Core Sugar Residues of the *N*-Linked Oligosaccharides of Russell's Viper Venom Factor X-Activator Maintain Functionally Active Polypeptide Structure

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ABSTRACT: We previously showed that the factor X activator of Russell's viper venom (RVV-X) contains six *N*-linked oligosaccharide chains: four in the heavy chain and one in each of the two light chains [Gowda, D. C., Jackson, C. M., Hensley, P., & Davidson, E. A. (1994) *J. Biol. Chem. 269*, 10644−10650).] In the present study, we have investigated the role of the carbohydrate moieties in the structure and functional activity of RVV-X. Sequential removal of sugar residues from the terminal ends by exoglycosidases, up to 50% of total carbohydrates, did not significantly alter the activity of RVV-X, demonstrating that the peripheral carbohydrate moieties are not involved in interactions with factor X. However, removal of whole oligosaccharide chains by *N*-glycanase caused an almost total loss of the ability of RVV-X to activate factor X to factor X_a. In parallel with these observations, circular dichroism spectroscopy showed that complete deglycosylation, but not the removal of peripheral sugars, caused a significant change in the secondary structure. Together, these data demonstrate that the oligosaccharide chains are necessary for the functional activity, and that the trimannosylchitobiose core residues are sufficient for the maintenance of the native polypeptide structure.

The carbohydrate moieties of glycoproteins are involved in a wide variety of biological functions (Dwek et al., 1993; Varki, 1993). In *N*-linked oligosaccharides, the outer sugar residues (antennae) often serve as recognition markers and modulate biological functions through surface interactions. The core sugar residues may then function primarily as supporting structures between the polypeptides and the outer sugar residues. However, recent studies suggest that in some glycoproteins the core sugar residues are required for the structural integrity of the polypeptide. For example, Wyss et al. (1995) have shown that the chitobiose core of the *N*-linked carbohydrate moiety of the cell-adhesion domain of human CD2 increases the stability and rigidity of the glycoprotein through a series of interactions with amino acids that are on a lysine-rich surface. Similarly, single *N*-acetylglucosamine residues at Asn^{152} and Asn^{173} are sufficient to support the functional ligand binding activity of rat luteinizing hormone receptor (Zhang et al., 1995). Here, we show that the core sugar residues of the *N*-linked oligosaccharides of the factor X activator of Russell's viper venom (RVV-X)[1] are also involved in maintaining functional polypeptide structure.

RVV-X is a glycoprotein that can activate coagulation factor X to X_a (Kisiel et al., 1976; Stocker, 1990; Takeya et al., 1992). This process involves cleavage of the same internal peptide bond (between Arg^{51} and Ile^{52}) as that cleaved by factor IX_a or by factor VII_a during physiological blood coagulation (Jackson & Nemerson, 1980). RVV-X is a 93 kDa glycoprotein that consists of three disulfide linked polypeptide chains: one heavy chain (α-chain, M_r 57 600) and two light chains (β-and γ-chains, M_r 19 400 and 16 400, respectively) (Gowda et al., 1994). RVV-X contains six *N*-linked oligosaccharides, four in the α-chain and one in each of the β- and γ-chains.

RVV-X is multifunctional. In addition to being a potent activator of factor X, RVV-X has been demonstrated to be a platelet aggregation inhibitor (disintegrin-like) (Takeya et al., 1992). Consistent with these properties, the sequence of the α-chain indicates the presence of three distinct domains: a metalloproteinase-like domain, a disintegrin-like domain, and a functionally unknown cysteine-rich domain. While the sequence of the β-chain is unknown, the γ-chain has homology to Ca^{2+}-dependent lectins. The activation of factor X is inhibited by a snake venom factor IX/factor X-binding protein with a C-type lectin structure (Atoda et al., 1991), suggesting that the γ-chain of RVV-X may participate in the recognition of factor X (Takeya et al., 1992).

Experimentally, site-directed mutagenesis has been a powerful approach to establish the roles of *N*-linked oligosaccharides but cannot be used to determine the contribution of individual sugars within the oligosaccharide chain. For analyzing the role of the core sugar residues, exoglycosidase

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[1] Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; *N*-glycanase, peptide-N^4-2-(*N*-acetyl-β-glucosaminyl)asparagine amidase; PAGE, polyacrylamide gel electrophoresis; RVV-X, factor X activating glycoprotein from Russell's viper venom; X, factor X; X_a, activated factor X.

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treatment remains the method of choice. However, this approach is not always feasible because even the outer sugar residues (except for the terminal residues) are frequently resistant to exoglycosidases. With RVV-X, we found that the majority of the antennae sugar residues can be removed by sequential treatment with exoglycosidases. After each treatment with a specific exoglycosidase, or the removal of whole carbohydrate chains by *N*-glycanase, the functional activity was measured. We also performed circular dichroism spectroscopy so as to correlate changes in the functional property with structural changes. Our results demonstrate that the core oligosaccharide chains of RVV-X are necessary and sufficient for the maintenance of both polypeptide structure and the ability to activate factor X.

EXPERIMENTAL PROCEDURES

Materials. RVV-X from *Vipera russelli russelli* venom and factor X from bovine serum were isolated as described previously by Kisiel et al. (1976) and Jackson et al. (1968), respectively. Concentrations were determined using extinction coefficients at 280 nm of $E^{1\%} = 14.7$ (Jackson et al., 1968) and $E^{1\%} = 12.4$ (Jackson et al., 1971) for RVV-X and factor X, respectively. Jack bean β-galactosidase (30 units/mg), bovine testis β-galactosidase (20 units/mg), chicken liver β-galactosidase (12 units/mg), jack bean β-*N*-acetylhexosaminidase (30 units/mg), jack bean α-mannosidase (25−30 units/mg), *Helix pomatia* β-mannosidase (70 units/mg), and *Clostridium perfringens* neuraminidase (250 units/mg) were from Oxford Glycosystems (Rosedale, NY). The glycan differentiation kit, peptide-*N*-glycosidase F (*N*-glycanase), rat liver 2,6-sialyltransferase, and CMP-*N*-acetylneuraminic acid were from Boehringer Mannheim (Indianapolis, IN).

Gel Electrophoresis. Discontinuous SDS−PAGE was performed according to Laemmli (1970). Samples were dissolved in 60 mM Tris-HCl/2% SDS/0.5 M 2-mercaptoethanol/10% glycerol (pH 6.8) and heated in a boiling water bath for 5 min.

Carbohydrate Analysis. RVV-X, deglycosylated RVV-X, and exoglycosidase-treated RVV-X (10−20 μg each) were hydrolyzed in 0.4 mL of 2.5 M trifluoroacetic acid at 100 °C for 4−5 h. The hydrolysates were evaporated to dryness in a Speed-Vac and then dissolved in water and analyzed for neutral sugars and hexosamines. Samples were also hydrolyzed in 0.1 M sulfuric acid at 80 °C for 1 h, neutralized with 0.2 M NaOH, and analyzed for sialic acid. Analyses were performed with a Dionex BioLC HPLC system (Dionex Corp., Sunnyvale, CA) coupled to pulsed amperometric detection using a CarboPac PA1 high-pH anion-exchange column (4 250 mm) (Hardy & Townsend, 1994). The eluents were (a) 20 mM NaOH for neutral sugars and hexosamines and (b) 100 mM NaOH/150 mM NaOAc for sialic acids. Chromatography peaks were quantitated using standard solutions of monosaccharides.

Treatment with Exoglycosidases. Treatment with exoglycosidases was performed by sequentially incubating 1−2 mg/mL solution of RVV-X with (i) *C. perfringens* neuraminidase (1−1.5 units/mL) in 50 mM sodium acetate (pH 5.5) at 37 °C for 4 h, (ii) bovine testis β-galactosidase (1−2 units/mL) in 50 mM sodium citrate/sodium phosphate (pH 6.0) at 37 °C for 24 h, (iii) jack bean β-*N*-acetylhexosaminidase (5 units/mL) in 50 mM sodium citrate/sodium phosphate (pH 6.0) at 37 °C for 48 h, (iv) *H. pomania* α-mannosidase (6 units/mL) in 50 mM sodium citrate/sodium phosphate (pH 6.0) at 37 °C for 36 h, and (v) jack bean β-mannosidase (1.2 units/mL) in 50 mM sodium citrate/sodium phosphate (pH 6.0) at 37 °C for 24 h.

Enzyme-treated RVV-X samples were filtered using Centricon-30 centrifugal microconcentrators (Amicon, Danvers, MA). Filtrates were then analyzed for the released sugars by high-pH anion-exchange chromatography using a Dionex BioLC HPLC system (Hardy & Townsend, 1994). Aliquots of the retentates were precipitated with 4 volumes of methanol and then analyzed for sugar composition as described above. Appropriate aliquots of the glycosidases were separately analyzed for their contribution to the total sugar composition. For circular dichroism spectroscopy, a second aliquot of each retentate was repurified on a 3 mL column of QAE-Sephadex A-50.

Sialylation of RVV-X. RVV-X was sialylated using CMP-*N*-acetylneuraminic acid and rat liver 2,6-sialyltransferase as described previously (Weinstein et al., 1982; Gowda et al., 1994).

Deglycosylation of RVV-X. RVV-X (1 mg/mL in 0.2 M sodium phosphate, pH 8.0) was incubated with peptide-*N*-glycosidase F (*N*-glycanase) (60 units/mL) at 37 °C for 40−48 h (Maley et al., 1989). The sample was then dialyzed against 50 mM HEPES/100 mM NaCl (pH 7.4).

Circular Dichroism Spectroscopy. Circular dichroism measurements were made in a Jasco J-500C spectropolarimeter, equipped with a DP-500N data processor (Japan Spectroscopic Co., Easton, MD). Protein solutions (0.2 mg/mL) in 50 mM sodium phosphate (pH 7.2) were held in 1 mm path length quartz cuvettes. The instrument was calibrated with a 0.7 mg/mL solution of L-10 camphorsulfonic acid (Chen & Yang, 1977). Each sample was scanned four times, with a time constant of 8 s, averaged, and corrected for baseline rotation. Signals were digitized at 0.22 nm intervals by a Q-3010 A/D converter (Quasitronics Inc., Houston, PA). Data were converted to mean residue ellipticity using the formula, [Ø] = {millidegrees (mean residue weight)} {path length concentration (mg/mL) 10}. The units of [Ø] are degrees cm^2 dmol^{-1}. Spectra were analyzed using the Contin (Provencher & Glockner, 1981) and Yang (Yang et al., 1986) programs.

Chromogenic Substrate Assay for Factor X Activation. Activation reactions were performed at 25 °C in 50 mM HEPES/100 mM NaCl/10 mM $CaCl_2$ (pH 7.4). To avoid adsorptive losses of Xa, reaction tubes were coated with 0.1% aqueous poly(ethylene glycol) (M_r 20 000) for 1−2 h, aspirated, and dried at 80 °C. For functional assays after treatment with exoglycosidases, factor X was 200, 1000, and 2500 nM. The concentration of factor X in other experiments is as specified in Figures 2 and 3. Factor X was activated with 0.1, 0.05, or 0.02 nM RVV-X. At the indicated times, 0.1 mL aliquots were quenched by rapid mixing with 0.1 mL of 50 mM HEPES/100 mM NaCl/50 mM EDTA (pH 7.4). Activation was then assessed by measuring the amount of active factor X_a, as follows. The quenched reaction solution was mixed with 20 volumes of 50 mM HEPES/0.1 M NaCl/0.5 mg/mL Brij 35/0.12 mM H,D-cyclohexylglycyl-Ala-Arg-*p*-nitroanilide (Pefachrome TH, Pentapharm Ltd., Basel) (pH 7.8) and incubated at 37 °C for 3 min. The formation of *p*-nitroaniline was quantified at 405 nm using an extinction coefficient of 9920 M^{-1} cm^{-1}.

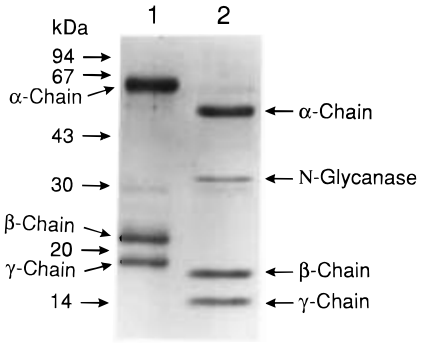

FIGURE 1: SDS−PAGE of RVV-X and deglycosylated RVV-X. Untreated RVV-X (5 μg, lane 1) and *N*-glycanase-treated RVV-X (5 μg, lane 2) were analyzed on a 12% SDS−polyacrylamide gel and then stained with Coomassie Blue. The positions of standard proteins are indicated on the left.

Table 1: Carbohydrate Composition of RVV-X before and after Deglycosylation with *N*-Glycanase

	carbohydrate content (pmol/μg of protein)	
	untreated RVV-X	*N*-glycanase-treated RVV-X
fucose	52	3
N-acetylglucosamine	351	1
galactose	177	5
mannose	188	8
N-acetylneuraminic acid	150	nd[a]

[a] nd, not determined.

Enzyme Kinetics. Initial velocities of X_a formation were estimated by linear regression. K_m and V_{max} were estimated by nonlinear least-squares regression to the Michaelis−Menten equation using MINSQ software (MicroMath Scientific Software, Salt Lake City, UT), as described previously (Carlisle et al., 1990).

RESULTS

Deglycosylation of RVV-X. *N*-Glycanase is a peptide *N*-glycosidase that is capable of removing both complex and high mannose type *N*-linked oligosaccharides from proteins. Deglycosylation of RVV-X by *N*-glycanase under nondenaturing conditions caused considerable shifts to lower apparent molecular weights for all three polypeptide chains (Figure 1). The observed mobilities closely correspond to the predicted molecular weights of 48 000, 17 000, and 14 000, for the deglycosylated α-, β-, and γ-chains, respectively (Gowda et al., 1994). There was only a trace amount of material at the mobility of the glycosylated α-chain, and no detectable material at the mobilities of the glycosylated β- and γ-chains, indicating a near complete level of deglycosylation.

The extent of deglycosylation of RVV-X upon treatment with *N*-glycanase was also determined by carbohydrate compositional analysis by high-pH HPLC using sensitive pulsed amperometric detection. Quantitative estimation of the sugars released by acid hydrolysis of the deglycosylated RVV-X revealed that *N*-glycanase removed >95% of the oligosaccharide chains (Table 1).

Activation of Factor X by RVV-X and Deglycosylated RVV-X. To determine the functional role of the carbohydrate moieties, the deglycosylated RVV-X was analyzed for its ability to activate factor X to X_a. The formation of X_a from factor X was measured at three different concentrations of either buffer-treated or deglycosylated RVV-X. The formation of X_a was linear with time and with the concentration of either form of RVV-X (Figure 2A). However, the rate of X_a formation by deglycosylated RVV-X was about 130-fold lower than for the buffer-treated sample (Figure 2B), indicating that the carbohydrate moieties are required for the functional activity.

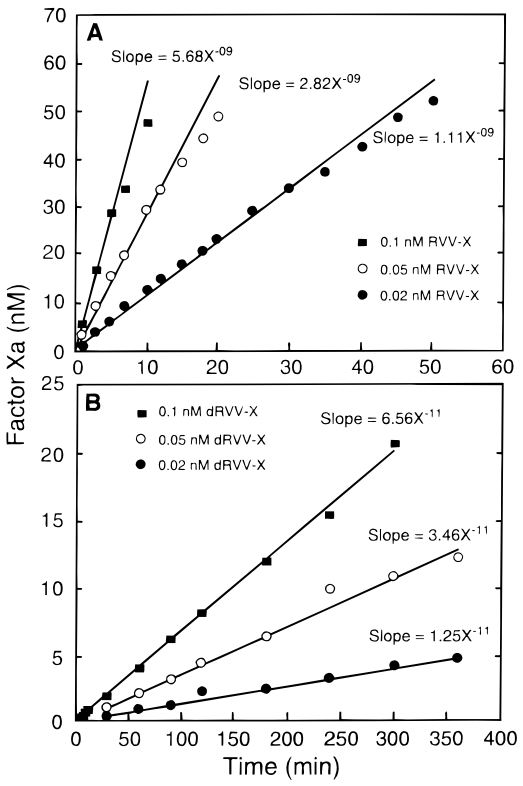

FIGURE 2: Activation of factor X by RVV-X and deglycosylated RVV-X. Factor X (200 nM) was activated with 0.02, 0.05, or 0.1 nM of either RVV-X (panel A) or deglycosylated RVV-X (panel B). The amount of X_a that was formed was measured at indicated time points using a synthetic peptide as a chromogenic substrate. The slope of the curves are in units of M^{-1} min^{-1}.

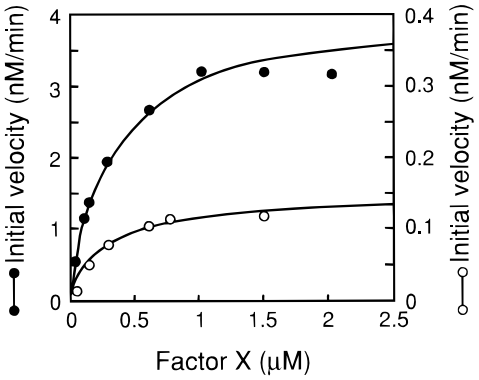

FIGURE 3: Activation of factor X by RVV-X and deglycosylated RVV-X. Factor X was activated with 0.02 nM of buffer-treated RVV-X (closed circles; left *y*-axis) or 0.1 nM of deglycosylated RVV-X (open circles; right *y*-axis). The velocity of formation of X_a is plotted against the initial concentration of factor X. Note that 5-fold more deglycosylated RVV-X was employed and that the right hand *y*-axis (deglycosylated RVV-X) has been expanded 10-fold relative to the left *y*-axis (buffer-treated RVV-X).

The kinetic constants for the activation of factor X were determined (Figure 3). The system obeyed Michaelis−Menten kinetics. With factor X as the substrate, the K_m for untreated RVV-X and deglycosylated RVV-X were similar

Table 2: Activity Analysis of Exoglycosidase-Treated RVV-X[a]

exoglycosidase	relative activity[b]
none	(1.0)
C. perfringens neuraminidase	1.0
bovine testis β-galactosidase	1.1
jack bean *N*-acetylhexosaminidase	1.0
jack bean α-mannosidase	0.9

[a] The activity was measured using three different concentrations of RVV-X as shown in Figure 2. [b] Ratio of activity (ratio of slopes of the time course of factor X to X_a activation) of exoglycosidase-treated RVV-X to that of control RVV-X.

(300 and 250 nM, respectively). Thus, the major difference is that the observed k_{cat} for untreated RVV-X (200 s^{-1}) was much higher than that for deglycosylated RVV-X (1.45 s^{-1}).

Sequential Removal of Sugar Residues of RVV-X by Exoglycosidases. As shown above, the near complete removal of the oligosacharides of RVV-X resulted in an almost total loss of function. In order to further characterize the importance of the oligosaccharides, RVV-X was treated with a series of exoglycosidases to sequentially remove the peripheral sugar residues.

Previously, we have shown using three different sialidases, *V. cholerae* neuraminidase, *C. pefringens* neuraminidase, and Newcastle virus disease neuraminidase, that RVV-X can be completely desialylated using the *C. perfringens* enzyme (Gowda et al., 1994). Two consecutive treatments with *C. perfringens* neuraminidase gave completely desialylated RVV-X.

After removal of the terminal sialic acid residues, the exposed β-galactosyl residues in RVV-X were released by β-galactosidase. In a preliminary experiment, β-galactosidase from bovine testis, jack bean, and chicken liver were compared. These removed 11.2, 3.4, and 1.7 mol of galactose per mol of RVV-X, respectively. Bovine testis β-galactosidase was therefore employed. Two consecutive treatments removed about 90% of the total β-galactosyl residues in RVV-X.

Treatment with jack bean β-*N*-acetylhexosaminidase removed approximately 16.7 mol of *N*-acetylglucosaminyl residues per mol of RVV-X. This corresponds to about 50% of the total *N*-acetylglucosaminyl residues (Gowda et al., 1994). A second treatment removed an additional 5% of the total *N*-acetylglucosamine. The sample was then treated with jack bean α-mannosidase and then with *H. pomatia* β-mannosidase. Jack bean α-mannosidase removed 5−6 mol of mannose per mol of RVV-X. *H. pomatia* β-mannosidase released only 1−2 mol of mannose per mol of RVV-X. Further removal of core sugars by exoglycosidases was not possible.

Activation of Factor X by Exoglycosidase-Treated RVV-X. RVV-X, treated as described above with exoglycosidases, was analyzed for the ability to activate factor X (Table 2). Removal of all sialic acid residues, 90% of β-galactosyl residues, and 55% of *N*-acetylglucosaminyl residues did not affect the activity of RVV-X. The activity was also essentially unchanged (the decrease was about 10%) when some of the core mannosyl residues were removed (Table 2).

Activation of Factor X by Sialylated-RVV-X. RVV-X contains a significant amount (2.3 mol/mol) of terminal, unsubstituted β-galactosyl residues. These are localized exclusively on the α- and γ-chains (Gowda et al., 1994).

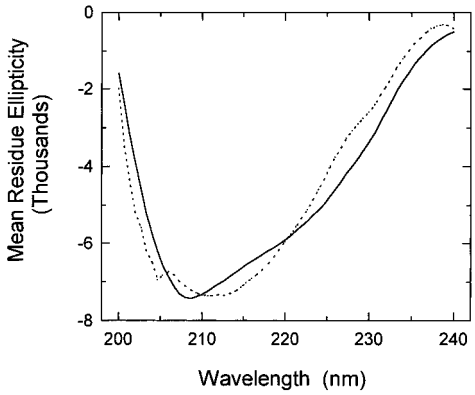

FIGURE 4: Circular dichroism spectra of RVV-X. Buffer-treated RVV-X (solid line); *N*-glycanase-treated RVV-X (dotted line).

Table 3: Circular Dichroism Analysis of Untreated and Glycosidase-Treated RVV-X

structure type[a]	RVV-X	A	B	C	D	E
α-helix	16	9	18	17	17	18
β-sheet	30	58	28	32	28	33
remainder	54	33	54	49	55	49

[a] Values (percent) are averages of two or three measurements. Treatments were with A, *N*-glycanase; B, sialidase; C, sialidase and β-galactosidase; D, sialidase, β-galactosidase and *N*-acetylhexosaminidase; E, sialidase, β-galactosidase, *N*-acetylhexosaminidase, and α-mannosidase.

Terminal β-galactosyl residues were enzymatically sialylated, thereby increasing the net negative charge of RVV-X; we previously demonstrated that 2.3 mol of sialic acid residues can be enzymatically added to RVV-X (Gowda et al., 1994). Kinetic analysis revealed that sialylation did not alter the activity of RVV-X (data not shown).

Secondary Structure of RVV-X. Circular dichroism measurements were carried out to study the effects of oligosaccharide modifications upon the polypeptide structure. The spectrum of untreated RVV-X has a wide negative minimum at 210−220 nm, consistent with a substantial amount of β-sheet (see Figure 4 and Table 3). Analysis indicated that the untreated RVV-X contains approximately 16% helix and 30% β-sheet.

Circular dichroism spectra of the sialylated RVV-X and various exoglycosidase-treated RVV-X samples were almost superimposable with that of the untreated glycoprotein (data not shown). Partial removal of the core mannosyl residues (by *H. pomatia* β-mannosidase) also resulted in no discernible spectral changes. However, complete removal of the carbohydrate moieties by *N*-glycanase caused a significant change in the circular dichroism spectrum, indicative of a roughly doubling of the β-sheet content, at the expense of the other secondary structural types (Figure 4 and Table 3).

DISCUSSION

We have previously shown that RVV-X contains six asparagine-linked oligosaccharide chains, four in the α-chain and one in each of β- and γ-chains (Gowda et al., 1994). In this study, we investigated the role of these carbohydrates in the conversion of factor X to X_a. We demonstrate that the complete removal of oligosaccharide chains markedly decreases the functional activity of RVV-X, whereas the removal of peripheral sugars has no effect. The loss of activity upon deglycosylation by *N*-glycanase is accompanied

by a significant increase in β-sheet content. In contrast, the removal of peripheral sugars by the exoglycosidases has no effect on either the activity or the secondary structure of RVV-X. Thus, we conclude that while the oligosaccharide chains are necessary for functional activity, the core structures (trimannosylchitobiose core, Man_3-$GlcNAc_2$−) are sufficient for the maintenance of structural integrity.

Complete deglycosylation of RVV-X using *N*-glycanase causes a marked loss (k_{cat} decreased 130-fold) in the ability to activate factor X. The decrease in function is paralled by changes in secondary structure, with an approximately doubling of β-sheet content. The simplest explanation is that the structural changes are the source of the functional loss. That is, deglycosylation causes a structural change that is characterized by a decrease in α-helix content, an increase in β-sheet content, and the essentially complete loss of activity.

RVV-X retained <1% of its activity upon treatment with *N*-glycanase. The residual activity could be due to a small amount of RVV-X that may have escaped being deglycosylated. These molecules could account for all of the residual activity and for the unaltered K_m. Neither the SDS−PAGE (Figure 1) nor the quantification of the remaining carbohydrates (Table 1) can rule out this possibility.

Alternatively, it is plausible that deglycosylated molecules of RVV-X have unaltered K_m toward factor X, accompanied by a roughly 130-fold lower k_{cat}. This is consistent with our observation that three separate preparations of deglycosylated (*N*-glycanase-treated) RVV-X had similar low levels of remaining activity (data not shown). In this case, the carbohydrate moieties do not contribute to the binding of factor X but are essential to the ability to process the bound X to X_a. If so, then binding and catalytic functions are likely to be contained in distinct domains.

The peripheral oligosaccharides in RVV-X are not required for either the ability to activate factor X or for the maintenance of secondary structure (Table 2). The enzymatic substitution of terminal β-galactosyl residues in the untreated RVV-X with sialic acid also did not affect the activity or the circular dichroism spectrum. Even the partial removal of core mannosyl residues resulted in only a small decrease in the ability to activate factor X and little, if any, change in the circular dichroism spectrum. Taken together, these data establish that the terminal sugar residues are not critical to the recognition and activation of factor X. The absence of functional effect upon the removal of the peripheral sugars also implies that they do not directly participate in the RVV-X/factor X interface. It is then highly unlikely that the core sugars are located within the RVV-X/factor X interface. Thus, the core structures are necessary and sufficient for structural integrity, and their role must be limited to the maintenance of functionally required structural features of the polypeptide components of RVV-X.

The structural and functional importance of the core sugar residues for RVV-X resembles the newly discovered roles for core sugar residues of *N*-linked oligosaccharides in rat luteinizing hormone receptor (Zhang et al., 1995) and in human CD2 (Wyss et al., 1995). For the luteinizing hormone receptor, the proximal *N*-acetylglucosamine residues of two functional oligosaccharide chains are sufficient to support the ligand binding activity (Zhang et al., 1995). In the case of CD2, the chitobiose core of the single *N*-linked carbohydrate moiety increases the stability and rigidity of the cell adhesion domain through a series of defined interactions with amino acids that are on a positively charged, lysine-rich surface (Wyss et al., 1995).

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