloidal Blue Staining kit (Invitrogen) were subjected to mass
Enzyme Assays—GalNAc transfer, GlcA transfer, and chondroitin polymerase activities of the recombinant enzymes were measured using radiolabelled donor substrates as described previously (13) with a slight modification; for the GalNAc transfer activity assay, a 50-µl mixture containing 50 mM Tris-HCl (pH 7.2), 20 mM MnCl₂, 0.15 M NaCl, UDP-[³H]GalNAc (3 nmol, 0.1 µCi) as the donor substrate and 1 nmol of chondroitin hexasaccharide as the acceptor substrate was incubated with the recombinant enzymes (2.0 µg) at 30 °C for 60 min and then heated in boiling water. For the GlcA transfer activity assay, UDP-[¹⁴C]GlcA (3 nmol, 0.1 µCi) and 1 nmol of chondroitin pentasaccharide were used as the donor and acceptor substrates, respectively. For the polymerase activity assay, UDP-[³H]GalNAc (3 nmol, 0.1 µCi) and UDP-GlcA (3 nmol) were used as the donor substrates, and 0.1 nmol of chondroitin pentasaccharide was used as the acceptor substrate. The radiolabelled saccharides were separated by a Superdex Peptide column and measured by a liquid scintillation counter. The enzyme activities were determined by calculating the amount of the incorporated radioactive sugars.

RESULTS

Effect of Deleting the N-terminal Peptide on K4CP Activity—The N-terminal peptide (residues 58–134), which wraps around the C-terminal domain of K4CPΔ57 in the x-ray crystal, comprises a linear structure that contains a random coil and three α-helices (see supplemental Fig. 1A for locations). This peptide was successively deleted to produce mutants K4CPΔ57, K4CPΔ101, K4CPΔ107, and K4CPΔ113 (Fig. 1). Subsequently, ITC was employed using these deletion mutants to determine their binding to the donor substrates UDP-GalNAc and UDP-GlcA. The K4CPΔ95 mutant, which removed the first two α-helices, retained similar $K_d$ values for binding to UDP-GalNAc and UDP-GlcA to those observed with K4CPΔ57 (Table 1). Therefore, K4CPΔ95 was further deleted by six amino acid residues at a time to produce K4CPΔ101, K4CPΔ107, and K4CPΔ113 (Fig. 1). The K4CPΔ113 mutant exhibited $K_d$ values for binding to UDP-GlcA and UDP-GalNAc similar to those of K4CPΔ57 and K4CPΔ95 (Table 1). Mutant K4CPΔ107 maintained a $K_d$ value for UDP-GlcA binding similar to that of the K4CPΔ95 mutant while exhibiting a significant decrease in that of UDP-GalNAc binding. With its further deletions, K4CPΔ113 lost binding to UDP-GalNAc while retaining UDP-GlcA binding. The donor substrate product UDP bound to K4CPΔ95 but not to K4CPΔ107 or K4CPΔ113 (Table 1).

Given these donor substrate interactions, the K4CP deletion mutants were then subjected to enzyme assays to determine GalNAc and GlcA transfer and polymerase activities (Table 2). K4CPΔ95 and K4CPΔ101 catalyzed these three activities as effectively as K4CPΔ57. K4CPΔ107 abrogated polymerase activity while fully retaining both GalNAc and GlcA transfer activities. K4CPΔ113 retained levels of UDP-GalNAc transfer activity that were decreased by 50% while virtually abrogating UDP-GlcA transfer activity; as expected, K4CPΔ113 did not catalyze the polymerization reaction. Thus, the deletions of the N-terminal peptide resulted in generating K4CP mutants with diverse enzymatic features. Among them, K4CPΔ107 provided the most critical insight into the nature of K4CP; K4CP needs to coordinate its two transfer activities to catalyze the polymerization reaction and residues 101–113 are critical for this coordination to occur.

Our previous ITC analysis of donor substrate binding demonstrated that UDP-GalNAc does not bind to the N-terminal active site where GalNAc transfer occurs unless the C-terminal binding motif DSD is inactivated by mutation to ASA (12). Therefore, the mutant constructs K4CPΔ101 ASA, K4CPΔ107 ASA, and K4CPΔ113 ASA were generated to examine UDP-GalNAc binding to their N-terminal active sites. ITC analysis on these ASA mutants confirmed that all of these ASA mutants bind UDP-GalNAc to their N-terminal active sites (Table 3), supporting the fact that K4CPΔ101, K4CPΔ107, and K4CPΔ113 catalyzed GalNAc transfer activity (Table 1). Noticeably, these ASA mutants exhibited $K_d$ values that were 6–20-fold lower for UDP binding compared to those for UDP-GalNAc binding. However, these higher UDP bindings
TABLE 1
Donor substrate binding of N-terminal truncated K4CP enzymes
The results obtained from ITC analysis are presented: thermodynamic parameters $\Delta S$ (cal/mol/degree), $n$ (number of binding sites), and the binding constant, $K_d$ ($\mu$M) for UDP, UDP-GlcA, and UDP-GalNAc at 20 °C. ND signifies a reaction in which no binding was detected.

| Enzyme | $\Delta S$ (cal/mol/degree) | $K_d$ ($\mu$M) | $n$ |
|--------|-----------------------------|----------------|-----|
| K4CPΔ57 | -22.61 | 358.17 ± 26.64 | 1.25 ± 0.32 |
| K4CPΔ95 | -1.84 | 494.56 ± 26.24 | 1.14 ± 0.33 |
| K4CPΔ101 | -6.20 | 429.18 ± 77.66 | 0.94 ± 0.04 |
| K4CPΔ107 | 0.29 | 61.73 ± 20.61 | 0.81 ± 0.06 |
| K4CPΔ113 | ND | ND | ND |

TABLE 2
GalNAc and GlcA transfer and polymerase activities
Transfer and chondroitin polymerase activities of the recombinant enzymes were measured using radioisotope donor substrates as described under “Experimental Procedures.” K4CPΔ57 activity is presented as having full (100%) activity, and the percentage of activity possessed by the mutant constructs is described relative to that of K4CPΔ57.

| Enzyme | GalNAc-T | GlcA-T | Polymerase |
|--------|----------|--------|------------|
| K4CPΔ57 | 1.54 ± 0.27 | 0.100 | 0.138 |
| K4CPΔ95 | 1.56 ± 0.30 | 0.101 | 0.141 |
| K4CPΔ101 | 1.54 ± 0.27 | 0.999 | 0.958 |
| K4CPΔ107 | 1.27 ± 0.10 | 0.823 | 0.784 |
| K4CPΔ113 | 0.84 ± 0.09 | 0.548 | 0.498 |

TABLE 3
UDP and UDP-GalNAc binding to the N-terminal active site of truncated K4CP enzymes utilizing their ASA mutant
The results obtained from ITC analysis are presented: thermodynamic parameters $\Delta S$ (cal/mol/degree), $n$ (number of binding sites), and the binding constant, $K_d$ ($\mu$M) for UDP and UDP-GalNAc at 20 °C.

| Enzyme | $\Delta S$ (cal/mol/degree) | $K_d$ ($\mu$M) | $n$ |
|--------|-----------------------------|----------------|-----|
| K4CPΔ101 ASA | -0.04 | 20.44 ± 7.26 | 1.06 ± 0.05 |
| K4CPΔ107 ASA | 3.53 | 18.97 ± 6.52 | 0.89 ± 0.13 |
| K4CPΔ113 ASA | 18.90 | 1.48 ± 0.74 | 0.89 ± 0.02 |

did not prevent them from catalyzing GalNAc transfer at the N-terminal active site.

The Peptides That Determine K4CP Activity—Given that K4CPΔ107 altered enzymatic activity, a partial proteolysis experiment was employed to test the hypothesis that deletion of residues 58–107 affected the K4CP structure in such a manner that resulted in altered enzyme activity. Peptide fragments, which were generated from digested K4CPΔ57 and K4CPΔ107, were then separated by SDS-PAGE and subjected to mass spectrometry (supplemental Fig. 2). A peptide fragment consisting of nine amino acids found in the C terminus beginning with Tyr-677 and ending with Leu-686 was uniquely generated from the K4CPΔ107 protein (supplemental Table 3). Analysis of the x-ray structure of K4CPΔ57 revealed that the peptide 677YTWKEI682 within this fragment forms an interface with the N-terminal peptide 113DWPSDL118 in the K4CP molecule: Tyr-677, Trp-679, and Lys-681 form hydrogen bonds with Asp-113, Trp-679, and Lys-681 form hydrogen bonds with Asp-113, Pro-115, Asp-117, and Leu-118 within 113DWPSDL118. This 113DWPSDL118 peptide appeared to be involved in determining K4CP enzyme activity.

The determining role of the interaction between 113DWPSDL118 and 677YTWKEI682 was further investigated by internally deleting these peptides from K4CPΔ57. K4CPΔDWPSDL was capable of binding to both UDP-GlcA and UDP-GalNAc (Table 4). Despite binding to UDP-GlcA, K4CPΔDWPSDL nearly abrogated all GlcA transfer and polymerase activities (Table 5). K4CPΔDWPSDL ASA confirmed that K4CPΔDWPSDL retained GalNAc transfer activity at the N-terminal active site, although ITC analysis did not detect UDP-GalNAc binding to this deletion mutant. As expected, K4CPΔDWPSDL ASA completely eliminated the ~1% residual GlcA transfer activity that remained in the K4CPΔDWPSDL mutant. UDP did not bind to either K4CPΔDWPSDL or K4CPΔDWPSDL ASA. Similar to K4CPΔDWPSDL, K4CPΔYTWEKI, which internally deleted 677YTWKEI682, bound to both UDP-GalNAc and UDP-GlcA and retained GalNAc transfer activity; however, GlcA transfer and polymerase activities were completely abrogated (Tables 4 and 5). Thus, K4CPΔDWPSDL and K4CPΔYTWEKI decoupled UDP-GlcA binding from GlcA transfer activity in K4CPΔ57. In an alternate to internal deletions, Tyr-677, Trp-679, and Lys-681 within 677YTWKEI682 were simultaneously substituted with Asp, Ala, and Asp, respectively, to disrupt the interactions between these two peptides. The triple mutants K4CP YWKp and K4CP YWKp ASA abolished all function of K4CP, and no donor substrate binding and no enzyme activities were detected (Tables 4 and 5). On the other hand, both K4CP YWKp and K4CP YWKp ASA bound UDP at $K_d$ values of around 1 $\mu$M at both N- and C-terminal active sites (Table 4).

Binding of Acceptor Substrates CH6 and CH7—ITC analysis for acceptor substrates with K4CPΔ57, K4CPΔ57 ASA, and
The bifunctional glycosyltransferase chondroitin synthase K4CP alternatively transfers GalNAc and GlcA at the N- and C-terminal active sites, respectively, to polymerize them into the chondroitin chain. A characteristic of this polymerization reaction is the fact that there is no template to assist K4CP with the reaction, as compared with the polymerization reactions catalyzed by DNA and RNA polymerases and peptide synthesis. Our study utilized K4CP mutants and defined the different states that occur during the polymerization reaction. Moreover, the peptide interaction between \textsuperscript{113}DWPSDL and \textsuperscript{677}YTWEKI has been characterized as an essential factor that determines transfer activities and enables the polymerization reaction. These findings are consistent with the hypothesis that K4CP possesses an intrinsic mechanism within itself to coordinate the two transfer reactions, enabling K4CP to extend the chondroitin chain.

Based on enzyme activities, K4CP\textsuperscript{Δ57} mutants can be organized into three different groups that possess structural features that could represent distinct stages of the enzyme reaction: I, II, and III (Fig. 2A). Stage I enzyme possesses both GlcA and GalNAc transfer activities but no polymerase activity; stage II possesses GalNAc transfer activity but no GlcA transfer or polymerase activities; stage III comprises an inert enzyme with no transfer or polymerase activities. Based on these stages the reaction cycle of the proposed polymerization cycle is depicted in Fig. 2B. In rejecting the notion that polymerization is a random reaction, the first compelling evidence in support of the concept of a coordinated reaction mechanism came when K4CP\textsuperscript{Δ107} was found to fully retain both donor and acceptor substrate binding as well as transfer activities, but polymeriza-

**TABLE 4**

Regulation of enzyme functions by the interaction between \textsuperscript{113}DWPSDL and \textsuperscript{677}YTWEKI

| Enzyme            | UDP-GalNAc | UDP-GlcA | UDP |
|-------------------|------------|----------|-----|
| K4CP\textsuperscript{Δ57} | 14.90      | 22.80    |     |
| K4CP\textsuperscript{Δ57} ASA | ND         | ND       |     |
| K4CP\textsuperscript{Y7W7K7} | 6.53       | 19.72    |     |
| K4CP\textsuperscript{Y7W7K7p} ASA | ND        | ND       |     |

**TABLE 5**

C6 acceptor substrate binding to the N-terminal active site of truncated K4CP enzymes utilizing their ASA mutants

| Enzyme            | C6 acceptor substrate | K_d | n   |
|-------------------|-----------------------|-----|-----|
| K4CP\textsuperscript{Δ57} | ND                    | ND  | ND  |
| K4CP\textsuperscript{Δ57} ASA | 28.40               | 0.25 | 0.06 |
| K4CP\textsuperscript{Δ107} | 10.80                | 8.78 | 0.92 |
| K4CP\textsuperscript{Y7W7K7} ASA | ND                  | ND  | ND  |
| K4CP\textsuperscript{Y7W7K7p} ASA | 12.90              | 8.47 | 1.23 |

K4CP\textsuperscript{Δ57} ACA determined that CH6 (GlcA at the non-reducing end) and CH7 (GalNAc at the non-reducing end) bind to the N- and C-terminal active sites, respectively (Table 4). Acceptor bindings remain constant in K4CP\textsuperscript{Δ107}, which abrogates polymerase activity while retaining transfer activities. In addition, the binding of CH6 to K4CP\textsuperscript{Δ57} ABA, but not to the K4CP\textsuperscript{Δ57}, indicates that CH6 binding to the N-terminal active site is regulated by the C-terminal active site. On the other hand, CH7 binding to the C-terminal active site is not controlled by the N-terminal active site. The characteristics of these acceptor bindings are reminiscent of the donor substrates. Although K4CP Y7W7K7p ASA neither binds to UDP-GalNAc nor catalyzes GalNAc transfer activity, this mutant retains binding to donor substrate CH6 (Table 6). Likewise, CH7 binding is retained by K4CP\textsuperscript{Y7W7K7} (Table 7), which does bind to the donor substrate UDP-GlcA but does not catalyze GlcA transfer activity. Thus, K4CP\textsuperscript{Y7W7K7} appears to alter the substrate binding conformation so that this mutant loses transfer activity.

**DISCUSSION**

The bifunctional glycosyltransferase chondroitin synthase K4CP alternatively transfers GalNAc and GlcA at the N- and C-terminal active sites, respectively, to polymerize them into the chondroitin chain. A characteristic of this polymerization reaction is the fact that there is no template to assist K4CP with the reaction, as compared with the polymerization reactions catalyzed by DNA and RNA polymerases and peptide synthesis. Our study utilized K4CP mutants and defined the different states that occur during the polymerization reaction. Moreover, the peptide interaction between \textsuperscript{113}DWPSDL and \textsuperscript{677}YTWEKI has been characterized as an essential factor that determines transfer activities and enables the polymerization reaction. These findings are consistent with the hypothesis that K4CP possesses an intrinsic mechanism within itself to coordinate the two transfer reactions, enabling K4CP to extend the chondroitin chain.
question as to why ITC analysis did not detect UDP-GalNAc binding to the N-terminal active site (Table 4), this donor binding could have occurred in a manner that did not allow for detection by ITC. Despite possessing binding ability to both UDP-GlcA and C7 substrates at the C-terminal active site, K4CPΔYTWEKI was unable to catalyze GlcA transfer activity (stage II). Noticeably, K4CPΔYTWEKI strengthened its UDP binding affinity (Kₐ values 6-fold lower than those observed with K4CPΔ57) to be equivalent to UDP-GlcA binding constant at the C terminus. These changes in UDP binding indicate that the deletion mutants alter a portion of the active site structure to where the UDP moiety of UDP-sugar molecule binds; this alteration may have repressed GlcA transfer activity in K4CPΔYTWEKI.

A simultaneous triple mutation of the peptide YTWEKI (K4CP YWKpm) appears to alter K4CP structure differently from complete deletion of the peptide (K4CPΔYTWEKI). K4CP YWKpm and K4CP YWKpm ASA have provided experimental evidence indicating that K4CP can adopt structural features that force the enzyme to be totally free from donor substrate binding as well as catalytic activities while retaining CH6 and CH7 acceptor substrate binding to their respective sites (stage III). Because K4CP YWKpm ASA retains C6 acceptor substrate binding, the acceptor substrate cannot be the direct cause of repression of transfer activity. It is intriguing that we did not encounter a K4CP mutant that represses GalNAc transfer at the N-terminal active site while proceeding with GlcA transfer activity at the C-terminal active site, which should exist during the polymerization reaction. K4CP YWKpm and K4CP YWKpm ASA exhibit high affinity UDP binding (Kₐ values around 1 μM) to each active site, possibly resetting K4CP for the next round of the catalytic cycle to elongate the chondroitin chain. Because the binding affinity of UDP-GlcA at the C-terminal active site in K4CPΔ57, but not UDP-GalNAc, is equivalent to the UDP binding in K4CP YWKpm, stage III may represent an enzymatic state that precedes a subsequent structural alteration allowing UDP-GlcA binding to initiate the new-round of the reaction. Therefore, these structural features may enable K4CP to coordinate transfer reactions to elongate the chondroitin chain. The possibility of these structural features being conserved in other bi-functional glycosyltransferases is intriguing. Given that K4CP is the only such transferase whose structure has been solved, future investigations will have to determine whether other bifunctional glycosyltransferases possess peptides that interact in a manner similar to that of K4CPΔYTWEKI.

In conclusion, these K4CP mutants exhibit at least three different states of the polymerization reaction that can be integrated into a hypothetical scheme for the overall reaction cycle used by K4CP to elongate chondroitin chains (Fig. 2). Because acceptor substrates remain bound to their respective active sites, donor substrate binding appears to be the determinant for K4CP’s ability to coordinate the two transfer activities and polymerize chondroitin chains. The reaction may start by UDP-GlcA binding to the C-terminal active site of Stage III, transferring GlcA to the non-reducing end of the oligosaccharide. Then the produced oligosaccharide moves into the N-terminal active site, which should exist during the polymerization reaction to catalyze GlcA transfer activity (stage II). The green circles with UDP denote the inactive sites that exhibited strong UDP binding (stage III). The observed transfer and polymerase activities are shown below for each stage. B, the proposed sequence of the enzyme reaction: red and blue circles show sites that are enzymatically active and inactive, respectively. The reaction starts at the C-terminal active site transferring GlcA to GalNAc at the non-reducing end of the oligosaccharide (n = 1), during which the N-terminal active site is inactive and could be occupied by UDP; after this first transfer reaction, the product moves into the N-terminal active site from which GalNAc is transferred, during which the C-terminal active site remains occupied by UDP-GlcA (because of the UDP-GlcA high binding affinity) and is inactive; the second transferred product then moves back to the C-terminal active site. Once a single reaction cycle is completed, then chondroitin chain is elongated n1 to n2. The data between the N-terminal peptide and the C-terminal domain indicate their interactions. Open circles with N indicate the N terminus of K4CPΔ57 molecule, and the N-terminal peptide interacts with the C-terminal domain.

Coordinated Transfer Reactions for Polymerization

FIGURE 2. Schematic representation of the proposed different stages of the polymerization reaction. A, boxes represent domains, and circles indicate their active sites. The red circles with DSD and DCD indicate an active site with donor binding (stage I). A blue circle with DCD signifies an inactive site with donor binding (stage II). The green circles with UDP denote the inactive sites that exhibited strong UDP binding (stage III). The observed transfer and polymerase activities are shown below for each stage. B, the proposed sequence of the enzyme reaction: red and blue circles show sites that are enzymatically active and inactive, respectively. The reaction starts at the C-terminal active site transferring GlcA to GalNAc at the non-reducing end of the oligosaccharide (n = 1), during which the N-terminal active site is inactive and could be occupied by UDP; after this first transfer reaction, the product moves into the N-terminal active site from which GalNAc is transferred, during which the C-terminal active site remains occupied by UDP-GlcA (because of the UDP-GlcA high binding affinity) and is inactive; the second transferred product then moves back to the C-terminal active site. Once a single reaction cycle is completed, then chondroitin chain is elongated n1 to n2. The dots between the N-terminal peptide and the C-terminal domain indicate their interactions. Open circles with N indicate the N terminus of K4CPΔ57 molecule, and the N-terminal peptide interacts with the C-terminal domain.
minal active site of Stage II by virtue of the regulation imposed by the C-terminal active site and GalNAc transfer follows. Then the second transferred product may move back to the C-terminal active site. K4CP is endowed with an intrinsic molecular mechanism that may utilize the interaction of the N-terminal 113DWPSDL118 with the 677YTWEKI682 peptide of the C-terminal domain to coordinate GalNAc and GlcA transfers and elongate the chondroitin chain. In this scheme, understanding the structural basis for why K4CP mutants possess both transfer activities but not polymerase activity will be most critical for us to determine the molecular mechanism of the polymerization reaction. With this in mind, solving the structural features that connects the 113DWPSDL118 peptide with the C-terminal domain, an area for which no electron density was detected in the current K4CP structure, may be critical to unifying these observed snapshots at stages during the polymerization process to fully decipher the molecular-based regulatory machinery that confers K4CP the ability to coordinate its polymerization reaction.

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