Structural Requirements for High-Affinity Heparin Binding: Alanine Scanning Analysis of Charged Residues in the C-Terminal Domain of Human Extracellular Superoxide Dismutase†

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ABSTRACT: An essential property of human extracellular superoxide dismutase (hEC-SOD) is its affinity for heparin and heparan sulfate proteoglycans located on cell surfaces and in the connective tissue matrix. The C-terminal domain of hEC-SOD plays the major role in this interaction. This domain has an unusually high content of charged amino acids: six arginine, three lysine, and five glutamic acid residues. In this study, we used alanine scanning mutagenesis of charged amino acids in the C-terminal domain to elucidate the requirements for the heparin/heparan sulfate interaction. As a tool in this study, we used a fusion protein comprising the C-terminal domain of hEC-SOD fused to human carbonic anhydrase II (HCAII). The interaction studies were performed using the surface plasmon resonance technique and heparin−Sepharose chromatography. Replacement of the glutamic acid residues by alanine resulted, in all cases, in tighter binding. All alanine substitutions of basic amino acid residues, except one (R205A), reduced heparin affinity. The arginine and lysine residues in the cluster of basic amino acid residues (residues 210−215), the RK-cluster, are of critical importance for the binding to heparin, and arginine residues promote stronger interactions than lysine residues.

Mammalian cells produce two different types of copper- and zinc-containing SODs,[1] one intracellular (CuZn-SOD) and the other extracellular (EC-SOD). A characteristic of hEC-SOD is its ability to bind to sulfated glycosaminoglycans (GAGs). The strong affinity of EC-SOD to heparin and heparan sulfate has been well documented (*1*). Due to its strongly basic nature, the C-terminal domain was, at an early stage, proposed to be responsible for this interaction (*2*). This hypothesis has been confirmed by a wide range of experiments (*3*−*6*), and the C-terminal domain has been shown to act as an independent heparin binding unit (*7, 8*).

Increasing numbers of cellular activities have been shown to be dependent on protein−oligosaccharide interactions. The functions of these proteins and complexes have been described and include protein activation, localization and protection of bound proteins, chaperone activity during secretion, and co-receptor roles (*9*). Moreover, the association of the amyloid precursor protein (implicated, in mutant forms, in Alzheimer's disease) with heparin and heparin sulfate has recently been proposed to be important in its interactions with nerve cells and for its metabolic functions (*10*).

The protein−heparin interactions are often very specific (*9*), but the nature of the interactions at a molecular level is poorly understood. The most intensively studied protein−GAG interactions are those involving heparin or heparan sulfate, which in most cases are the biological ligands. However, there are as yet only two cases where the X-ray structure of protein−heparin complexes has been determined (*11, 12*). In the absence of more structural information, the mechanism by which heparin operates at the molecular level still remains uncertain.

In a few cases, both the amino acids and the oligosaccharide residues that participate in the interaction between heparin binding proteins and heparin have been defined. Certain distribution patterns of basic amino acids (Lys and Arg) are known to be common features of heparin binding regions and sequences (*13*). Definitions of consensus sequences for heparin binding at the protein level have been attempted several times, but none of the published sequences are valid for all cases of heparin binding (*9, 13*−*15*). Caldwell et al. investigated the prevalence of amino acids in protein−heparin/heparan sulfate binding sites (*16*). They found that besides arginine and lysine residues, glycine and

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[1] Abbreviations: EC-SOD, extracellular superoxide dismutase; GAG, glycosaminoglycan; HCAII, human carbonic anhydrase II; SEC, size exclusion chromatography; SOD, superoxide dismutase; SPR, surface plasmon resonance; TFE, 2,2,2-trifluoroethanol; RU, response unit; K_D, equilibrium dissociation constant; k_a, association rate constant; k_d, dissociation rate constant. Enzymes: CuZn-SOD, intracellular Cu- and Zn-containing superoxide dismutase, EC 1.15.1.1; EC-SOD, extracellular Cu- and Zn-containing superoxide dismutase, EC 1.15.1.1; HCAII, human carbonic anhydrase II, EC 4.2.1.1; FusCC, a fusion protein comprising the 25 C-terminal amino acids from human EC-SOD fused to the C-terminal of HCAII.

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serine residues were also commonly involved in the heparin binding, and serine and proline commonly featured in the interaction with heparan sulfate. Heparin and heparan sulfate binding sites have been found within both α-helical and *β*-sheet frameworks, but Margalit et al. proposed that two basic amino acids, separated by a distance of 20 Å, are essential for association, regardless of the tertiary structure.

In our previous studies, the C-terminal domain of hEC-SOD was analyzed using various fusion proteins and a synthesized peptide corresponding to the C-terminal sequence. The results of these studies suggested that an α-helical structure is essential for the heparin interaction, and that the oligomeric state of the protein does not significantly affect heparin affinity (*7*, *8*, *17*).

In the present study, our aim was to identify amino acids that are of critical importance for the heparin interaction. The charged amino acids were the focal points of this study because of the high content of these in the C-terminal sequence, and the documented role of charged amino acids in this type of interaction. The main strategy we applied was alanine scanning mutagenesis. We also introduced amino acid substitutions as an attempt to disturb the secondary structure. The effect on the heparin binding properties of each variant was investigated.

MATERIALS AND METHODS

Chemicals and Proteins. The heparin−albumin−biotin complex and avidin used in the SPR experiments to generate the heparin binding surface were obtained from Sigma, and TCEP, tris(2-carboxyethyl)phosphine hydrochloride, was from Pierce. An amino-coupling kit and CM5 sensor chips were obtained from BIAcore, Uppsala, Sweden. Oligonucleotides for mutagenesis were from DNA Technology. The ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit used came from PE Applied Biosystems.

Construction and Purification of Fusion Proteins. The construction of the fusion protein FusCC has been described earlier (*8*). Eighteen variants of FusCC were constructed using site-directed mutagenesis according to the method of Kunkel (*18*) with a slight modification as described by Mårtensson et al. (*19*). The DNA sequences of the variants were confirmed by DNA-sequencing of the entire coding region. The fusion proteins were produced essentially as described earlier (*19*), except that 0.5 mM $ZnSO_4$ was added at the time of induction. Cultures were harvested by centrifugation and resuspended in a homogenization buffer containing 0.1 M Tris−SO_4, pH 9.0, 0.2 M K_2SO_4, 0.3 mM PMSF, 1 mM EDTA. The resuspended bacteria were homogenized by use of a bead-beater or by ultrasonication. After centrifugation for 30 min at 15 000 rpm, the supernatant was collected and applied to an affinity column according to the method of Khalifah (*20*). Fractions containing fusion protein were collected and concentrated by ultrafiltration, using YM10 (10K) membranes in a stirred Amicon cell. The variant C219S was further purified by a heparin−Sepharose chromatographic step.

SDS−PAGE. SDS−PAGE was performed on a discontinuous polyacrylamide gel system (*21*) containing 14.9% acrylamide and 0.1% SDS. Prior to analysis, the protein samples were boiled in an SDS-cocktail that contained *β*-mercaptoethanol except when nonreducing conditions were required. The gels were stained in 0.025% Coomassie Brilliant Blue R.

Size-Exclusion Chromatography (SEC). SEC was performed on Superose 12 HR 10/30, or Superdex 75 HR 10/30, columns using an FPLC-system (Amersham Pharmacia Biotech). The mobile phase for the SEC experiments was 20 mM Hepes buffer, pH 7.4, 0.15 M NaCl, 0.005% w/v Tween 20, and the flow rates were 0.5 and 0.75 mL/min for the Superose 12 and Superdex 75 columns, respectively. SEC under reducing conditions was performed by incubating the protein for 1 h in the running buffer with the addition of 0.4 mM TCEP.

Analytical Heparin−Sepharose Chromatography. The heparin−Sepharose chromatography was carried out on a 1 mL HiTrap, heparin-Sepharose column (Amersham Pharmacia Biotech) with a flow rate of 1 mL/min. The equilibration buffer was composed of 20 mM Hepes, pH 7.4, 50 mM NaCl, and 0.005% w/v Tween-20 to minimize unspecific binding, as in the SPR experiments (see below). After application and before elution of the fusion proteins, the column was washed with this buffer. Bound fusion protein was eluted with a linear gradient of 50 mM to 1 M NaCl in the equilibration buffer. Heparin−Sepharose chromatography under reducing conditions was performed using the above buffers with the addition of 0.4 mM TCEP and incubation for 1 h at room temperature.

SPR Experiments. Interaction analyses were performed on a Biacore 2000 instrument (Biacore AB, Uppsala, Sweden) at 25 °C using 20 mM Hepes buffer, pH 7.4, 0.15 M NaCl, and 0.005% w/v Tween 20 as the running buffer.

The detection system uses SPR to monitor changes in refractive index at the surface as the binding event occurs. The processed signal vs time is then expressed in terms of arbitrary resonance units (1 RU, 1 pg/mm^2 for protein), in the form of a sensorgram. Sensorgrams provide information about the kinetics [association rate (k_a), dissociation rate (k_d)] and the equilibrium dissociation constant [affinity (K_D)] of the interaction.

In an initial binding experiment, avidin was immobilized through primary amino groups to the activated carboxyl groups on four flow cells on a CM5 research-grade sensor chip, according to procedures suggested by the instrument manufacturer, at an average density of 5600 response units (RU). Residual active groups were inactivated by ethanolamine. After the immobilization, a heparin−albumin−biotin complex was captured on an avidin surface at a density of 650 RU. To test for nonspecific binding of the fusion proteins to the albumin part of the heparin complex, a biotin−albumin complex was captured on a flow cell at a density of 540 RU. The binding of FusCC to the different surfaces (avidin, avidin−biotin−albumin, and avidin−biotin−albumin−heparin) was then investigated. At a protein concentration of 10 μM, nonspecific binding to the avidin and avidin−biotin−albumin surfaces was 127 and 128 RU, respectively. The binding response to the avidin−biotin−albumin−heparin surface at the corresponding protein concentration was 2800 RU. Thus, a sensor surface with immobilized avidin or avidin−biotin−albumin can be used as a reference surface to correct for nonspecific binding.

In the binding experiments, the heparin−albumin−biotin complex was captured at different densities on two flow cells. One flow cell was prepared with a high density (on average

1000−1500 RU), and the other with a lower density (200−400 RU). Only a minor effect of the densities on the rate constants was observed while the effects upon altering the flow rate (5−50 μL/min) were more pronounced. These results suggest that the interaction to some extent is limited by mass transport. Differences in transport limitation between nonspecific and specific binding sites might also contribute to the observed flow rate dependence. To determine the equilibrium dissociation constant (K_D) for the protein/heparin interactions, concentration series of FusCC and mutants were injected at a flow rate of 5 μL/min over the heparin and avidin surfaces. Each concentration series included 8−10 protein concentrations ranging from 0.05 to 200 μM (depending on the protein studied). The heparin biosensor data were corrected by subtracting the nonspecific binding to the reference surface (avidin). To determine the equilibrium dissociation constant (K_D), the responses at equilibrium (ΔR_{eq}) for each protein concentration were fit to a 1:1 binding model. This 1:1 binding model is described by the equation:

$$\Delta R_{eq} = \frac{\Delta R_{max} C}{K_D + C}$$

where ΔR_{eq} is the increase of the response value at equilibrium, ΔR_{max} is the capacity of the immobilized heparin to bind a protein, and C is the concentration of injected protein.

The dissociation rate constants appear to be fast, especially for the K- and R-variants, and in some cases of the same order of magnitude as the time required for effective change of the buffer in the flow-cell, in particular when using the relatively low flow rate of 5 μL/min.

To minimize effects of mass transport, we choose to compare apparent dissociation rates from experiments using a low-density heparin chip. Assuming that the dissociation is described by a simple exponential function, we calculate an apparent half-life for the dissociation phase. In Table 2, the effect of each substitution on the apparent dissociation half-life is expressed as the ratio $t_{1/2variant}/t_{1/2\ FusCC}$. Due to the limitations mentioned above, these ratios should be taken as rough estimates of the effects of the amino acid substitutions on dissociation.

We have used slightly different conditions in this study as compared to earlier studies (*7*). In this study, we, as suggested by the manufacturer, added 0.005% w/v Tween 20 to the buffer to avoid unspecific binding. However, according to experiments on FusCC binding in the presence and absence of 0.005% Tween 20, the effect of Tween 20 on the K_D values was marginal. Another difference between the studies is the use of albumin-coupled heparin in the present study while the heparin was directly modified by avidin in the former study. This might affect the density of heparin on the sensor chip and might be the cause for the difference in affinity of FusCC for heparin in the two studies.

Circular Dichroism. Circular dichroism (CD) spectra were recorded at 4 °C on a CD6 spectrodichrograph (Jobin-Yvon Instruments SA, Longjumeau, France). Far-UV spectra for the proteins were recorded in a 0.5 mm quartz cell at a protein concentration of 0.5−0.7 mg/mL in 10 mM potassium phosphate, pH 7.0, 0.15 M NaCl. Due to the absorbance of 0.15 M NaCl, CD spectra were collected down to 200 nm. Difference spectra, representing the CD signal originating from the C-terminal domains, were obtained by subtracting the HCAII spectrum in molar ellipticities from the spectra of the fusion proteins. The resulting difference spectra were recalculated to give mean residue weight ellipticities for the C-terminal domain of FusCC (i.e., 32 amino acid residues, comprising 28 from the hEC-SOD N-terminal domains and 4 from the spacer). Secondary predictions of the difference spectra were performed according to Chakrabartty et al. (*22*)

Table 1: [NaCl] Required To Elute Dimeric FusCC and Variants from Heparin−Sepharose

protein	C-terminal sequence	[NaCl] (M)[a]
FusCC	WERQAREHSERKKRRRESECKAA	0.48
R202A	WE**A**QAREHSERKKRRRESECKAA	0.42
R205A	WERQA**A**EHSERKKRRRESECKAA	0.42
R210A	WERQAREHSE**A**KKRRRESECKAA	0.34
R213A	WERQAREHSERKK**A**RRESECKAA	0.35
R214A	WERQAREHSERKKR**A**RESECKAA	0.33
R215A	WERQAREHSERKKRR**A**ESECKAA	0.37
K211A	WERQAREHSER**A**KRRRESECKAA	0.32
K212A	WERQAREHSERK**A**RRRESECKAA	0.38
E201A	W**A**RQAREHSERKKRRRESECKAA	0.52
E206A	WERQAR**A**HSERKKRRRESECKAA	0.54
E209A	WERQAREHS**A**RKKRRRESECKAA	0.55
E216A	WERQAREHSERKKRRR**A**SECKAA	0.53
E206P	WERQAR**P**HSERKKRRRESECKAA	0.55
K212P	WERQAREHSERK**P**RRRESECKAA	0.37
R215P	WERQAREHSERKKRR**P**ESECKAA	0.35
E201A:E209A	W**A**RQAREHS**A**RKKRRRESECKAA	0.57
K212A:R215	AWERQAREHSERK**A**RR**A**ESECKAA	0.23
C219S	WERQAREHSERKKRRRESE**S**KAA	0.32
CC mono	WERQAREHSERKKRRRESECKAA	0.32

[a] The reproducibility of the experiments was investigated by performing each experiment in triplicate, resulting in a similar standard deviation for each protein of about 0.003 M in the NaCl concentration.

Matrix-Associated Laser Desorption Ionization Mass Spectrometry (MALDI-MS) Analysis. Mass spectral analysis of FusCC (the proteins) was begun by preparing a 1:1 dilution of an aqueous 10 μM stock solution of FusCC with a saturated solution (10 mg/mL) of sinapinic acid in 50% acetonitrile with 0.05% TFA. The sample was spotted on a seeded surface and dried under an air-steam. Mass spectra were acquired in the linear mode with delayed extraction using a Voyager-DE STR MALDI time-of-flight instrument (PerSeptiveBiosystems, Framingham, MA) with a 337 nm nitrogen laser. Spectra were acquired using a 1700 ns delay and 25 000 acceleration voltage. The noise filter was 0.7. Mass spectra were calibrated externally with human carbonic anhydrase II (HCAII) and bovine serum albumin (BSA).

RESULTS

Protein Purification and Characterization. FusCC and the C-terminal variants (Table 1) were expressed in *E. coli*. After cell disruption, essentially all of the fusion protein remained in the supernatant, indicating that it is highly soluble in the bacteria cytoplasm. All fusion proteins were purified in a single step by affinity chromatography using the HCAII-part of the fusion protein as an affinity tag (*20*).

Analysis of purified FusCC and FusCC variants on SEC identified two FusCC-containing fractions, in all cases except C219S, corresponding to a dimer and monomer, respectively. SEC under reducing conditions resulted in just a monomeric fraction. Since HCAII is a monomeric protein and the only cysteine present in our fusion protein is C219, our conclusion is that an S−S bridge in FusCC is responsible for the

dimerization. This hypothesis is strengthened by the observation that both the FusCC variant C219S and the reduced form of FusCC are purely monomeric proteins, according to MALDI-MS analysis and SEC (not shown). We have also observed that the C-terminal is sensitive to proteolysis, which results in monomeric FusCC with lower (or no) heparin affinity, indicating extensive degradation of the C-terminal domain. The dimeric form was therefore used as an indicator of a full-length C-terminal, and the fraction containing the dimer was used in all our experiments except where otherwise indicated.

Analysis on Heparin−Sepharose. A linear gradient of increasing NaCl concentration was used to elute FusCC and variants from the heparin−Sepharose column. Elution of the variants E201A, E206A, E209A, and E216A from the heparin−Sepharose column required 0.52−0.55 M NaCl. This is a higher concentration than needed for elution of FusCC (0.48 M) (Table 1). Replacement of arginine or lysine residues with an alanine residue (R202A, R205A, R210A, K211A, K212A, R213A, R214A, and R215A) caused the opposite effect on heparin−Sepharose binding (i.e., it reduced the affinity), but with greater variation (Table 1). Proline residues replaced amino acid residues in three of the variants (E206P, K212P, and R215P). These variants show almost the same affinity as their counterparts with corresponding alanine replacements (E206A, K212A, and R215A; see Table 1). The effect of the two double mutations E201A:E209A and K212A:R215A is not strictly additive (Table 1). The variant K212A:R215A displayed markedly lower affinity, and eluted at 0.23 M NaCl, while E201A:E209A displayed an increased affinity, and eluted at 0.57 M. Finally, the affinity for heparin−Sepharose shown by monomeric FusCC and the monomeric variant, C219S, was identical but reduced, since the NaCl concentration needed to elute these variants was 0.32 M, compared to 0.48 M for the "dimeric" FusCC (Table 1).

In the present study, two major changes in the chromatographic conditions were introduced, as compared to the heparin−Sepharose chromatography analysis in previous work (*7*, *8*): (i) the flow rate was 1 mL/min in this study as compared to the 0.05 mL/min used previously; (ii) the equilibration buffer was 20 mM Hepes buffer, pH 7.4, 50 mM NaCl with 0.005% w/v Tween-20, as compared to the 15 mM sodium cacodylate, pH 6.5, with 50 mM NaCl used in earlier studies. In both cases, bound protein was eluted using a gradient of 50 mM to 1 M NaCl in the equilibration buffer. This results in slightly lower NaCl concentrations being needed for elution of EC-SOD and FusCC as compared to previous work (0.55 M in both cases) (*8*).

SPR Experiments. All proteins except the variant K212A:R215A bound to the heparin chip in such a way that it was possible to calculate K_D values. Figure 1 shows the sensorgrams of FusCC in a binding experiment. The responses were concentration dependent and are shown for concentrations of 0.19, 0.38, 0.96, 1.91, 3.83, 19.17, and 38.33 μM. All responses approached equilibrium within 300 s, and the responses from each curve at $t = 290$ s (ΔR_{eq}) were plotted versus FusCC concentrations, shown in the binding plot in the inset graph. Fitting these data to a 1:1 binding model yielded an apparent K_D of 8.5 ± 0.9 μM. The spike observed in the beginning of the sensorgrams at high protein concentrations is observed only after subtraction of the reference signal. Since the observed nonspecific binding to the reference surface is slower than the binding to the heparin-activated surface, a spike is generated upon subtraction.

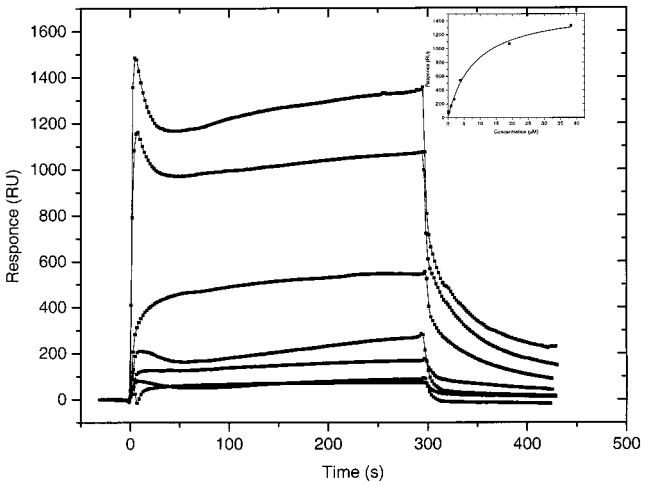

FIGURE 1: SPR sensorgrams for the interaction of FusCC with heparin−albumin−biotin captured on an avidin surface. The responses at equilibrium (ΔR_{eq}) were fit to a 1:1 binding model. The responses are shown for FusCC concentrations of 0.19, 0.38, 0.96, 1.91, 3.83, 19.17, and 38.33 μM. The inset graph shows the binding isotherm.

Table 2: Binding Parameters for FusCC and Variants Determined in the SPR Experiments (K_D Presented with Standard Deviation)

protein	K_D (μM)	$t_{1/2(FusCC)}/t_{1/2(variant)}$[b]	ΔG (kcal mol^{-1})	$\Delta\Delta G$[b] (kcal mol^{-1})
FusCC	8.5 ± 0.9	1	6.9	−
R202A	20.5 ± 2.5	1.1	6.4	0.5
R205A	7.0 ± 2.3	0.3	7.0	−0.1
R210A	51.0 ± 2.5	0.3	5.9	1.1
R213A	52.0 ± 8	0.3	5.8	1.1
R214A	47.0 ± 7	0.4	5.9	1.0
R215A	48.0 ± 2.5	0.4	5.9	1.0
K211A	30.0 ± 3.5	0.6	6.2	0.7
K212A	21.0 ± 2.5	0.5	6.4	0.5
E201A	1.0 ± 0.1	0.6	8.2	−1.3
E206A	2.2 ± 0.2	1.4	7.7	−0.7
E209A	1.8 ± 0.4	0.9	7.8	−0.9
E216A	2.5 ± 0.1	1.1	7.6	−0.7
E206P	1.4 ± 0.1	1.4	8.1	−1.2
K212P	19.5 ± 3.5	0.3	6.4	0.5
R215P	48.0 ± 8	0.2	5.9	1.0
E201A:E209A	0.4 ± 0.04	3.7	8.7	−1.8
E201A:E209A[a]	0.21		9.1	−2.2
K212A:R215A	−	−	−	−
K212A:R215A[a]	113	−	5.4	1.5
C219S	49.0 ± 13	0.3	5.9	1.0
CC mono	36.0 ± 2	0.4	6.1	0.9

[a] Calculated K_D from the sum of the $\Delta\Delta G$ values of the single mutations assuming additivity. [b] The apparent half-life has been calculated from experiments using a low-density heparin chip. The effect of the mutations is expressed as the ratio $t_{1/2(FusCC)}/t_{1/2(variant)}$.

As can be seen in Table 2, all fusion proteins with the exception of K212A:R215A have affinities in the 0.2−52 μM range, representing medium to strong binding. FusCC displayed a K_D value of 8.5 μM. The variants E201A, E206A, E209A, and E216A displayed K_D values of 1.0, 2.2, 1.8, and 2.5 μM, respectively (Table 2).

In all cases where arginine was replaced by an alanine residue except R205A ($K_D = 7.0$ μM), heparin affinity was reduced. The R202A, R210A, R213, R214A, and R215A variants displayed K_D values ranging from 20 to 52 μM

(Table 2). The changes in affinities for K211A (K_D = 30 μM) and K212A (K_D = 21 μM) were less pronounced as compared to the arginine residues in the RK-cluster (R210−R215), but they still represent reductions in affinity. The effects of the substitutions on the apparent half-lives of the complexes are somewhat different (Table 2). In all the cases where arginine was replaced by an alanine residue or a lysine residue, except R202A, the apparent half-lives were decreased. In the R202A variant, the apparent half-life was unaffected. In the variants where a glutamic acid residue has been replaced, the apparent half-lives of the complexes were virtually unaffected except for E201A where the apparent half-life was decreased. Moreover, comparing the replacements E206P, K212P, and R215P to the corresponding alanine substitutions (E206A, K212A, and R215A), K212P and R215P caused no change in heparin affinity.

The double substitutions (E201A:E209A and K212A:R215A) displayed clear changes in affinity. E201A:E209A displayed a 20-fold increase in affinity. The K212A:R215A variant, on the other hand, displayed such a low affinity that it was impossible to determine any specific binding at all to heparin. The monomeric FusCC (K_D = 36 μM) and the monomeric variant C219S (K_D = 49 μM) had lower affinity compared to dimeric FusCC (K_D = 8.5 μM).

Heparin Affinity As Measured by Heparin−Sepharose Affinity Chromatography. In our earlier work (*7*), we demonstrated that the salt concentration needed to elute different proteins from heparin−Sepharose cannot always be used to estimate their relative heparin affinity. The results of the present study support this conclusion; especially data related to FusCC and the variants R202A and R205A (Table 1). R202A and R205A both eluted at 0.42 M NaCl, a somewhat lower salt concentration than that needed to elute FusCC (0.48 M). However, the K_D for R202A is 20.5 μM while the K_D values for FusCC and variant R205A are 8.5 and 7 μM, respectively (Table 2). In addition, the chromatography in the experiments reported here (see Materials and Methods) was performed under different conditions from those reported earlier (*7*, *8*). Thus, it seems that the binding behavior of the proteins in the heparin−Sepharose chromatography is strongly dependent on the chromatographic parameters.

Circular Dichroism Spectra. CD spectra of HCAII, FusCC, and the mutated proteins were recorded in the far-UV region. The contributions from the C-terminal domain to the far-UV CD spectra were obtained by calculating the difference between the spectra of the FusCC variants and HCAII. The resulting difference spectra for FusCC and the proline variants (E206P, K212P, and R215P) are shown in Figure 3. The negative bands observed at 205 and 222 nm are strongly indicative for the presence of α-helical elements in the C-terminal domain. The α-helical content of the C-terminal domain at +4 °C in FusCC was 27% according to the method of Chakrabartty (*22*). The variants E206P, K212P, and R215P have slightly lower α-helix content (Figure 3).

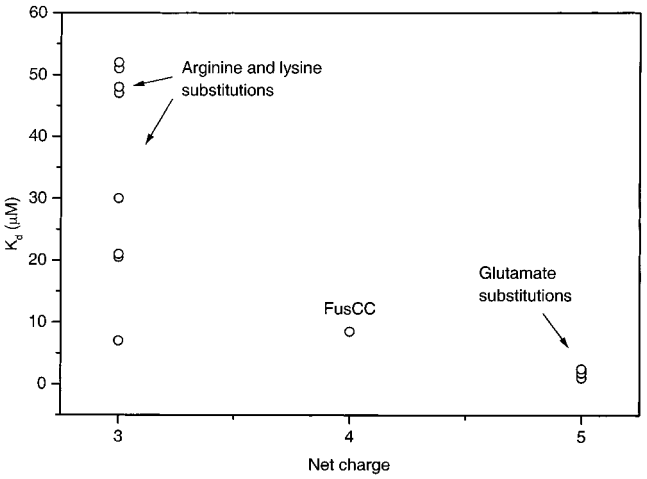

FIGURE 2: K_D values for the variants versus total net-charge of the C-terminal domain.

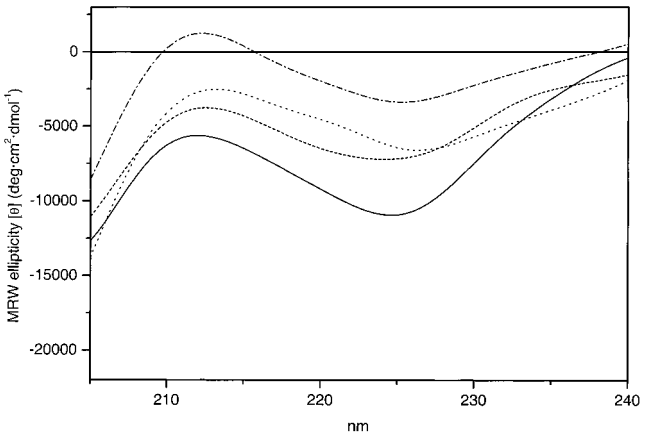

FIGURE 3: Difference spectra in far-UV (200−240 nm) representing the CD signal from the C-terminal domains of FusCC (—), E206P (•••), K212P (− • −), and R215P (− − −).

DISCUSSION

Dimerization, Full-Length C-Terminal Domain, and Heparin Affinity. FusCC eluted as a dimer when analyzed on SEC, although we have earlier reported the protein to be monomeric (*7*, *8*). The discrepancy is probably due to slow proteolytic degradation of the C-terminal and/or the fact that the SEC analysis was performed on reduced protein in the previous study (see the discussion below). In the earlier study, the binding analysis by heparin−Sepharose and SPR was done on freshly prepared protein, whereas the SEC analysis was done on stored material.

Since HCAII (the fusion partner to the C-terminal domain of EC-SOD) is a monomeric enzyme, the most probable explanation of the FusCC dimerization is that cysteine residue 219 in the C-terminal domain (Table 1) is involved in an intermolecular S−S bridge, linking two fusion protein molecules together. To test this hypothesis, we performed two types of experiments. First, we reduced the dimeric FusCC and second we substituted cysteine residue 219 with a serine residue (C219S) to hinder S−S bridge formation. The effects on the heparin binding and oligomeric states were then investigated. Both the C219S and reduced FusCC were shown by SEC as well as by MALDI-MS to be monomeric proteins. We considered dimerization to be a good indicator of an intact C-terminal in our fusion proteins because only full-length proteins can form dimers. In the present investigation, we therefore used freshly prepared dimeric fusion protein, or freshly reduced dimeric protein (to ensure full-length monomeric protein) in all our binding experiments. The K_D value for C219S was reduced by a factor of

approximately 5, compared to the dimeric FusCC (Tables 1 and 2). The binding kinetics of the monomeric (reduced) FusCC indicated a slightly tighter binding compared to the C219S variant (Table 2). It should be noted that the relatively small effect of the dimeric state on the affinity for heparin is not due to multimeric binding (see discussion below).

The participation of cysteine residue 219 in S−S bridge formation in hEC-SOD cannot be deduced from these experiments, but it has been proposed by Enghild et al. and Oury et al. (*23*, *24*). In addition, there are examples in which heparin is sandwiched between two α-helices in the bound state (*25*, *26*). However, Sandström et al. have shown that substituting alanine for cysteine 219 (C219A) in EC-SOD had almost no effect on the binding (*5*). A possible S−S bridge does not appear, in this case, to be favorable for heparin binding.

We also investigated the effect of the redox state on hEC-SOD. The protein was shown by SEC to be tetrameric under nonreducing, as well as under reducing, conditions (results not shown). This was expected since it is well established that the subunit interaction in hEC-SOD, which is mediated by the N-terminal domain, is unusually stable (*27*, *28*). The observation of dimers when analyzing hEC-SOD with SDS−PAGE under nonreducing conditions (*23*, *24*) might be due to S−S bridging between cysteine 219 residues from two subunits (results not shown). However, since the protein contains six cysteines per subunit, dimer formation can be equally well explained by the formation of other intersubunit S−S bridges after the denaturation in SDS.

Replacement of Charged Amino Acids. The C-terminal domain of hEC-SOD has an unusually high content of charged amino acids. Of the 25 amino acid residues closest to the C-terminal, 6 are arginines (202, 205, 210, 213, 214, and 215), 3 are lysines (211, 212, and 220), and 5 are glutamates (201, 206, 209, 216, and 218). We have earlier demonstrated by NMR experiments that arginine residues are involved in the heparin interaction (*8*), but the putative involvement of the lysine residues could not be confirmed by these experiments.

In the alanine scanning studies of the charged amino acid residues in the C-terminal domain reported here, we excluded glutamate 218 and lysine 220 because Sandström et al. (*5*) had already shown that the substitutions K220A and E218A had only a marginal effect on the binding of the corresponding protein variants to a heparin−Sepharose column.

Alanine substitutions of the arginines in the "RK-cluster", positions 210−215, have the strongest effect on the affinity. The effects of all of these substitutions are very similar, and their K_D values are about 6-fold higher than that of FusCC, which correspond to a difference in free binding energy ($\Delta\Delta G$) of approximately 1 kcal mol^{-1} (Table 2). Substitution of lysine residues (K211A and K212A) in the RK-cluster has a smaller effect than the arginine substitutions (Table 2). In summary, the RK-cluster is essential for high heparin affinity, and the arginine residues promote stronger interactions with heparin as compared to lysine residues. The latter conclusion is consistent with earlier studies on synthetic peptides and fibroblast growth factor-1 (*16*, *29*, *30*).

It was not possible to determine the kinetic parameters for K212A:R215A because the specific binding of this variant was too weak. In an attempt to determine its binding constant, we used a protein concentration of 130 μM. Also at this high protein concentration, the observed SPR response was low, about twice the unspecific background binding and corresponding to about 5−10% of the maximal RU at corresponding concentrations of other binding variants. Assuming additively, theoretical calculation of the binding constant from the sum of the $\Delta\Delta G$ values of the variants K212A and K215A (Table 2) results in a K_D value of 113 μM. However, the observed week binding of the variant K212A:R215A indicates a significantly larger K_D than 113 μM.

In a simple electrostatic interaction model, glutamate residues will cause repulsion in the heparin binding event, resulting in unstable interactions and slower association kinetics. Indeed, all substitutions of alanine for glutamate residues resulted in stronger binding. The $\Delta\Delta G$ values of all glutamate substitutions are in the range 0.7−1.3 kcal mol^{-1} (Table 2). The variant E201A displayed the most pronounced effect with 8.5-fold stronger binding. The substitutions in positions 206 and 216 have smaller effects on the binding constants, and the binding constant of variant E209A falls between those of E201A and the other two variants. These substitutions, except for the E201A variant, appear to have a small effect on the apparent half-life for the complex with heparin. The double mutant E201A:E209A displays a K_D which is lower than that of FusCC by a factor of about 20 (Table 2). It can be noted that this experimentally determined affinity is only half as strong as that expected from the K_D value calculated from the sum of the $\Delta\Delta G$ of E201A and E209A (Table 2). Thus, a simple charge interaction model is insufficient to explain the effect of the substitutions E201A:E209A.

In all cases, except for the C219S variant, the SPR studies of the protein−heparin interaction were performed on S−S-bridged dimeric protein to ensure the presence of a full-length C-terminal. Previous studies by us (*7*) have shown that a monomeric form of the C-terminal peptide in α-helical conformation binds specifically to heparin with $K_D = 0.64$ μM. Our interpretation of the present results on S−S cross-linked variants of FusCC therefore assumes specific binding to one of the peptides only, as a starting point. One may then ask what effects the highly positively charged second peptide has on the binding. The difference in the free energy of binding ($\Delta\Delta G$) between the dimeric form of FusCC and the monomer forms, C219S and reduced FusCC, is 0.9−1 kcal mol^{-1} (Table 2). It is clear from this small affinity difference between monomer and dimer that the binding energies are not additive (from monomer to dimer), and that the charged cluster (+4) in the second (nonbinding) C-terminal domain contributes a general electrostatic free energy of binding that is similar to the contribution of one charged residue in the C-terminal that binds. Hence, heparin binds only to one of the C-terminal domains with high affinity, and the other only modulates the binding.

In Figure 2, the total charge of the C-terminal domain in the different variants is plotted against their corresponding K_D values. The dispersion of the K_D values of variants with the same net charge indicates that different charged amino acid residues have different roles, which is most clearly seen in the arginine to alanine replacement (Figure 2). Similar results have been observed by Lellouch et al. (*31*) in a peptide derived from antithrombin III.

It is noteworthy that all glutamic acid residues in the sequence, except E218, have an arginine as a neighboring amino acid residue. A possible explanation is that the negatively charged glutamate residues act as a filter, so that less specific interactions with other biological ligands do not occur. Another explanation for this is that the arrangement allows beneficial ion pair formation between neighboring amino acid residues with different charges, or between side chains that are close in a possible α-helical structure (*32*). Assuming an α-helical structure, ion pairing between residues such as E206/R210 and E209/R213 would have a stabilizing influence.

Structural Implications. Secondary structure predictions, produced by the GOR IV (*33*) method, suggest an α-helical structure from positions 201 to 217 in the C-terminal domain. In addition, a previous study by us (*7*) showed that a synthetic peptide corresponding to the C-terminal adopts an α-helical structure when TFE is added and the affinity for heparin increased in parallel with the increase in α-helical content. At 10% TFE, the synthetic peptide binds to heparin with a K_D value close to that of hEC-SOD (*7*), indicating that the C-terminal domain adopts a helical conformation when present in its original position in EC-SOD. Thus, an α-helical conformation of the C-terminal domain appears to be an important factor promoting heparin affinity. Notably, the far-UV CD spectrum of FusCC recorded at room temperature indicates very little helical content, which is reflected in a 20 times weaker heparin binding than EC-SOD. However, analysis of the far-UV CD difference spectrum recorded at +4 °C suggests 27% α-helix in the C-terminal domain of FusCC (Figure 3). Apparently the C-terminal domain can readily adopt an α-helical conformation also in the setting of the fusion protein FusCC.

In an attempt to disturb the possible α-helical structure in the C-terminal sequence, proline was introduced at positions 206, 212, and 215. The far-UV difference spectra of all these proline variants indicate a slight lowering of the α-helical structure (Figure 3). When comparing the heparin binding properties of the proline variants to the corresponding alanine variants, they have virtually identical effects on the heparin affinity (Tables 1 and 2). There may be enough flexibility in the arginine and lysine side chains to compensate for a change in structure.

The effect of the replacements in positions 201, 202, and 205 all indicate that flexibility is restricted in the N-terminal part of the C-terminal domain. This conclusion is based on the following observations. The most N-terminal replacement, E201A, is unique among the E substitutions in that it decreases the apparent half-life of the complex (Table 2). In contrast to the other R and K substitutions, the substitution R202A does not affect the apparent half-life of the complex while K_D indicates weaker affinity. Consequently, the association rate must be decreased compared to FusCC. However, most strikingly, unlike all other variants, the R205A variant displayed almost identical K_D compared with of FusCC (Table 2). Thus, according to the SPR experiments, R205 does not seem to be involved in the heparin interaction and must be positioned in an orientation facing away from heparin. If so, the structure around position 205 is not flexible.

In summary, it is apparent from our study that most of the charged residues of the C-terminal domain have an impact on the interaction of the protein with heparin, but their roles in the interaction differ somewhat. The N-terminal end of the C-terminal domain appears to be well structured, and the basic amino acids in the RK-cluster contribute most of the binding energy. However, the precise molecular contacts of the different charged amino acid residues in the C-terminal domain cannot be deduced from this study.

Further studies of the heparin interaction of the C-terminal domain will include structural studies and NMR spectroscopy of variants and peptides to elucidate the precise structural requirements and limitations. We will also investigate the specific requirements for the binding of the C-terminal domain to heparan sulfate from different sources.

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