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Fluorescence studies on the interaction of some ligands with carcinoscorpin, the sialic acid specific lectin, from the horseshoe crab, *Carcinoscorpius rotundacauda*

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Abstract. The binding affinities of some ligands towards the sialic acid-specific lectin carcinoscorpin, from hemolymph of the horseshoe crab *Carcinoscorpius rotundacauda* have been determined by protein fluorescence quenching in presence of ligands. Among the ligands studied, the disaccharide O-(N-acetylneuraminyl)-(2→6)-2-acetamido-2-deoxy-D-galactitol has the highest K_a(l.15 × 10^6 M^{-1}) for carcinoscorpin. Studies on the effect of pH on K_a values of disaccharide suggests the possible involvement of amino acid residues having pK_a values around 6.0 and 9.0 in the binding activity of carcinoscorpin. There were distinct changes in the accessibility of the fluorescent tryptophan residues of carcinoscorpin by ligand-binding as checked through potassium iodide quenching.

Keywords. Horseshoe crab lectin; sialic acid; fluorescence; ligand-binding.

Introduction

Lectins are good analytical tools to undertake diverse studies on cell-membranes and to isolate and characterize specific cell-surface glycoconjugates (Lis and Sharon, 1977). They have served as good models to study protein-carbohydrate interactions (Goldstein and Hayes, 1978). Many of the physical and biological functions of oligo-, polysaccharides, glycoproteins and glycolipids occurring in the body fluids and in the structural elements of cells have been attributed to their sialic acid components (Lloyd, 1975). Although lectins with specificity towards sialic acid have not been as well characterized as those with specificity for other sugar residues such as D-galactose, D-mannose, D-glucose, N-acetylglucosamine, etc. (Goldstein and Hayes, 1978), a few sialic acid specific lectins have been reported– limulin from the horseshoe crab *Limulus polyphemus* (Marchalonis and Edelman, 1968); a lectin isolated from the serum of the lobster, *Homarus americanus* (Hall and Rowlands, 1974); an agglutinin from the albumen gland of certain snails (Voigtmann *et al.,* 1971); a protein extracted from the prickly lettuce plant, *Lectura scariola* (Yen *etal.,*1980); and our laboratory has also isolated a sialic acid-specific lectin, carinoscorpin, from the hemolymph of the marine horseshoe crab,

Abbreviations used: Ca^{2+}-free carcinoscorpin, Carcinoscorpin dialysed exhaustively against 0.05 M Tris-HCl, pH 8.0 containing 0.1 M NaCl; EDTA-carcinoscorpin, carcinoscorpin dialysed exhaustively against 0.05 M Tris-HCl, pH 8.0 containing 0.1 M NaCl and 0.01 M Na_2-EDTA; NeuNAC, N-acetylneuraminic acid.

Carcinoscorpius rotundacauda (Bishayee and Dorai, 1980). Information on the interaction of this lectin with ligands and the specificity towards 2-oxo-3-deoxyoctonate and some physical aspects of its binding with sialoglycoproteins have been reported recently (Dorai *et al.,* 1982; Mohan *et al.,* 1982).

In view of the possible use of carcinoscorpin in the study of cellular sialoglycoconjugates, it was essential to characterize the mechanism of binding of this lectin with its ligands. Physicochemical methods provide a better resolution of binding specificities than inhibition of hemagglutination which is semi-quantitative. We report in this paper the fluorescence studies of carcinoscorpin binding with various ligands.

Materials and methods

N-Acetylneuraminic acid (NeuNAc), N-glycoloylneuraminic acid, 2-oxo-3-deoxyoctonate, D-glucuronic acid, neuraminyl lactose and phosphorylcholine (Ca^{2+}-salt) were purchased from the Sigma Chemical Company, St. Louis, Missouri, USA. All other chemicals used were of the analytical grade.

Purification of carcinoscorpin

The sialic acid binding lectin, carcinoscorpin of *Carcinoscorpius rotundacauda* hemolymph was purified to homogeneity by the procedure of Dorai *et al.* (1981). Purified carcinoscorpin was kept in small aliquots at a protein concentration less than 2 mg/ml and stored at – 20°C until further use.

Preparation of carcinoscorpin-specific disaccharide

The disaccharide, O-(N-acetylneuraminyl)-(2→6)-2-acetamido-2-deoxy-D-galactitol, was prepared from sheep submaxillary mucin as described earlier (Mohan *et al.,* 1982).

Fluorescence measurements (ligand titration)

The ligand-induced quenching of protein fluorescence was studied in an Aminco-Bowman spectrofluorometer at 25°C. The association constants (K_a) for various ligands were determined according to Jolley and Glaudemans (1975). Carcinoscorpin solution of fixed protein concentration (0.1×10^{-6}M; A_{280} 0.05) in 0.05 M Tris-HCl, pH 8.0 containing 0.1 M NaCl and 0.01 M $CaCl_2$ was titrated with the ligands of appropriate concentration. The excitation wavelength was 280 nm and the quenching of fluorescence intensity was measured at 332 nm at which it was maximal. The buffer solution and the ligand solutions did not have any fluorescence between 290 nm and 400 nm. The spectra of ligand-carcinoscorpin solution after completion of titration was recorded for each ligand.

The free energy change of binding for each ligand was calculated by using the question, $\Delta G° = -RT\ln K_a$.

Effect of pH on K_a

Titration of native carcinoscorpin with disaccharide was done as described above at different pHs (4.5-9). The log values of K_a were plotted against pH.

Accessibility of tryptophan residues

The accessibility of tryptophan residues in the presence and absence of ligands (disaccharide and phosphorylcholine) was studied by KI quenching. Native lectin (0.93×10^{-7}M) and lectin in presence of the ligands, disaccharide (8×10^{-6}M) and phosphorylcholine (0.01 M), were titrated with 5 M KI, the addition being done in small aliquots. KI solution contained 200 mM sodium thiosulphate to suppress tri-iodide formation. A control titration with 5 M NaCl was performed simultaneously for non-specific changes in relative fluorescence intensity due to ionic-strength variation. After correcting for dilution effect, the fluorescence intensity data was plotted as F_0/F against [KI] where F_0 and F are the fluorescence intensities of native and ligand-bound carcinoscorpin, respectively, of same protein concentration.

Determination of protein

The concentration of lectin solution was determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard.

Results and discussion

Emission spectra of carcinoscorpin, Ca^{2+}-free carcinoscorpin and EDTA carcinoscorpin

Since Ca^{2+} is a prerequisite for the biological activity of carcinoscorpin (Mohan *et al.,* 1982) the effect of Ca^{2+} on the structure of carcinoscorpin was checked by fluorometry. The emission spectra of carcinoscorpin and its Ca^{2+}-free derivatives were recorded (figure 1). The fluorescence intensity at the emission maximum (332 nm) is lower in case of Ca^{2+}-free carcinoscorpin and EDTA-carcinoscorpin compared to the native protein indicating a change in the micro-environment of fluorescent aromatic amino acid residues upon removal of Ca^{2+} from the lectin.

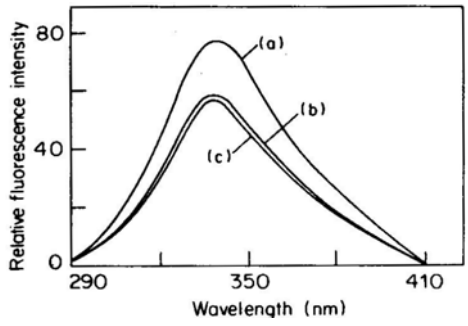

Figure 1. *Fluorescence spectra of native carcinoscorpin, Ca^{2+}-free-carcinoscorpin and EDTA-carcinoscorpin.*

The emission spectra of native carcinoscorpin,Ca^{2+}-free-carcinoscorpin and EDTA-carcinoscorpin (see 'Abbreviations') was measured at 25°C in 0.05 M Tris-HCl pH 8.0 containing 0.1 M NaCl. Protein concentration was 0.1×10^{-6} M in each case. Excitation wavelength was 280 nm (a) Native carcinoscorpin (b) Ca^{2+}-free-carcinoscorpin (c) EDTA-carcinoscorpin.

From circular dichroism measurements, Roche *et al.* (1978) have reported changes in the structure of limulin in absence of Ca^{2+}. With the addition of 0.01M Ca^{2+} the fluorescence intensity of Ca^{2+}-free carcinoscorpin however did not increase. The decrease in fluorescence intensity upon removing Ca^{2+}, therefore, does not seem to be reversible. But Bull biological activity.(as checked by binding to fetuin) could be restored in Ca^{2+}-free-carcinoscorpin or EDTA-carcinoscorpin with the addition of 0.01 M Ca^{2+} (Mohan *et al.,*1982). This is corroborated by the findings that disaccharide could quench, in presence of 0.01 M Ca^{2+}, the fluorescence intensity of Ca^{2+}-free and EDTA-carcinoscorpin to the same extent as that of native carcinoscorpin (see "binding parameters for ligands"). The observation suggests a structure for carcinoscorpin that could tolerate minor perturbations without losing the ability to regain Bull biological activity.

Binding parameters for ligands

In the presence of some ligands, the fluorescence intensity of carcinoscorpin decreased by 8-12% and the emission maximum (332nm) underwent red shift of 5 nm. Taking advantage of this quenching the K_a values of various ligands for carcinoscorpin were calculated (table 1). A representative Scatchard plot for the binding of disaccharide and NeuNAc is given in figure 2. The higher K_a value of

Table 1. Binding constants (K_a), maximal fluorescence change (ΔF_{max}) and free energies of binding ($-\Delta G^o$) for various ligands-with carcinoscorpin.

Ligands	K_a (M^{-1})	$-\Delta G^\circ$ (KJ/mol)	ΔF_{max} (%)	Minimum concn. needed for inhibition of one hemag-glutination dose (mM)[a]
N-Acetylneuraminic acid	8.56×10^3	2.53	10	1.0
N-Glycoloylneuraminic acid	3.6×10^3	20.37	13	0.5
2-Oxo-3-deoxyoctonate	ND[b]	–	<2	0.84
D-Glucuronic acid	3.64×10^3	20.40	7	0.8
N-Acetylneuraminyl galactitol	1.15×10^6	34.72	12	0.002
N-Acetylneuraminyl lactose	6.2×10^3	21.73	8.5	0.12
Phosphorylcholine	ND[b]	–	<3	10.0

[a] Values taken from Mohan *et al.* (1982).

[b] Not determined.

disaccharide (approximately 100 times than that of NeuNAc) reflects the high specificity of carcinoscorpin for the disaccharide which is complemented by our earlier studies on hemagglutination (Mohan *et al.,* 1982). While screening various ligands to inhibit carcinoscorpin-fetuin interaction and also hemagglutination by carcinoscorpin, it was noted that apart from NeuNAc and the disaccharide, D-glucuronic acid and 2-oxo-3-deoxyoctonate were also good inhibitors (Mohan *et al.,* 1982) which promoted us to determine the K_a values for these ligands. The association constant for D-glucuronic acid is found to be 3.64×10^3 M^{-1}. But

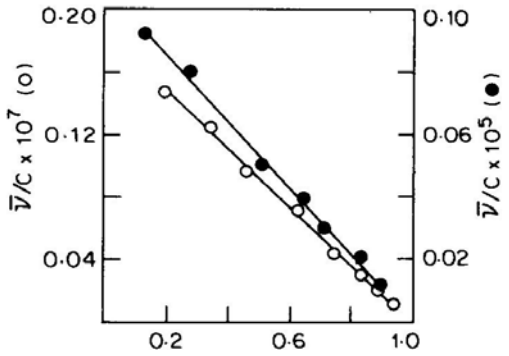

Figure 2. *Scatchard plots of the binding data of carcinoscorpin titrated with ligands.*

Carcinoscorpin solution (0.1×10^{-6} M, A_{280} 0.05) in 0.05 M Tris-HCl pH 8.0 containing 0.1 M NaCl and 0.01 M $CaCl_2$ was titrated at 25°C with disaccharide (O) and NeuNAc (=). The left side ordinate is for the disaccharide and right side ordinate is for NeuNAc.

unfortunately too low a quenching (<2.0%) of carcinoscorpin fluorescence was observed in the presence of 2-oxo-3-deoxyoctonate and hence its K_a could not be determined. However, in presence of 0.01 M2-oxo-3-deoxyoctonate, the K_a of the disaccharide became 0.9×10^5 M^{-1}, a decrease of 10 fold of the value found in absence of 2-oxo-3-deoxyoctonate. At this stage, it is only possible to suggest that 2-oxo-3-deoxyoctonate binds at a site contiguous to (overlapping site) or the same site as that of the disaccharide but with a different orientation.

Recently Robey and Liu (1981) have shown that limulin, a sialic acid-specific lectin, could bind to phosphorylcholine which is at a site different from that of sialic acid binding site. Our earlier results showed that phosphorylcholine does not inhibit the binding of carcinoscorpin to sialoproteins or its hemagglutinating activity (Mohan *et al.,* 1982) which does not, however, rule out its. binding to carcinoscorpin. When we measured the fluorescence of carcinoscorpin in presence of phosphorylcholine, a very small decrease in fluorescence intensity was observed (table 1) similar to that seen with 2-oxo-3-deoxyoctonate. However, unlike 2-oxo-3-deoxyoctonate, phosphorylcholine, even at concentration as high as 0.1 M does not alter the K_a of carcinoscorpin for disaccharide. The result does not establish whether phosphorylcholine is capable of binding to carcinoscorpin. But it suggests that, similar to limulin, the binding site for phosphorylcholine, if there is any in carcinoscorpin, is different from that for the sialic acid derivatives.

pH dependence of binding

The variation in the K_a values with pH is shown in figure 3. The optimum pH for binding was found to be around pH 8.0. We could not observe any intrinsic fluorescence quenching by the disaccharide above and below the pHs 9.0 and 5.0, respectively, suggesting the lack of interaction of lectin with the ligand around these extremes of pHs. The lectin, however, could regain its binding activity, as observed through fluorescence quenching, when brought back to pH 8.0 from pH 9.0 and pH 5.0. The ionizing groups at pK_a around 6.0 and 9.0 are possibly involved in the binding process. It is relevant to note that his residues (pK_a 6.0) have been proposed to be involved in the Ca^{2+} binding site of Concanavalin A (Hardman and

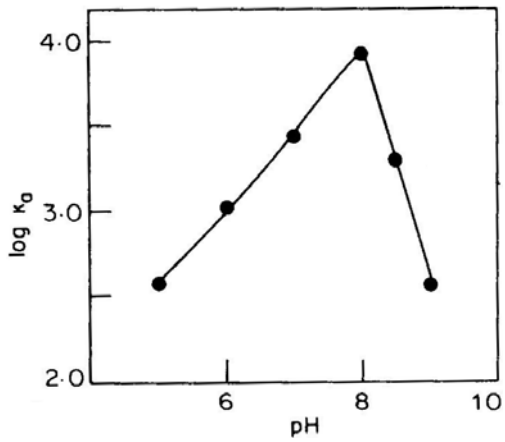

Figure 3. *Effect of pH on the binding of disaccharide to carcinoscorpin*

Titration of native carcinoscorpin (0.93×10^{-7} M) in 0.05 M Tris-HCl, 8.0 containing 0.1 M NaCl and 0.01 M $CaCl_2$ was done at different pHs with disaccharide. The K_a values calculated in each case were plotted as their log values against pH.

Goldstein, 1977). Chemical modification studies of carcinoscorpin employing group-specific reagents should help in identifying the amino acid residues required for binding Ca^{2+} and/or sialic acid.

KI quenching

The accessibility of tryptophan residues in proteins and the effect of ligand on the accessibility of tryptophan could be studied through iodide quenching of protein

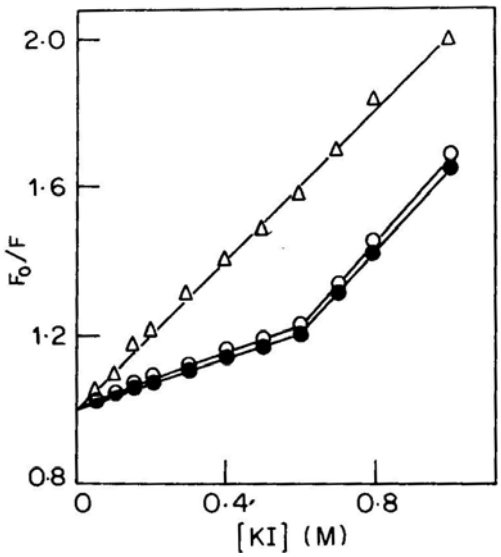

Figure 4. *KI quenching of carcinoscorpin in the presence and absence of disaccharide.*

Stern-Volmer plot of the quenching of carcinoscorpin (0.93×10^{-7} M) in 0.05 M Tris-HC , pH 8.0 containing 0.1 M NaCl and 0.01 M $CaCl_2$ with 5 M KI. Excitation and emission wavelengths were 280 and 332 nm, respectively. (=) native carcinoscorpin alone, (O) native carcinoscorpin in presence of 0.01 M phosphorylcholine, (Δ) native carcinoscorpin in presence of disaccharide (8×10^{-6} M).

fluorescence (Lehrer, 1967). This method has been utilized to probe the effect of the disaccharide binding on carcinoscorpin (figure 4). The Stern-Volmer plot of KI quenching data revealed difference in accessibility among the fluorescent tryptophan residues of carcinoscorpin in the absence of ligand (figure 4, (=). In presence of the disaccharide, interestingly, all the tryptophans behaved as a single homogeneous class, and a substantial increase in the accessibility compared to the unliganded carcinoscorpin was noticed (figure 4, Δ). The tryptophan residues of low accessibility in unliganded lectin seems to be perturbed by disaccharide with the effect that these residues acquired higher accessibility. The disaccharide could do so, either by directly binding in the vicinity of tryptophan residues and/or, through a change in the conformation of carcinoscorpin structure subsequent to ligand binding. However, in the presence of phosphorylcholine, no change in the accessibility of tryptophan residues was observed, and the plot was coincident with that of the lectin not combined with the ligand (figure 4, O). The quenching data of other lectins like wheat germ agglutinin and *Abrus precatorius* seed agglutinin showed that ligand decreased the accessibility of fluorescent residues in them (Privat and Monsigny, 1975; Herman and Behnke, 1980). But binding of lactose to *Momordica charantia* lectin caused a slight increase in the accessibility of tryptophan residues but without affecting the pattern of heterogeneity (Das *et al.,* 1981).

To summarize, we have characterized the binding of disaccharide with carcinoscorpin through fluorescence measurements. Due to low changes in fluorescence, K_a values for 2-oxo-3-deoxyoctonate and phosphorylcholine could not be determined. However, it is inferred that 2-oxo-3-deoxyoctonate could bind at the same site as that of disaccharide or to an overlapping site with a possible difference in its orientation. In contrast, phosphorylcholine, if at all it binds to carcinoscorpin, would bind at a site different from that of sialic acid derivatives.

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References

Bishayee, S. and Dorai, D. T. (1980) *Biochim. Biophys. Acta,* **623,** 89.

Das, M. K., Khan, M. I. and Surolia, A. (1981) *Biochem. J.,* **195,** 341.

Dorai. D. T., Bachhawat, B. K., Bishayee, S., Kannan, K. and Rao, D. R.(1981) *Arch. Biochem. Biophys.,* **209,** 325.

Dorai, D. T., Srimal, S., Mohan, S., Bachhawat, B. K. and Balganesh, T. S. (1982) *Biochem. Biophys. Res. Commun.,* **104,** 141.

Goldstein, I. J. and Hayes, C. E. (1978) *Adv. Carbohydr. Chem. Biochem.,* **35,** 127.

Hall, J. L. and Rowlands, D. T. Jr. (1974) *Biochemitry,* **13,** 828.

Hardman,K. D. and Goldstein, I.J. (1977) in *Immunochemistry of Proteins,* ed. M.Z. Atassi (New York: Plenum Press) Vol. 2, p. 373.

Herman, M. S. and Behnke, W. D. (1980) *Biochim. Biophys. Acta,* **621,** 43.

Jolley, M. E. and Glaudemans, C. P. J. (1975) *Carbohydr. Res.,* **33,** 377.

Lehrer, S. S. (1967) *Biochem. Biophys. Res. Commun.,* **29,** 767.

Lis, H. and Sharon, N. (1977) in *The Antigen,* ed. M. Sela (New York: Academic Press) Vol. 4, p. 429.

Lloyd, C. W. (1975) *Biol. Rev.,* **50,** 325.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.,* **193,** 265.
Marchalonis, J. J. and Edelman, G. M. (1968) *J. Mol. Biol.,* **32,** 453.
Mohan, S., Dorai, D. T., Srimal, S. and Bachhawat, B. K. (1982)*Biochem. J.,* **203,** 253.
Privat, J. J. and Monsigny, M. (1975) *Eur J. Biochem.,* **60,** 555.
Robey, F. A. and Liu, T. Y. (1981) *J. Biol. Chem.,* **256,** 969.
Roche, A. C., Maurizat, J. C. and Monsigny, M. (1978) *FEBS Lett.,* **91,** 233.
Voigtmann, R., Salfner, B. and Uhlenbruck, G. (1971) *Z. Immun-Forech.,* **141,** 488.
Yen, S. E., Manfield, J. M. and Wallace, J. H. (1980) *Int. Arch. Allergy Appl. Immunol.,* **61,** 32.