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The Chondroitin Polymerase K4CP and the Molecular Mechanism of Selective Bindings of Donor Substrates to Two Active Sites*

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Bacterial chondroitin polymerase K4CP is a multifunctional enzyme with two active sites. K4CP catalyzes alternative transfers of glucoronic acid (GlcA) and *N*-acetylgalactosamine (GalNAc) to elongate a chain consisting of the repeated disaccharide sequence GlcAβ1–3GalNAcβ1–4. Unlike the polymerization reactions of DNA and RNA and polypeptide synthesis, which depend upon templates, the monosaccharide polymerization by K4CP does not. To investigate the catalytic mechanism of this reaction, we have used isothermal titration calorimetry to determine the binding of the donor substrates UDP-GlcA and UDP-GalNAc to purified K4CP protein and its mutants. Only one donor molecule bound to one molecule of K4CP at a time. UDP-GlcA bound only to the C-terminal active site at a high affinity (K_d = 6.81 μM), thus initiating the polymerization reaction. UDP-GalNAc could bind to either the N-terminal or C-terminal active sites at a low affinity (K_d = 266–283 μM) but not to both sites at the same time. The binding affinity of UDP-GalNAc to a K4CP N-terminal fragment (residues 58–357) was profoundly decreased, yielding the average K_d value of 23.77 μM, closer to the previously reported K_m value for the UDP-GalNAc transfer reaction that takes place at the N-terminal active site. Thus, the first step of the reaction appears to be the binding of UDP-GlcA to the C-terminal active site, whereas the second step involves the C-terminal region of the K4CP molecule regulating the binding of UDP-GalNAc to only the N-terminal active site. Alternation of these two specific bindings advances the polymerization reaction by K4CP.

Chondroitin sulfate is a glucosaminoglycan that is a polysaccharide chain composed of the sugar molecules glucuronic acid (GlcA)[2] and *N*-acetylgalactosamine (GalNAc). A disaccharide unit (GlcAβ1–3GalNAcβ1–4) continually repeats to form a nonbranching polysaccharide chain that can consist of over a hundred individual monosaccharides, each of which can be sulfated in various positions and quantities (1). Chondroitin chains are usually found attached to proteins, forming proteoglycans, and are present in the extracellular matrix and cell surfaces of various human tissues including the cartilage, aorta, skeletal muscle, eye, lung, and brain tissues (2–4). Through interaction with various extracellular proteins, chondroitin plays a critical role in the regulation of a variety of cellular activities including growth, development, and response to injury in the nervous system (5, 6). For example, the loss of chondroitin sulfate from the cartilage is a major cause of osteoarthritis and intervertebral disc and cartilage degeneration (7, 8). Multifunctional polymerases responsible for the synthesis of chondroitin chains have been cloned and characterized for their transfer reactions (9–12). However, the molecular mechanism of the polymerization reaction catalyzed by these enzymes remains virtually unknown.

Bacterial cells also synthesize a chondroitin chain. Although mammalian chondroitin polymerases are Golgi membrane-bound enzymes, those of bacterial polymerases are soluble enzymes. For instance, the K4 strain of *Escherichia coli* contains an enzyme, encoded by the KfoC gene of the K4 gene cluster, that catalyzes the synthesis of the chondroitin chain, named K4 chondroitin polymerase or K4CP (9). The reduced amino acid sequence of K4CP revealed two conserved UDP-sugar binding motifs (D*X*D), one located in the N-terminal region and the other located in the C-terminal region of the molecule. Comparative sequence analysis shows that the system of two active sites is conserved in the other bacterial and mammalian chondroitin polymerases as well as the other polysaccharide polymerases such as the heparan polymerase Exostosins (10, 13–16). We have previously employed isothermal titration calorimetry (ITC) to investigate the specific binding of donor substrate to UDP-*N*-acetylhexosaminyltransferase Exostosin Like-2 that possesses only one active site (17). Using the water solubility of the bacterial K4CP enzyme to our advantage, we have now extended our study to include the ITC measurement of binding specificity of donor substrates to the two active sites of K4CP to explicate the catalytic mechanism of how this enzyme executes the polymerization process.

We bacterially expressed a truncated enzyme of K4CP (from residues 55 to 686) that retained all of the original polymerase activity, from which the D*X*D motifs were mutated to generate various mutant enzymes. In addition, a deletion mutant lacking

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[2] The abbreviations used are: GlcA, glucuronic acid; GalNAc, *N*-acetylgalactosamine; ITC, isothermal titration calorimetry.

the C-terminal half of the K4CP was constructed. Wild type K4CP and these mutants were expressed in bacterial cells, purified, and subjected to ITC analysis to determine the nature of binding for the donor substrates UDP-GlcA, UDP-GlcNAc, and UDP-GalNAc. UDP binding was also investigated. Here we now present key elements of the specific donor bindings that are consistent with the catalytic mechanism of the alternative transfer reactions by K4CP.

EXPERIMENTAL PROCEDURES

Expression and Purification of Proteins—*E. coli* BL21 (DE3) cells transformed with pGEX K4CP were grown and harvested as previously reported (17). The cells were then disrupted in a French Press at room temperature under pressures varying between 8,000 and 24,000 p.s.i. Following ultracentrifugation, the K4CP protein was then purified from the supernatants by absorption onto glutathione-Sepharose resin (GE Healthcare) and eluted via thrombin cleavage overnight at 4 °C. The eluted protein was then dialyzed overnight into 25 mM HEPES (pH 7.5), 100 mM NaCl, 20 mM $MnCl_2$, and 1 mM $CaCl_2$. Purification of protein was confirmed by taking 10 μl of eluted protein and combining it with 4 μl of NuPage 4× LDS sample buffer (Invitrogen), 2 μl of 2-mercaptoethanol (Sigma-Aldrich) and then running the sample on a NuPage 4–12% Bis-Tris gel (Invitrogen). The gel was then stained with Coomassie Brilliant Blue G-250 (Fluka) and then destained to make the protein bands more clearly visible.

Isothermal Titration Calorimetry—Isothermal titration calorimetry measurements were carried out in HEPES buffer using a VP-ITC MicroCalorimeter (Micro Cal, Inc.) at 20 °C. Substrate solution was injected into a reaction cell containing the protein. For substrate solutions containing UDP-GlcA, thirty injections of 3 μl at 180-s intervals were performed. For substrate solutions containing UDP, UDP-GlcNAc, or UDP-GalNAc, 30 injections of 3 μl at 300-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 (18). Following data analysis, K (M^{-1}) is then converted to K_d (μM).

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. The pairs of primers used to mutate the DCD-binding motif found in the N terminus region to ACA, DCE, and DCK were: TGGGTTCGGAGCCATAGCACAAGCCAGAATTGCAACATA and TATGTTGCAATTCTGGCTTGTGCTATGGCTCCGAACCCA; TGGGTTCGGAGCCATTTCACAATCCAGAATTGC and GCAATTCTGGATTGTGAAATGGCTCCGAACCCA; and TGGGTTCGGAGCCATTTTACAATCCAGAATTGC and GCAATTCTGGATTGTAAAATGGCTCCGAACCCA, respectively. The pairs of primers used to mutate the DSD-binding motif found in the C terminus region to ASA, DSE, DSK, DSA, ASD, and KSD were: TGGTTCAAGAAAGTCAGCAGAGGCTAACTGACCTATATA and TATATAGGTCAGTTAGCCTCTGCTGACTTTCTTGAACCA; TTCAAGAAAGTCTTCAGAGTCTAACTGACCTAT and ATAGGTCAGTTAGACTCTGAAGACTTTCTTGAA; TTCAAGAAAGTCTTTAGAGTCTAACTGACCTAT and ATAGGTCAGTTAGACTCTAAAGACTTTCTTGAA; TTCAAGAAAGTCAGCAGAGTCTAACTGACCTAT and ATAGGTCAGTTAGACTCTGCTGACTTTCTTGAA; TTCAAGAAAGTCATCAGAGGCTAACTGACCTAT and ATAGGTCAGTTAGCCTCTGATGACTTTCTTGAA; and TTCAAGAAAGTCATCAGATTTTAACTGACCTAT and ATAGGTCAGTTAAAATCTGATGACTTTCTTGAA, respectively. The primers used to generate the ACA and ASA mutants were also used to generate the double mutant, thereby mutating the D*X*D motifs found in both regions to A*X*A. The primers used to generate the ACA and DSA mutants were used to create the double mutant in which the N terminus-binding site was mutated to ACA and the C terminus-binding site was converted to DSA. The primers used to delete the C-terminal region of the enzyme were: CGCGGATCCAAAGCTGTTATTGATATTGAT and CGGAATTCTTAATGCGTAAACTCTTCATCAAA. The mutations were confirmed by sequencing with the Big Dye terminator cycle sequencing reaction kit (Applied Biosystems).

Enzyme Assays—Hydrolysis was determined as follows. 53 μg of protein were tested in an assay mixture containing 25 mM HEPES (pH 7.5), 100 mM NaCl, 20 mM $MnCl_2$, 1 mM $CaCl_2$, and 1 μl of either UDP-[^{3}H]GlcNAc (36.0 Ci/mmol; PerkinElmer Life Sciences), UDP-[^{3}H]GlcA (20.0 Ci/mmol; American Radiolabeled Chemicals, Inc.), or UDP-[^{3}H]GalNAc (20.0 Ci/mmol; American Radiolabeled Chemicals, Inc.). Assay mixtures containing radioactive plus nonradioactive substrates at final concentrations of 1.6, 3.2, 6.4, 9.6, 16.0, 32.0, 64.0, 96.0, 128.0, and 200.0 μM were incubated for 1 h at 30 °C. The incubation mixtures were boiled for 2 min, and the precipitated protein was removed by centrifugation at 20,400 × g for 5 min. The resulting supernatants were subjected to a Hi-Load 16/60 Superdex 30pg column (GE Healthcare) equilibrated with 250 mM NH_4HCO_3 containing 7% 1-propanol using the ÄKTA Purifier (GE Healthcare). Fractions were collected at a rate of 1 ml/min, and the amount of the hydrolyzed product UDP was quantified with a scintillation counter. Data analysis was performed using the Origin version 7.0 software programmed to fit data to an *s/v-s* plot to calculate the K_m and V_{max} values using Michealis-Menten kinetics.

RESULTS

Binding of UDP-GlcA—ITC was performed to examine the binding of UDP-GlcA to the truncated K4CP (hereafter referred to as K4CP). The calorimetric profile showed that UDP-GlcA bound to K4CP at a ratio of one donor molecule to one enzyme molecule (Fig. 1). The binding of UDP-GlcA was tested at various temperatures ranging from 5 to 40 °C to ascertain the nature of donor binding (Table 1), from which the true values for the changes in entropy (ΔS) and enthalpy (ΔH) were directly determined by plotting Log K *versus* 1000/K°: $\Delta S = -25.18 \pm 1.43$ cal/mol/deg and $\Delta H = -14{,}000.45 \pm 419.29$ cal/mol (19). Using these values, it was found that the $T\Delta S$ was lower in value than the ΔH value, suggesting that this binding is an enthalpy-driven reaction. In addition, the Gibbs free energy

(ΔG) values remained constant over the various temperatures, implying that the nature of this binding reaction is spontaneous and that the maximum potential of K4CP to perform the binding remains the same over a range of temperatures. As expected, the K_d values varied from 3.26 to 55.09 μM depending upon the temperatures used for the assays, whereas the number of the UDP-GlcA molecules that bound to the K4CP molecule was the same at all temperatures (Table 1).

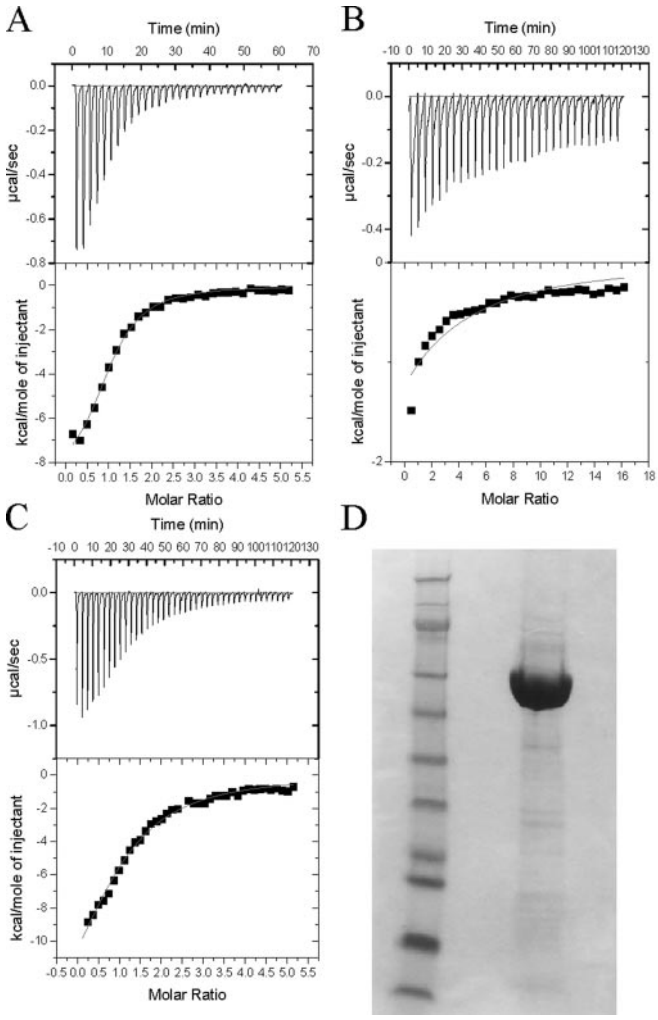

FIGURE 1. *A*, calorimetric profile of UDP-GlcA binding to K4CP. The reaction cell contained a solution of K4CP (1.4 ml and 124.8 μM) in 25 mM HEPES buffer (pH 7.5) containing 20 mM $MnCl_2$, 0.1 M NaCl, and 1 mM $CaCl_2$. The syringe contained 1 mM UDP-GlcA in the same buffer. *Top panel*, raw calorimetric data obtained from the injection of 3 μl aliquots of UDP-GlcA at 3-min intervals. The *lower panel* shows the integrated binding isotherm with the experimental points (■) and best fit. *B*, calorimetric profile of UDP-GalNAc binding to K4CP. *C*, calorimetric profile of UDP-GlcA binding to the K4CP mutant construct ^{239}ACA^{241}. *D*, gel showing the purity of K4CP used in ITC experiments and enzyme assays. The *first lane* contains the SeeBlue Plus 2 prestained molecular marker (Invitrogen), and the *second lane* contains purified K4CP.

Binding of UDP-GalNAc and UDP—ITC was performed at 20 °C for determining the binding of UDP-GalNAc and UDP. Both UDP-GalNAc and UDP bound to K4CP at a one to one molecular ratio (Table 2). UDP-GalNAc was characterized by its extremely high K_d value, 358.17 μM at 20 °C. This value was more than 40-fold higher when compared with the K_d value of 8.5 μM at 20 °C yielded by UDP-GlcA binding and the averaged K_d value (6. 81 $\pm$ 1.72 μM) for UDP-GlcA, a number arrived at by calculating from five different binding assays. The K_m values of UDP-GlcA and UDP-GalNAc transfer reactions were previously reported (9). Intriguingly, the K_d value of 358.17 μM was over 10-fold higher than the reported K_m value (31.6 μM) of the UDP-GalNAc transfer reaction catalyzed by K4CP, whereas the corresponding K_d and K_m values for UDP-GlcA were similar. The inactive donor substrate UDP bound to K4CP at a K_d value of 89.53 μM, which was higher than the K_d value of UDP-GlcA binding but was lower than that of UDP-GalNAc binding. Only one UDP molecule was found to bind to the K4CP molecule, which was unexpected given the fact that there are two binding sites for donor substrates. These experimental observations are at first glance puzzling but may provide the insightful clues necessary to understand the catalytic mechanism of the polymerization reaction; both UDP and UDP-GalNAc bind to K4CP at a one to one molecular ratio, and the K_d value of UDP-GalNAc was higher than the reported K_m value.

Delineating the Specific Donor-binding Site—Site-directed mutagenesis was employed to mutate the aspartic acid residues of the K4CP D*X*D motifs: either the N-terminal ^{239}DCD^{241} or the C-terminal ^{519}DSD^{521} to ^{239}ACA^{241} or ^{519}ASA^{521}. In addition, both ^{239}DCD^{241} and ^{519}DSD^{521} were simultaneously mutated to ^{239}ACA^{241} and ^{519}ASA^{521}, hereafter just referred to as D*X*Ddm. These mutants were subjected to ITC analysis (Table 3). UDP-GlcA bound to the ^{239}DCD^{241} mutant but not to the ^{519}DSD^{521} mutant, and moreover, the K_d value of this binding was close to that of the UDP-GlcA binding to K4CP. These results unequivocally identified the C-terminal DSD as the sole binding site of UDP-GlcA. UDP-GalNAc was found to bind to the both ^{239}ACA^{241}and ^{519}ASA^{521} mutants at similar affinities; their K_d values were within the range of the K_d value (358.18 μM) of UDP-GalNAc binding to K4CP. Thus, UDP-GalNAc is capable of binding to either the N- or C-terminal D*X*D motif. This tantalizing observation lead us to measure UDP-GalNAc transfer activity by the D*X*D mutants: K4CP and the ^{519}DSD^{521} mutant catalyzed the reaction at 5.895 and 5.469 pmol/min/μg protein, respectively, but the ^{239}ACA^{241} exhibited no transfer activity (Table 4). Because UDP-GalNAc was

TABLE 1

Thermodynamic parameters, ΔG, ΔH, ΔS, number of binding sites (n), and the binding constant, K_d for UDP-GlcA binding with wild type K4CP at five different temperatures

The substrate and K4CP concentrations were 0.8 mM and 42 μM, respectively.

Temperature	ΔG	ΔH	ΔS	K_d	n
	cal/mol	*cal/mol*	*cal/mol/deg*	μM	
5 °C	−6983.4 ± 1064	−10,350 ± 1064	−12.11	3.26 ± 1.78	1.05 ± 0.08
10 °C	−6815.3 ± 2557	−12,280 ± 2557	−19.31	5.55 ± 2.13	0.85 ± 0.14
20 °C	−6652.3 ± 1973	−11,100 ± 1973	−15.18	11.04 ± 3.33	0.82 ± 0.11
30 °C	−6331.4 ± 703.8	−14,370 ± 703.8	−26.53	27.32 ± 10.94	0.93 ± 0.25
40 °C	−6107.4 ± 4077	−18,690 ± 4077	−40.20	55.09 ± 8.24	0.97 ± 0.19

TABLE 2

Thermodynamic parameters, ΔG, Δ*H*, Δ*S*, number of binding sites (*n*), and the binding constant, K_d for donor binding with wild type K4CP in solution at 20 °C

UDP-GlcA and UDP-GalNAc concentrations were 1 mM; UDP and UDP-GalNAc concentrations were 4 mM. K4CP concentration was 124.8 μM. All of the experiments were conducted in triplicate to confirm the results obtained. Fresh enzyme was prepared for each experiment.

Substrate	Δ*G*	Δ*H*	Δ*S*	K_d	*n*
	cal/mol	*cal/mol*	*cal/mol/deg*	μM	
UDP-GlcA	−7209.1 ± 1124	−11,270 ± 1124	−13.86	8.05 ± 1.52	0.95 ± 0.07
UDP-GalNAc	−4779.2 ± 483.9	−11,630 ± 483.9	−22.61	358.17 ± 26.64	1.25 ± 0.32
UDP	−5431.7 ± 1953	−8643 ± 1953	−10.96	89.53 ± 22.77	0.91 ± 0.09

TABLE 3

Comparison of the binding constants K_d of UDP, UDP-GalNAc, UDP-GlcNAc, and UDP-GlcA for wild type K4CP to the mutant constructs ^{239}ACA^{241}, ^{519}ASA^{521}, and DXDdm

In ^{239}ACA^{241} the DXD-binding motif of the N-terminal region was eliminated by mutating the existing DCD amino acid sequence to ACA. In ^{519}ASA^{521} the DXD-binding motif of the C-terminal region was eliminated by converting the existing DSD amino acid sequence to ASA. In DXDdm, both DXD-binding motifs were eliminated by mutating the N-terminal binding site to ACA and the C-terminal binding site to ASA. All of the ITC experiments were performed at 20 °C and in triplicate to confirm results using a fresh batch of enzyme produced for each experiment. ND, no detectable binding.

Enzyme	UDP-GlcA	UDP-GalNAc	UDP
Wild			
K_d	8.05 ± 1.52	358.17 ± 26.64	89.53 ± 22.77
n	0.95 ± 0.07	1.25 ± 0.32	0.91 ± 0.09
^{239}ACA^{241}			
K_d	14.12 ± 1.50	266.74 ± 57.58	54.23 ± 11.18
n	0.99 ± 0.08	0.94 ± 0.11	0.72 ± 0.34
^{519}ASA^{521}			
K_d	ND	283.69 ± 30.76	16.70 ± 2.04
n	ND	1.15 ± 0.09	0.99 ± 0.08
DXDdm			
K_d	ND	ND	ND
n	ND	ND	ND

TABLE 4

Hydrolysis and transfer of donor substrates by wild type K4CP, and the mutant constructs ^{239}ACA^{241} and ^{519}ASA^{521}

The hydrolysis of K_m (μM) of UDP-GalNAc and UDP-GlcA and the transfer activity (pmol/min/μg of protein) of UDP-GalNAc and UDP-GlcNAc by wild type, ^{239}ACA^{241} and ^{519}ASA^{521} are shown. ND, no detectable transfer. Assay mixtures containing radioactive plus nonradioactive substrates at final concentrations of 1.6, 3.2, 6.4, 9.6, 16.0, 32.0, 64.0, 96.0, 128.0, and 200.0 μM were incubated with 53 μg of protein for 1 h at 30 °C.

	Substrate	Wild	^{239}ACA^{241}	^{519}ASA^{521}
Hydrolysis	UDP-GlcA	ND	ND	ND
	UDP-GalNAc	65.09	135.31	24.27
Transfer activity	UDP-GalNAc	5.895	ND	5.469
	UDP-GlcNAc	0.394	ND	0.534

found to bind to K4CP at a one to one molecular ratio, the N-terminal ^{239}DCD^{241} should be the binding site of UDP-GalNAc during the transfer reaction. However, the present binding assays using ITC could not determine the binding site of UDP-GalNAc. In a fashion similar to UDP-GalNAc, UDP bound to both the ^{239}ACA^{241} and ^{519}ASA^{521} mutants. Because the K_d value for the former mutant was closer to that for K4CP (89.53 μM) than that for the latter mutant, this one molecule of UDP appeared to bind to the C-terminal ^{519}DSD^{521} of K4CP. As expected, when both ^{75}DCD^{77} and ^{519}DSD^{521} were mutated, the D*X*Ddm possessed no capability to bind any of the donor substrates.

To further elucidate the binding of UDP-GalNAc, we attempted to construct deletion mutants by separating the K4CP molecule into N- and C-terminal fragments (residues 58–357 and residues 358–686, respectively). Although the C-terminal fragment did not express in our bacterial system, we were able to express and purify the N-terminal fragment for ITC analysis (Table 5). Consistent with our previous data, the N-terminal fragment abrogated the binding of UDP-GlcA. Conversely, both UDP-GalNAc and UDP retained their binding to the fragment. Most interestingly, the K_d value of UDP-GalNAc binding became similar to the reported K_m value of UDP-GalNAc transfer activity by K4CP. To reutilize the correlation of this K_d value with the catalytic activity of K4CP, we measured hydrolysis of K4CP and the D*X*D mutants using UDP-GlcA and UDP-GalNAc as substrates (Table 4). No hydrolysis activity was observed with UDP-GlcA with any of these enzymes. On the other hand, UDP-GalNAc was hydrolyzed by all enzymes. The K_m value of the hydrolysis by the ^{519}ASA^{521} mutant was nearly identical to the K_d value of UDP-GalNAc binding to the N-terminal fragment, and moreover, the corresponding value for hydrolysis by K4CP was closer to this K_d value. These results are consistent with the conclusion that the N-terminal ^{239}DCD^{241} is the binding site of UDP-GalNAc. Furthermore, the relatively high affinity of UDP-GalNAc binding provides critical information for us to hypothesize about the molecular mechanism by which K4CP regulates the binding of UDP-GalNAc to N-terminal ^{239}DCD^{241}.

Defining the Functions of the C-terminal DSD Motif—Recent x-ray crystal structures of the glycosyltransferases GlcAT1 and EXTL2 have demonstrably shown that the first aspartic acid residue in the D*X*D motif interacts with the sugar moiety of the donor substrate and that the second aspartic acid residue interacts with the UDP portion of the donor substrate (20, 21). Using this information as a guide, the first aspartic acid of the D*X*D motif was substituted with a negatively charged glutamic acid and a positively charged lysine to produce the following mutants: ^{239}DCE^{241}, ^{239}DCK^{241}, ^{519}DSE^{521}, and ^{519}DSK^{521}. These mutants were subjected to ITC analysis to decipher the role of aspartic acid in the regulation of donor substrate binding (Table 6). UDP-GlcA was able to bind to all four mutants at a one to one molecular ratio. Thus, the GlcA moiety solely determined the binding of UDP-GlcA. Moreover, the results indicated that the mutations of the N-terminal ^{239}DCD^{241} had no effect on the binding of UDP-GlcA to the C-terminal ^{519}DSD^{521}. UDP-GalNAc bound to the ^{239}DCE^{241}, ^{239}DCK^{241}, and ^{519}DSE^{521} mutants (Table 6), which is consistent with the facts that the second aspartic acid does not directly interact with the sugar moiety and that UDP-GalNAc can bind to either the N-terminal or C-terminal D*X*D motif. A surprising finding was that the ^{519}DSK^{521} mutant abrogated the bindings of UDP-GalNAc as well as that of UDP. Because the second aspartic acid is the primary residue responsible for the ability of the enzyme to interact with the UDP moiety, it may be reasonable to con-

TABLE 5

Thermodynamic parameters, ΔG, ΔH, ΔS, number of binding sites (n), and the binding constant K_d for donor binding with the K4CP mutant construct KctDel

The experiments were performed in solution at 20 °C and in triplicate to confirm the results using a fresh batch of enzyme produced for each experiment. In this mutant the entire C-terminal region is deleted, isolating the N-terminal region for analysis. The UDP, UDP-GlcA, UDP-GlcNAc, and UDP-GalNAc concentrations were 2 mM. KctDel concentration was 31 μM. ND, no detectable binding.

Substrate	ΔG	ΔH	ΔS	K_d	n
	cal/mol	*cal/mol*	*cal/mol/deg*	μM	
UDP-GlcA	ND	ND	ND	ND	ND
UDP-GalNAc	−6184.3 ± 5325	−7463 ± 5325	−4.22	34.72 ± 9.14	0.82 ± 0.20
UDP	−6361.4 ± 1721	−9249 ± 1721	−9.53	26.00 ± 6.36	0.96 ± 0.16

TABLE 6

Binding constants K_d (μM) of UDP, UDP-GalNAc, and UDP-GlcA

All of the ITC experiments were performed at 20 °C and in triplicate to confirm results using a fresh batch of enzyme produced for each experiment. ND, no detectable binding.

Enzyme	UDP-GlcA	UDP-GalNAc	UDP
^{239}DCE^{241}			
K_d	5.68 ± 0.81	194.48 ± 54.74	106.49 ± 24.21
n	0.84 ± 0.03	0.81 ± 0.21	0.81 ± 0.19
^{239}DCK^{241}			
K_d	3.57 ± 0.37	157.36 ± 39.91	45.64 ± 3.98
n	1.00 ± 0.02	0.96 ± 0.26	0.96 ± 0.05
^{519}DSE^{521}			
K_d	4.42 ± 0.45	155.62 ± 26.67	75.13 ± 11.58
n	0.89 ± 0.01	0.81 ± 0.16	0.77 ± 0.24
^{519}DSK^{521}			
K_d	12.09 ± 1.62	ND	ND
n	0.99 ± 0.03	ND	ND
^{519}DSA^{521}			
K_d	ND	222.12 ± 70.26	9.56 ± 1.07
n	ND	0.75 ± 0.14	0.76 ± 0.02
^{519}KSD^{521}			
K_d	ND	ND	35.77 ± 4.61
n	ND	ND	0.95 ± 0.22
^{519}ASD^{521}			
K_d	ND	ND	23.61 ± 2.31
n	ND	ND	1.22 ± 0.09
^{239}ACA^{241}/^{519}DSA^{521}			
K_d	ND	ND	ND
n	ND	ND	ND

clude that UDP does not bind to the C-terminal ^{519}DSK^{521} site. UDP-GalNAc bound to the C-terminal ^{519}DSD^{521} site weakly, and its sugar moiety may not be able to overcome the change to the second aspartic acid residue, thereby resulting in its inability to bind to the ^{519}DSK^{521} site. The most surprising finding, however, was that no binding was detected for UDP-GalNAc and UDP despite the fact that the N-terminal ^{239}DCD^{241} site remained unaltered, because both UDP-GalNAc and UDP bind to the N-terminal ^{239}DCD^{241} site of the ^{519}ASA^{521}mutant (Table 3).

The second alanine residue (Ala^{241}) was responsible for binding the two donors to the N-terminal ^{239}DCD^{241}, because the ^{519}DSA^{521} mutant retained the binding ability of UDP-GalNAc and UDP while losing its binding ability to UDP-GlcA (Table 6). These results suggested that, depending on the type of residue replacing the second aspartic acid, the C-terminal DSD motif differentially regulates the binding of UDP-GalNAc and UDP to the N-terminal DCD motif. Given this conclusion, the first aspartic acid residue of the C-terminal DSD motif was then mutated to lysine and alanine to produce the ^{519}KSD^{521} and ^{519}ASD^{521} mutants. This aspartic acid residue is the primary determinant for the binding of the sugar moiety of a donor substrate (20, 21). The subsequent loss of UDP-GlcA binding was consistent with the fact that the C-terminal DSD motif is the only site for which UDP-GlcA had an affinity (Table 6). It would be reasonable to observe no binding of UDP-GalNAc to these mutants, if indeed the C-terminal DSD was the only binding site in the K4CP molecule. However, the fact that UDP-GalNAc did not bind to the N-terminal DCD motif of the ^{519}KSD^{521} and ^{519}ASD^{521} mutants reiterated the possibility that the C-terminal DSD motif may regulate donor binding to the N-terminal DCD motif, thus becoming a primary regulator of enzyme function and activity of K4CP. It was reasonable that a continuous binding of UDP to these mutants is observed because UDP does not interact with the first aspartic acid residue, and the double mutant ^{239}ACA^{241}/^{519}DSA^{521} did not bind any of the donor substrates.

DISCUSSION

The chondroitin polymerase K4CP is a multifunctional enzyme that transfers GlcA and GalNAc from the donor substrates UDP-GlcA and UDP-GalNAc to synthesize the (GlcAβ1–3GalNAcβ1–4)$_n$ chain. The K4CP molecule contains the two potential binding sites for donor substrates. We have used bacterially expressed and purified K4CP enzymes and have employed ITC to investigate the specific donor bindings. One insightful result is the fact that K4CP is capable of binding to only one donor molecule at a given time regardless of the type of donor substrates: UDP, UDP-GalNAc, or UDP-GlcNAc. Another significant result is the observation suggesting that the C-terminal ^{519}DSD^{521} site provides K4CP with not only the sole binding site for UDP-GlcA but also the ability to regulate binding of UDP-GalNAc to the N-terminal donor-binding site ^{239}DCD^{241}. Thus, the chondroitin polymerase K4CP appears to utilize a fairly sophisticated mechanism to catalyze the polymerization reaction.

A critical question, the answer of which is required for understanding the catalytic mechanism of the polymerization, is whether K4CP has one active site at which both the UDP-GlcA and UDP-GalNAc transfer reactions take place or has two distinct active sites. K4CP catalyzes an inverting reaction, in which the α-configuration of the C1 bond of the donor sugar is inverted to the β-configuration in a product formed (9). X-ray crystal structures of various inverting glycosyltransferases have determined the conserved structural signatures and the unique conformation and orientation of substrates at the active site (21). The structures of mammalian glycosyltransferases are represented by the so-called GT-A fold that is a single globular protein consisting of two subdomains (21–24). The N-terminal subdomain has a Rossman fold and provides the binding site for the donor substrate, whereas the C-terminal subdomain consists of mixed β-sheets and constitutes the acceptor substrate binding site. At the active site

that resides in the open cleft across the two subdomains, the leaving oxygen of the phosphate group and the C1 atom of the donor sugar and the acceptor OH group align to advance a bimolecular nucleophilic substitution type in line displacement reaction. Also the inverting glycosyltransferases are characterized by the presence of a catalytic base residue that deprotonates the acceptor OH group. The x-ray structure of K4CP has now been solved and has revealed two distinct domains. Both domains are characterized by having all of the structural features of the GT-A fold, and both can be perfectly superimposed with known structures of other glycosyltransferases. In addition, the revealed K4CP structure has the two GT-A folds that orient the two active site clefts at an 180° angle, suggesting that the two domains function independently, with nonsequential and random binding and release of donor substrates, as the catalytic mechanism of the polymerase reaction.

Our ITC study has concluded that UDP-GalNAc and UDP-GlcA specifically bind to the N- and C-terminal D*X*D motifs of the K4CP enzyme, respectively. The presence of two independent binding sites also been implicated in another glycosyltransferase, the *Pasteurella multocida* hyaluronan synthase (25). Consistent with this conclusion, the K4CP structure has led to the conclusion that UDP-GalNAc and UDP-GlcA are present at the N- and C-terminal GT-A domains, respectively.[3] In our ITC study, however, only one molecule of UDP is found to bind to one molecule of a given K4CP enzyme. Although the binding of UDP to the ^{239}ACA^{241} and ^{519}ACA^{521} mutants indicates that UDP could bind to either the N- or the C-terminal D*X*D site, in fact UDP appears to bind to the C-terminal DSD of the wild type K4CP enzyme. During the GlcA transfer reaction, this binding of UDP can be transient because its K_d value is more than 10 times higher that the corresponding value of UDP-GlcA binding. Various experimental features associated with the use of protein crystals to solve structures, such as cocrystallization of K4CP with a high concentration of UDP, which may have led to UDP binding to both D*X*D sites. Intriguingly, structural analysis of multiple forms of the x-ray crystal structures found that either there is binding of two UDP molecules or no UDP binding at all. This finding may suggest that there is structural cross-talk between the N- and C-terminal D*X*D sites to regulate UDP binding.

Our binding assays demonstrated that UDP-GalNAc could bind to either the N-terminal or C-terminal D*X*D sites at similar affinities. Thus, during the polymerization reaction, UDP-GalNAc should preferentially bind to the N-terminal DCD site because the UDP-GalNAc transfer reaction is catalyzed by the N-terminal active site. We observed the selective binding of GalNAc to the N-terminal DCD only when the C-terminal DSD was mutated. Contrary to what analysis of the x-ray crystal structural data suggests, the C-terminal domain of K4CP appears to regulate donor binding and probably the transfer activity of the N-terminal domain. K4CP exhibited high affinity binding of UDP-GlcA to the C-terminal DSD motif only and did not hydrolyze UDP-GlcA, so that the enzyme can effectively convert the donor binding activity to its transfer activity. The inability of UDP-GlcA to bind to the N-terminal DCD motif and the constant occupation at the C-terminal DSD motif ensure that this enzyme will never transfer two glucuronic acids sequentially to an acceptor substrate and force UDP-GalNAc binding to the N-terminal DCD motif.

In conclusion, ITC analyses have now determined the specific binding of donor substrates to the bacterial chondroitin polymerase K4CP. In solution, only one donor substrate binds to K4CP at a given time. The C-terminal DSD motif binds to UDP-GlcA with a high affinity, apparently initiating the polymerase reaction and coordinating the N-terminal DCD motif to bind to UDP-GalNAc. The underlying molecular mechanism for control of the N-terminal domain by the C-terminal domain with subsequent transfer reactions is a subject ripe for further scrutiny and investigations.

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