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Thermodynamic Characterization of the Interaction between the C-Terminal Domain of Extracellular Superoxide Dismutase and Heparin by Isothermal Titration Calorimetry[†]

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ABSTRACT: Extracellular superoxide dismutase (ECSOD) interacts with heparin through its C-terminal domain. In this study we used isothermal titration calorimetry (ITC) to get detailed thermodynamic information about the interaction. We have shown that the interaction between ECSOD and intestinal mucosal heparin (M_w 6000−30000 Da) is exothermic and driven by enthalpy at physiological salt concentration. However, the contribution from entropy is favorable for binding of small isolated heparin fragments. By studying different size-defined heparin fragments, we also concluded that a hexasaccharide moiety is sufficient for strong binding to ECSOD. The binding involves proton transfer from the buffer to the ECSOD−heparin complex, and the results indicate that the number of ionic interactions made between ECSOD and heparin upon binding varies from three to five for heparin and an octasaccharide fragment, respectively. Surprisingly and despite the many charges found on both the protein and the polysaccharide, our results indicate that the nonionic contribution to the binding is large. From the temperature dependence we have calculated the constant pressure heat capacity change (ΔC_p) of the interaction to -644 J K^{-1} mol^{-1} and -306 J K^{-1} mol^{-1} for heparin and an octasaccharide, respectively

Extracellular superoxide dismutase (EC 1.15.1.1) is a homotetrameric glycoprotein that scavenges superoxide anion radicals in the extracellular matrix (*1*). ECSOD[1] plays a major role in blood pressure regulation and vascular function, at least in part, through modulating the levels of extracellular superoxide anion radicals and nitric oxide bioactivity in the vasculature (*2*, *3*). For optimal protection of the cells the enzyme has to associate to the cell surfaces, and it does so by interacting with heparan sulfate proteoglycans (HSPG) (*4*−*7*). Heparan sulfate is the major biological ligand for ECSOD, and it also associates with heparin which is structurally analogous to heparan sulfate while interactions with other sulfated glycosaminoglycans (GAGs) such as chondroitin sulfate and dermatan sulfate are weak (*5*).

Recently, the X-ray structure of hECSOD was reported (*8*). Unfortunately, only the central catalytic domain could be determined, and it was found to have large similarities to intracellular hCuZnSOD. The three-dimensional structure of the N- and C-terminal domains is still unknown. However, the structure−function relationships of both the N-terminal and the C-terminal domains have been mapped by our group (*7*, *9*−*11*). The tetramer is formed by interactions between the N-terminal parts of the subunits (*9*, *10*), and the C-terminal domain, which has a high α-helical content, has been shown to be responsible for the interaction with heparin and heparan sulfate (*5*, *6*, *12*).

Heparan sulfate (HS) is a ubiquitous molecule present on the cell surfaces of most mammalian cells and in the extracellular matrix. Heparan sulfate has been shown to interact with a wide range of proteins with different functions, for example, cytokines (*13*, *14*), chemokines (*15*−*17*), growth factors (*18*, *19*), lipases and lipoproteins (*20*, *21*), extracellular matrix proteins (*22*, *23*), and different enzymes, including ECSOD (*5*). Interaction with heparan sulfate affects the destination, half-life, and bioactivity of the proteins (*24*) and is therefore critical for the function of many biological pathways in the cell.

Most studies of interactions with proteins have been performed by using heparin instead of heparan sulfate as the binding partner, mainly because of the commercial availability of heparin. The chemical composition of heparin and heparan sulfate is very similar. The major difference is that heparan sulfate has a lower degree of sulfation, a higher degree of GlcNAc residues, and a lower content of epimerized uronic acids (IdoA) (*4*, *25*, *26*). Variable patterns of substitution of the disaccharide subunits with *N*-sulfo, *O*-sulfo, and *N*-acetyl groups give rise to a large number of complex sequences in both heparin and HS, which are structurally the most complex members of the glycosaminoglycan (GAG) family of polysaccharides (*25*). This heterogeneity complicates the structural determination of heparin−protein complexes. No specific consensus sequence in the primary sequences has been found among the heparin-binding proteins; however, the interactions include interactions between clusters of

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[1]Abbreviations: ΔC_p, constant pressure heat capacity change; ECSOD, extracellular superoxide dismutase; GAG, glycosaminoglycan; GlcA, β-D-glucuronic acid; GlcN, β-D-glucosamine; GlcNAc, *N*-acetyl-D-glucosamine; GlcNS, *N*-sulfo-D-glucosamine; ΔH, enthalpy change; hCuZnSOD, human intracellular Cu- and Zn-containing superoxide dismutase; hECSOD, human extracellular superoxide dismutase; HEPES, *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; IdoA, α-L-iduronic acid; ITC, isothermal titration calorimetry; K_d, dissociation constant; *n*, reaction stoichiometry; ΔS, entropy change; SASA, solvent-accessible surface area; SPR, surface plasmon resonance.

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positively charged amino acids in the protein and negatively charged groups of the heparan sulfate chain (*27*, *28*). Growing evidence suggests that specific and selective interactions play an important role (*25*, *27*−*30*).

The heparin-binding domain of hECSOD is composed of the 26 C-terminal amino acids (197−222) with sequence PGLWE**R**QA**R**EHSE**RKKRRR**ESEC**K**AA, 9 of which are positively charged at physiological pH. The role of the charged residues in the heparin-binding domain has been systematically investigated by our group by using fusion proteins (*11*, *31*, *32*) and by using synthetic peptides (*11*). However, a thorough thermodynamic characterization of the interaction in solution has not previously been performed.

In this study we have used ITC to perform a detailed thermodynamic characterization of the interaction between ECSOD and heparin or different heparin-derived oligosaccharides. By performing analyses at different experimental conditions, important information regarding the interaction, such as ionic/nonionic contribution, proton transfer, and the constant heat capacity change, was obtained.

MATERIALS AND METHODS

Chemicals and Proteins. The extracellular superoxide dismutase used in the experiments was produced in Chinese hamster ovary cells as described by Tibell et al. (*12*). The recombinant ECSOD has the same characteristics as human ECSOD and will therefore simply be called ECSOD in this study. Since the C-terminus is sensitive to proteolytic degradation, the protein was analyzed by affinity chromatography on heparin−Sepharose. Normally 90−99% of the protein bound to the column, indicating little or no proteolytic degradation. The monomeric concentration of the protein was calculated from activity measurements with the pyrogallol autoxidation assay (*33*). Since the protein may contain less than 100% Cu(II) ions, the concentration of monomers may be underestimated by calculations based on activity only. Therefore, the protein was titrated with an octasaccharide heparin fragment by using ITC, and the concentration of intact monomers was calculated from the obtained *n*-values assuming a 1:1 binding between the octasaccharide and the ECSOD monomers. The assumption of 1:1 (and not 1:2) binding is reasonable because of the strong binding to hexa- and octasaccharides and the very weak binding of tetrasaccharides reported by Lookene et al. (*11*). Generally, the calculation showed that the protein concentration was 1.5 times higher than the estimate from activity measurements.

Porcine intestinal mucosal heparin, ranging from 6000 to 30000 Da in molecular weight, was purchased from Sigma (H3393). The isolated heparin fragments used in the ITC studies had molecular masses ranging from 1800 to 11800 Da and were a kind gift from Per Östergaard, Novo Nordisk A/S, Denmark. All other chemicals were obtained from VWR International (Stockholm, Sweden).

Isothermal Titration Calorimetry. ITC experiments were performed using a MicroCal VP-ITC microcalorimeter (MicroCal, Northampton, MA) (*34*). ECSOD (4.5−7.5 μM monomer) was dialyzed against the appropriate buffer (20 mM, pH 7.4), and the final exchange buffer was then used to prepare the heparin solutions.

The concentration of the heparin and heparin fragments was 200 μM, in the case of heparin calculated from an average molecular mass of 18000 Da. The protein solution was placed in the calorimeter cell, and the heparin solution was loaded into the syringe injector. The titrations involved injections of 5 μL of heparin solution into the protein solution, with a delay of 210 s between injections. The analyses were performed at different experimental conditions such as temperature (15−37 °C), buffer system (HEPES, imidazole, and phosphate), and salt concentrations (0.15−0.45 M).

The calorimetric titration data were fit to a model involving identical and independent binding sites as described earlier (*34*, *35*) to give the stoichiometry (n), the dissociation constant (K_d), and the change in binding enthalpy (ΔH), using the Origin 5.0 nonlinear least-squares program supplied with the Microcal VP-ITC instrument. The system also gave information of the change in entropy (ΔS). The reported parameters are the average of duplicate measurements. To correct for any discrepancies in the baseline outlined by the software, a manual adjustment was performed. The effect of dilution of the heparin solution in the titration cell was controlled for by performing a blank titration, which consisted of titration of heparin into buffer solution. However, in order to not introduce any noise into the measurement of the sample, an average value from the saturated upper part of the binding isotherm of the sample, corresponding to the heat of dilution of heparin, was subtracted. The cell was washed with 0.1 M NaOH, carefully rinsed with Milli-Q water (Millipore AB, Sweden), and equilibrated with buffer before each set of experiments. Between the duplicate measurements the cell was washed with the appropriate buffer.

RESULTS

A typical binding isotherm for the interaction between ECSOD and heparin is shown in Figure 1. The peaks correspond to an exothermic reaction. A model with a single set of identical and independent binding sites was used to calculate the thermodynamic parameters.

Titrations at Different Salt Concentrations. The effects of ionic concentration upon the energetics of the binding between ligands and biological macromolecules have mainly been studied with nucleic acids (*36*). In those studies the outcome is based on the fact that the nucleic acid is a linear negatively charged polyelectrolyte in which the charge density is reduced by counterion condensation on the molecule, and that cationic counterions are released upon binding of ligands (*37*).

Heparin has the highest charge density of all known biomolecules (*4*), with a fraction of its negative charges neutralized by positively charged counterions, e.g., Na^+, in solution (*38*). The assumption that heparin behaves like a polyelectrolyte in solution has successfully been used by Hileman et al. in earlier ITC studies of protein−heparin interactions (*39*). Thus the binding of ECSOD to heparin can, according to the molecular theory of polyelectrolyte solutions (*37*), be regarded as an ion-exchange process, involving stoichiometric release of bound Na^+ ions from the heparin chain. The equilibrium is described as follows:

$$L + H(Na^+)_x \overset{K_d}{\longleftrightarrow} L - H(Na^+)_{x-Z\Psi} + Z\Psi Na^+$$

where L represents ligand and H represents heparin. K_d is the equilibrium dissociation constant of the binding, and Z is the number of purely ionic interactions between the two species, which results in the displacement of $Z\Psi$-bound Na^+ ions from heparin. Ψ represents the apparent fraction of Na^+ bound to heparin per anionic charge. This dimensionless constant depends

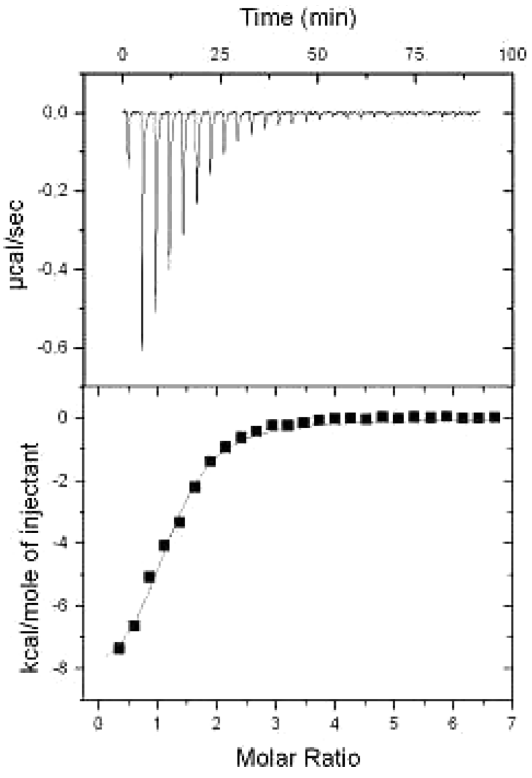

FIGURE 1: Binding isotherm for the interaction of ECSOD with heparin. In the top panel, the peaks indicate the heat released after each addition of heparin into the protein solution. In the bottom panel, the integrated peak area is plotted as a function of molar ratio (heparin/ECSOD). The line represents the best fit of the ITC data, which was used to calculate the thermodynamic parameters. In this experiment 5 μL injections of 200 μM heparin were made into 7 μM ECSOD. Both reactants were diluted in 20 mM HEPES and 0.15 M NaCl, pH 7.4. The experiment was performed at 20 °C.

only on the axial charge density of the polyelectrolyte. For heparin a value of 0.8 has been reported (*40*). The dependence of the dissociation constant on the sodium concentration is given by eq 1

$$\log K_d = \log K_{d(\text{nonionic})} + Z\Psi \log [Na^+] \quad (1)$$

where $K_{d(\text{nonionic})}$ is a constant reflecting the contribution of nonionic interactions to the binding. A plot of the log of the dissociation constant of the complex versus the log of the sodium ion concentration gives a straight line (Figure 2), where the slope indicates the number of Na^+ ions being released from heparin upon binding to ECSOD. The data from the experiments performed at different sodium chloride concentrations are shown in Table 1.

In the interaction between heparin and ECSOD the slope of the line (Figure 2) indicated that 2.3 sodium ions were released from heparin upon binding. By dividing this value with 0.8, the number of ionic interactions was calculated to 2.8, meaning that approximately three purely ionic interactions are made between ECSOD and heparin upon complex formation.

The intercept at 1.0 M NaCl gives an estimate of the contribution of nonionic interactions to the binding since much of the purely ionic interactions may be screened by the small ions at this concentration (*40*, *41*). In our system the K_d at 1 M NaCl was calculated to 33 μM ($10^{-4.48}$), which corresponds to a free energy (ΔG) of −25.6 kJ/mol. A comparison to the free energy calculated for the interaction at 0.15 M NaCl (−33.4 kJ/mol) indicates that contributions from electrostatic interactions other than the ionic ones are important.

A similar experiment was performed with the octasaccharide, and the results which are reported in Table 2 and Figure 2 indicated that 3.6 sodium ions were released upon binding and that four to five ionic interactions were made between ECSOD and the octasaccharide. The K_d at 1 M NaCl was calculated to

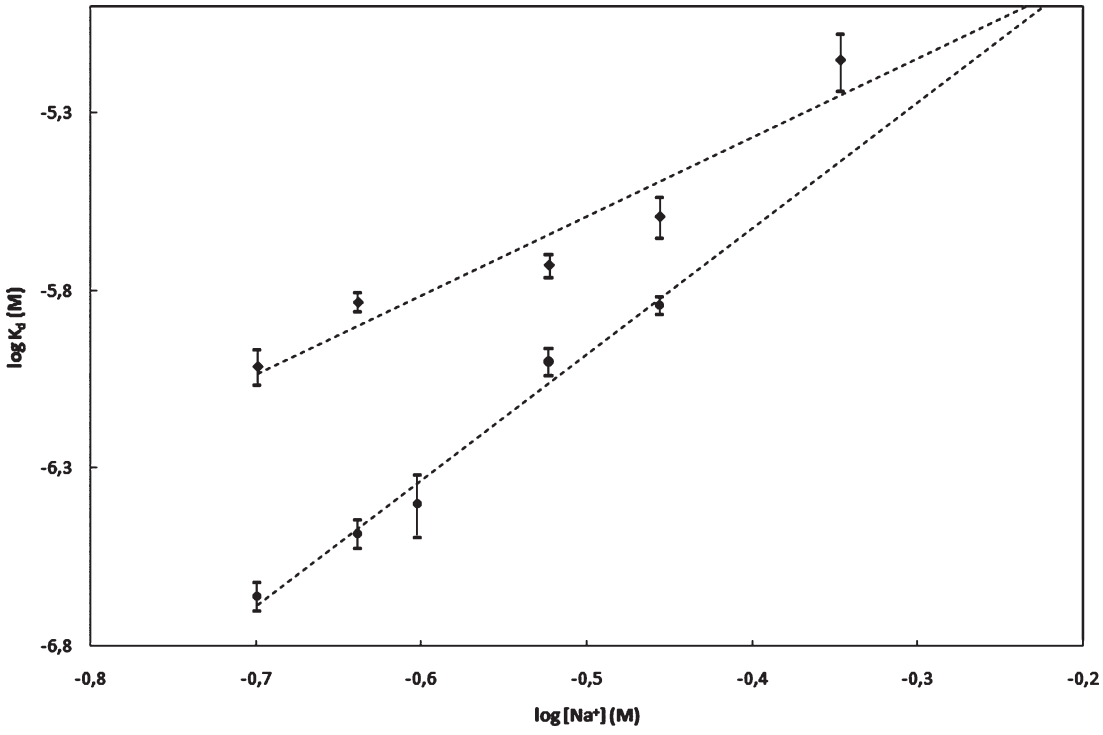

FIGURE 2: Effect of salt concentration on the interaction between ECSOD and heparin (◆) and octasaccharide (●). Linear regression analysis was used to fit the values to a line. The regression coefficient was 0.93 for the interaction with heparin and 0.97 for the interaction with octasaccharide. The slopes correspond to the number of Na^+ ions released from heparin/octasaccharide upon binding.

Table 1: Thermodynamic Parameters for the Interaction between ECSOD and Heparin at Different Salt Concentrations[a]

[NaCl] (M)	$K_{d(obs)}$[b] (μM)	$\Delta H_{(obs)}$[b] (kJ/mol)	$T\Delta S_{(obs)}$[b] (kJ/mol)	$n_{(obs)}$[b]	$K_{d(site)}$[c] (μM)	$\Delta H_{(site)}$[c] (kJ/mol)	$T\Delta S_{(site)}$[c] (kJ/mol)
0.20	0.26 ± 0.03	−90.54 ± 3.01	−52.91 ± 2.08	0.30 ± 0.01	0.97 ± 0.11	−24.09 ± 0.80	10.26 ± 0.67
0.23	0.39 ± 0.02	−76.53 ± 1.66	−39.92 ± 1.79	0.31 ± 0.01	1.47 ± 0.09	−20.34 ± 0.44	12.96 ± 0.59
0.30	0.71 ± 0.11	−70.02 ± 4.50	−34.89 ± 2.82	0.29 ± 0.01	1.87 ± 0.14	−16.14 ± 0.42	16.56 ± 0.17
0.35	0.84 ± 0.12	−61.46 ± 5.43	−26.74 ± 0.56	0.27 ± 0.02	2.56 ± 0.34	−14.75 ± 1.49	17.20 ± 0.24
0.45	2.60 ± 0.41	−33.16 ± 11.75	−1.23 ± 1.44	0.16 ± 0.05	7.06 ± 1.29	−5.64 ± 1.30	23.83 ± 0.08

[a]All experiments were performed at 25 °C in 20 mM HEPES, pH 7.4. [b]Observed data from the titrations. [c]Calculated parameters for the interaction between ECSOD and the individual binding sites of heparin. Since the average stoichiometry of the interactions is 0.26, the values were obtained by multiplying the heparin concentration of the experiments with a factor of 3.85 (= 1/0.26) so as to achieve an n value near 1. Data are reported as the mean of two separate titrations ± 1 SD.

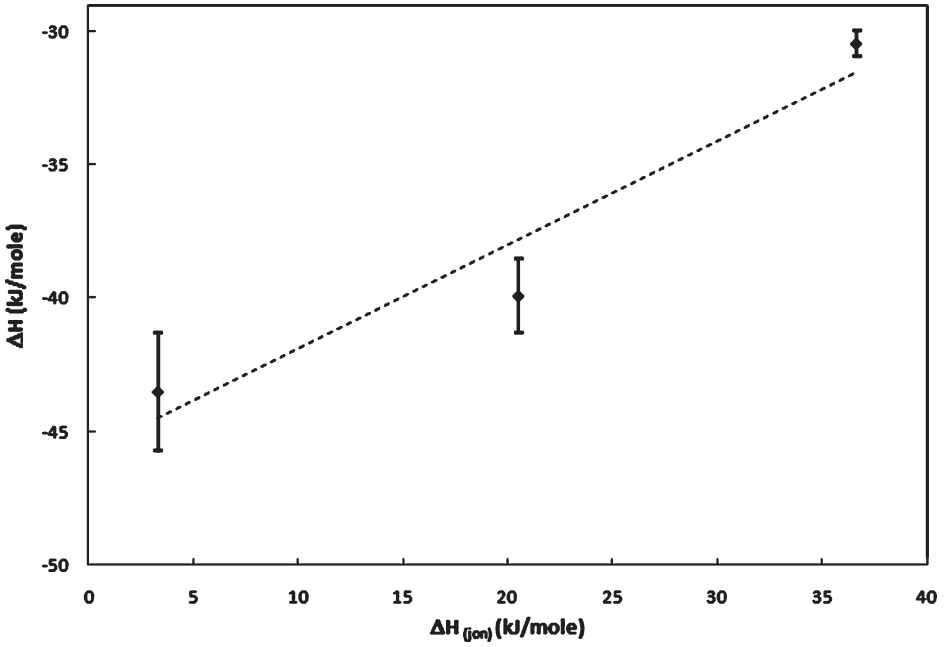

FIGURE 3: Effect of buffer system on the interaction between ECSOD and heparin. Linear regression analysis was used to fit the values to a line. The regression coefficient was 0.92. The positive value of the slope (0.39) indicates proton transfer from the buffer to the ECSOD−heparin complex upon binding.

Table 2: Thermodynamic Parameters for the Interaction between ECSOD and Octasaccharide at Different Salt Concentrations[a]

[NaCl] (M)	K_d (μM)	ΔH (kJ/mol)	$T\Delta S$ (kJ/mol)	n
0.20	0.22 ± 0.02	−20.61 ± 0.46	17.44 ± 0.42	1.77 ± 0.57
0.23	0.33 ± 0.03	−19.02 ± 0.37	18.01 ± 0.21	1.06 ± 0.01
0.25	0.40 ± 0.08	−19.01 ± 0.63	17.59 ± 0.62	0.87 ± 0.05
0.30	1.00 ± 0.09	−16.31 ± 0.35	17.96 ± 0.12	1.10 ± 0.02
0.35[b]	1.44 ± 0.08	−14.10 ± 0.18	19.25 ± 0.32	1.75 ± 0.02

[a]All experiments were performed at 25 °C in 20 mM HEPES, pH 7.4. Data are reported as the mean of two separate titrations ± 1 SD. [b]This measurement represent one titration.

69 μM (ΔG = −23.7 kJ/mol). Thus the free energy differs only by 1.9 kJ/mol from the corresponding value for heparin, which indicates that the contributions from nonionic interactions to the binding are very similar in heparin and the octasaccharide.

Titrations in Different Buffers. From analysis of the interaction in different buffer systems, it is possible to determine if the formation of the heparin−ECSOD complex involves any protonation events. This information can be obtained by plotting the observed enthalpy change against the enthalpy change associated with the ionization of the buffer (Figure 3). The buffer systems were chosen based on their pK_a values (around 7) and on their different heats of ionization. The heat of ionization for imidazole, HEPES, and phosphate are 36.6, 20.5, and 3.3 kJ/mol, respectively (*42*). The data for these experiments are reported in Table 3.

The observed enthalpy change per site (ΔH_{site}) includes components from both the heat of binding (ΔH_{bind}) and the protonation effect (ΔH_{ion}). These terms are related by eq 2 (*43*).

$$\Delta H_{site} = \Delta H_{bind} + (\Delta n)\Delta H_{ion} \quad (2)$$

The slope of the curve, Δn, corresponds to the number of protons being released or taken up by the buffer upon binding. A negative slope indicates a net uptake of protons, while a positive slope indicates a net release of protons from the buffer (*44*). In our study we obtained a value of 0.39, indicating proton release by the buffer and transfer to the formed complex. The intercept represents the buffer-independent heat associated with the binding (*45*), which in our experiment was −45.83 kJ/mol.

Titrations at Different Temperatures. By performing the titrations of heparin into protein solution at different temperatures, the constant pressure heat capacity change (ΔC_p), going from the free state to the bound state, can easily be measured. This term is simply the temperature derivative of ΔH, as shown in eq 3 (*43*).

$$\Delta C_p = (\Delta H_{T2} - \Delta H_{T1})/(T_2 - T_1) \quad (3)$$

Table 3: Thermodynamic Parameters for the Interaction between ECSOD and Heparin in Different Buffers[a]

buffer	K_d[b] (μM)	ΔH[b] (kJ/mol)	$T\Delta S$[b] (kJ/mol)	n[b]	$K_{d(site)}$[c] (μM)	$\Delta H_{(site)}$[c] (kJ/mol)	$T\Delta S_{(site)}$[c] (kJ/mol)
imidazole	0.23 ± 0.01	−79.58 ± 1.27	−41.66 ± 0.70	0.36 ± 0.01	0.60 ± 0.04	−30.43 ± 0.49	5.11 ± 0.29
HEPES	0.53 ± 0.05	−104.12 ± 3.75	−68.29 ± 0.95	0.40 ± 0.01	1.42 ± 0.14	−39.99 ± 1.39	−6.58 ± 0.19
phosphate	0.63 ± 0.08	−112.78 ± 5.42	−77.34 ± 0.75	0.38 ± 0.03	1.69 ± 0.22	−43.49 ± 2.20	−10.50 ± 0.31

[a]All experiments were performed at 25 °C in 20 mM buffer and 0.15 M NaCl, pH 7.4. [b]Observed data from the titrations. [c]Calculated parameters for the interaction between ECSOD and the individual binding sites of heparin. Since the average stoichiometry of the interactions is 0.38, the values were obtained by multiplying the heparin concentration of the experiments with a factor of 2.63 (= 1/0.38) so as to achieve an n value near 1. Data are reported as the mean of two separate titrations ± 1 SD.

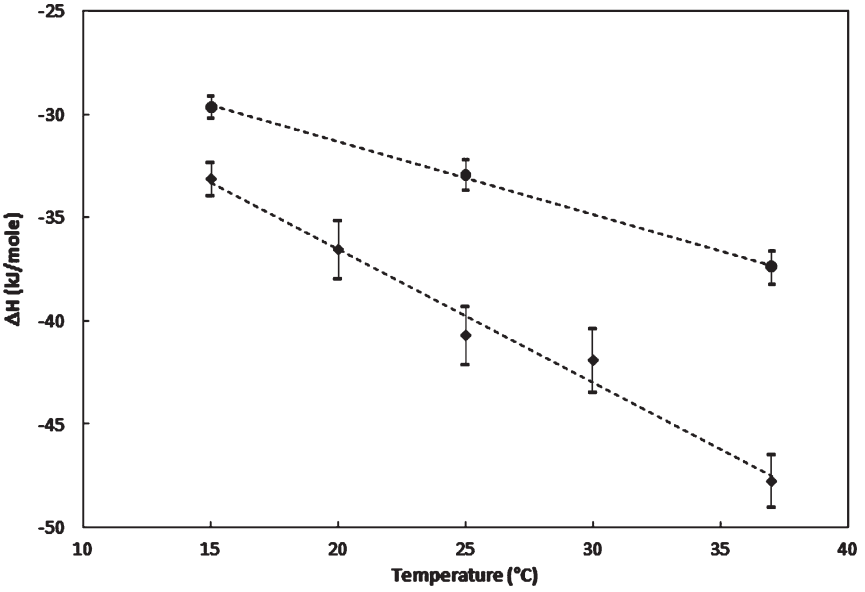

FIGURE 4: Effect of temperature on the interaction between ECSOD and heparin (◆) and octasaccharide (●). Linear regression analysis was used to fit the values to a line. The regression coefficient was 0.96 for the interaction with heparin and 0.98 for the interaction with octasaccharide. The slopes indicate the constant pressure heat capacity change (ΔC_p) of the binding.

Table 4: Thermodynamic Parameters for the Interaction between ECSOD and Heparin at Different Temperatures[a]

T (°C)	K_d[b] (μM)	ΔH[b] (kJ/mol)	$T\Delta S$[b] (kJ/mol)	n[b]	$K_{d(site)}$[c] (μM)	$\Delta H_{(site)}$[c] (kJ/mol)	$T\Delta S_{(site)}$[c] (kJ/mol)
15	0.20 ± 0.06	−86.69 ± 3.80	−49.56 ± 2.52	0.47 ± 0.04	0.47 ± 0.20	−33.11 ± 0.86	2.02 ± 0.37
20	0.42 ± 0.07	−93.87 ± 3.59	−58.02 ± 1.21	0.42 ± 0.02	1.08 ± 0.17	−36.53 ± 1.41	−2.99 ± 0.64
25	0.53 ± 0.05	−104.12 ± 3.75	−68.29 ± 0.95	0.40 ± 0.01	1.39 ± 0.14	−40.69 ± 1.42	−5.28 ± 0.15
30	0.31 ± 0.03	−107.28 ± 2.81	−69.49 ± 0.55	0.37 ± 0.01	0.81 ± 0.10	−41.89 ± 1.53	−6.50 ± 1.37
37	0.27 ± 0.02	−122.72 ± 3.24	−83.74 ± 2.31	0.32 ± 0.01	0.70 ± 0.06	−47.76 ± 1.26	−11.21 ± 0.96

[a]All experiments were performed in 20 mM HEPES and 0.15 M NaCl, pH 7.4. [b]Observed data from the titrations. [c]Calculated parameters for the interaction between ECSOD and the individual binding sites of heparin. Since the average stoichiometry of the interactions is 0.396, the values were obtained by multiplying the heparin concentration of the experiments with a factor of 2.53 (= 1/0.396) so as to achieve an n value near 1. Data are reported as the mean of two separate titrations ± 1 SD.

In a plot of the enthalpy change versus the experimental temperature, the resulting slope gives the ΔC_p. In our case the ΔC_p of the system was calculated to -644 J K^{-1} mol^{-1} (Figure 4). The parameters obtained from these experiments are found in Table 4.

The temperature dependency was also measured in the interaction between ECSOD and the octasaccharide. In this case the ΔC_p of the system was calculated to -306 J K^{-1} mol^{-1} (Figure 4). The parameters obtained from these experiments are found in Table 5.

Titration with Heparin Fragments of Different Sizes. The results from the analyses of the interaction between ECSOD and different size-defined heparin fragments generally indicated slightly stronger binding than to long-chain heparin. The positive values of the entropy change mean that the entropic factor contributes to stronger binding when smaller fragments are used. By the use of different heparin fragments, we also showed that a hexasaccharide moiety is sufficient for strong binding to ECSOD. The parameters obtained from these experiments are found in

Table 5: Thermodynamic Parameters for the Interaction between ECSOD and the Octasaccharide at Different Temperatures[a]

T (°C)	K_d (μM)	ΔH (kJ/mol)	$T\Delta S$ (kJ/mol)	N
15	0.11 ± 0.02	−29.61 ± 0.52	8.79 ± 0.14	0.80 ± 0.02
25	0.21 ± 0.06	−32.91 ± 0.72	5.37 ± 0.76	0.93 ± 0.03
37	0.13 ± 0.04	−36.37 ± 0.81	4.67 ± 1.19	0.76 ± 0.02

[a]All experiments were performed in 20 mM HEPES and 0.15 M NaCl, pH 7.4. Data are reported as the mean of two separate titrations ± 1 SD.

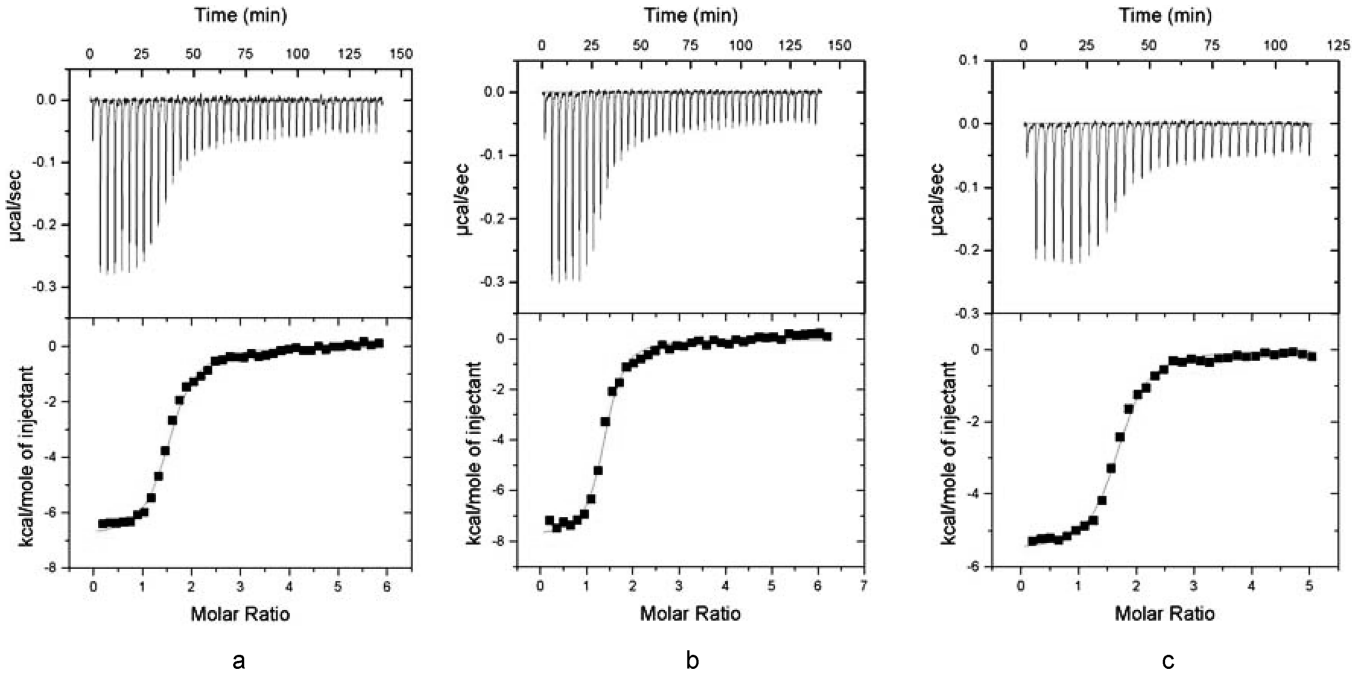

FIGURE 5: Binding isotherms for the interaction between ECSOD and different oligosaccharides: (a) hexasaccharide, (b) octasaccharide, and (c) decasaccharide.

Table 6: Thermodynamic Parameters for the Interaction between ECSOD and Heparin Fragments of Different Sizes[a]

size of ligand	K_d[b] (μM)	ΔH[b] (kJ/mol)	$T\Delta S$[b] (kJ/mol)	N[b]	$K_{d(site)}$[c] (μM)	$\Delta H_{(site)}$[c] (kJ/mol)	$T\Delta S_{(site)}$[c] (kJ/mol)
1800 (hexa)	0.24 ± 0.03	-28.06 ± 0.86	9.77 ± 0.96	1.01 ± 0.01			
2400 (octa)	0.21 ± 0.06	-32.91 ± 0.72	5.37 ± 0.76	0.93 ± 0.03			
3000 (deca)	0.21 ± 0.02	-23.23 ± 0.27	14.92 ± 0.06	1.08 ± 0.02			
9400	0.53 ± 0.07	-51.71 ± 1.89	-15.87 ± 0.60	0.57 ± 0.02	0.99 ± 0.15	-29.93 ± 1.31	4.39 ± 0.98
11800	0.29 ± 0.04	-58.14 ± 2.32	-20.81 ± 1.83	0.51 ± 0.03	0.46 ± 0.09	-28.73 ± 2.22	7.49 ± 2.61

[a]All experiments were performed at 25 °C in 20 mM HEPES and 0.15 M NaCl, pH 7.4. [b]Observed data from the titrations. [c]Calculated parameters for the interaction between ECSOD and the individual binding sites of the heparin fragments. The values were obtained by multiplying the heparin concentration of the experiments with the factor $1/n$ so as to achieve an n value near 1. Data are reported as the mean of two separate titrations $\pm$ 1 SD.

Table 6, and the binding isotherms for the interactions between ECSOD and the smaller oligosaccharides are shown in Figure 5.

DISCUSSION

Contributions from Ionic and Nonionic Interactions. The binding of proteins, including ECSOD, by heparan sulfate and heparin probably involves electrostatic interactions between positively charged groups of the amino acids and negatively charged groups of the polysaccharide (*25*). However, the situation may be more complex, since the results from several studies indicate that specific sequences in the GAGs are designed for the selective interactions with certain proteins and that these interactions can result in the regulation of the protein activities (*27*−*30*). In the case of human ECSOD, the interaction does not affect the activity of the protein; instead, it lengthens the physiological half-life of the enzyme and facilitates optimal protection of the cell surfaces (*4*, *46*, *47*). To clarify the nature of the binding and to determine the ionic and nonionic components of the interaction between heparin and ECSOD, the effect of sodium ion concentration on the protein−heparin interaction was examined.

The results from our experiments performed at different salt concentrations (Figure 2) indicate that the number of purely ionic interactions made between ECSOD and heparin is approximately three. Since the structure of the binding complex is unknown, evidence for which amino acids that are actually interacting with heparin is not available. However, earlier SPR experiments performed by our group, focusing on the role of the basic amino acids, show that the arginines in the RK cluster (amino acids 210−215) contribute much to the binding energy, since the dissociation constant increased approximately 4 times in the substitutions R210A, R213A, R214A, and R215A as compared to the K_d measured for the interaction between heparin and the unmodified protein (*32*). Thus from comparison with present results, it seems reasonable to assume that heparin forms three ion-pair interactions with the arginine residues in the RK cluster upon binding and that some of the remaining basic amino acids contribute to the binding by forming hydrogen bonds. The values obtained are thus in good agreement with current data.

The release of sodium ions upon binding between ECSOD and heparin is an entropically favorable event, termed the polyelectrolyte effect (*36*), which contributes to the overall entropy change in a positive way. However, positive values of ΔS are only seen when ECSOD interacts with smaller oligosaccharides, e.g., hexa-, octa-, and decasaccharides (Table 6). This could be due to a large negative entropy contribution associated with more degrees of freedom being lost in heparin upon binding than for the smaller and relatively rigid oligosaccharides. For heparin, this effect apparently dominates over the contribution from the

sodium ion release. Considering that the short oligosaccharides bind stronger (K_d = 0.21−0.24 μM) than heparin (K_d = 1.42 μM), it is tempting to speculate that a more ordered structure in heparin is induced by unspecific and nonproductive interactions with the protein, outside the primary binding site, upon complex formation. This is also consistent with the observation of a smaller ΔC_p value for the octasaccharide, which may indicate that a larger surface area is being hidden in the interaction between ECSOD and heparin than in the binding to the octasaccharide (see also below).

Analyses of the interaction at different salt concentrations (see Figure 2) show that the contribution from nonionic interactions to the binding between ECSOD and heparin at physiological salt concentration (0.15 M) is substantial. Interestingly, similar results on nonionic contributions to binding have been reported in studies of some other heparin-binding proteins such as antithrombin III (*48*), cardiotoxin (*49*), basic fibroblast growth factor (*41*), and brain natriuretic peptide (*39*). The nonionic interactions may involve hydrogen bonding and/or hydrophobic interactions between the amino acids in the C-terminus of ECSOD and the heparin chain. For example, W200 and L199 and the long carbon chains of both lysine and arginine residues could contribute to hydrophobic interactions. However, since the hydrophobic parts of heparin is limited to the rare acetamide group (*39*), hydrophobic interactions are likely to give a relatively small contribution to the nonionic interactions observed. Instead, the nonionic interactions should mainly come from hydrogen bonding. Notably, all charged and polar amino acids are potential candidates to serve as hydrogen-bonding partners.

The analysis of the interaction between ECSOD and the octasaccharide at different salt concentrations showed that about four sodium ions were released upon binding and that four to five ionic interactions were made between ECSOD and the octasaccharide. The free energy of interaction at 1 M NaCl differs only by 1.9 kJ/mol between heparin and the octasaccharide, indicating that the contributions from nonionic interactions to the binding are very similar. The observation that the binding of the octasaccharide involves additional ionic interactions can have different reasons. First, the octasaccharide may have a charge density and distribution of negative charges that differ from heparin in general and which allow formation of these additional ion pairs. Second, the small size of the octasaccharide may allow conformational rearrangements that favor formation of more ionic bonds. The binding of octasaccharide is stronger than to heparin (K_d = 0.21 μM compared to 1.42 μM), which may be an effect of the additional ionic interactions or it may be coupled to an entropic contribution in contrast to the entropic cost observed for heparin. It is possible that the purification procedure for the heparin fragments, which includes gel filtration and ion-exchange chromatography, has led to an enrichment of fragments that have a higher charge density than the average heparin molecule. Notably, the oligosaccharide fragments were isolated from heparin, indicating that long-chain heparin must contain regions with corresponding binding affinity. However, our attempts to resolve the heparin binding into distinct binding sites with different affinities have failed, probably because the differences are too small to be resolved by ITC.

Proton Transfer upon Formation of the ECSOD−Heparin Complex. The results from the ITC titrations in buffers with different heats of ionization show that 0.39 proton is transferred from the buffer upon formation of the ECSOD−heparin complex. If the proton is being transferred to the protein, the most likely acceptor would be the histidine residue (H207) in the C-terminal domain of ECSOD, since it may have a pK_a value near pH 7.4, which was used in the experiments. Thus, the results indicate that the histidine becomes protonated upon formation of the complex, which may result from an increase of its pK_a (approximately from 7.4 to 8.4). Since the proton transfer occurs upon binding, it is most probable that a negative charge in the heparin molecule stabilizes the positively charged histidine residue.

Burial of Accessible Surface Area upon Complex Formation. By performing the titrations at different temperatures, the constant pressure heat capacity change (ΔC_p) can be measured. This term has been shown to correlate to the burial of surface area (*43*). A large negative heat capacity change often indicates dehydration, cavity formation, and/or change in water ordering (*50*, *51*). Because of the lack of structural information regarding both heparin and the heparin-binding domain in ECSOD, detailed conclusions about the molecular rearrangements cannot be drawn. Furthermore, the choice of buffer and pH can influence the heat capacity change (*44*); thus caution must be used in interpreting these data. However, the value of ΔC_p (−644 J K^{-1} mol^{-1}) is similar to values obtained for other proteins binding to heparin (*52*).

Earlier studies (*7*) and unpublished data indicate that the C-terminus of ECSOD rearranges from a dynamic random coil state to an α-helical conformation upon binding to heparin. The observed ΔC_p may thus have contributions from several events: (i) a conformational shift in the C-terminusl as described above, (ii) a burial of the surface of the part of the C-terminal domain that interacts with heparin, (iii) the burial of the corresponding surface on heparin in the complex, and (iv) a temperature-dependent protonation reaction.

Measurements of the binding between the octasaccharide and ECSOD made at different temperatures resulted in a constant pressure heat capacity change of −306 J K^{-1} mol^{-1}, which is roughly half of the observed value for the interaction with heparin. This value is similar to values obtained for other proteins binding to heparin fragments (*53*). The smaller value, compared to those obtained for heparin, supports a hypothesis that heparin makes interactions beyond those involved in the binding, thus burying more surface area.

Stoichiometry of the Binding. Based on the fact that one hexasaccharide binds per protein subunit and that the molecular mass of the oligosaccharide is 10 times smaller than the average heparin molecule used in the study, theoretically, heparin should be able to interact with 10 ECSOD protein subunits. However, the *n* value of the titration indicates that heparin only contains 2.5 binding sites on average. Two factors may contribute to this finding. First, the ECSOD molecule is large compared to the binding site; thus the protein sterically hinders binding of a second protein molecule near the first binding site. Second, the sequence of sugar moieties in heparin is heterogeneous, which may reduce the number of potential binding sites.

Concluding Remarks. A comparison of the binding of ECSOD to long-chain heparin and to oligosaccharides shows clear differences in the enthalpic and entropic contributions. The enthalpy is larger for binding to heparin (−40 kJ/mol; see Table 3) than for binding to the oligosaccharides (−23 to −33 kJ/mol; see Table 6) at 25 °C. This indicates that long-chain heparin make more contacts with the ECSOD molecule than the oligosaccharides. However, since the binding to the oligosaccharides is stronger, the result indicates that these interactions which occur

outside the C-terminus are unproductive; i.e., the entropic cost is higher than the enthalpic gain. The observation that the stoichiometry for the binding of long-chain heparin to ECSOD subunits is only 2.5 on average seems to support the interpretation that much of the heparin molecule is shielded from binding to other sites by essentially unproductive interactions with the large ECSOD molecule. This unproductive interaction might occur within a groove in the central part of the ECSOD molecule which from molecular docking has been shown to have the ability to harbor heparin (*8*). When smaller heparin fragments are used, the entropy term becomes positive, meaning that the entropic factor contributes to stronger binding. There are fewer interactions, as indicated by the enthalpy term, but the ones made are productive and contribute to the binding. Apparently, the small oligosaccharides lose fewer degrees of freedom upon binding than the larger heparin molecules. The concomitant release of sodium ions and hydration water leads to a positive contribution to binding from entropy (see Table 3). The observation that more sodium ions are released from the octasaccharide than from heparin upon binding indicates that the stronger binding to the hexa-, octa-, and decasaccharides might emanate from formation of more ionic interactions with the fragments than with heparin. A study by Larnkjaer et al. on lipoprotein lipase has shown that the affinity for a hexasaccharide with nine sulfo groups was approximately 5 times higher than for a hexasaccharide with seven sulfo groups (*54*). A hexasaccharide fragment that binds to basic fibroblast growth factor with a dissociation constant of 100 nM has also been isolated (*41*).

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