The Conformation of the Sialyl Lewis X Ligand Changes upon Binding to E-Selectin

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ABSTRACT: High-field NMR spectroscopy has been used to study the complex formed by the tetrasaccharide sialyl Lewis X and its receptor, E-selectin. Transferred NOEs demonstrate a specific interaction between the protein and ligand and enable measurement of the dissociation constant for the complex to be between approximately 1.1 and 2.0 mM. Differences between Overhauser spectra for free and bound sialyl Lewis X highlight a conformational change upon binding. This can be pinpointed to a change in the torsion angle of the glycosidic link between the sialyl and galactosyl residues and used to select a likely "bound" conformation from four low-energy species. Docking the bound form of sialyl Lewis X onto a model of the lectin domain of E-selectin suggests that the conformational change upon binding results primarily from steric interactions.

An early event in the response to tissue damage or infection is the attachment of leukocytes to cells of the vascular endothelium proximal to the site of trauma. The initial recruitment of neutrophils and monocytes from the bulk blood flow is believed to result from the interaction between the E-selectin protein and oligosaccharides of the sialyl Lewis X class [reviewed by Lasky (1992)]. E-Selectin (also known as ELAM-1 and LECAM2) is expressed on the surface of endothelial cells in response to TNF, IL-1β, and endotoxins (Bevilacqua *et al.*, 1989). Sialyl Lewis X is presented by glycoproteins and/or glycolipids on the leukocyte surface (Lowe *et al.*, 1990; Phillips *et al.*, 1990).

The discovery of the sequence of E-selectin (Bevilacqua *et al.*, 1989) led to its characterization as a 582-residue, multidomain protein. The N-terminal domain is homologous to C-type lectins and is believed to be the primary site of carbohydrate recognition. Then follows an EGF[1]-like domain, six SCR-like domains, a putative membrane anchor, and a small cytoplasmic domain. The chief differences between E-, P-, and L-selectins lie in the number of SCR domains (Lasky, 1992). On the basis of the homology with mannose binding protein (MBP; Weis *et al.*, 1991), models for the lectin domain of E-selectin have been proposed (Erbe *et al.*, 1992; Mills, 1993), and the subsequent X-ray structure of MBP complexed with mannose enabled prediction of a possible mode of interaction of sialyl Lewis X with the selectins (Weis *et al.*, 1992). The recently reported X-ray diffraction structure of the lectin and EGF domains of E-selectin (Graves *et al.*, 1994) provided the first detailed insight into the means by which this protein recognizes its ligand.

The sialyl Lewis X epitope (SLeX) was demonstrated to be a ligand for E-selectin by Lowe *et al.* (1990) and Phillips *et al.* (1990) with the fucosyl and sialyl residues required for activity (Tiemeyer *et al.*, 1991). SLeX has since been shown to bind P- and L-selectins (Foxall *et al.*, 1992). NMR studies of the solution structure of SLeX (Ball *et al.*, 1992; Ichikawa *et al.*, 1992; Lin *et al.*, 1992) are in general agreement, with rotating-frame Overhauser enhancements (ROEs) being used to locate the fucosyl ring against the galactosyl ring and define a relative orientation for the sialyl and galactosyl rings. In this paper we report the use of transferred nuclear Overhauser enhancements (tNOEs) to monitor the interaction between recombinant E-selectin and SLeX and demonstrate that the conformation of SLeX bound to E-selectin differs from the commonly perceived solution conformation.

EXPERIMENTAL PROCEDURES

Sialyl Lewis X [Sial-α-2,3-Gal-β-1,4-(Fuc-α-1,3)-GlcNAc] was obtained from Oxford Glycosystems (Abingdon, U.K.) and Dextra Laboratories Limited (Reading, U.K.). E-Selectin was produced in baculovirus-infected SF9 cells as a modified fusion protein (Cavegn & Bernard, 1992): residues 1–429 of human E-selectin were followed by two IgG binding domains (termed Z; Nilsson *et al.*, 1987). The culture supernatant from a 36-L culture of baculovirus-infected *Spodoptera frugiperda* SF9 cells was passed through a column of immobilized human IgG. Retained material was eluted with 3 M ammonium thiocyanate and exchanged into 20 mM sodium phosphate, pH 7.2, by dialysis or gel filtration using G25 Sepharose. The protein was further purified by anion-exchange chromatography using POROS Q, Millipore Mem-sep Q, or Pharmacia Q Sepharose resins equilibrated with 20–50 mM Tris-HCl, pH 8.0. Recombinant E-selectin eluted in 100–200 mM sodium chloride when a linear gradient was applied. Adherence of HL60 cells to E-selectin bound to IgG-coated wells was used to demonstrate activity. Bovine serum albumin (fraction V) was obtained from Sigma and was dialyzed repeatedly before use.

Protein for NMR studies was exchanged into 1 mM d-Tris and 10 mM calcium chloride, in D_2O, pH 7–7.5 (uncorrected

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[1] Abbreviations: BSA, bovine serum albumin; D_2O, deuterium oxide; DQF-COSY, double-quantum-filtered correlation spectroscopy; d-Tris, deuterated Tris buffer; EGF, epidermal growth factor; Fuc, fucose; Gal, galactose; GESA, geometry of saccharides algorithm; GlcNAc, *N*-acetylglucosamine; MBP, mannose binding protein; NOE, nuclear Overhauser enhancement; NOESY, nuclear Overhauser enhancement spectroscopy; ROE, rotating-frame Overhauser enhancement; ROESY, rotating-frame Overhauser enhancement spectroscopy; SCR, short consensus repeat; Sial, sialic acid; SLeX, sialyl Lewis X; tNOE, transferred nuclear Overhauser enhancement; TOCSY, total correlation spectroscopy; TPPI, time-proportional phase incrementation.

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meter readings), by ultrafiltration or passage through a PD10 desalting column. Protein concentrations were estimated from absorbance at 278 nm (E_{max} = 86 500 M^{-1} cm^{-1}).

All NMR experiments were recorded at 25 °C on a Bruker AMX 600-MHz spectrometer with presaturation of the residual solvent resonance. Two-dimensional DQF-COSY (Rance *et al.*, 1983), NOESY (Jeener *et al.*, 1979), ROESY (Bothner-By *et al.*, 1984), and TOCSY (Braunschweiler & Ernst, 1983) experiments were acquired in the phase-sensitive mode with quadrature detection in t_1 achieved with TPPI (Bodenhausen *et al.*, 1979). The phase cycle for DQF-COSY was modified to incorporate suppression of rapid pulsing artifacts (Derome & Williamson, 1990). Two-dimensional spectra were typically acquired with 48 or 64 scans of 2048 complex data points per increment and 450–512 increments per data set. Spin-locking was achieved in TOCSY spectra using MLEV-17 (Bax & Davis, 1985), and mixing times varied from 44 to 176 ms. Mixing times in NOESY and ROESY spectra ranged from 80 to 300 ms. Data were processed and analyzed using the NMRZ package from New Methods Inc., Syracuse, NY.

The dissociation constant for the SLeX/E-selectin interaction was determined by recording a series of NOESY spectra with 5 μM protein and at ligand concentrations ranging from 0.44 to 3.85 mM. Under these conditions the total ligand concentration $[L]_T$ approximates the free ligand concentration [L], and the dissociation constant K_D can be expressed as

$$K_D = \frac{[P]_T[L]_T}{[PL]} - [L]_T$$

where $[P]_T$ is the total protein concentration and [PL] is the concentration of the protein/ligand complex. This can be rearranged to yield

$$[L]_T = \frac{[P]_T[L]_T}{[PL]} - K_D$$

The relative concentration of the complex, k[PL], can be determined from the cross-peak intensity in the NOESY spectrum. Plotting $[P]_T[L]_T$/[cross-peak intensity] versus $[L]_T$ yields a straight line with an intercept of $-K_D$. Cross-peak intensities from NOESY spectra were determined using the manual integration facility within NMRZ and scaled to the intensities of resonances from 100 μM imidazole added as a standard. The contributions from the free ligand were estimated from the ratio of cross-peak to diagonal-peak intensities in the NOESY spectrum of the ligand, multiplied by the intensity of the related diagonal peak in the NOESY spectrum of the complex. Data were analyzed using the linear regression routine within Microsoft EXCEL.

Molecular modeling was performed using the software packages QUANTA and CHARMM from Molecular Simulations Inc. and SYBYL from Tripos Associates running on Silicon Graphics Personal IRIS or INDIGO workstations. A model for the lectin domain of human E-selectin was constructed from the X-ray structure of rat mannose binding protein (Weis *et al.*, 1991) using QUANTA. The sequences were aligned so that insertions and deletions were restricted to surface regions of MBP, in turns where possible: residues 1–43, 47–56, 57–66, 70–96, and 102–121 of E-selectin were aligned with residues 110–152, 153–162, 165–174, 175–201, and 202–221 of MBP, respectively. Coordinates for corresponding atoms were copied from the MBP structure to the E-selectin model, the insertions and deletions were manually manipulated to give reasonable geometries, and the model was subjected to conjugate gradient minimization using CHARMM. A calcium ion was inserted in a position corresponding to cation site 2 reported by Weis *et al.* (1991), and the protein was further energy minimized.

RESULTS AND DISCUSSION

The ^{1}H NMR assignments for SLeX obtained using DQF-COSY, TOCSY, and ROSEY spectra were found to be in general agreement with those reported by Lin *et al.* (1992), although a second set of GlcNAc resonances were observed due to the presence of both α and β anomers of this ring. The anomeric mixture does not affect resonances of other rings, and thus their average conformations, consistent with suggestions that the GlCNAc ring is not important for activity (Tyrrell *et al.*, 1991). As anticipated for a molecule of this size, cross-peak intensities in NOESY spectra are extremely weak, in accordance with the observations of Ball *et al.* (1992) and Lin *et al.* (1992). Several interresidue cross peaks were observed in ROESY spectra, and connectivities were identified across glycosidic linkages between the galactosyl H1 resonance and the α-GlcNAc and β-GlcNAc H4 resonances and between the fucosyl H1 resonance and the β-GlcNAc H3 resonance (the connectivity to the α-GlcNAc H3 resonance was obscured). Unambiguous ROEs were also identified between the galactosyl H3 and sialyl $H3_{ax}$ resonances, the fucosyl CH_3 and galactosyl H2 resonances, the fucosyl H5 and galactosyl H2 resonances, and the fucosyl H1 and GlcNAc CH_3 resonances. Each of these connectivities has been reported previously (Ball *et al.*, 1992; Ichikawa *et al.*, 1992; Lin *et al.*, 1992).

Essentially identical chemical shifts and ROESY cross peaks were observed for SLeX in the presence of E-selectin. NOESY cross peaks between SLeX resonances were also clearly observed in the presence of E-selectin, at higher intensity than in the absence of protein. The enhanced NOE intensities in the presence of E-selectin are indicative of a transferred NOE (tNOE) situation (Balaram & Bothner-By, 1972), in which the lifetime of the ligand bound to a more slowly tumbling protein allows NOEs to develop. It should be noted that the relative concentrations of protein and ligand used in the studies are more in keeping with those favored by Campbell and Sykes (1993) for observaton of tNOEs via NOESY spectra, rather than those utilized by Clore and Gronenborn (1982) for 1D steady-state tNOEs.

The intensities of several NOESY cross peaks for SLeX resonances in the presence and absence of E-selectin are compared in Table 1. Minimal NOEs for sialyl Lewis X were observed when bovine serum albumin was substituted for E-selectin, suggesting that sample viscosity could not account for the enhanced NOE intensity. Extremely weak NOEs were observed for a solution of E-selectin alone, but not at the same location as those assigned to SLeX resonances. The intensities of the SLeX NOESY cross peaks are reduced by the addition of EDTA, consistent with the reported calcium dependence of the interaction. The intensities of ROESY cross peaks for each solution are included for comparison in Table 1—their lack of variation indicates little variation in the bulk ligand.

Transferred NOEs are typically observed where the affinity between ligand and protein is in the micromolar to millimolar range (Clore & Gronenborn, 1982). The dissociation constant for E-selectin and SLeX was estimated from the variation (with SLeX concentration) in the intensities of NOESY cross peaks between the following resonances (listed as F_2–F_1): sialyl $H3_{ax}$–$H3_{eq}$; sialyl $H3_{ax}$–H5; sialyl $H3_{eq}$–$H3_{ax}$; α-GlcNAc H2–H1; galactosyl H3–H1 (Figure 1). Regression analysis gave

Table 1: Intensities of Representative Cross Peaks in Overhauser Spectra of Sialyl Lewis X[a]

	SLeX + E-selectin[b]		SLeX[c]		SLeX + E-selectin + EDTA[d]		SLeX + BSA[e]	
resonance	ROESY	NOESY	ROESY	NOESY	ROESY	NOESY	ROESY	NOESY
Sial $H3_{ax}$–$H3_{eq}$	0.409	0.136	0.424	0.048	0.393	0.043	0.369	0.030
Sial $H3_{ax}$–H5	0.045	0.044	0.032	nm	0.040	nm	0.032	nm
Sial $H3_{ax}$–Gal H3	0.029	nm	0.042	nm	0.031	nm	0.030	nm
Gal H1–H5	0.092	0.051	0.081	0.022	0.065	0.039	0.080	nm
Gal H2–Fuc Me	0.040	0.024	0.039	nm	0.034	nm	0.039	nm
Gal H2–Fuc H5	0.025	0.011	0.022	nm	0.030	nm	0.024	nm
Gal H3–H1	0.058	0.025	0.063	0.017	0.055	0.022	0.053	nm

[a] Peak intensities were measured by manual integration within NMRZ. Cross peaks are labeled with the format $F_2 - F_1$, and their intensities are normalized by dividing by the intensity of the diagonal resonance with the same F_2 frequency. Errors in these values are approximately ±0.01. nm = not measurable. [b] Solution contained 21 μM E-selectin, 1.55 mM SLeX, 1 mM d-Tris, and 10 mM $CaCl_2$, pH 7. [c] Solution contained 1.62 mM SLeX, 1 mM d-Tris, and 10 mM $CaCl_2$, pH 7. [d] Solution contained 19 μM E-selectin, 1.41 mM SLeX, 1 mM d-Tris, 10 mM $CaCl_2$, and 15.5 mM EDTA, pH 7. [e] Solution contained 25 μM BSA, 1.5 mM SLeX, 1 mM d-Tris, and 10 mM $CaCl_2$, pH 7.

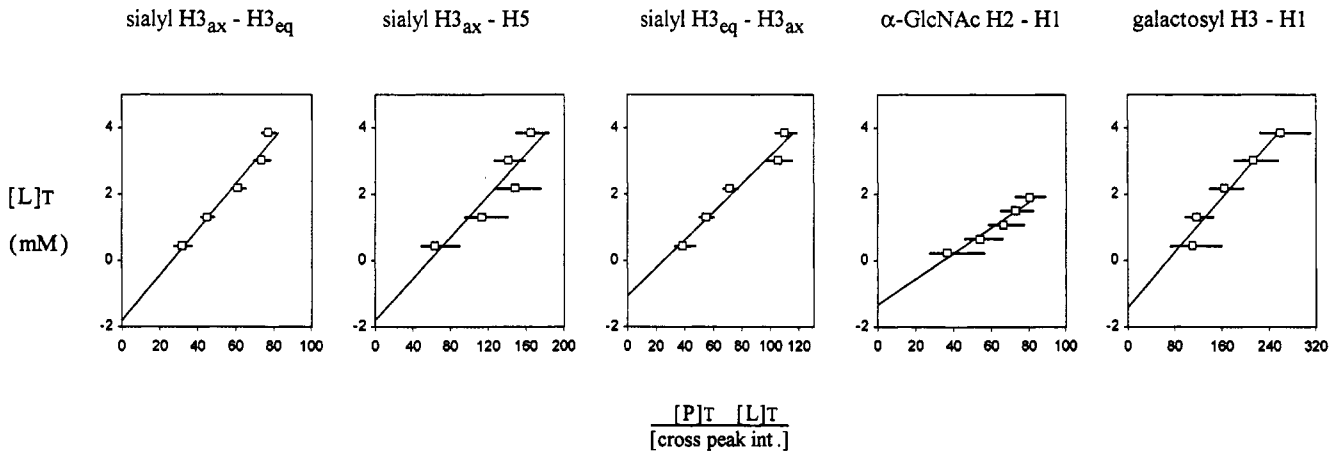

FIGURE 1: Variation in NOESY cross-peak intensities with ligand concentration. Cross peaks were analyzed as described in Experimental Procedures. Cross peaks are referred to by the format $F_2 - F_1$. The x axis is dimensionless as it is partly described by cross-peak intensity. K_D is determined from the absolute value of the y intercept.

values for the K_D of 1.8 ± 0.4, 1.8 ± 1.0, 1.1 ± 0.4, 1.3 ± 0.3, and 1.4 ± 0.5 mM, respectively. The affinity of E-selectin for sialyl Lewis X has been reported to be 750 μM (Nelson *et al.*, 1993) using an ELISA system.

The pattern of cross peaks in NOESY spectra of SLeX with E-selectin is generally similar to the pattern of ROESY cross peaks. In particular, interresidue NOEs are observed between the galctosyl H2 resonance and the fucosyl H5 and CH_3 resonances. An additional cross peaks between the fucosyl H5 and galactosyl H6 resonances appears in the NOESY spectrum but not in the ROESY; this occurs at mixing times of 80, 150, and 300 ms. Other cross peaks are also present in NOESY and not ROESY spectra, but only at the longest mixing time, suggesting they result from spin diffusion. The most significant absence in the NOESY spectrum is the cross peak between the sialyl $H3_{ax}$ and galactosyl H3 resonances which is observed in the ROESY spectrum (Figure 2). This difference occurs at mixing times of 80, 150, and 300 ms.

The concentrations of ligand and protein in these experiments imply that connectivities present in ROESY spectra represent predominantly the free ligand, due to the overwhelming proportion of free to bound ligand. This is supported by the fact that chemical shifts and ROESY cross-peak intensities were reasonably independent of other components in solution (Table 1 and Figure 2). The fact that negligible NOEs are observed for the free ligand, however, suggests that cross peaks in NOESY spectra of the ligand/protein mixture are representative of the bound form of SLeX, developing via the transferred NOE phenomenon. This is consistent with the observation that NOESY cross peaks for SLeX are minimal except in the presence of E-selectin and the absence of EDTA. The differences in cross-peak patterns between NOESY and ROESY spectra thus arise from different conformations for the free and bound forms of SLeX. NOEs between the galactosyl and fucosyl resonances in the bound form of SLeX suggest the stacking of these rings is similar to that in the solution state. The absence of an NOE between the sialyl and galactosyl resonances, however, suggests the relative orientation of these rings changes upon binding.

The solution conformations for SLeX so far reported have been in general agreement (Ball *et al.*, 1992l; Ichikawa *et al.*, 1992; Lin *et al.*, 1992), but it is important to note that these have been generated with ROE restraints, including one between the sialyl and galactosyl rings. This ROE was observed in the current study, but comparing its intensity (at 80-ms mixing time) with intraresidue ROEs between the galactosyl H1, H3, and H5 resonances, where distances are known, suggested a separation for the sialyl $H3_{ax}$ and galactosyl H3 protons of 2.6–3.0 Å. This is significantly greater than the separation of approximately 2 Å predicted from the published models (Ichikawa *et al.*, 1992; Lin *et al.*, 1992). The reduction in ROE intensity could result from specific mobility about the galactosyl–sialyl link and possibly population of other conformational states. Flexibility about the galactosyl–sialyl link has been suggested by NMR and molecular dynamics studies of SLeX in solution (Mukhopadhyay *et al.*, 1994; Rutherford *et al.*, 1994). Calculations suggesting a single solution conformation for SLeX may be influenced by including ROE restraints which are appropriate

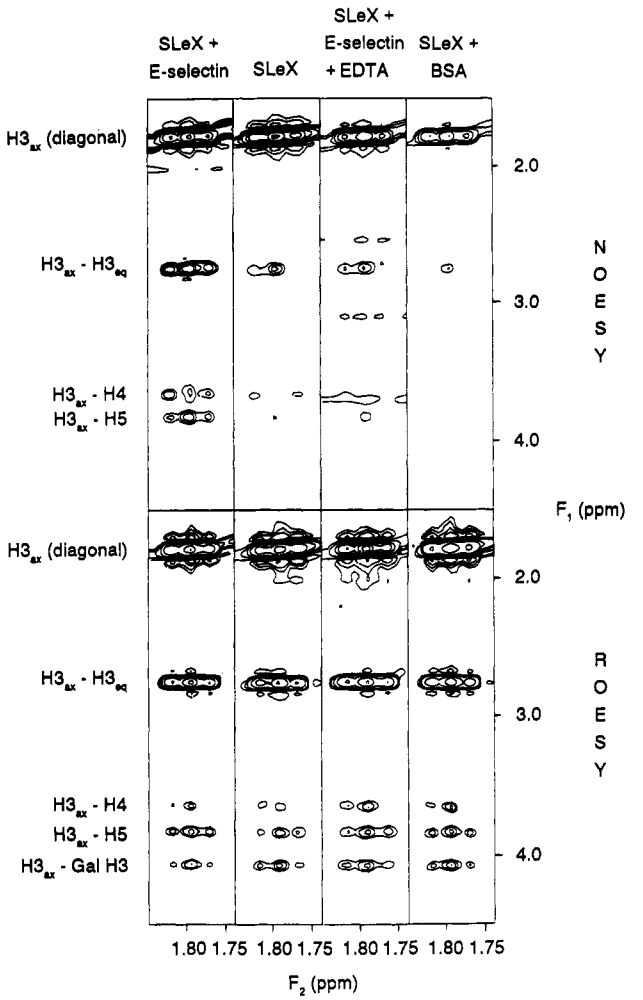

FIGURE 2: Portions of NOESY (upper row) and ROESY (lower row) spectra for the solutions of SLeX described in Table 1 (except for the solution of SLeX alone in which its concentration was 0.49 mM). In each case the diagonal peak corresponding to the sialyl $H3_{ax}$ resonance and cross peaks involving it are displayed.

for only part of the population.

The existence of multiple solution conformations for SLeX was considered by Ichikawa *et al.* (1992), who, using the GESA algorithm, generated four low-energy structures (designated GESA-A to -D) in the absence of ROE restraints. These differed mainly in the conformation of the galactosyl–sialyl link, with GESA-A and GESA-B approximating the conformations consistent with observed ROE restraints (Ball *et al.*, 1992; Ichikawa *et al.*, 1992; Lin *et al.*, 1992). Conformational analysis of SLeX *in vacuo*, using the SYBYL force field, produced two distinct families (S. G. Lister, unpublished observations): the fucosyl–GlcNAc–galactosyl core in all cases was similar to the GESA structures, but the galactosyl–sialyl link in one family approximated GESA-B, while in the other it approximated GESA-C (Figure 3). A recently reported molecular dynamics simulation of SLeX in solution utilizing NMR restraints also produced one cluster of low-energy conformations with galactosyl–sialyl dihedral angles similar to GESA-B and another cluster similar to GESA-C (Rutherford *et al.*, 1994). The GESA-C and GESA-D structures were discounted by Ichikawa *et al.* (1992) because they would not display Overhauser effects between the sialyl $H3_{ax}$ and galactosyl H3 resonances. These structures thus meet the transferred NOE criterion characteristic of the bound form of SLeX. GESA-D, however, would be further characterized by NOEs between the sialyl $H3_{eq}$ resonance and the galactosyl H3 and H4 resonances; as neither of these is observed, this is unlikely to represent the bound conformation. GESA-C (Figure 3), which fits the observed data for the bound form of SLeX, is thus deemed to be closest to the "bound" conformation, although it could be somewhat modified by interactions with the protein. E-Selectin may recognize the bound conformation from several interconverting species—other conformations of SLeX must be present in solution to give rise to the sialyl $H3_{ax}$–galactosyl H3 ROE. Recognition of a single oligosaccharide conformation from a solution ensemble has been inferred from NMR studies of a disaccharide/antibody complex (Glaudermans *et al.*, 1990) and the X-ray structure of a biantennary oligosaccharide with a plant lectin (Bourne *et al.*, 1992).

Models of SLeX, based on the dihedral angle restraints for the GESA structures of Ichikawa *et al.* (1992), were constructed using SYBYL and docked with the model of E-selectin using QUANTA. Weis *et al.* (1992) suggested that one of the two calcium binding sites in MBP is maintained in selectins and SLeX binds to this via the 2-OH and 3-OH groups of the fucosyl ring. Attempts to dock the GESA-C structure of SLeX with the E-selectin model starting from this premise gave unsatisfactory results on two grounds: (i) the glycosidic link from C1 of the GlcNAc ring was directed toward the core of the protein, from where it could not lead to the remainder of the glycoconjugate; (ii) the sialyl ring was remote from the protein, which could not explain its requirement for activity (Tiemeyer *et al.*, 1991). More satisfactory models were obtained when the 3-OH and 4-OH of the fucosyl ring were coordinated to the calcium. In these cases the C1 glycosidic link from the GlcNAc was directed away from the protein and the sialic acid was located near Tyr 48, Lys 111, and Lys 113; these residues are conserved in all known selectin sequences, and their importance in ligand recognition has previously been implicated (Erbe *et al.*, 1992, 1993; Hollenbaugh *et al.*, 1993; Bajorath *et al.*, 1994). The likely interaction of another functionally important residue, Tyr 94, with the galactosyl residue can be predicted from the X-ray structure of E-selectin (Graves *et al.*, 1994). It should be noted, however, that Graves *et al.* (1994) used the solution conformation of SLeX for modeling the SLeX/E-selectin complex, which should be modified in the disposition of the sialyl ring. Although the resolution of our model is limited, it appears that binding the GESA-A or GESA-B conformation of SLeX would produce unfavorable steric interactions between the glycerol and amide substituents of the sialyl ring and the protein, while with GESA-C, which approximates the bound form, these substituents are directed toward solvent, consistent with suggestions that they are not required for activity (Tyrrell *et al.*, 1991).

CONCLUSIONS

The results presented in this paper provide the first direct measurement of the dissociation constant for the sialyl Lewis X/E-selectin complex and the first example of the use of transferred NOEs for such measurements. The Overhauser enhancements differ for free and complexed sialyl Lewis X, suggesting the oligosaccharide conformation changes upon binding or that only one of a number of solution species is bound. Comparison of the experimental results with previously reported low-energy structures enabled identification of a conformation of sialyl Lewis X likely to approximate the bound form.

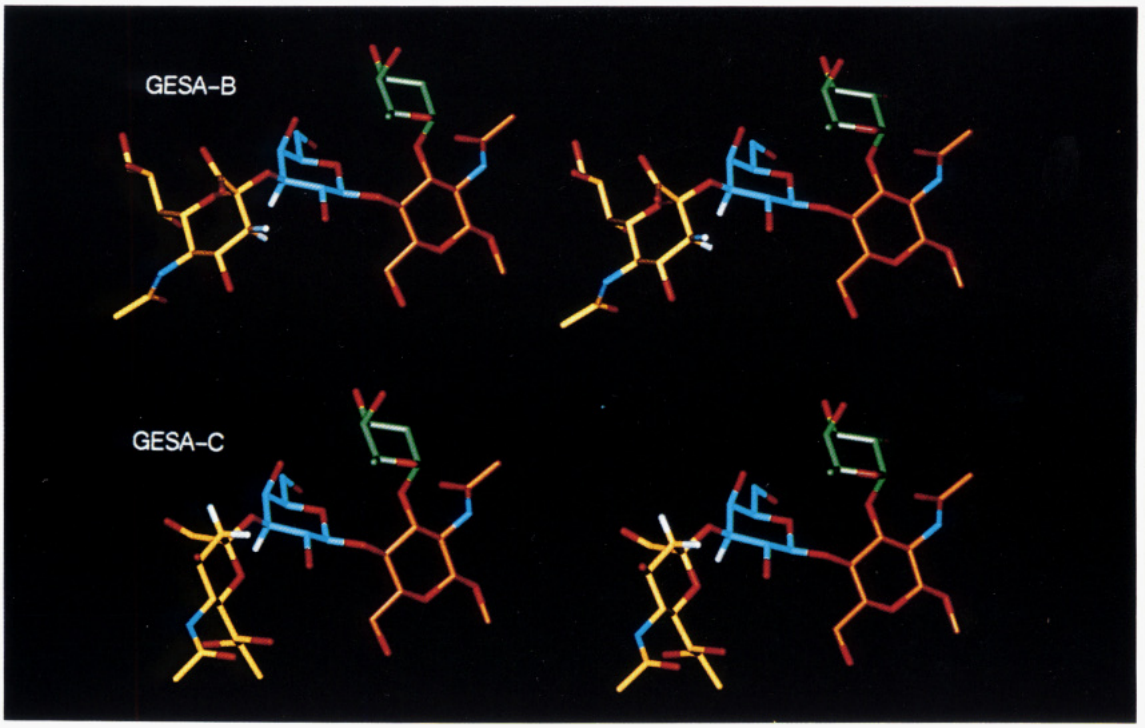

FIGURE 3: Stereoviews of the conformations of SLeX described by Lin *et al.* (1992) as GESA models B and C. Carbon atoms of each residue are colored as follows: sialyl, yellow; galactosyl, blue; fucosyl, green; GlcNAc, orange. The only hydrogen atoms shown are those on the C3 atoms of the galactosyl and sialyl rings. The GlcNAc is shown as a methoxy derivative at the anomeric position (β configuration). The fucosyl–GlcNAc–galactosyl cores are virtually identical in the two structures, but the ϕ and ψ dihedral angles for the sialyl–galactosyl link are respectively – 170° and –8° in GESA-B and –79° and 7° in GESA-C (ϕ is defined by Sial C1–Sial C2–O–Gal C3; ψ is defined by Sial C2–O–Gal C3–Gal H3). These dihedral angles are similar to those reported for minima B and A by Rutherford *et al.* (1994).

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SUPPLEMENTARY MATERIAL AVAILABLE

The amino acid sequence of the modified E-selectin used in these studies (1 page). Ordering information is given on any current masthead page.

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