Different Affinities of Glycosaminoglycan Oligosaccharides for Monomeric and Dimeric Interleukin-8: A Model for Chemokine Regulation at Inflammatory Sites†

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ABSTRACT: The binding of interleukin-8 (IL-8) to heparan sulfate (HS) proteoglycans on the surface of endothelial cells is crucial for the recruitment of neutrophils to an inflammatory site. Fluorescence anisotropy measurements yielded an IL-8 dimerization constant of 120 nM. The binding affinities, obtained by isothermal fluorescence titration, of size-defined heparin and HS oligosaccharides to the chemokine were found to depend on the oligomerization state of IL-8: high affinity was detected for monomeric and low affinity was detected for dimeric IL-8, referring to a self-regulatory mechanism for its chemoattractant effect. The highest affinity for monomeric IL-8 was detected for the HS octamer with a K_d < 5 nM whereas the dissociation constants of dimeric IL-8 were found in the medium micromolar range. No indication for increasing affinities for monomeric IL-8 with increasing oligosaccharide chain length was found. Instead, a periodic pattern was obtained for the dissociation constants of the GAG oligosaccharides with respect to chain length, referring to optimum and least optimum chain lengths for IL-8 binding. GAG disaccharides were identified to be the minimum length for chemokine binding. Conformational changes of the dimeric chemokine, determined using CD spectroscopy, were detected only for the IL-8/HS complexes and not for heparin, pointing to an HS-induced activation of the chemokine with respect to receptor binding. Thermal unfolding of IL-8 yielded a single transition at 56 °C which was completely prevented by the presence of undigested HS or heparin, indicating structural stabilization, thereby prolonging the biological effect of the chemokine.

The importance of glycosaminoglycans (GAGs)[1] in numerous biological processes has attracted increasing attention over the past few years (*1, 2*). Chemically, GAGs are polyanionic linear polysaccharides which are part of cell surface and extracellular matrix proteoglycans (*3, 4*). They have been ascribed a variety of functions including the organization of the extracellular matrix, the mediation of cell adhesion and migration, the regulation of proliferation and differentiation, etc. (*5, 6*). Heparan sulfates (HS) belong to the GAG family and are, compared to heparin, structurally more heterogeneous as a result of relatively discrete and variable postpolymerization modifications (*7, 8*). These include N- and O-sulfation and epimerization of the precursor D-glucuronic acid (GlcA)/L-iduronic acid (IdoA)−D-glucosamine (GlcN) copolymer. Interactions of proteins with GAGs are thought to be highly specific for a particular oligosaccharide sequence as has been recently demonstrated for antithrombin (*9*) and for the basic fibroblast growth factor (bFGF) (*10, 11*). The binding of heparin to bFGF facilitates both oligomerization of the protein and receptor binding (*12*) although monomeric bFGF−saccharide complexes have been identified as the minimal active unit (*13*). Similarly, the interaction of the chemokine interleukin-8 (IL-8) with cell surface GAGs on the endothelium was shown to promote the oligomerization of the chemokine (*14*) and thus the presentation to its high-affinity receptor on migrating neutrophils.

IL-8 is an 8 kDa protein which belongs to the structurally related C-X-C family of proinflammatory chemokines and is produced by many different types of immune and nonimmune cells (*15, 16*). Being chemotactic for neutrophils, IL-8 is thought to establish a chemotactic gradient across the vascular endothelium in infected or damaged tissues. For this purpose, it is supposed that IL-8 is presented on cell surface heparan sulfate proteoglycans (HSPGs) to the approaching leukocyte, thereby increasing the local chemokine concentration and thus enhancing its effect on the IL-8 specific G-protein-coupled transmembrane receptors on the neutrophil (*17*). IL-8 is biologically active as a monomer but dimerizes readily at higher concentrations (*18*). Consequently, the 3D structure of IL-8 initially determined by NMR spectroscopy (*19, 20*) revealed a homodimer in which two symmetry-related antiparallel α helices are lying on top of a six-stranded antiparallel β-sheet which forms the dimerization region. It has been shown recently that the structure of IL-8 is essentially independent of its quaternary

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[1] Abbreviations: CD, circular dichroism; GAG, glycosaminoglycan; HS, heparan sulfate; IL-8, interleukin-8; GlcA, glucuronic acid; IdoA, iduronic acid; GlcN, glucosamine; dp, degree of polymerization.

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structure, except for some distortion in the C-terminal part which forms an α-helix in the dimer and is less structured in the IL-8 monomer (*21*).

Kuschert et al. have recently identified the amino acids of IL-8 which are involved in HS binding using site-directed mutagenesis and NMR studies (*22*). The amino acids involved in GAG binding include Arg60, Lys64, Lys67, and Arg68 which are contained within the C-terminal α-helix of IL-8 as well as Lys20 contained in the proximal loop. On the other hand, Spillmann et al. reported a specific domain of HS which binds to IL-8 (*23*). It was found to consist of 2 sulfated subdomains (S-domains) which are 6 monosaccharide units in length and which are connected by a flexible 14 monosaccharide unit stretch of low sulfation that enables the sequence to fold into a horseshoe so that each S-domain engages the antiparallel IL-8 dimer with a common polarity. The structure is somewhat analogous to the extended HS sequences that bind to IFN-γ (*24*) and platelet factor 4 (*25*). IL-8 binding was reported to correlate with the occurrence of the di-O-sulfated disaccharide IdoA(2-OSO_3)−$GlcNSO_3$-(6-OSO_3).

By using ligand-induced fluorescence quenching, we show here that a wide range of different size-fractionated heparin and HS oligosaccharides display high affinity for monomeric and lower affinity for dimeric IL-8. Structural changes of dimeric IL-8 induced by HS, but not by heparin, refer to an "activation" of the chemokine which is hypothesised to be important for the migration of neutrophils. The inhibition of thermal IL-8 unfolding by GAGs is indicating a major role of GAGs for structurally stabilizing the chemokine.

EXPERIMENTAL PROCEDURES

Preparation of Heparin and HS Oligosaccharides. Size-defined HS and heparin saccharides were prepared from porcine mucosal HS and low molecular weight heparin-Innohep (Leo Laboratories) by scission with commercially available (SIGMA) heparitinase (HS) and heparinase (heparin). Separation of the product oligosaccharides was achieved using a Bio-Gel P-6 gel filtration column (170 × 1.5 cm) for the HS fragments and a Bio-Gel P-10 gel filtration column (140 × 1.0 cm) for the heparin fragments as previously described (*13, 26*). A typical electrophoretic and gel filtration profile of heparinase-generated oligosaccharides is displayed in Figure 1.

Cloning, Expression, and Purification of IL-8. Total RNA from IL-1-stimulated HaCaT cells was isolated and reverse-transcribed into first-strand cDNA. This was used as template for the PCR amplification of the IL-8 cDNA, which was then 5′-filled-in using T4 DNA polymerase and blunt-end-cloned into the T7 promoter-containing pET-3a expression vector. For expression and purification, this construct was transformed into calcium-competent BL21 cells, and IL-8 expression was induced by adding 1mM IPTG after an OD = 0.6 was reached. One gram of *E. coli* paste was resuspended in 2 mL of 20 mM TRIS/HCl (50 mM NaCl, pH 8.0), sonicated (5 × 20 s at 100 W), and centrifuged for 20 min at 10000*g*. The inclusion bodies (1 g) were resuspended in 10 mL of guanidine hydrochloride (6 M) and stirred at 4 °C overnight. After extensive dialysis against 0.5% acetic acid, anion exchange of IL-8 was performed using a Mono S column which was developed with a linear gradient from 50 mM MES (pH 6.5) to 50 mM MES (1 M NaCl, pH 6.5) in 60 min. IL-8 was detected at 310 mM NaCl. This was followed by reversed-phase HPLC of IL-8 on a C4 column which was developed with a linear gradient from 20% to 55% acetonitrile (0.1% TFA).The retention time of IL-8 was found to be 20 min. Refolding of IL-8 was accomplished on a Mono S column using the same conditions as described above. The overall yield was 8−10 mg of pure (>99%) IL-8 per liter of culture. All methods used for the cloning, expression, and purification of IL-8 followed established protocols (*27, 28*).

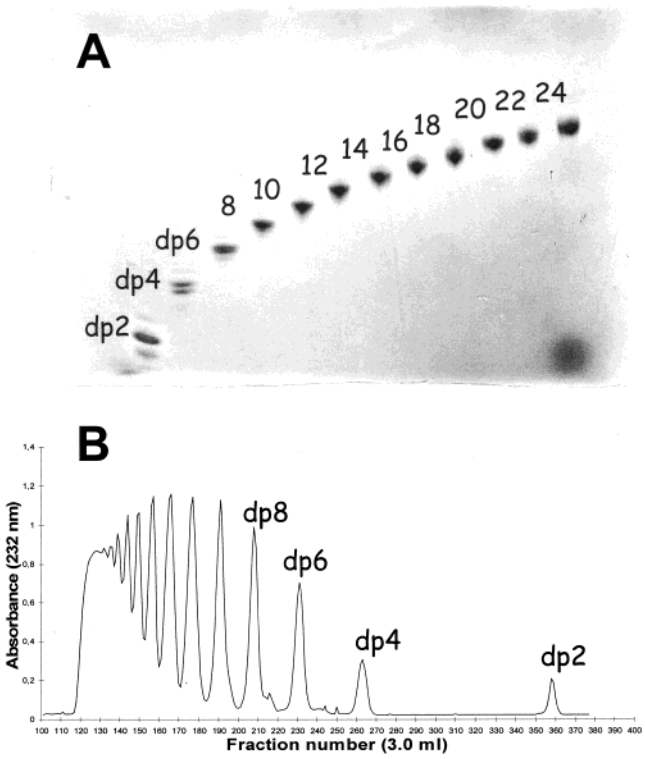

FIGURE 1: Partial heparinase cleavage of low molecular weight heparin. Polyacrylamide gel electrophoresis and Azure A staining (curve A) of the fragments (dp2−dp24) obtained after gel filtration (curve B).

Fluorescence Measurements and Data Analysis. Steady-state fluorescence measurements were recorded on a Perkin-Elmer (Beaconsfield, England) LS50B fluorometer as described previously (*29, 30*). For the fluorescence anisotropy measurements, IL-8 was dialyzed into freshly prepared PBS and concentrated to a final concentration of 220 μM. From this stock solution, the various dilutions needed for determining the dimerization constant were prepared directly in the cuvette. To achieve an equilibrium in a reasonable length of time, the solution was constantly stirred during the measurements.

The fluorescence anisotropy of IL-8 was collected at an emission wavelength of 336 nm, and the changes in quaternary structure were followed by increasing the protein concentration. The fluorescence anisotropy is defined as

$$r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH}) \quad (1)$$

where I_{VV} is the fluorescence intensity recorded with excitation and emission polarizers in vertical positions and I_{VH} is the fluorescence intensity recorded with the emission polarizer aligned in a horizontal position. The *G* factor is

the ratio of the sensitivities of the detection system for vertically and horizontally polarized light: $G = I_{HV}/I_{HH}$. For each point in the dimerization experiments, the anisotropy was recorded over 30 s, and the mean r values for each IL-8 concentration were used for the determination of the IL-8 dimerization constant.

In the fluorescence intensity titrations, the emission of a 50 nM or a 700 nM IL-8 solution upon excitation at 282 nm was recorded over the range of 300−400 nm following the addition of an aliquot of the respective HS ligand and an equilibration period of 2 min. The use of very concentrated GAG oligosaccharide stock solutions (2−5 mg/mL) ensured a dilution of the protein sample of less than 10%. Molar concentrations of the GAG oligosaccharides were calculated assuming an average molecular mass of 493 Da for a heparin disaccharide unit and 452 Da for a HS disaccharide unit (this is based on an average degree of sulfation of 2 per heparin disaccharide and 1.5 per HS disaccharide). The slit widths were set at 10 nm when 50 nM IL-8 was used and at 2 nm when 700 nM IL-8 was used, and the spectra were recorded at 100 nm/min. A 290 nm cutoff filter was inserted into the emission path to avoid stray light. The samples were stirred during the measurements, and the temperature was maintained at 22 °C by coupling to an external water bath. After background subtraction, the fluorescence intensity, F, was integrated, and the mean values resulting from three independent experiments were plotted against the volume-corrected concentration of the added ligand. The resulting binding isotherms were analyzed by nonlinear regression using the program Origin (Microcal Inc., Northampton, MA) to the following equation describing a bimolecular association reaction:

$$F = F_i + F_{max}\left[\frac{K_d + [\text{IL-8}] + [\text{L}] - \sqrt{(K_d + [\text{IL-8}] + [\text{L}])^2 - 4[\text{IL-8}][\text{L}]}}{2[\text{IL-8}]}\right] \quad (2)$$

where F_i is the initial and F_{max} is the maximum fluorescence value, K_d is the dissociation constant, and [IL-8] and [L] are the total concentrations of IL-8 and HS ligand, respectively. The fitted parameters were F_{max} and K_d. This equation is based on the general solution for a bimolecular association reaction (*31*).

Circular Dichroism Measurements and Analysis. The CD spectra of aqueous IL-8 and IL-8/HS complex solutions were recorded in cuvettes with a path length of 0.1 cm on a Jasco J-710 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan). Spectra were collected with a response time of 0.25 s and with a data point resolution of 0.1 nm. Commonly, five scans were averaged to yield smooth spectra. The concentration of IL-8 was held constant at 5 μM for all CD measurements. The spectra were background-corrected with respect to the CD signal of the HS oligosaccharides. Analysis of the CD spectra with respect to secondary structural elements was accomplished using the program SELCON (*32*). For thermal unfolding, a 5 μM IL-8 solution, in the absence or in the presence of excess amounts of undigested HS or heparin, was placed into a sealed cuvette with a path length of 0.1 cm, and three scans (250−190 nm) were recorded after thermal equilibration was reached (5 min).

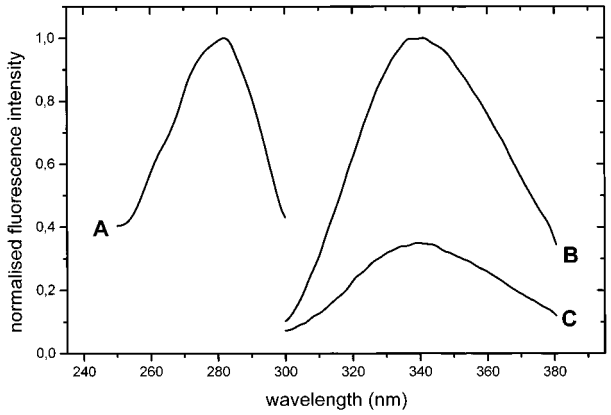

FIGURE 2: Normalized fluorescence excitation (curve A) and emission (curve B) spectra of a 200 nM IL-8 solution in PBS. Curve C represents the saturation emission spectrum after titration with heparin dp12 (see text). The spectra presented were corrected for background emission.

RESULTS

The steady-state fluorescence spectra of IL-8 are shown in Figure 2. The fluorescence emission maximum at 336.5 nm is typical for a tryptophan residue in a slightly solvent-shielded environment like that of Trp57 at the top of the C-terminal α-helix of IL-8 (*20*). The steady-state spectra did not change significantly whether monomeric or dimeric IL-8 was investigated because the dimerization interface is provided by the central β-sheets and is thus not in the vicinity of the tryptophan fluorophore. With the intrinsic tryptophan chromophore located in the proximity of the C-terminal α-helix, the proposed site of interaction with GAGs (*22*), a concentration-dependent change in the fluorescence emission properties upon binding of GAGs was expected. The fluorescence quenching resulting from GAG binding is illustrated in curve C (Figure 2). Depending on the GAG oligosaccharide under investigation, the emission of IL-8 was quenched to an extent of 60−80%. In addition, the emission maximum was blue-shifted by 1.1 nm. This refers to a significant conformational change of IL-8 upon GAG binding which leads to a rearranged microenvironment of the tryptophan residue. The fluorescence quenching of the tryptophan residue was therefore used as a GAG concentration-dependent signal in isothermal binding studies.

Since the quaternary structure of IL-8 was shown to influence the affinity of the chemokine for its GAG ligands (*33*), conditions were sought where binding to the monomer and to the dimer could be separately investigated. Different values have been reported by various groups for the dimerization constant of IL-8 using a variety of methods (*18*). By recording the concentration-dependent fluorescence anisotropy of the chemokine resulting from the intrinsic chromophore Trp57, which is a measure of the rotational motion of the protein due to its specific hydrodynamic volume, a dimerization constant of 120 nM for IL-8 was calculated by fitting the data to eq 2, with [L] representing in this special case the IL-8 "ligand" concentration for the homodimerization reaction (see Figure 3). To carry out fluorescence binding experiments of heparin and HS oligosaccharides to monomeric compared to dimeric IL-8, chemokine concentrations of 50 and 700 nM were chosen.

The results of isothermal fluorescence binding experiments of heparin and HS oligosaccharides with different chain

Table 1: Dissociation Constants (K_d) and Respective Standard Errors of GAG Oligosaccharides Binding to Monomeric and Dimeric IL-8 (Data Analysis Was Accomplished As Described under Experimental Procedures)

	HS oligosaccharides		heparin oligosaccharides	
	K_d (IL-8 = 50 nM) ($\times 10^{-9}$ M)	K_d (IL-8 = 700 nM) ($\times 10^{-6}$ M)	K_d (IL-8 = 50 nM) ($\times 10^{-9}$ M)	K_d (IL-8 = 700 nM) ($\times 10^{-6}$ M)
dp2	113 ± 18	87.7 ± 10	380 ± 55	104 ± 3
dp4	602 ± 303[a]	63.4 ± 2.8	57 ± 24[a]	60 ± 2.8
dp6	170 ± 24	34 ± 3.2	597 ± 112	116.6 ± 4.3
dp8	<5[b]	16.4 ± 2.5	32 ± 17[a]	103.7 ± 4.2
dp10	199 ± 114[a]	57.6 ± 5.2	96 ± 28	122.3 ± 3.5
dp12	147 ± 132[a]	64.1 ± 23.6	183 ± 35	102.2 ± 2.5
dp14	448 ± 93	23.8 ± 2.6	1657 ± 670	93.1 ± 1.9
dp16	250 ± 73	1.7 ± 0.9	135 ± 28	93.9 ± 3.3

[a] K_ds were obtained by fitting only the initial phase of the fluorescence saturation curve to eq 2 (for details, see text). [b]Due to the low signal-to-noise ratio at this low protein concentration combined with the immediate saturation obtained for the HS octasaccharide, this K_d value represents an estimate rather than a precisely calculated parameter.

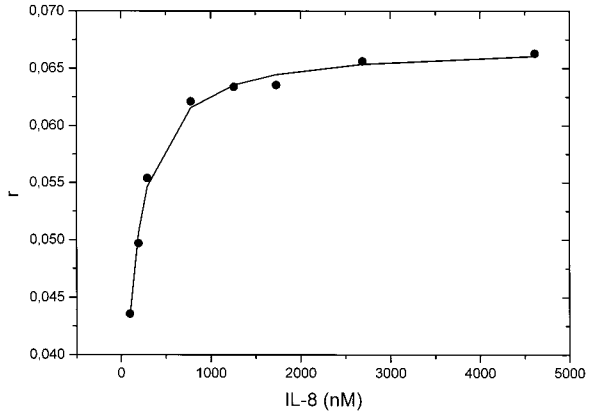

FIGURE 3: Dependence of the fluorescence anisotropy of IL-8 in PBS on the chemokine concentration. For each data point, the anisotropy signal, r, recorded at λ_{ex} = 282 nm and λ_{em} = 336 nm, was averaged over 60 s, and the mean of three independent measurements is shown.

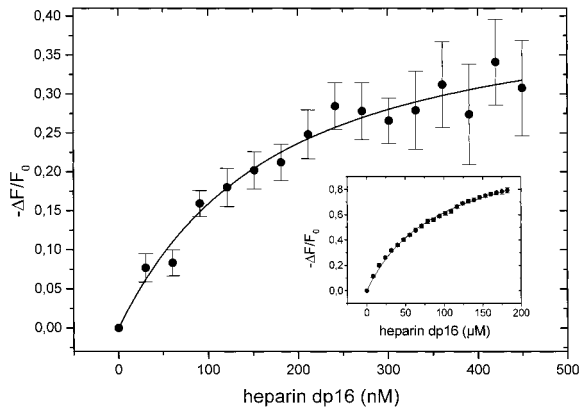

FIGURE 4: Fluorescence binding isotherms for the interaction of monomeric IL-8 (main graph) and of dimeric IL-8 (inset) with heparin dp16. The changes in fluorescence are plotted as the normalized change in fluorescence ($-\Delta F/F_0$, where F_0 is the initial fluorescence intensity before addition of ligand) vs the concentration of oligosaccharide. The data represent mean values of three independent experiments and were fitted as described under Experimental Procedures.

lengths to monomeric and dimeric IL-8 are summarized in Table 1. Based on our dimerization experiments (see Figure 3), IL-8 concentrations of 50 and 700 nM were chosen to represent monomeric and dimeric IL-8, respectively. The assumption of one binding site per IL-8 monomer for GAG oligosaccharides for the data fitting was justified by the molecular architecture of the GAG binding site, which was recently identified by site-directed mutagenesis studies (*22*) and for which we have put forward a three-dimensional model by molecular dynamics computer simulations (Krieger et al., manuscript submitted for publication). Two different situations were identified in the GAG oligosaccharide binding experiments: high-affinity binding when using monomeric IL-8 and low-affinity binding when dimeric IL-8 was used (see Figure 4). In some size-defined GAG oligosaccharide fractions (marked with an asterisk in Table 1), a clearly separated second phase was detected in the fluorescence titrations after an intermediate plateau was reached. This is interpreted as low-affinity GAG binding concomitantly to high-affinity binding. In the cases where this occurred, more data points were collected for the initial saturation phase, and the dissociation constants of the respective oligosaccharides to monomeric IL-8 were obtained by fitting the data to eq 2, disregarding the clearly separated second phase.

A closer look at the dissociation constants obtained for the IL-8 monomer reveals that the K_d values of the size-fractionated GAG oligosaccharides dp2−dp16 did not differ by orders of magnitude between the two GAG classes. However, profound differences in K_d values were found among the size-fractionated GAG oligosaccharides (see Table 1) with the HS octasacharide yielding the lowest K_d ($<5 \times 10^{-9}$ M) and the heparin 14-mer giving the highest K_d ($=1.6 \times 10^{-6}$ M) value. In addition, the K_d values of the oligosaccharides were found to display an interesting periodicity with respect to chain length, giving low K_d values for HS dp2 and dp8 as well as for heparin dp4 and dp8, and high K_d values for HS dp4 and dp14 as well as for heparin dp6 and dp14 (see Table 1). The optimum and the least optimum chain lengths for IL-8 binding were thus identified. Interestingly, both heparin and HS disaccharides were found to bind to monomeric IL-8 with high affinity (see Table 1), with the HS disaccharides yielding a 10-fold lower K_d value than the heparin disaccharides. This indicates a GAG disaccharide to be the minimum chain length for IL-8 binding.

In the case of low-affinity binding to dimeric IL-8, the heparin oligosaccharides were found to interact with generally lower affinity compared to the HS oligosaccharides (see Table 1). These results must, however, be regarded as preliminary since in some of the IL-8/HS oligosaccharide fluorescence titrations saturation had to be extrapolated

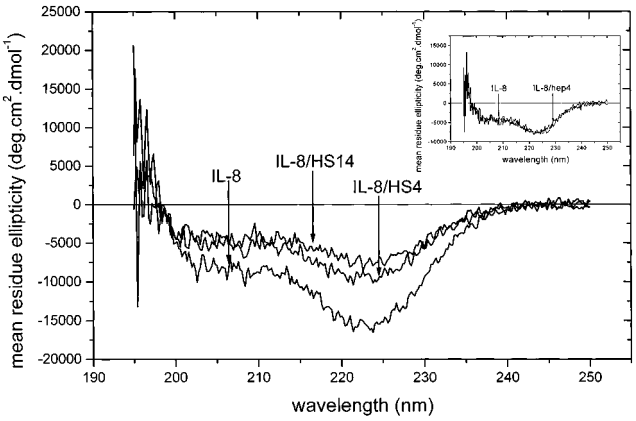

FIGURE 5: CD spectra of 5 μM IL-8 in complex with 25 μM HS oligosaccharides (main graph) and with 25 μM heparin oligosaccharides (inset). The spectra presented here were corrected for the background signal of buffer plus ligand. Five scans were recorded to obtain smooth spectra.

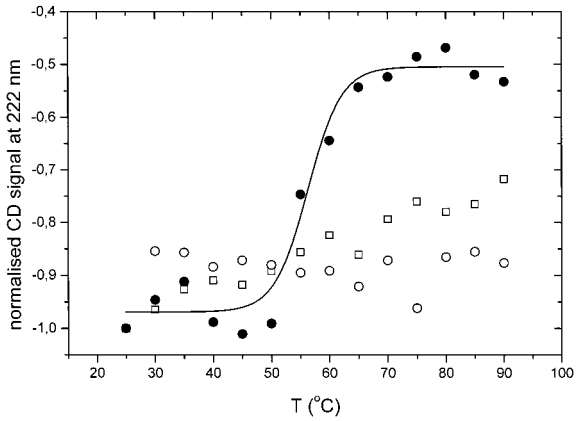

FIGURE 6: Thermal unfolding of IL-8 in the absence (●) and in the presence of undigested heparin (□) and HS (○). The CD signal of a 5 μM IL-8 solution at 222 nm at increasing temperature is displayed. The concentration of the GAGs was approximately 25 μM.

because of the limiting amount of HS oligosaccharide available. Nevertheless, the K_d values of the HS oligosaccharides for IL-8 binding were found to again exhibit a periodicity with respect to HS chain length peaking at dp2 and dp12 and showing minima at dp8 and dp16 whereas in the case of the heparin oligosaccharides only one minimum K_d value for dp4 was observed (see Table 1).

The structural change of IL-8 upon GAG oligosaccharide binding as revealed by the fluorescence quenching of the protein's single tryptophan residue was further investigated by CD spectroscopy. For this purpose, the CD spectra of IL-8 in complex with various GAG oligosaccharides were recorded. Unfortunately, only the conformational changes of dimeric IL-8 could be investigated under these conditions because for sufficient signal intensity the concentration of the chemokine had to be adjusted to 5 μM. Only the HS oligosaccharides were found to induce a secondary structure change of IL-8, mainly in the α-helical part of the protein as can be seen by the reduction of the CD signal at 222 nm upon ligand binding (see Figure 5). The magnitude of this effect was found to be dependent upon the length of the oligosaccharide under investigation. The CD spectra of IL-8 in the heparin complexes showed virtually no change compared to the unliganded protein (see Figure 5, inset). As in the fluorescence titration experiments of dimeric IL-8, heparin and HS oligosaccharides yielded different results in the CD experiments, indicating different structural changes of the chemokine induced by the two GAG classes and thus referring to different modes of binding.

As a concluding step of this study, thermal unfolding experiments of IL-8 in the presence and in the absence of GAGs were performed using CD spectroscopy. In Figure 6, the CD signal at 222 nm corresponding to the α-helical signal of the protein at increasing temperature is shown. An almost identical curve was obtained if the CD signal at 216 nm was plotted against increasing temparature, corresponding to the β-sheet content of the chemokine (data not shown). IL-8 was found to unfold with a single transition at 56 °C (see Figure 6), indicating no stable folding intermediate. Unfolding was found to be completely reversible (data not shown). Unfolding was completely prevented by the addition of undigested heparin or HS to IL-8 (see Figure 6). Interestingly, unfolding was partly reversed by the addition of heparin or HS to the denatured chemokine (data not shown). This refers to a specific stabilizing effect of GAGs on the IL-8 structure.

DISCUSSION

Inflammatory diseases are characterized by leukocyte emigration from the circulation to the affected tissue. Chemoattractants such as IL-8 are thought to drive the egress of leukocytes because injection of IL-8 into tissues was found to emulate the entire process of inflammatory neutrophil recruitment (*34*), the leukocyte for which IL-8 is chemotactic. A major prerequisite for neutrophil emigration is their attachment to the surface of endothelial cells. This interaction involves a stepwise participation of adhesion molecules located on neutrophils as well as on the endothelial cells: the first step is selectin-mediated neutrophil tethering and rolling followed by β2 integrin-mediated firm neutrophil adhesion to the endothelium (*35*). The rapid activation of β2 integrins is stimulated by chemoattractants such as IL-8 (*36*). However, the most prominent in vitro effect of chemoattractants is the induction of directed leukocyte migration, i.e., chemotaxis. Consequently, adhesion of leukocytes to endothelial cells as well as emigration have traditionally been thought to be induced by soluble chemoattractants (*37*). It was shown only recently that IL-8 immobilized via its C-terminus is required for the induction of neutrophil emigration (*38*). This immobilization, as well as oligomerization in order to increase the local concentration of IL-8 on endothelial cells, was reported to be accomplished by GAGs (*14*).

Because of the relatively low concentration of IL-8 in the lumen of a vessel even in the vicinity of inflamed tissue, the initial encounter on endothelial cell surfaces is presumed to be between monomeric chemokine and GAG. IL-8 was shown to be biologically fully active as monomer and as dimer (*39*). Consequently, the structural requirements for receptor binding on the neutrophil must be met by monomeric and by dimeric IL-8. The structure determination of a chemically modified monomeric IL-8 did indeed yield a structure very similar to the IL-8 dimer with slight distortions in the C-terminal part of the monomer (*21*). Various methods were therefore employed to determine the dimerization constant of IL-8, yielding a rather broad concentration range

for the dimerization to occur depending on the method used (*18*). We have performed fluorescence anisotropy experiments which gave a dimerization constant for the chemokine of 120 nM. Since so far the affinites of IL-8 monomers and dimers for GAGs have not been measured and since this is a key question in the context of IL-8 accumulation at inflammatory sites where there may be an increasing tendency for dimers to form as the local chemokine concentration rises in response to inflammation triggers (e.g., IL-1, TNF-α), we have applied isothermal fluorescence titration to monomeric and dimeric IL-8.

As can be seen from Table 1, the affinities of GAG oligosaccharides for monomeric and dimeric IL-8 differed by several orders of magnitude depending upon the length and the nature (HS or heparin) of the oligosaccharide under investigation. Different modes of GAG binding are thus assumed for the IL-8 monomer and for the dimer. We therefore suggest that in the IL-8 monomer the dimer interface becomes (partly) involved in GAG binding, leading to high affinities, and/or that in the IL-8 dimer the actual GAG binding site on the chemokine becomes less accessible for GAG binding, leading to low affinites. Evidence for this "communication" between the dimerization interface and GAG binding site stems from investigating the dimerization of IL-8 in the presence of GAGs which yielded a higher dimerization constant than in the absence of GAG (Kungl, unpublished results). The involvement of the dimer interface of monomeric IL-8 in GAG binding either can be by a direct interaction of GAGs with amino acids constituting the interface or can be indirect by a structural change of the dimer interface in monomeric IL-8 upon GAG binding. High GAG affinities for monomeric IL-8, taken together with the fact that monomeric IL-8 is able to activate its protein receptor on neutrophils, indicate that monomeric IL-8 is most probably the biologically active form of the chemokine which binds with high affinity to GAGs on the endothelium, thus presenting itself to the approaching neutrophil. Dimerization could therefore be a concentration-dependent means for reducing the affinity of IL-8 to the GAGs on the endothelium surface, thereby inducing the chemokine to diffuse away from the GAGs. This could represent a self-regulatory mechanism for the dissolution of an IL-8 chemotactic gradient which was established based on the high affinity of monomeric IL-8 for GAGs.

No evidence for increasing IL-8 affinities with respect to GAG oligosaccharides with increasing chain length was found. Instead, optimum chain lengths for IL-8 binding were identified (see Table 1). This is similar to the situation found for DNA binding proteins which interact with oligonucleotides well-defined with respect to both length and sequence. Since due to the enzymatic preparation the oligosaccharides are not expected to differ from each other in a specific way except in length (only the HS di- and tetrasaccharides differ from the rest in that they are mainly N-acetylated), the optimum GAG chain lengths are most probably the result of the ability of certain oligosaccharides to adopt a favorable 3-D structure for IL-8 binding. Our results are somewhat contradictory to the traditional view that the affinity of GAG binding proteins for their GAG ligands increases with increasing GAG chain length. A possible explanation for our different finding is that we used direct binding for the determination of dissociation constants whereas in other studies radioactive competition assays with heparin-bound proteins were used (*23, 33*). In these experiments, beside the affinity of the actually binding oligosaccharide, the affinity of the prebound saccharide being competed off the protein under investigation must be taken into account, rendering a direct comparison of binding and competition elusive.

Kuschert et al. (*22*) have identified GAG binding amino acids in IL-8 by NMR shift experiments using heparin disaccharides. Likewise, in our fluorescence isothermal binding experiments, significant affinities of IL-8 for HS and for heparin disaccharides were detected (see Table 1). A GAG disaccharide is therefore suggested to represent the minimum chain length for IL-8 binding [we have further evidence for this finding as the GAG interaction site in our recently computed molecular model of an IL-8/GAG complex consists of a disaccharide (Krieger et al., manuscript submitted for publication)]. Interestingly, the di- and tetrasaccharides occurring in natural HS were found to be mainly N-acetylated (*40*). We thus put forward a model for monomeric IL-8 being "captured" in vivo by such unmodified, nonsulfated segments which comprise the majority of the HS chain. Then, by analogy with DNA−protein interactions, the chemokine could "slide" to a high-affinity S-domain, constituted by an octasaccharide core sequence which is able to adopt a conformation favorable for IL-8 binding, where a more stable association could develop. The predominant structure of such domains is ΔGlcA-GlcNSO$_3$-[IdoA(2-OSO$_3$)-GlcNSO$_3$]$_2$-IdoA-GlcNAc (*40*). Thus, the presence of two internal sulfated iduronates seems to give the best fit to the GAG binding site of IL-8. By occupying the high-affinity sites on the endothelial HS, a local threshold concentration of the chemokine would be achieved to trigger the IL-8 receptor on the approaching neutrophils. Once the local concentration of IL-8 reaches a certain level exceeding the value of the dimerization constant, the chemokine will begin to form homodimers which will, by their significantly lower affinity for GAGs compared to the monomers, diffuse away from the pericellular HS. By this means, the chemoattractant effect of IL-8 could be dampened.

The impact of heparin and HS on the secondary structure of IL-8 was found to be quite different (see Figure 5) although the affinities for the two classes did not differ by orders of magnitude (see Table 1). Assuming that the HS oligosaccharides adopt, depending upon their length, certain conformations—favorable or unfavorable for IL-8 binding—in solution, unlike heparin which was shown to exhibit a rather rigid helical conformation (*41*), the significant change in the IL-8 secondary structure upon HS but not upon heparin binding can be explained by an induced fit. This leads to structural rearrangements in the case of HS binding which cannot be achieved by heparin binding. The conformational effect of HS could be the reason for the recent observation that HS but not heparin induces neutrophil migration (*42*).

Thermal unfolding experiments revealed a single transition temperature of the chemokine at 56 °C (see Figure 6). No stable folding intermediates were detected which means either that monomerization of the chemokine could not be detected by CD spectroscopy or that denaturation and monomerization occur concomitantly. The addition of HS or heparin to IL-8 was shown to prevent IL-8 from unfolding, thereby indicating the role of GAGs on IL-8 stability. In vivo,

this could result in a prolonged IL-8 response and/or in preventing the chemokine from proteolytic degradation.

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