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FLUORESCENCE POLARIZATION STUDIES OF SACCHARIDE BINDING TO WHEAT GERM AGGLUTININ AND LYSOZYME

Paul J. Stein and K. Craig Heehn

Department of Chemistry, College of St. Scholastica, Duluth, MN

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SUMMARY:

N-acetyl-D-glucosamine causes only slight increases in the polarization of Wheat germ agglutinin fluorescence at saturating levels whereas the disaccharide and trisaccharide produce increases in the polarization value from 0.116 to 0.151 and 0.154 respectively. These increases suggest that rotational motions of the tryptophan residue at the binding sites are being restricted by an interaction between these tryptophans and the bound sugars. A model of the nature and location of these interactions is discussed.

Comparable results are obtained with lysozyme, which shows a larger effect upon binding of N-acetyl-D-glucosamine, but a maximal increase in polarization upon binding the corresponding disaccharide or trisaccharide.

INTRODUCTION:

Wheat germ agglutinin is a dimer with a molecular weight of approximately 35,000 (1). It is capable of binding N-aceytl-D-glucosamine[1] and its β-1,4 linked oligomers at two sites per subunit (2,3). Each site is capable of accomodating at least three linked monosaccharide units (4,5). Each subunit has three tryptophan residues of which only two are fluorescent, and these behave homogeneously toward potassium iodide quenching and N-bromo-succinimide oxidation (6,7). The oxidation of one tryptophan residue per subunit leads to almost total loss of hemagglutinating activity (7). Binding of GlcNAc, $(GlcNAc)_2$, $(GlcNAc)_3$, or $(GlcNAc)_4$, causes an increase in the fluorescence intensity of WGA and the oligosaccharide binding also results in a blue shift of the emission wavelength (8,5).

This paper reporst new information on the nature of the WGA-saccharide interaction using intrinsic fluorescence polarization. This fluorescent par-

[1]Abbreviations used: GlcNAc, N-acetyl-D-glucosamine; $(GlcNAc)_2$, di-N-acetylchitobiose; $(GlcNAc)_3$, tri-N-acetylchitotriose; $(GlcNAc)_4$, tetra-N-acetylchitotetraose; WGA, Wheat germ agglutinin; P, fluorescence polarization; λmax, fluorescence emission maximum wavelength; GuHCl, guanidinium chloride; DTT, dithiothreitol.

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ameter is dependent upon any molecular motion of a fluorophore during its excited state which changes the angle of its emission oscillator. In the case of a tryptophan residue in a protein this may include contributions from fast localized motions of the tryptophan as well as from the gross rotational movement of the protein. Theoretical considerations concerning fluorescence polarization and applied localized motions of fluorophores attached to macromolecules have been described by Dale and Eisinger (9).

Significant changes are observed in P when $(GlcNAc)_2$ or $(GlcNAc)_3$ are bound to WGA. These changes are compared to similar changes upon adding the oligosaccharides to lysozyme, an enzyme in which tryptophan-sugar interactions at the active site have been well established (10,11).

MATERIALS AND METHODS:

Hen egg white lysozyme was purchased from Sigma Chemical Co. WGA was purified according to the method of Bassett (12). Both polyacrylamide gel electrophoresis and sodium dodecyl sulfate gel electrophoresis produced a single band. The agglutinating activity of WGA was determined by mixing serial dilutions of protein with a 3% suspension of human type A+ red blood cells of 20 mM phosphate buffered saline, pH 7.4 (7). Half maximal agglutination was obtained with 2.8×10^{-7} M WGA.

GlcNAc and $(GlcNAc)_2$ were purchased from Sigma Chemical Co. $(GlcNAc)_2$ and $(GlcNAc)_3$ were also purified by the method of Rupley (13), and crystals washed with methanol.

Fluorescence results were obtained on an Aminco spectrofluorometer (SPF) which was not corrected for emission monochromator or photomultiplier response. Polarization results were obtained using Thomson Glans-prism polarizers supplied by Aminco for use with the SPF. Polarization values were determined by the method of Azumi and McGlynn (14), with excitation at 295 nm.

The average rotational correlation time (ρ) for WGA fluorescence was determined by a form of the Perrin equation (15,16):

$$1/P - 1/3 = (1/P_o - 1/3)(1 + 3\tau/\rho) \qquad (1)$$

The average fluorescence lifetime, τ, for WGA excited at 295 nm was taken as 4.3 nsec (17). The polarization in the absence of Brownian motion, P_o, was determined both in 90% glycerol assuming no rotation occurred and by extrapolation of a Perrin plot to infinite viscosities, in which the polarization values were measured at varying concentrations of sucrose. Both methods produced the same value.

RESULTS:

The effect of sugars on WGA was determined by adding incremental additions of the sugar until a constant fluorescence intensity or polarization value was obtained. The initial concentration of WGA was 1.5×10^{-6} M. Table I shows the observed fluorescence intensity enhancements, correcting for

TABLE I
Fluorescence Effects of Saturating Levels of Saccharides on WGA.[a]

Sample	Fl. Enhancement[b]	λmax	P[c]	ρ[e]
WGA	---	350	.116 ± .006	18 ± 3
WGA + 22mM GlcNAc	12%	349	.123 ± .003	23 ± 2
WGA + 7.9mM $(GlcNAc)_2$	50%	343	.151 ± .003	62 ± 5
WGA + 5.3mM $(GlcNAc)_3$	54%	342	.154 ± .002	69 ± 4
WGA in 90% glycerol	---	350	.191 ± .003	------
denatured WGA[d]	---	350	.112 ± .005	------

[a]Measurements made with 1.5×10^{-6} M WGA in 20mM phosphate buffer, pH 7.4, at 28°C with excitation at 295nm, and emission at the λmax value.

[b]Fluorescence enhancement measured from increase in intensity at λmax.

[c]The P value for no added saccharide is the average from 8 measurements, all others are the average of 2 or 3 measurements and agree with measurements at a different saturating level of saccharide.

[d]WGA denatured in 6 M GuHCl with .01% DTT.

[e]Assumes τ directly proportional to fluorescence intensity.

concentration changes of the protein, at saturating levels of the saccharides. These values are in close agreement with those found in previous studies. The blue shift in λmax also correlated well with these results although our emission maxima values are slightly higher, most likely due to a lack of correction for emission monochromator or photomultiplier response (5,8).

The polarization values increase slightly when GlcNAc is added to WGA but large increases are observed upon addition of $(GlcNAc)_2$ or $(GlcNAc)_3$. This is in contrast to a previous study which indicated that $(GlcNAc)_3$ had no effect on the intrinsic fluorescence polarization (6). The polarization value for WGA denatured in 6 M GuHCl and .01% DTT and the intrinsic polarization (P_o) for WGA indicates the minimum and maximum values for P respectively, and are included in Table I.

The increase in P from adding incremental additions of $(GlcNAc)_2$ or $(GlcNAc)_3$ followed a hyperbolic plot which leveled off at relatively low sugar concentrations. This shows that the polarization changes are caused by bind-

ing and not simply by an increase in solution viscosity. This was also confirmed when additions of similar concentrations of sucrose produced no increase in P.

Since the saccharides also enhanced the fluorescence intensity which usually indicates an increase in the fluorescence lifetime, the changes in P may contain contributions from changes in τ rather than fluorophore rotations. However, according to equation 1, increases in τ would result in decreases in P with constant rotational motion. The opposite change is observed indicating that rotational changes are causing the polarization differences. Values of ρ calculated assuming the intensity increases reflect corresponding increases in τ are included in Table I.

The same type of experiments were repeated adding GlcNAc and its corresponding dimer and trimer to lysozyme. The results are shown in Table II. The fluorescence intensity changes and λmax shifts are similar to previous studies (18,19). The polarization changes were qualitatively similar to those obtained with WGA, displaying some increase with saturating levels of GlcNAc, and a larger and nearly equivalent increase in P upon addition of $(GlcNAc)_2$

TABLE II

Fluorescence Effects of Saturating Levels of Saccharides on Lysozyme.[a]

Sample	Fl. Enhancement[b]	λmax	P[c]
Lysozyme	---	340	.141 ± .003
Lysozyme + 8.3mM GlcNAc	-12%	340	.150 ± .005
Lysozyme + 8.6mM $(GlcNAc)_2$	33%	332	.176 ± .008
Lysozyme + 7.6mM $(GlcNAc)_3$	33%	334	.169 ± .005
Lysozyme in 90% Glycerol	---	340	.189 ± .003
denatured Lysozyme[d]	---	350	.090 ± .003

[a] Measurements made with 1.0×10^{-6} M lysozyme with all other conditions the same as noted in Table I.

[b] Same as for results in Table I.

[c] The P value with no saccharide added is based on the average of 5 measurements, all others based on the average of 3 measurements.

[d] Lysozyme denatured in 6 M GuHCl with .01% DTT.

and $(GlcNAc)_3$. In contrast to WGA, the P value for denatured lysozyme is considerably lower than the value for the native enzyme.

DISCUSSION:

Consistent with other fluorescent properties of WGA the polarization results also indicate the high solvent exposure of the tryptophan residues, since there is little difference in the mobility of these residues in native WGA or denatured, reduced WGA. In addition, this suggests that the tryptophans in native WGA have little interaction with neighboring side chains. Saturation of WGA with GlcNAc produces only slight decreases in the mobility of the tryptophans, even though proton NMR studies show that the N-acetyl-methyl group of GlcNAc is located near the face of a tryptophan residue (20). Saturating the protein with $(GlcNAc)_2$ or $(GlcNAc)_3$ causes severe restrictions of tryptophan mobility as judged by increases in P. These changes imply a direct saccharide-tryptophan interaction as opposed to an indirect protein conformational change particularly in light of CD experiments on saccharide binding to WGA which showed changes in the aromatic region but not in the peptide backbone region (21).

These results suggest a picture of a tryptophan residue in each sugar binding site which has a relatively large conformational range in solution and moves very rapidly between a series of positions. The conformational range in this model includes the B and/or A binding subsites proposed by Allen et al. (4), where the second sugar residue in $(GlcNAc)_2$ and the second and third sugar resides in $(GlcNAc)_3$ bind. It does not include the monosaccharide binding site labeled as subsite C in Allen's model. This site, according to the NMR study (20), must be immediately adjacent to one position the tryptophan can assume. Upon binding $(GlcNAc)_2$ or $(GlcNAc)_3$ the tryptophan is restricted to occupying a much smaller conformational range, resulting in higher polarization values. This may be due in part to hydrogen bonding interactions with the sugar, as is observed for lysozyme from the crystal structure (10).

For lysozyme, the polarization values upon binding saccharides reflect a similar process, however, interpretation is more difficult due to the presence

of 6 tryptophan residues and the unique fluorescent properties of Trp 108 in the active site (22). That binding of the monosaccharide elicits a stronger response in lysozyme than in WGA probably occurs because the β form of GlcNAc binds nearer the B and C subsites where tryptophans 62 and 63 are located (10). Since the H-bonding interactions between these two tryptophans and bound substrate occur at the third sugar unit, the dimer may also bind in the location corresponding to the second and third sugar units on $(GlcNAc)_3$ causing the same polarization effect. This same ambiguity may apply to $(GlcNAc)_2$ binding to WGA.

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