PROTEIN–SUGAR INTERACTIONS. ASSOCIATION OF β-(1→4) LINKED *N*-ACETYL-D-GLUCOSAMINE OLIGOMER DERIVATIVES WITH WHEAT GERM AGGLUTININ (LECTIN)

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1. Introduction

Wheat germ agglutinin (WGA) is a lectin [1–3] which binds selectively *N*-acetyl-D-glucosamine, *N,N'*-diacetyl-chitobiose $(GlcNAc)_2$ *N,N',N''-triacetyl*-chitotriose $(GlcNAc)_3$ and *N,N',N'',N'''*-tetraacetyl chitotetraose $(GlcNAc)_4$ [4,5]. These mono- and oligosaccharides have been shown to be the best inhibitors of the agglutination of erythrocytes and other types of cells [1–3]. LeVine et al. [6] reported that WGA has *one N*-acetyl-D-glucosamine binding site per 23 000 g of the protein, on the basis of equilibrium dialysis experiments. The same conclusion was also deduced from fluorescence experiments in the case of β- (1→4) linked oligomers of *N*-acetyl-D-glucosamine [4]. We report here that WGA has *two independent and homogeneous binding sites* for reduced chitotetraose per mole of protein, on the basis of equilibrium dialysis experiments, and that WGA forms a precipitate with chitobiose-bovine serum albumin conjugate, even under conditions where it is known that the protein is in its monomeric form.

2. Materials and methods

WGA was purified as previously described and is devoid of neutral and amino sugars [5]. The molecular weight of the polypeptide chain, as determined by disc gel electrophoresis at pH 7.2, in the presence of 0.1% mercaptoethanol and 0.1% sodium dodecylsulfate was found to be 21 000 ± 1000 [5]. This value is close to the value (23 000) obtained by Wright et al. [7] on the basis of X-ray crystallographic studies and to the value (23 500) obtained by Nagata and Burger [8]. We have used this last value in our calculations as already done [5,9]. Protein concentration was determined using $E^{1\%}_{280}$: 15.0. Freeze dried WGA was dissolved in the appropriate buffer and the concentration was determined from absorbance measurements at 280 nm in a Cary 14 spectrophotometer using a 1 cm pathlength cell. All solutions were passed through Millipore filters (HAWP, 0.45) using a Swinnex-13 millipore adapter.

The oligosaccharides $(GlcNAc)_n$ were isolated from chitin as previously described [10]. Amino acids, amino sugar and glucosaminitol contents were determined on a Beckman amino acid analyzer, model 120 C, after hydrolysis with redistilled HCl in sealed tubes in vacuo at 105°C for 24 hr (unless otherwise stated). Values for glucosamine and glucosaminitol were corrected for destruction during hydrolysis [11]. Amino sugar content was also determined by the modification [12] of the original Elson–Morgan procedure [13].

2.1. *Equilibrium dialysis experiments*

$NaBT_4$-reduced-$(GlcNAc)_4$ was prepared by adding tritium labelled BH_4Na (10 mCi, 26.6 Ci/mM, CEA) to a solution of 11 mg of *N,N',N'',N'''*-tetraacetyl-chitotetraose in 2 ml of $HNaCO_3$ 0.01 M aqueous solution, pH 8.2. After 4 hr, the excess of BH_4Na was destroyed by adding acetic acid until pH 4.0, then $NaBT_4$-reduced-$(GlcNAc)_4$ was purified by gel filtration on Sephadex G 25 (2 × 50 cm) column. The specific radioactivity of $NaBT_4$-reduced-$(GlcNAc)_4$ ($6 \cdot 10^7$ cpm/μmole) was calculated as the ratio of the

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radioactivity of an aliquot and the glucosamine (and glucosaminitol) content determined after hydrolysis in HCl 4N, at 100°C for 4 hr. Membranes (Visking) used for equilibrium dialysis were washed twice with aqueous solutions of $HNaCO_3$ 0.6 M, EDTA 1 mM at 100°C for 30 min, and then with distilled water. The washed membranes were kept at 4°C in an ethanol–water mixture (50:50, v/v). Before use, the membranes were hydrated in distilled water and sponged dry. Altuglass plastic cells of 0.5 ml volume in each chamber, designed essentially according to the specifications of Myer and Schellman [14] were used. Dialysis cells were prepared by placing 0.3 ml of WGA (10^{-5} M) on one side and 0.3 ml of $NaBT_4$-reduced $(GlcNAc)_4$ (various concentrations) on the other side. The cells were sealed with plastic screws and shaken at 20°C. After equilibrium was attained (24 hr), samples were removed from each chamber, and 50 μl samples were dried on millipore filters (HAWP, 0.45 μ). Samples were counted in 5 ml of the following mixture (PPO: 5 g, POPOP:0.2 g, toluene 1 litre) [15] in a liquid scintillation counter (Beckman).

2.2. *Chitobiose-BSA conjugate*

The *O-p*-nitrophenyl-glycoside of *N,N'*-diacetyl-β-chitobiose has been prepared according to the procedure described by Zurabyan et al. [16]. Reduction of *O-p*-nitrophenyl-*N,N'*-diacetyl-β-chitobioside was carried out by catalytic hydrogenation under atmospheric pressure and at 25°C in methanol in the presence of palladium charcoal and one equivalent of HCl [17]. The *O-p*-aminophenyl-*N,N'*-diacetyl-β-chitobioside was coupled, after diazotization, to the amino-acyl side chains of bovine serum albumine (Pentex) according to the procedure of Tabachnik and Sobotka [18]. The protein-carbohydrate conjugate was purified by gel filtration on Sephadex G 50 column (2 × 80 cm), equilibrated with NaCl 0.1 M, Tris–HCl 0.05 M buffer pH 7.2. The carbohydrate content of carbohydrate-protein conjugate was determined after hydrolysis (HCl 5.6 N, 105°C, 24 hr) by analysis on an amino acid auto-analyzer. The gel diffusion procedure of Goldstein and So [19,20] was employed for studying WGA chitobiose-BSA-conjugate interaction.

Before use, the gel plates were maintained 24 hr in various buffers, either in the absence and in the presence of sodium dodecyl-sulfate and mercaptoethanol. A solution of chitobiosa–BSA conjugate (5 mg/ml or 2 mg/ml) in the appropriate buffer was placed in the central well (40 μl) of the agar plate; peripheral wells (5 μl) contained various amounts of WGA (1 mg/ml, 2 mg/ml, 4 mg/ml or 8 mg/ml). Similar experiments were done with WGA solutions in the central well and various amounts of carbohydrate–BSA conjugate in peripheral wells, or of free BSA or of free *O-p*-nitrophenyl-*N,N'*-diacetyl-chitobioside. The plates were incubated at 25°C for 12 hr to 24 hr, then they were rinsed with salt solution, and dyed with Lissamine Green (K and K).

3. Results and discussion

3.1. *Equilibrium dialysis*

In a previous paper [5] it was shown that maximum affinity of *N*-acetyl-D-glucosamine derivatives for WGA was obtained with *N,N',N''*-triacetyl-chitotriose and *N,N',N'',N'''*-tetraacetyl-chitotetraose. This result implied the presence of, at least, three subsites in the binding site of WGA, as suggested by Allen et al. [9] on the basis of inhibition of agglutination. In order to determine the number of site(s) of the protein, equilibrium dialysis experiments were made with the $NaBT_4$-reduced *N,N',N'',N'''*-tetraacetyl-chitotetraose. Preliminary experiments with WGA alone and with reduced chitotetraose alone, showed that less than 1% of the protein crossed the membrane, and that sugar equilibrium was attained within 24 hr. We verified that the number of counts is a linear function of the amount of labelled carbohydrate applied to the millipore filter. Fig. 1 shows a Scatchard plot [21] of the binding of $NaBT_4$-reduced-$(GlcNAc)_4$ to WGA. The least square resultant straight line intercepts the abscissa axis near 2. The association constant of $NaBT_4$-reduced-$(GlcNAc)_4$ was calculated to be 5.3×10^4 M^{-1}. This value is about twice that found on the basis of fluorescence experiments [4,5] for $(GlcNAc)_3$ which was calculated assuming *one* binding site per molecule of protein. This difference might also be due either to a difference in the affinities of α- and β-$(GlcNAc)_3$, since the anomeric hydroxyl group of the last GlcNAc is free in $(GlcNAc)_3$, or to a weak affinity of *N*-acetyl-glucosaminitol for WGA. Allen et al. [9] showed on the basis of inhibition of the agglutinating activity of WGA that GlcNAc was 3 times less efficient than methyl-α(or -β)-GlcNAc. The binding of monosaccharides

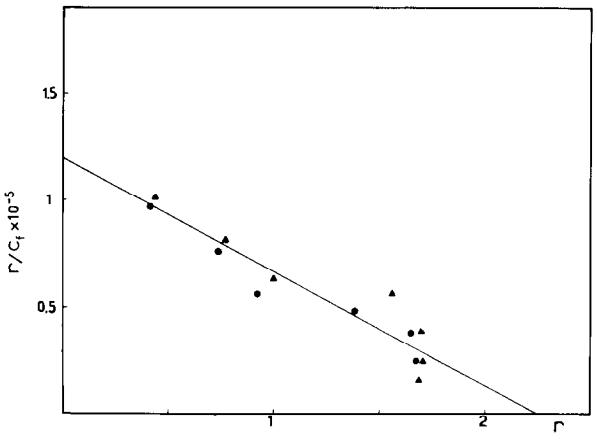

Fig. 1. Scatchard plot of binding of $NaBT_4$-reduced-$(GlcNAc)_4$ to WGA. The binding experiments were done in an equilibrium dialysis cell for 24 hr at 25°C in 0.2 M NaCl 0.05 M Tris–HCl, pH 7.2. Protein concentration was $2 \cdot 10^{-5}$ M (0.46 mg/ml) and Na-BT_4-reduced-$(GlcNAc)_4$ concentrations were varied between 10^{-5} and 15×10^{-5} M; r is the average number of $NaBT_4$-reduced-$(GlcNAc)_4$ molecules bound per mole of the agglutinin molecule; C_f is the concentration of free $NaBT_4$-reduced $(GlcNAc)_4$. ▲——▲ and ○——○ independent experiments.

to concanavalin A is dependent on the sugar anomery and on the nature of the aglycones: the association constants of *O*-methyl-, *O*-*p*-nitrophenyl- and *O*-(4-methyl-umbelliferyl)-α-D-mannopyranosides are 2.06×10^4 [22], 3.9 or 3.6×10^4 [23] and 3.54×10^4 M^{-1} [24], respectively; those of D-glucose, *O*-methyl-α-, *O*-methyl-β- and *O*-*p*-nitrophenyl-α-D-glucosides were 5.88×10^2, 1.76×10^3, 4.94×10^3 [22] and 1×10^4 [23]. LeVine et al. [6] have reported that WGA has one binding site per 23 000 g of the protein. Recently Nagata and Burger [25] found that WGA has two binding sites for *N*-acetyl-D-glucosamine per mole of polypeptide chain (mol. wt. 17 000 ± 1000). These authors suggested two explanations to the discrepancy between the report of LeVine et al. [6] and their own work: 1) It might be a technical difference, for LeVine et al. used 40 times less protein than Nagata and Burger and a different counting procedure; however, our own experiments were done with a protein 10 times less concentrated than Nagata and Burger, and the counting procedure, we used, was similar to that used by LeVine et al. [6]. 2) *N*-acetyl-D-glucosamine which is a small ligand might be bound to two subsites of the single binding site of the protein; however, the results we report here, are not consistent with this explanation, since we used an oligomer of *N*-acetyl-D-glucosamine.

Moreover, the existence of two sites per monomer is also supported by fluorescence measurements with *O*-(4-methyl-umbelliferyl)-glycosides reported in the following paper [26].

Our results are thus in good agreement with those of Nagata and Burger [25]. It can be concluded that the protein monomer has *two independent binding sites* for *N*-acetyl-D-glucosamine and its β-1-4 linked oligomer and that *N*-acetyl-D-glucosamine does not bind to two subsites of the binding sites of the protein.

3.2. *Agar gel diffusion experiments*

In 0.2 M NaCl, 0.05 M Tris–HCl buffer pH 7.2, the *N,N'*-diacetyl chitobiose-bovine serum albumine conjugate inhibits a maximum absorption at 350 nm with a molar extinction coefficient 4.3×10^4. This carbohydrate–BSA conjugate contained 4 chitobiose units per mole of BSA. As shown in fig. 2, WGA, $(GlcNAc)_2$–BSA conjugate and $(GlcNAc)_2$ interact like antibody, antigen and hapten, respectively in an

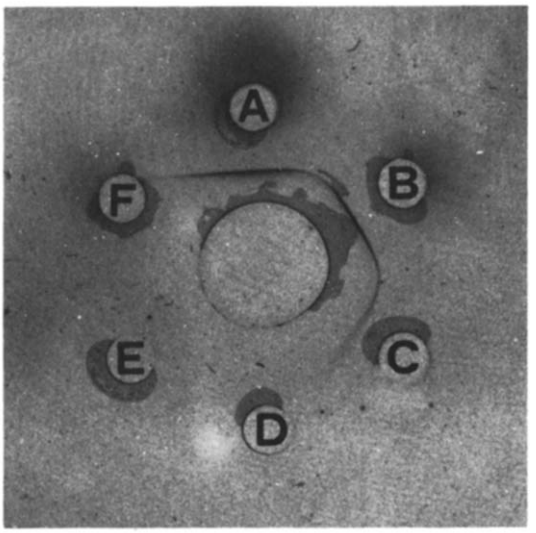

Fig. 2. Agar gel diffusion patterns of WGA with chitobiose–BSA conjugate; central well: WGA 1 mg/ml; A, B, C, D: chitobiose–BSA conjugates: 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.3 mg/ml respectively; E: 1-*O*-*p*-nitrophenyl-*N,N'*-diacetyl-chitobioside: 1 mg/ml + chitobiose–BSA conjugate 5 mg/ml; F: free BSA 5 mg/ml. The precipitation bands corresponding to C and D were present before coloration but vanished partially during the dying operation.

agar diffusion assay since a precipitation band was produced between $(GlcNAc)_2$–BSA conjugate and WGA, and since $(GlcNAc)_2$ was able to inhibit the precipitation. The $(GlcNAc)_2$–BSA conjugate was used to study the interaction with WGA at various pH and in presence of sodium dodecylsulfate and mercaptoethanol.

In neutral medium, the finest precipitation bands were obtained, when chitobiose–BSA conjugate (5 mg/ml) solutions were placed in the central well and when WGA solutions were in the peripherical wells. The minimal WGA concentration needed to observe a precipitation band was about 1 mg/ml. When WGA solution was placed in the central well, finest results were obtained with the following concentration: WGA solutions 1 mg/ml, chitobiose–BSA conjugate: 4 mg/ml. The same results were obtained in the presence of sodium dodecylsulfate alone (0.1% to 1%) in the presence of mercaptoethanol alone (0.1%) and in the presence of mercaptoethanol (0.1%) and sodium dodecylsulfate (0.1%). Since in the last conditions the dissociation of WGA into monomers is complete, as shown by disc gel electrophoresis [5,9,25] it can be concluded that the monomer contains more than one binding site, and that neither mercaptoethanol, nor sodium-dodecylsulfate induce a dramatic modification of the structure of the binding sites.

By fluorescence measurements [5] it was not possible to provide any evidence for interaction between WGA and GlcNAc or its derivatives below pH 2.8. The absence of precipitation band in agar gel diffusion experiments at low pH (up to 4.2) indicates that a three dimensional lattice of sufficient size cannot form. Since the protomer contains two binding sites, it can be concluded that the binding sites (or at least one of them) are not able to bind *N,N'*-diacetylchitobioside in acidic medium. A similar behaviour was shown for concanavalin A by So and Goldstein [27] who showed that no precipitation occured between concanavalin A and polysaccharide at pH below 4. However, concanavalin A is still able to bind the monosaccharide at pH 2.4 [23,28].

In neutral medium, but not in acidic medium, the $(GlcNAc)_2$–BSA conjugate is a valuable pseudo-antigen for WGA, as is the polychitobiosylaspartate prepared by Shier [29]. It can be used for quantitative precipitation studies as done by Iyer and Goldstein [20] with concanavalin A and various carbohydrate-protein conjugates, and for preparation of antichitobiose antibodies. This work is in progress.

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