Proc. Natl. Acad. Sci. USA
Vol. 75, No. 3, pp. 1324–1328, March 1978
Biophysics

Ligands containing heavy atoms: Perturbation of phosphorescence of a tryptophan residue in the binding site of wheat germ agglutinin

(heavy atom effect/lectin/protein–sugar interaction)

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Communicated by R. B. Setlow, January 3, 1978

ABSTRACT Information on the structure of binding sites of wheat germ agglutinin was obtained on the basis of fluorescence and phosphorescence changes of tryptophan residues induced by the binding of several thiomercuribenzoate derivatives of glycosides. The thiomercuribenzoate derivatives bind selectively to wheat germ agglutinin in the same way as the corresponding sugars. Using the thiomercuribenzoate of di-*N*-acetyl-β-chitobiose, it was found that: (*i*) the fluorescence of tryptophan residues was drastically quenched at both 298 and 77 K; (*ii*) the phosphorescence intensity was strongly enhanced at 77 K; (*iii*) the phosphorescence lifetime was markedly decreased. A similar effect was observed with the thiomercuribenzoate of *N*-acetyl-β-D-glucosamine. These changes were completely reversed upon addition of 1-*O*-methyl-di-*N*-acetyl-β-chitobioside. The thiomercuribenzoate of β-D-glucose had no effect at all, and the thiomercuribenzoate of tri-*N*-acetyl-β-chitotriose had a limited effect. These results are interpreted as a specific heavy atom effect due to a close contact between one tryptophan residue of the protein and the heavy atom of the bound ligand. They are consistent with the view that: (*i*) binding sites of wheat germ agglutinin may be divided in three subsites, A, B, and C; (*ii*) a tryptophan residue is in the binding site at subsite C; and (*iii*) this residue and the ligand are in close contact. This new method, using the enhancement of spin-orbit coupling due to the selective perturbation induced in a tryptophan residue by a ligand containing a heavy atom, has proved to be suitable for locating the tryptophan residue in the binding site of wheat germ agglutinin and can probably be extended to other sugar-binding proteins.

Wheat germ agglutinin (WGA) is a plant lectin which agglutinates various types of animal cells, in particular malignant cells and protease-treated normal cells (1–3). The agglutination of cells is inhibited by *N*-acetylglucosamine and its β-1-4-linked oligomers (2, 4). This lectin binds chitin (5), cell wall polymers, and bacterial cells (6) and inhibits fungal growth (7). It was shown that under physiological conditions WGA is a dimer that can be dissociated into two identical subunits of 18,000 molecular weight at low pH (8, 9). We demonstrated that the dimer binds four *N*-acetylglucosaminide ligands with equal affinity, so that each protomer binds saccharides at two different locations (10, 11) as already suggested (8). Luminescence studies of WGA showed that two out of three tryptophans of each protomer were fluorescent (12) and that their fluorescence was modified upon binding oligosaccharides containing *N*-acetylglucosamine (13). However, although the specific binding of ligands alters tryptophan fluorescence (12, 14, 15) as well as the near ultraviolet absorbance (16) and the circular dichroism (17) of WGA, and even though the crystal structure of WGA is now known at 2.2-Å resolution (18), we have no information as yet to locate the tryptophan residue(s) inside the binding site(s).

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Using oligosaccharide substituted by a heavy atom, we report here that one tryptophan residue is in the binding site of WGA and that it is located at the third subsite. Since the observation of Kasha (19), the influence of heavy atoms on the luminescence properties of aromatic molecules has been the subject of many studies (see, e.g., refs. 20 and 21). Heavy atoms are known to enhance spin-orbit coupling in organic molecules. This phenomenon can be intra- or intermolecular, i.e., the heavy atom can be either covalently linked to the organic molecule or in the vicinity of this molecule without any covalent bond. The heavy atom effect leads to three effects on the spectroscopic properties of the organic molecule: fluorescence quenching, enhanced intersystem crossing, and shortening of the phosphorescence lifetime. An increase in phosphorescence quantum yield is usually observed if the increase in the intersystem crossing rate is not overcompensated by the increase in the triplet state deactivation rate (responsible for the decreased phosphorescence lifetime). It was observed that the effect is larger when the heavy atom is heavier, and that it is obtained only when heavy atoms and chromophore interact over distances of the order of van der Waals radii, as illustrated by the emission analysis of proflavin bound to the copolymer poly(dA-BrdU) (22). A strong effect can be expected when a heavy atom such as mercury gives a complex with the chromophore, as observed in the case of nucleotides (23, 24). A ligand substituted with a mercury atom is expected to perturb the spectroscopic properties of aromatic residues in proteins if such residues are present in the binding site. To obtain information on the presence of tryptophan residue(s) in the binding site of WGA, the thiomercuribenzoate derivatives of β-D-glucose, *N*-acetyl-β-D-glucosamine, di-*N*-acetylchitobiose, and tri-*N*-acetylchitotriose were synthesized, and their effects on the fluorescence and the phosphorescence of WGA were studied.

MATERIAL AND METHODS

WGA, prepared as previously described (25) was purchased from Industrie Biologique Française, Gennevilliers, France. *N*-Acetyl tryptophanamide, benzoic acid, and *p*-hydroxymercuribenzoate were purchased from Sigma Chemical Co. The 1-thio-*p*-mercuribenzoates of β-D-glucose, *N*-acetyl-β-D-glucosamine, di-*N*-acetyl-β-chitobiose and tri-*N*-acetyl-β-chitotriose were obtained by reaction of the thiouronium peracetylglycosides (26) and *p*-hydroxymercuribenzoate. The 1-thio-*p*-mercuribenzoate glycosides (Fig. 1) were de-*O*-acetylated, using sodium methoxide, and were then crystallized.

Abbreviations: WGA, wheat germ agglutinin; CB-SHgBzO$^-$, 1-*S*-thiomercuribenzoate of di-*N*-acetyl-β-chitobiose; CT-SHgBzO$^-$, 1-*S*-thiomercuribenzoate of tri-*N*-acetyl-β-chitotriose; GlcNAc-SHgBzO$^-$, 1-*S*-thiomercuribenzoate of *N*-acetyl-β-D-glucosamine; CB-OCH_3, 1-*O*-methyl-di-*N*-acetyl-β-chitobioside; BzO$^-$, benzoate.

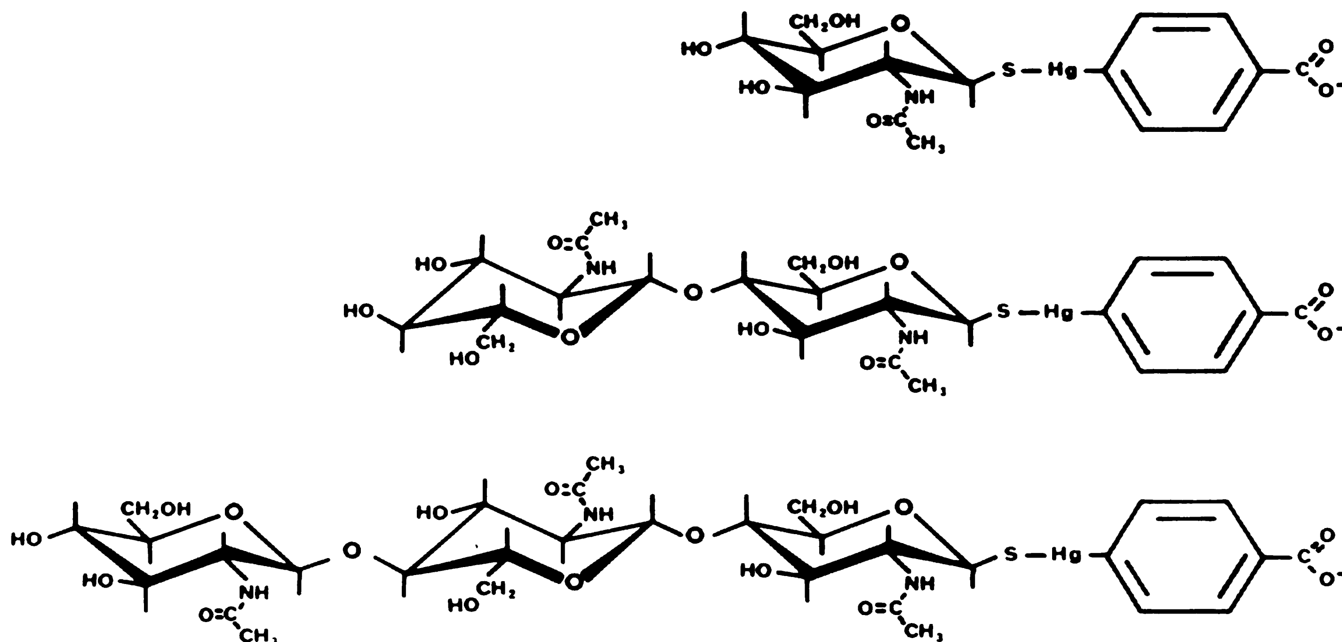


FIG. 1. Formulas, top to bottom, of 1-*S*-thiomercuribenzoate derivatives of *N*-acetyl-β-D-glucosamine (GlcNAc-SHgBzO⁻, molecular weight 556.6), di-*N*-acetyl-β-chitobiose (CB-SHgBzO⁻, molecular weight 759.6), and tri-*N*-acetyl-β-chitotriose (CT-SHgBzO⁻, molecular weight 962.6).

The details of these syntheses and the physical properties of these derivatives will be described elsewhere. The thiomercuribenzoate sugar derivatives have a maximum absorbance at 235 nm and did not absorb at wavelengths higher than 270 nm (Fig. 2).

Fluorescence Measurements. Excitation and emission spectra were recorded with a Jobin–Yvon spectrofluorimeter, modified as previously described (27). The solutions were contained in 5- × 5-mm quartz cells. All the solutions were filtered on Millipore HAWP 0.45-μm pore diameter filters. The protein concentration was about 1 μM, so that the absorbance at the excitation wavelength (295 nm) was always very low ($A_{295}^{1\,cm} \leq 0.05$). The temperature was maintained at 298 K.

Luminescence Measurements at 77 K. The Jobin–Yvon spectrofluorimeter was modified to work at 77 K. The sample was contained in a Suprasil quartz tube of 2-mm inner diameter. The quartz tube was maintained in a Dewar flask filled with liquid nitrogen. Total luminescence spectra were recorded in the absence of the phosphoroscope, using an excitation wavelength of 275 nm. Phosphorescence spectra were recorded using a rotating can phosphoroscope. The excitation wavelength was 280 nm. Phosphorescence decays were measured with a Tektronix oscilloscope equipped with a Polaroid camera.

RESULTS

The thiomercuribenzoate sugar derivatives were found to be stable in aqueous solution at neutral pH. No spectral change and no degradation product was detected within one month in 0.05 M Tris·HCl/0.15 M NaCl buffer, pH 7.2, at 277 K.

The thiomercuribenzoate derivative of di-*N*-acetyl-β-chitobiose (CB-SHgBzO⁻) was able to inhibit the agglutination of rabbit erythrocytes induced by WGA. The minimum concentration (80 μM) required to give inhibition was close to that of the 1-*O*-methyl-di-*N*-acetyl-β-chitobioside (70 μM).

The thiomercuribenzoate derivatives are well suited for spectroscopic studies because they absorb only weakly above 270 nm (Fig. 2). This enabled us to excite tryptophan residues of WGA rather selectively. At room temperature, the tryptophan fluorescence of WGA was quenched upon addition of thiomercuribenzoate derivatives (GlcNAc-SHgBzO⁻, CB-SHgBzO⁻, or CT-SHgBzO⁻). As shown in Fig. 3, CB-SHgBzO⁻ quenched WGA fluorescence nearly completely. Addition of 1-*O*-methyl-di-*N*-acetyl-β-chitobioside (CB-OCH_3) restored the fluorescence spectrum expected for the WGA–disaccharide complex: the fluorescence intensity was enhanced and the fluorescence maximum was shifted toward shorter wavelength. When the thiomercuribenzoate derivative of β-D-glucopyranose was added, no fluorescence quenching occurred. Furthermore, the fluorescence of *N*-acetyltryptophanamide (10 μM) was not affected by the presence of the thiomercuribenzoate derivatives used in the same concentration range. The thiomercuribenzoate derivatives of *N*-acetyl-β-D-glucosamine and of tri-*N*-acetyl-β-chitotriose quenched partially the tryptophan fluorescence of WGA (40% and 60%, respectively).

These results demonstrate that: (*i*) Thiomercuribenzoate derivatives that do not serve as ligands do not quench the

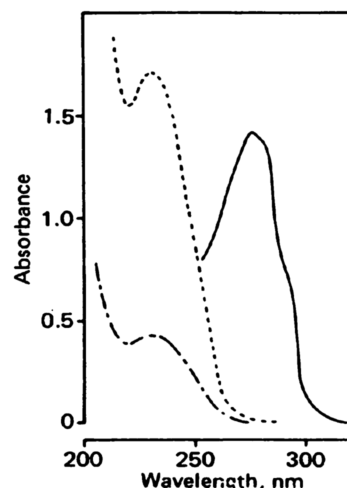


FIG. 2. Absorption spectra of: ——, WGA (28 μM); - - -, 1-*S*-thiomercuribenzoate derivatives of di-*N*-acetyl-β-chitobiose (0.1 mM); and - — -, benzoate (0.5 mM) in 0.15 M NaCl/0.05 M Tris·HCl, pH 7.2; 298 K. Optical path, 1 cm.

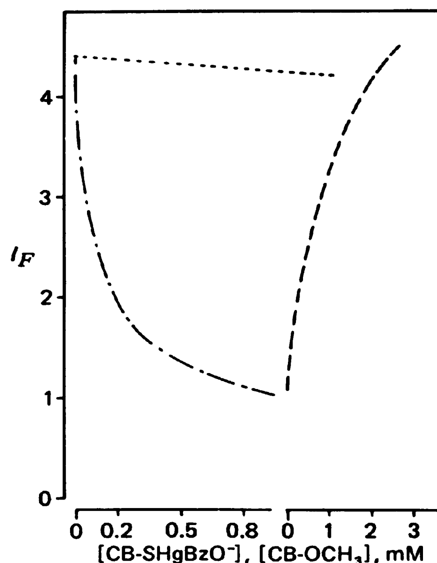


FIG. 3. Change of fluorescence of WGA (1.4 μM) upon adding 1-*S*-thiomercuribenzoate-β-D-glucopyranose (- - -); upon adding 1-*S*-thiomercuribenzoate derivatives of di-*N*-acetyl-β-chitobiose (- — -); the subsequent addition of 1-*O*-methyl-di-*N*-acetyl-β-chitobioside reversed the mercuribenzoate quenching (— — —). Buffer: 0.15 M NaCl/0.05 M Tris-HCl, pH 7.2; 298 K. I_F, fluorescence intensity in arbitrary units.

tryptophan fluorescence of WGA or the fluorescence of free tryptophan. (*ii*) In contrast, the specific ligands substituted by the heavy metal do quench the tryptophan fluorescence of WGA, and the effect is maximum with CB-SHgBzO$^-$. (*iii*) CB-SHgBzO$^-$ and CB-OCH_3 compete for the same binding site of WGA.

At low temperature (77 K), WGA emits a fluorescence whose maximum is at 325 nm and a phosphorescence whose vibronic structure is characteristic of tryptophan (Fig. 4). The addition of CB-OCH_3 shifted the fluorescence maximum slightly to shorter wavelengths and the phosphorescence maximum to longer wavelengths. By using a phosphoroscope, the fluorescence contribution could be eliminated from the emission spectrum. The phosphorescence spectrum thus recorded (Fig. 5) contained very little of the tyrosine triplet state emission (seen at wavelengths shorter than 390 nm) and could be ascribed mainly to tryptophan. The phosphorescence of benzoate and CB-SHgBzO$^-$ was also recorded (Fig. 5). The vibronic structure was clearly apparent in the case of CB-SHgBzO$^-$ but not in the case of benzoate.

At room temperature, several studies (12, 14, 16) have shown that only two out of the three tryptophan residues per subunit emit fluorescence. At low temperature, it is reasonable to assume that the same situation prevails. However, we do not know how many tryptophans emit phosphorescence. Upon addition of CB-SHgBzO$^-$, dramatic effects were observed on the low-temperature emission spectrum (Fig. 6). Whereas fluorescence was nearly completely quenched, the phosphorescence emission of WGA was strongly enhanced. There was a 6-fold difference in the phosphorescence intensities in the absence and in the presence of an equimolar concentration of CB-SHgBzO$^-$. The shapes of the phosphorescence spectra in the absence and in the presence of CB-SHgBzO$^-$ were essentially identical. When an excess of CB-OCH_3 was added to the WGA–CB-SHgBzO$^-$ complex, the phosphorescence intensity was similar to that of free WGA.

Similar qualitative results were obtained with the monosaccharide derivative (GlcNAc-SHgBzO$^-$): the fluorescence

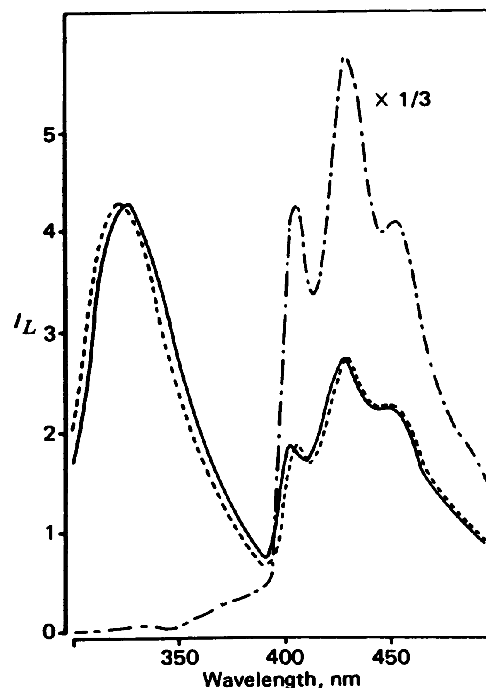


FIG. 4. Uncorrected luminescence spectra at 77 K of free WGA (14 μM) (——); WGA (14 μM) + 1-*O*-methyl-di-*N*-acetyl-β-chitobioside (1 mM) complex (- - -); and WGA (12.5 μM) in the presence of the 1-*S*-thiomercuribenzoate derivative of di-*N*-acetyl-β-chitobiose (280 μM) (- — -). I_L, fluorescence or phosphorescence intensity in arbitrary units.

emission was nearly totally quenched, while the phosphorescence intensity was increased 5-fold. However, if CT-SHgBzO$^-$ was used as a ligand the effect was much less important than with CB-SHgBzO$^-$. The phosphorescence intensity was increased only 2-fold.

The enhancement of the phosphorescence intensity in the presence of CB-SHgBzO$^-$ was accompanied by a decrease of the phosphorescence lifetime (Table 1) from 5.6 sec for free WGA to 0.4 sec for WGA in the presence of CB-SHgBzO$^-$. The phosphorescence decay was exponential and no long-lived

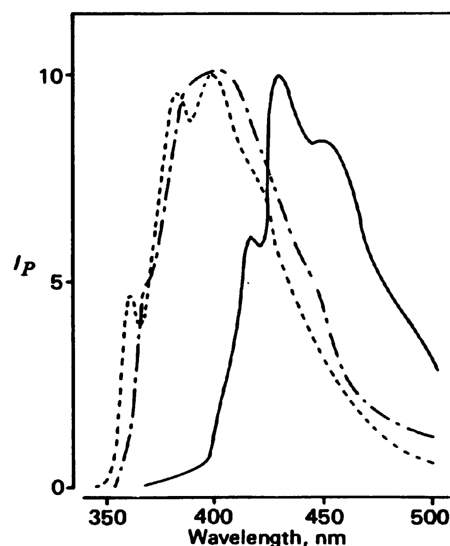


FIG. 5. Normalized phosphorescence emission spectra of WGA (——), benzoate (- - -), and the 1-*S*-thiomercuribenzoate derivative of di-*N*-acetyl-β-chitobiose (- — -), in 0.15 M NaCl/0.05 M Tris-HCl, pH 7.2; 77 K. Excitation wavelengths: 270 nm for WGA, 240 nm for benzoate and CB-SHgBz; 77 K. I_P, phosphorescence intensity in arbitrary units.

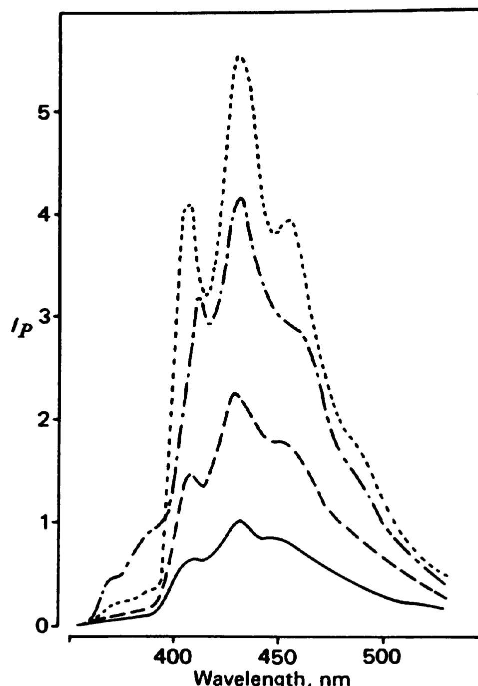


FIG. 6. Uncorrected phosphorescence spectra of free WGA (13 μM) (——) and WGA (12.5 μM) in the presence of 1-*S*-thiomercuribenzoate derivatives of tri-*N*-acetyl-β-chitotriose (280 μM) (— —); 1-*S*-thiomercuribenzoate derivatives of di-*N*-acetyl-β-chitobioside (500 μM) (- - -); and the 1-*S*-thiomercuribenzoate derivative of *N*-acetyl-β-D-glucosamine (1 mM) (- — -); in 0.15 M NaCl/0.05 M Tris-HCl, pH 7.2, at 77 K. Excitation wavelength: 270 nm; I_P, phosphorescence intensity in arbitrary units.

component was detected in the presence of equimolar concentrations of protein and ligand. Addition of CB-OCH_3 to the WGA–CB-SHgBzO$^-$ complex led to a reversal of the above effect. Upon addition of GlcNAc–SHgBzO$^-$ the phosphorescence decay was not exponential, but it could be represented as a superposition of two decays with lifetimes of 0.4 and 3.3 sec. Furthermore, if CT-SHgBzO$^-$ was used as a ligand, the phosphorescence lifetime was reduced from 5.6 to 4.4 sec. In all cases, the thiomercuribenzoate derivatives exhibited some phosphorescence emission whose contribution could be clearly seen at wavelengths shorter than 390 nm, especially if the excitation was set at wavelengths shorter than 290 nm. This phosphorescence was characterized by a low quantum yield, and by a lifetime of 0.26 sec that did not depend on whether or not the thiomercuribenzoate derivative was bound to WGA. For comparison, the lifetime of benzoate was 0.92 sec.

DISCUSSION

The binding of the thiomercuribenzoate derivatives of oligosaccharides to WGA is related to the nature of the sugar moiety. In spite of the large size of the mercury atom (van der Waals radius 1.54 Å), the binding of the thiomercuribenzoate ligands is not precluded.

The emission properties of WGA are drastically affected by the binding of the thiomercuribenzoate derivative of di-*N*-acetylchitobiose. At room temperature (298 K) the fluorescence of the tryptophyl residues of WGA is strongly quenched. In contrast to the effect of di-*N*-acetylchitobiose, which increases the fluorescence intensity and shifts the maximum toward shorter wavelengths (13, 15), the progressive addition of CB-SHgBzO$^-$ leads to a decrease of the fluorescence intensity without any effect on the spectrum. The fluorescence

Table 1. Phosphorescence lifetime of WGA and various ligand–WGA complexes at 77 K

Molecules	Lifetime, sec
WGA	5.6
WGA + CB-OCH_3	5.9
WGA + GlcNAc-SHgBzO$^-$	0.4 (0.9) and 3.3 (0.1)
WGA + GlcNAc-SHgBzO$^-$ + CB-OCH_3	5.6
WGA + CB-SHgBzO$^-$	0.4
WGA + CB-SHgBzO$^-$ + CB-OCH_3	5.7
WGA + CT-SHgBzO$^-$	4.4
WGA + CT-SHgBzO$^-$ + CB-OCH_3	5.7
BzO$^-$	0.92
CT-SHgBzO$^-$	0.26

Numbers in parentheses refer to the proportion of the species. Phosphorescence decay was recorded at 437 nm for WGA and at 390 nm for BzO$^-$ and CT-SHgBzO$^-$.

quenching was reversed upon addition of CB-OCH_3, indicating that the binding of the thiomercuribenzoate derivative is fully reversible and that binding of both ligands occurs at the same binding site.

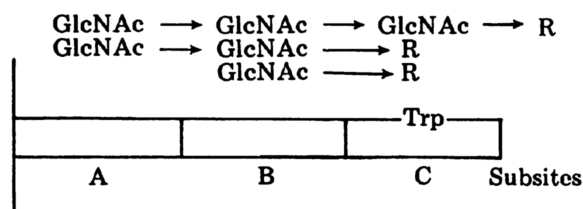
Each protomer of WGA has three tryptophan residues. Only two of them are fluorescent at room temperature. There are two binding sites per protomer and there is one fluorescent tryptophan per site (12). At low temperature, fluorescence quenching by CB-SHgBzO$^-$ is nearly quantitative, indicating that—as at room temperature—the third tryptophan does not fluoresce. Furthermore, fluorescence quenching is accompanied by an enhancement of phosphorescence emission (6-fold) together with a drastic shortening of the phosphorescence lifetime (14-fold). These results are characteristic of an increased spin-orbit coupling probability. The phosphorescence spectra of the WGA–CB-SHgBzO$^-$ complex and the free WGA are both characteristic of the phosphorescence of a tryptophan residue. Therefore, all these modifications are typical of an external heavy atom effect on the spectroscopic properties of tryptophan residues.

The observed effects (fluorescence quenching, phosphorescence enhancement, and lifetime decrease) of CB-SHgBzO$^-$ on the luminescence properties of WGA are reversed upon addition of CB-OCH_3. These results indicate that—at low temperature as well as at room temperature—the binding of the thiomercuri-derivative occurs at the same site as the normal ligand, and that the tryptophan luminescence is perturbed only when the heavy atoms may reach the binding site.

The monosaccharide derivative (GlcNAc-SHgBzO$^-$) has similar effects as the disaccharide. However, at room temperature fluorescence quenching is not quantitative, and at 77 K the phosphorescence enhancement is smaller than with the disaccharide derivative. It should be noted that the affinity constant of GlcNAc-SHgBzO$^-$ is quite low, and that the concentration required to reach the saturation is quite high, so secondary effects of the free ligand may not be excluded. However, at low temperature the shorter lifetime component was the main one, and the lifetime of this component was identical to that of the disaccharide. Therefore, the heavy atom of the monosaccharide derivative might be located at the same position in the binding site as the heavy atom of the disaccharide derivative. In contrast, the trisaccharide (CT-SHgBzO$^-$) had nearly no effect at all on the luminescence properties of WGA, indicating that the heavy atom does not occupy the same position in the binding site. Indeed, the perturbation of spin-orbit coupling of an aromatic compound induced by an external

heavy atom (here, the mercury atom) is strongly dependent upon the distance between the atom and the luminescent residue (here, the tryptophyl residue of the WGA binding site).

From the results reported above, it can be concluded that the mercury atom is in close contact with the tryptophan residue, when the di-*N*-acetylchitobiose derivative (or the *N*-acetylglucosamine derivative) is present in the binding site. But, when the tri-*N*-acetylchitotriose derivative binds to WGA, the mercury atom is farther away from the tryptophan. Because the binding site of WGA has a higher affinity for the trisaccharide than for the disaccharide and for the dissaccharide than for the monosaccharide (3, 4, 13, 15, 28), we may divide it into three subsites, A, B, and C. Each subsite may accept one *N*-acetylglucosamine residue. The results presented above are consistent with the presence of one tryptophan residue in subsite C. According to our present and previous results, the various ligands may be positioned in the binding site as shown in the following scheme:



in which R is GlcNAc, 4-methylumbelliferyl (10, 28), or thiomercuribenzoate. The free monosaccharide GlcNAc should be bound in subsite B, because, in contrast with the disaccharide or the trisaccharide, GlcNAc does not modify the fluorescence spectrum of WGA (13). According to the three-dimensional structure of WGA (18), tryptophan residue 21 could be the tryptophan of subsite C of one of the binding sites.

The method that we have described above, using the enhancement of spin-orbit coupling due to the perturbation induced in tryptophan residue by ligand containing heavy atom, has proved to be useful in locating the tryptophyl residue in the binding site of WGA. This method can be extended to other sugar-binding proteins. Preliminary measurements have shown that the thiomercuribenzoate of tri-*N*-acetyl-β-chitotriose enhances intersystem crossing in one or more tryptophan residues of lysozyme. The galactose-binding site of *lac* repressor might also be studied with this technique.

This work was supported by Institut National de la Santé et de la Recherche Médicale Grant 75.4.074.3, Délégation Général à la Recherche Scientifique et Technique Grant 77.7.0252, and a grant from the Fondation pour la Recherche Médicale Française to M.M.

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