Physicochemical studies on achatinin_H, a novel sialic acid-binding lectin

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We have purified a sialic acid-binding lectin, achatinin_H, in a single step by affinity chromatography, having high affinity for 9-*O*-acetylneuraminic acid. The physicochemical characterization of the interaction of achatinin_H with bivalent metal ions and sialic acid derivatives by the use of spectrofluorimetry, spectropolarimetry and precipitin reaction is reported. From fluorescence quenching studies the binding of Ca²⁺ ($K_a = 251 \pm 9 \text{ M}^{-1}$) and of Mn²⁺ ($K_a = 86 \pm 5 \text{ M}^{-1}$) was found to be weak, but their presence is absolutely necessary for sugar binding as well as biological activity. The nature and position of the substituent group play a very important role in the binding affinity. Achatinin_H shows a high affinity for 9-*O*-acetylneuraminic acid ($K_a = 1.20 \times 10^3 \pm 0.07 \times 10^3 \text{ M}^{-1}$) compared with that for the 4-*O*-acetyl derivative. In oligomers the binding strength increases in the order monosaccharide < disaccharide < trisaccharide. The binding affinity of achatinin_H for the disaccharide was found to reach a peak around pH 8. From c.d. spectral studies achatinin_H was found to have a high β -sheet content (46%) and a low α -helix content (24%). From precipitin analysis at least one sugar-binding site on each of the 16 monomer subunits of the protein is indicated.

INTRODUCTION

Lectins, the multivalent carbohydrate-binding proteins, have recently evoked considerable interest owing to their potential usage as specific probes to study the expression and architecture of cell-surface carbohydrates during development, differentiation and malignant transformation (Novogrodsky & Katchalski, 1971; Goldstein & Hayes, 1978; Barondes, 1981; Lis & Sharon, 1986). Various derivatives of sialic acid, e.g. *N*-acetylneuraminic acid (NeuAc), *N*-glycolylneuraminic acid (NeuGc) and 4-, 7-, 8- and 9-*O*-acetylated derivatives are very important constituents of cell-surface architecture, and the *O*-substituted sialic acids (OAc-NeuAc) exhibit interesting species- and tissue-specific distribution in animals (Schauer, 1982). The presence of 9-OAc-NeuAc in gangliosides from rat brain tumour and human malignant melanoma cells (Haverkamp *et al.*, 1977) and its influence on enzyme catabolism of glyco-conjugates, inhibition of bacterial and viral sialidase, alteration of the immunopotency of sialoglyco-conjugates (Schauer *et al.*, 1980) as well as the change in the degree of *O*-acetylation in transformation and other alteration in the environment of cells (Varki & Kornfeld, 1980) have drawn special attention to the expression and distribution of these *O*-acetylated derivatives on the cell surface.

Relatively few sialic acid-binding lectins have been identified (Marchalonis & Edelman, 1968; Hall & Rawlands, 1974; Bishayee & Dorai, 1980; Miller *et al.*, 1982; Chowdhury *et al.*, 1985; Ravindranath *et al.*, 1985; Ahmed *et al.*, 1986), among which only one is commercially available (Marchalonis & Edelman, 1968), and most others show broad specificity. None of them has been characterized by physicochemical studies, except carnoscorpin. Recently we have reported the purifi-

cation of a novel sialic acid-binding lectin achatinin_H (ATN_H) in a single step by affinity chromatography from the haemolymph of a land snail, *Achatina fulica* (Basu *et al.*, 1986). This lectin (M_r 242000) is highly specific for sialic acid, having high affinity for 9-OAc-NeuAc (Mandal & Basu, 1987). Its specificity is even narrower than that of the crab lectin, which shows specificity for both 4- and 9-*O*-acetyl derivatives. This lectin, therefore, differs from all other sialic acid-binding lectins reported so far. In view of its limited specificity ATN_H may prove to be a valuable tool for the localization and assessment of the functions of glyco-conjugates containing 9-OAc-NeuAc and has a tremendous potential in serodiagnosis. In order to use lectins as sensitive probes it is necessary to determine the binding parameters of the lectins with their complementary sugars and metal ions to elucidate their specificities and dimensions of their combining regions.

During our previous studies on ATN_H an important characteristic of this lectin did emerge, its absolute dependence on either of the bivalent metal ions, Ca²⁺ or Mn²⁺, for its interaction with the cell-surface glyco-conjugates containing sialic acid moieties. However, little information is available so far on the nature of binding of carbohydrate to ATN_H. The understanding of binding of metal ions and carbohydrates by a lectin is important for studies involving cell-surface probes and affinity chromatography of glyco-conjugates. In the present paper we describe our attempts to identify the role and nature of bivalent ion binding in maintaining its secondary structure and binding to sialic acid derivatives by physicochemical techniques such as spectrofluorimetry, spectropolarimetry and precipitin reaction. The aim is to widen the applicability of this lectin in surveying microheterogeneity of many biologically important sialoglyco-

Abbreviations used: ATN_H, achatinin_H; SSM, sheep submaxillary mucin; HA, haemagglutination.

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proteins and to use it as a probe in the study of membrane sialoglyco-conjugates.

MATERIALS AND METHODS

Chemicals

The chemicals were of analytical grade or of the purest material available. *N*-Acetylneuraminic acid (type IV), *N*-glycolylneuraminic acid, neuraminyl-(2-3)-lactose, bovine serum albumin, fetuin (type III) and colominic acid were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. α_1 -Acid glycoprotein, human serotransferrin, human lactotransferrin and *N*-acetylneuraminyl-(2-3)- β -D-galactopyranosyl-(1-4)-D-glucopyranose were generously given by Dr. H. Debray, Université des Sciences Techniques de Lille, Lille, France. Sheep submaxillary mucin (SSM) was isolated from the submaxillary glands of sheep obtained from a local slaughterhouse, according to the procedure of Murphy & Gottschalk (1961). 4-OAc-NeuAc and 9-OAc-NeuAc were generously given by James C. Paulson, Department of Biochemistry, U.C.L.A., Los Angeles, CA, U.S.A., and Professor R. Schauer, Biochemisches Institut, Universität Kiel, Kiel, Germany. Na¹²⁵I was from the Bhaba Atomic Research Centre, Trombay, India. The disaccharide [*N*-acetylneuraminyl- α -(2-6)-*N*-acetyl-D-galactosaminitol] was prepared according to the method of Dorai *et al.* (1981). The agglutinin was isolated and purified from the haemolymph of *Achatina fulica* snails by affinity chromatography on SSM-Sepharose 4B as described previously (Basu *et al.*, 1986). The protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Haemagglutination activity

Haemagglutination (HA) tests (Sarkar *et al.*, 1984) were performed with a Takatsy micro-titrator (Laxbro Mfg. Co., Puna, India) with 2% (v/v) rabbit erythrocytes in the presence of 30 mM-Ca²⁺ or 50 mM-Mn²⁺. HA titre is reported as the reciprocal of the highest dilution of lectin giving complete agglutination. Specific inhibitory effect of a sialic acid was determined by adding the material in serial dilutions to constant amounts of Ca²⁺ and ATN_H giving a minimum of 16 HA units.

Fluorescence measurements

Fluorescence measurements were made on a Perkin-Elmer MPF-44B fluorescence spectrometer with 10 nm slit width at 25 °C with the use of 3 ml quartz cuvettes of path length 1 cm. Excitation was at 285 nm and emission spectra were scanned in the range 300–400 nm. The titrations were carried out by successive addition of concentrated solution of the ligand (sample) to the protein solution (0.3 μ M in 0.15 M-NaCl/50 mM-Tris/HCl buffer, pH 8.0) followed by thorough mixing and recording of fluorescence intensities. Simultaneously control titrations of protein with buffer alone were also done. The values of corrected quenching (Q_c) were obtained from the relative intensities (at 330 nm) of sample and control titrations. The maximum extent of quenching (Q_{max}) was obtained from the extrapolation of the double-reciprocal plot ($1/Q_c$ versus $1/[L]_T$), $[L]_T$ being the total concentration of the ligand (Mandal & Karush, 1981). The fraction of sites occupied (α) was calculated at each titration point. The binding constants were obtained

from the intercept of the plot of $\log[\alpha/(1-\alpha)]$ versus $\log[L]_T$ according to the following relation (Chipman *et al.*, 1967):

$$\log\left(\frac{\alpha}{1-\alpha}\right) = \log[L]_T + \log K_a$$

Free energies of binding were calculated by using $\Delta G = -RT \cdot \ln K_a$, where R is the gas constant and T is the absolute temperature.

Effect of pH

Titration of native ATN_H at different pH values (in the range 4.0–9.0) with the disaccharide was carried out at 25 °C and the association constant at each pH was calculated.

C.d. measurements

C.d. spectra were recorded with a JASCO J20A recording spectropolarimeter under constant N₂ flush at 25 °C with a 1 ml cuvette of path length 2 mm. Spectra were scanned with two protein concentrations (50 μ g and 100 μ g in 0.15 M-NaCl/50 mM-Tris/HCl buffer, pH 8.0) in the far-u.v. region (204–240 nm), and average values were used for the calculation of mean residue ellipticity $[\theta]$ taking 110 as the mean residue weight. The percentages of α -helix, β -form and random coil were calculated by using a computer program of curve-fitting based on minimum standard deviation. Effectively, the program constructed theoretical curves for all the combinations of integral percentages of α -helix, β -form and random coil by using the reference values from Chen *et al.* (1974), obtained empirically from X-ray-crystallographic data, at intervals of 3 nm followed by the calculation of standard deviation for each combination and finally identifying the combination having the lowest standard deviation.

Iodination of ATN_H

The iodination of purified ATN_H (20 μ g) was carried out by following the method of Hunter (1978) with Na¹²⁵I (1 mCi). The labelled lectin was purified by gel filtration and affinity chromatography (Basu *et al.*, 1986) followed by dialysis against 0.15 M-NaCl/50 mM-Tris/HCl buffer, pH 8.

Glycoprotein binding assay

Binding assays were performed in glass tubes in 0.15 M-NaCl/50 mM-Tris/HCl buffer, pH 8.0, containing 30 mM-CaCl₂ and desialylated bovine serum albumin (10 mg/ml) (incubation buffer). One titration set contained 0.39 nM unlabelled ATN_H, a fixed amount of labelled lectin (3×10^4 c.p.m.) and various amounts of sialoglycoproteins. After 3 h of incubation at 25 °C, the mixture was filtered rapidly through 0.45 μ m-pore-size membrane filters with a Millipore Swinex 13 filter unit, washed twice with 0.2 ml of incubation buffer. The radioactivities of the filter papers were then counted directly on a γ -radiation counter. After the subtraction of control radioactivity (without sialoglycoproteins), the percentages of ATN_H precipitated were calculated. The precipitin curves were plotted as percentage of ATN_H precipitated versus amount of sialoglyco-conjugates (plots not shown). The amounts of glycoprotein needed for maximum precipitation were obtained from these plots (Table 4).

Fig. 1. Quenching of the fluorescence spectra of ATN_H.

Experiments were carried out in 50 mM-Tris/HCl/0.15 M-NaCl buffer, pH 8.0 at 25 °C. The fluorescence spectra were recorded in the absence (spectrum a) and after the addition of several samples from a stock of 1 M-CaCl₂ and of 39 mM-disaccharide to 1.4 ml (0.31 μM) of ATN_H solution: spectrum b, 0.005 ml of Ca²⁺; spectrum c, 0.02 ml of Ca²⁺; spectrum d, 0.005 ml of disaccharide; spectrum e, 0.01 ml of disaccharide; spectrum f, 0.02 ml of disaccharide; spectrum g, 0.03 ml of disaccharide; spectrum h, 0.05 ml of disaccharide; spectrum i, 0.1 ml of disaccharide. After each addition of the ligand, part of the spectrum was recorded and the total spectrum was given for the final addition. The lowest spectrum is for standard buffer. Buffer showed negligible fluorescence (< 5%), subtracted from control and sample readings for calculation.

RESULTS AND DISCUSSION

Effect of bivalent metal ions

The titration of ATN_H with Ca²⁺ (Fig. 1) and Mn²⁺ resulted in the quenching of the intrinsic fluorescence, with $Q_{\max}$ values 23% and 40% respectively. Binding was almost complete with the addition of 30 mM-Ca²⁺ (90%) and 50 mM-Mn²⁺ (80%). Titration with Mg²⁺ did not show any appreciable quenching. As presented in Table 1, the binding of Ca²⁺ ($K_a = 251 \pm 9 \text{ M}^{-1}$) and of Mn²⁺ ($K_a = 86 \pm 5 \text{ M}^{-1}$) was very weak, and Ca²⁺ binding was 3 times stronger than that of Mn²⁺. When the lectin was titrated with various concentration of Mn²⁺ in the presence of Ca²⁺ at three concentrations the binding constants decreased with the increased amounts of Ca²⁺. This decrease may be due to either a competition between Ca²⁺ and Mn²⁺ ions for the same site or an allosteric effect of one metal ion on the binding site of the

other. This question was resolved by quantitative analysis with the use of the relevant equations. The observed binding constants (Table 1) fitted very well (S.D. = 1.6 M^{-1}) to the allosteric model, with the value of the binding constant of Mn²⁺ to ATN_H-Ca²⁺ complex being $36 \pm 1 \text{ M}^{-1}$. But the competitive model yielded a very poor fit (S.D. = 33.6 M^{-1}). The constancy in $Q_{\max}$ of Mn²⁺ binding in the presence of increasing amounts of Ca²⁺ is in agreement with the allosteric model. In comparison with other lectins, concanavalin A contains separate binding sites for Ca²⁺, Mn²⁺ and sugar, but the binding of Mn²⁺ or Ni²⁺ opens up the site for Ca²⁺ and after the binding of these metal ions the sugar-binding site is activated (Kalb & Levitzki, 1968). Recent studies on the binding of Mn²⁺ and Ca²⁺ ions to lima-bean lectin indicate that there are two Ca²⁺-binding sites and one equivalent and independent Mn²⁺-binding site per monomer of the lectin (Nissen & Magnuson, 1986). With lima-bean lectin and concanavalin A the values of binding constants with bivalent metal ions, e.g. Ca²⁺, Mn²⁺, Ni²⁺ etc., are of the order of 10^3 – 10^5 M^{-1} but the binding constants of ATN_H are rather low.

No HA activity could be detected in the absence of Ca²⁺, indicating that its presence was absolutely necessary for the interaction of ATN_H with the erythrocytes. When Ca²⁺ was replaced with Mn²⁺ there was a drastic decrease (64-fold) in the HA activity (Table 1). In the presence of both the metal ions there was a 32-fold decrease in HA activity compared with that with Ca²⁺ alone, indicating that Mn²⁺ strongly influenced the interaction of ATN_H with cell surface. Binding studies with a disaccharide (defined in the Materials and methods section) also showed a similar pattern, as described below.

When ATN_H was titrated with the disaccharide in the absence of Ca²⁺ or Mn²⁺ there was no appreciable change in the intrinsic protein fluorescence, indicating that their presence was a necessary condition for the interaction of ATN_H with the sugars. However, when titrated after the addition of Ca²⁺ or Mn²⁺ to their respective optimum concentrations (30 mM for Ca²⁺ and 50 mM for Mn²⁺) the titration resulted in an additional maximum quenching of 28–50%. Fig. 1 shows the changes in the fluorescence spectra of the lectin by the addition of the disaccharide in the presence of Ca²⁺. Fig. 2 shows the representative plots of various titration parameters leading to the determination of $Q_{\max}$ and K_a for the binding of the disaccharide. The binding parameters are presented in Table 1. The values clearly indicate that the highest value of K_a was obtained with Ca²⁺, being 22-fold more than that with Mn²⁺. When both were present, the binding constant diminished. HA-inhibition experiments also gave a similar pattern to that shown in Table 1.

Sugar binding

The values of association constants (K_a) of the interaction of sugars containing a neuraminic acid moiety with ATN_H are presented in Table 2 along with the corresponding free-energy changes (ΔG) and $Q_{\max}$ values. In these cases the saturation of the binding sites by the sugars gave a maximum quenching of 25–30% in addition to that caused by Ca²⁺ binding. The main point to be noted here is that the monosaccharides NeuAc and NeuGc showed smaller values of binding constants ($K_a = 300 \pm 40 \text{ M}^{-1}$ and $K_a = 700 \pm 60 \text{ M}^{-1}$ respectively) than that of the disaccharide ($K_a = 1.86 \times 10^3 \pm 0.19 \times$

Table 1. Binding constants (K_a) and free energies of binding ($-\Delta G$) for various ligands with ATN_H at 25 °C

Specific activity is defined as the total HA titre/mg of protein. The reciprocal of the highest dilution of the agglutinin that produces visible agglutination was taken as the titre. A concentration of 0.085 mg/ml was used for HA experiments. Effective concentrations of Ca^{2+} and Mn^{2+} were 30 mM and 50 mM respectively. K_a and $Q_{\max}$ in fluorescence for the binding of various metal ions and saccharide were calculated as described in the Materials and methods section.

Ligand(s)	$10^{-3} \times K_a$ (M^{-1}) (mean $\pm$ S.E.M.)	$Q_{\max}$	$-\Delta G$ (kJ/mol)	$10^{-5} \times$ Specific activity (titre/mg of protein)	Minimum concentration for 50% inhibition of 16 HA units (mM)
Ca^{2+}	0.251 ± 0.009	23	13.7	7.50	—
Mn^{2+} with no Ca^{2+}	0.086 ± 0.005	40	11.1	0.12	—
Mn^{2+} with 7.5 mM- Ca^{2+}	0.054 ± 0.005	39	10.0	—	—
Mn^{2+} with 15 mM- Ca^{2+}	0.046 ± 0.006	42	9.5	—	—
Mn^{2+} with 30 mM- Ca^{2+}	0.040 ± 0.004	43	9.2	0.23	—
Disaccharide with 30 mM- Ca^{2+}	1.86 ± 0.19	28	18.8	—	0.41
Disaccharide with 50 mM- Mn^{2+}	0.085 ± 0.005	40	11.1	—	13.0
Disaccharide with 30 mM- Ca^{2+} and 50 mM- Mn^{2+}	0.20 ± 0.01	50	13.2	—	6.6

Fig. 2. (a) Plot of $1/Q_c$ versus $1/[L]_T$ for the determination of $Q_{\max}$ and (b) plot of percentage of quenching (Q_c) versus total ligand concentration ($[L]_T$)

(a) Plot of $1/Q_c$ versus $1/[L]_T$ for the determination of $Q_{\max}$. Samples of a solution of disaccharide (39 mM) were added to a 1.4 ml of ATN_H solution (0.31 μM) containing 30 mM- Ca^{2+} . (b) Plot of Q_c against $[L]_T$. Inset gives a graphical representation of the plot of $\log[\alpha/(1-\alpha)]$ versus $\log[L]_T$ for the determination of association constant.

10^3 M^{-1}), which was also the best inhibitor of HA activity. Of the two monosaccharides, binding of NeuGc was twice as strong as the binding of NeuAc, which differ only in the substitution at the 5-amino group of neuraminic acid. Binding with two other monosaccharides having *O*-acetyl substitutions at two different positions showed that, whereas 9-*O*-acetylation resulted in a very strong binding ($K_a = 1.20 \times 10^3 \pm 0.07 \times 10^3 \text{ M}^{-1}$), 4-*O*-acetylation decreased it to a negligible value. The same nature was revealed by the HA-inhibition experiments (Table 2). The trisaccharide NeuAc-(2-3)-lactose showed a higher association constant ($K_a = 3.12 \times 10^3 \pm 0.28 \times 10^3 \text{ M}^{-1}$) than that of the disaccharide. The negative free energies of binding of the sugars were in the range 8.4–20.0 kJ/mol (2–4.8 kcal/mol). These results indicate the importance of the position and nature of substituent groups on the free energy of binding of the interaction of the lectin with sugar moieties. The binding strength increases in the order monosaccharide < disaccharide < trisaccharide. The inhibition of HA follows a similar pattern but with an exception that the trisaccharide turns out to be a poor inhibitor. This may be taken as an indication that the binding strength may not be the only factor that governs the inhibitory activity, steric factors also playing some role. A study on lectin binding with chito-oligosaccharides showed that the binding strength increases steadily with increasing number of sugar units (Anantharam *et al.*, 1986).

Effect of pH

The HA of erythrocytes by ATN_H was found to be affected by the pH of the buffer. Table 3 presents the K_a values and the specific activities at different pH values, showing that the pH strongly affected the binding strength, having a narrow optimum pH around 8.0. It may be inferred that an ionizing group having $\text{p}K_a$ around 8 is involved in the binding. But since the $\text{p}K_a$ of an ionizing group of an amino acid is expected to be influenced by its environment, no amino acid can be singled out from this observation. However, our recent studies by chemical modification demonstrated the

Table 2. Binding constants (K_a) and free energies of binding ($-\Delta G$) for various ligands with ATN_H

Lectin solutions were preincubated with optimum concentration of Ca²⁺ (30 mM) at 25 °C.

Ligand	$10^{-3} \times K_a$ (M ⁻¹) (mean $\pm$ S.E.M.)	$Q_{\max}$	$-\Delta G$ (kJ/mol)	Minimum concentration needed for 50 % inhibition of 16 HA units (mM)
NeuAc (5-N-Ac)	0.30 ± 0.04	25	14.2	3.81
NeuGc (5-N-Gc)	0.70 ± 0.06	26	16.3	1.92
4-OAc-NeuAc	—	—	—	> 50.0
9-OAc-NeuAc	1.20 ± 0.07	30	17.7	1.35
Disaccharide	1.86 ± 0.19	28	18.8	0.41
Trisaccharide	3.12 ± 0.28	28	20.1	25.0

Table 3. Binding constants (K_a) and the specific activities of ATN_H at different pH values

pH	$10^{-3} \times K_a$ (M ⁻¹) (mean $\pm$ S.E.M.)	$10^{-5} \times$ Specific activity (titre/ mg of protein)
5.0	0.20 ± 0.02	0.0073
7.0	0.70 ± 0.06	0.014
8.0	1.86 ± 0.19	7.5
8.5	1.60 ± 0.12	7.5
9.0	0.92 ± 0.05	7.5
10.0	0.85 ± 0.05	7.5

involvement of tryptophan and histidine residues in sugar binding (Basu *et al.*, 1988). No change in the intrinsic fluorescence was observed above pH 10.0 or below pH 5.0.

C.d. spectra of ATN_H

The c.d. spectra of ATN_H in the presence and in the absence of Ca²⁺ are shown in Fig. 3. In the absence of Ca²⁺ the c.d. spectrum showed a negative maximum around 215 nm. In the presence of Ca²⁺ (50 mM) the spectrum showed small but significant shift to the shorter wavelength with a decrease in the negative values of $[\theta]$ in the range from 240 nm down to 210 nm. Calculation of the fractions of secondary structures by using standard values from Chen *et al.* (1974) gave 24% α -helix, 46% β -sheet and 30% random coil (standard deviation was 3.8% of the maximum) in the absence of Ca²⁺ whereas in its presence the respective values were 20%, 50% and 30% (with a standard deviation of 4.7% of the maximum). These results suggest that in the presence of Ca²⁺ about 4% α -helix is converted into β form. In view of the uncertainty in the prediction of secondary structure from c.d. measurement this conversion of 4% may not represent an exact situation, but some sort of rearrangement upon Ca²⁺ binding is evident from the significant difference in the two spectra in the u.v. region (210–240 nm). ATN_H thus contains a high amount of β structure. Many lectins are known to contain high amounts of β structure, namely *Helix pomatia* agglutinin (Hammerstrom, 1974), concanavalin A (Pflumm *et al.*, 1971), *Pisum sativum* lectin (Bures *et al.*, 1972), *Ricinus communis* lectin (Shimazaki *et al.*, 1975) and *Dolichos*

Fig. 3. C.d. spectra of ATN_H in the far u.v.

The Figure shows the c.d. spectra of ATN_H in the absence of Ca²⁺ (—) and in the presence of 50 mM-Ca²⁺ (---) along with the calculated points (O, in the absence of Ca²⁺; Δ, in the presence of Ca²⁺) obtained from the best-fitted structural composition. The ellipticity curves were calculated from six c.d. recordings at two protein concentrations (50 μg/ml and 100 μg/ml). The experiments were carried out in 50 mM-Tris/HCl/0.15 M-NaCl buffer, pH 8.0, at 25 °C.

biflorus and *Ribinia pseudoacacia* lectins (Pere *et al.*, 1975). However, potato lectin shows no evidence of β -sheet conformation (van Holst *et al.*, 1986), and wheat-germ agglutinin contains only 10% β structure (Thomas *et al.*, 1977).

Table 4. Determination of the binding mole ratios of sialoglycoproteins versus ATN_H

The reaction procedure as described in the Materials and methods section was followed. A 0.39 nmol portion of ATN_H was taken for each experiment. Values for binding ratios are those obtained at equivalence point.

Glycoprotein	Amount of added glycoprotein at equivalence point (μg)	At the point of maximum precipitation		Mole ratio of sialoglycoprotein versus ATN _H
		Amount of sialic acid (nmol)	Amount of sialoglycoprotein (nmol)	
Fetuin (<i>M_r</i> 48000)	300	43.92	6.25	16
Serotransferrin (<i>M_r</i> 80000)	500	133.0	6.25	16
Lactotransferrin (<i>M_r</i> 76000)	250	36.0	3.30	8.46
α ₁ -Acid glycoprotein (<i>M_r</i> 44000)	114	16.68	2.60	6.66
Human choriongonadotropin (<i>M_r</i> 33000)	100	34.0	3.00	7.69
SSM (<i>M_r</i> 1300000)	61	36.5	0.047	0.12

Precipitin studies with glycoproteins

Table 4 presents the titrating amounts of sialoglycoproteins along with the mole ratios of sialic acid contents and of native proteins to ATN_H at their respective equivalence points obtained from the maxima of the precipitin curves. The titrating amounts of sialic acid at the point of maximum precipitation differed for different glycoproteins, but their mole ratios to ATN_H exhibited an interesting pattern. For fetuin and serotransferrin the binding mole ratio was 16:1, indicating that 16 molecules of these glycoproteins are bound to one molecule of native ATN_H at the equivalence point. Since ATN_H has 16 subunits, one binding site on each subunit is implied. The mole ratios for lactotransferrin, α₁-acid glycoprotein and human choriongonadotropin to ATN_H were found to be around 8:1. The binding of 8 molecules of glycoprotein to one molecule of ATN_H indicates one binding site per two subunits. This situation may arise if the binding of one protein to a site excludes the binding to another site. At equivalence point the mole ratio of SSM to ATN_H was found to be 0.12:1 (or approx. 1:8), being very different from others. This result indicates that one molecule of SSM binds to 8 molecules of ATN_H at the point of maximum precipitation. Because of the very high *M_r* of SSM and the linear structure of its carbohydrate side chains, a precipitating complex may be thought to form by the binding of 8 molecules of ATN_H to one molecule of SSM. Hence from these studies it may be concluded that there is at least one sugar-binding site per subunit of ATN_H. It may be relevant to point out that attempts to determine the number of sites for sugar and metal ions from fluorescence measurements in accordance with Halfman & Nishida (1972) were unsuccessful, presumably because of low binding affinity.

From these physicochemical studies it may be inferred that the binding of a metal ion (Ca²⁺ or Mn²⁺) causes a small perturbation in the structure of the lectin, leading to some changes in the microenvironment of the native protein that facilitate the binding of sialic acids. Finally, these studies have provided information on the physicochemical basis of the biological phenomena of the interaction of this novel sialic acid-binding lectin with the cell-surface glyco-conjugates, having special structural features. The sharp specificity of ATN_H should provide opportunities for studying the expression of

specific glyco-conjugates in biological processes such as development, differentiation and malignant transformation.

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