Vol. 110, No. 3, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 10, 1983 Pages 926-933

PROTEIN-SUGAR INTERACTIONS : ENVIRONMENTAL EFFECT ON THE FLUORESCENCE OF *O*-(4-METHYLUMBELLIFERYL)-GLYCOSIDES

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Received December 27, 1982

We have investigated the effect of 12 solvents and several amino acids on the fluorescence of *O*-(4-methylumbelliferyl)-glycosides. We showed that : i) the fluorescence quenching is not related to the dielectric constant of the solvents : the fluorescence intensity was maximal in water (d=80) and in acetic acid (d=6.2) and was at least ten times lower in acetone (d=21) and in dioxane (d=2.2); ii) the fluorescence of *O*-(4-methylumbelliferyl)-*N*-acetyl-β-glucosaminide is not quenched in the presence of various amino acids including arginine, asparagine, aspartate, histidine, leucine, phenylalanine and proline ; iii) the fluorescence of *O*-(4-methylumbelliferyl)-glycoside is quenched by sulfur, phenol and indole amino acids or derivatives containing sulfur, phenol or indole groups. The changes in fluorescence intensities of *O*-(4-methylumbelliferyl)-glycosides upon binding to concanavalin A, wheat germ agglutinin and lysozyme are discussed with regard to the amino acid content of their binding sites.

O-(4-methylumbelliferyl)-glycosides have been successfully used to investigate the interaction of saccharides with lectins or enzymes (1-6). It was often observed that the ligand emission is quenched upon complex formation with lectins. It was suggested that this effect is related to the dielectric nature of the ligand environment (1,3). A similar interpretation was also proposed in the case of the interaction between 4-carboxymethylcoumarin and a specific antibody (7). In order to ascertain whether the observed fluorescence quenching of *O*-(4-methylumbelliferyl)-*N*-acetyl-β-D-glucosaminide (MUF-GlcNAc) upon binding to wheat germ agglutinin could be due to an hydrophobic environment we undertook a systematic investigation of the emission of MUF-GlcNAc , in various solvents and in the presence of various amino acids and related compounds.

MATERIALS AND METHODS

O-(4-methylumbelliferyl)-*N*-acetyl-β-D-glucosaminide and the peracetylated derivative were prepared as previously described (8). Concentrations of *O*-

Abbreviations : MUF : 4-methylumbelliferyl ; WGA : wheat germ agglutinin ; GlcNAc : *N*-acetyl-D-glucosamine

0006-291X/83/030926-08$01.50/0

(4-methylumbelliferyl)-glycosides in water were determined using a molar absorbance of 1.28×10^4 l x mole^{-1} x cm^{-1} at 316 nm (8). All amino acids were obtained from Sigma (Saint-Louis U.S.A.) ; glycylglycine from Serva (Heidelberg, G.F.R.) ; Urea from Merck (Darmstadt, G.F.R.), sodium 3,3'-dithiodipropionate from Aldrich (Beerse, Belgium), sodium indolacetate from Behring and guanidinium chloride from Sigma. Amino acids were used without further purification.

Wheat germ agglutinin prepared as previously described (9) was purchased from Pointed Girard, I.B.F.-Reactifs, Villeneuve-la-Garenne, France. Concentrations of WGA were determined by absorbance measurements at 280 nm $E^{1\%}_{280nm}=15$ and were expressed on the basis of four sugar binding sites per molecule (2,10,11). Solvents of analytical or spectroscopic grade were freshly distilled ; 1,4-dioxane was peroxide-free. Spectroscopic measurements in aqueous solutions were performed in 0.15M NaCl, 0.05M phosphate buffer, pH 7.2. Excitation and emission spectra were recorded at 25°C with a Fica MKII spectrofluorimeter (Fica, Le Mesnil Saint-Denis, France). Solutions were contained in 5 x 5 cm quartz cuvettes. The excitation wavelength was 336 nm which is isosbestic point of a mixture of β MUF-GlcNAc and WGA (3). All aqueous solutions were filtered on Millipore HAWP 0.45µm pore diameter filters before use. Interaction experiments were conducted at a constant MUF-GlcNAc concentration in the presence of various amounts of the tested compounds.

RESULTS

Fluorescence spectra of *O*-(4-methylumbelliferyl)-*N*-acetyl-β-D-glucosaminide were recorded in twelve solvents. The wavelength of maximum intensity was found to be independent of the solvent. The fluorescence intensity was maximum when the MUF-glycoside was dissolved in water. The relative fluorescence intensity is not related to the dielectric constant of the solvent as shown in figure 1.

Indeed, the fluorescence intensity in acetic acid (dielectric constant : 6.2) is almost as large as that in water (dielectric constant : 80) ; those in acetone (dielectric constant : 21) or in nitromethane (dielectric constant : 38) are lower than in 1,4-dioxane (dielectric constant : 2.2). The scatter of experimental points is slightly reduced when Kosower's Z-value (12) is the independent variable.

As a further check, absorption and excitation spectra were recorded using the same solvent (table 1).

The absorption is somewhat solvent dependent at wavelengths shorter than 300 nm. The spectra are however unchanged in shape at wavelengths higher than 300 nm. Similarly, excitation spectra recorded at 377 nm (wavelength of the maximum emission) have the same shape. Thus, the energy levels of MUF-GlcNAc which correspond to absorption in the range 300-350 nm are solvent independent.

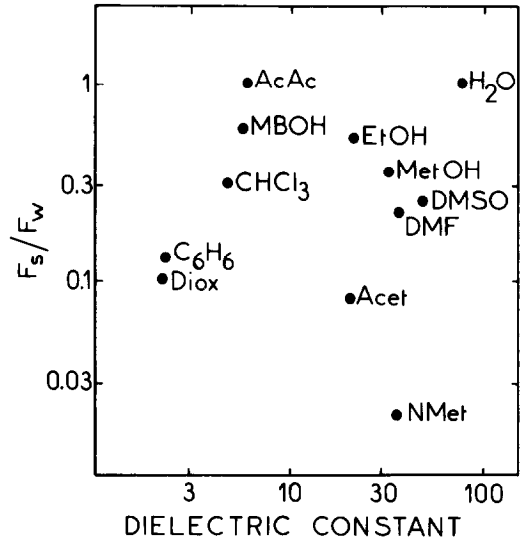

FIGURE 1 : Effect of various solvents on the fluorescence intensity of *O*-(4-methylumbelliferyl)-glycosides. F_s : fluorescence intensity in a given solvent. F_W : fluorescence intensity in water. Peracetylated MUF-GlcNAc (5 x 10^{-6}M) in C_6H_6 : benzene ; $CHCl_3$: chloroform ; Acet : acetone ; NMet : nitromethane. MUF-GlcNAc (5 x 10^{-6}M) in Diox : dioxane ; MBOH : 2-methyl-2-butanol ; AcAc : acetic acid ; EtOH : ethanol ; MetOH : methanol ; DMF : *N*-dimethylformamide ; DMSO : dimethylsulfoxide ; H_2O : water.

However the exctinction coefficient ε does depend on the solvent ; in dimethylsulfoxide and in dioxane, ε is significatively lower than in water or in acetic acid, while the dielectric constant of the former is close to that of water and the latter is lower than that of acetic acid. So, the fluorescence intensities which are strongly dependent on the solvent are neither related to the dielectric

Table 1 : Characteristic parameters of the absorption and excitation spectra of *O*-(4-methylumbelliferyl)-*N*-acetyl-β-D-glucosaminide in various solvents

Solvent	λ^{abs}_{max}	λ^{exc}_{max}	ε max	ε 336
	nm		$mM^{-1} \times cm^{-1}$	
Water	316	316	12.8	4.96
Ethanol	316	316	12.8	5.28
N-dimethylformamide	316	316	11.4	4.96
Dimethylsulfoxide	316	316	9.4	4.16
Acetic acid	316	316	12.8	5.12
Dioxane	316	316	10.1	4.00

λ^{abs}_{max} : wavelength of the maximum of absorption spectrum ; λ^{exc}_{man} : maximum of exicitation spectrum ; ε max : millimolar absorbance at the wavelength of the maximum absorption ; ε 336 : millimolar absorbance at the excitation wavelength, 336 nm.

Table 2 : Relative fluorescence intensity of *O*-(4-methylumbelliferyl)-*N*-acetyl-β-D-glucosaminide in the presence of 0.1 M L-amino acids and related compounds

Compounds	F/F_o (*)	Compounds	F/F_o
		Arginine	1.03
Glycine	1.00	Histidine	1.04
Alanine	1.01	Phenylalanine	1.16
Leucine	1.01	Tyrosine-OCH_3	0.60
Serine	0.98	*N*-Ac tryptophan	0.33
Threonine	0.97	Indolacetate	0.33
Aspartate	1.08	Methionine	0.81
Glutamate	1.02	3,3'-dithiodipropionate**	0.81
Asparagine	0.98	Glycylglycine	1.00
Glutamine	1.01	Urea	1.03
Proline	1.00	Guanidinium chloride	1.01

(*) Fluorescence intensity of MUF-GlcNAc (4.4×10^{-6}M) in the presence (F) and in the absence (F_o) of amino acids or related compounds, λ exc : 336 nm ; s.d. : ± 0.05.
(**) MUF-GlcNAc (1.4×10^{-4}M), λ exc : 350 nm.

constant of the solvent nor to the effect of the solvent on the extinction coefficient.

Since the fluorescence quantum yield of MUF-GlcNAc is apparently not related to some bulk property, we have investigated whether a specific interaction could be responsible for the observed effects. Indeed, the fluorescence of MUF-GlcNAc was found sensitive to the presence of certain amino acids.

As shown in table 2, most amino acids do not affect the MUF-GlcNAc fluorescence, with some notable exceptions : some aromatic compounds (indolacetate, tyrosine, tryptophan) and compounds bearing a thioether or a disulfide group (methionine and dithiodipropionate). Due to the low solubility of cysteine, tyrosine and tryptophan, the following compounds 3,3'-dithiodipropionate, L-tyrosine methylester and *N*-acetyl-L-tryptophan were respectively used to determine the quenching extent and the apparent association constants (figure 2).

At 25°C, the fluorescence of MUF-GlcNAc was quenched quantitatively (95±5p100) by 3,3'-dithiodipropionate, L-tyrosine methylester, indoacetate and *N*-acetyl-L-tryptophan and whatever the quencher was no shift of the emission maximum was observed. This indicates the formation of 1:1 non fluorescent complexe with MUF-GlcNAc and these compounds ; the association constant was found to be 5, 7, 21 and 23 l x $mole^{-1}$ respectively.

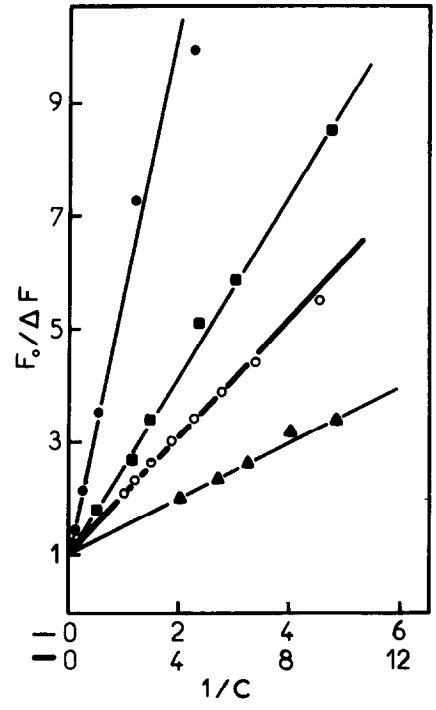

FIGURE 2 : Double reciprocal plot of the emission intensity of MUF-GlcNAC, as a function of quencher concentration.
(λ exc : 350 nm) :● —— ● MUF-GlcNAc (1.4 x 10^{-4}M) and 3,3'-dithiodipropionate (0.042 to 0.68M).
(λ exc : 336 nm) :■ —— ■ MUF-GlcNAc (4.4 x 10^{-6}M) and L-tyrosine methylester (0.02 to 0.2 M) ;○ —— ○ MUF-GlcNAc (3.8 x 10^{-6}M) and wheat germ agglutinin (2.8 x 10^{-5} to 2.8 x 10^{-4}M) ;▲ —— ▲ MUF-GlcNAc (4.4 x 10^{-6}M) and *N*-acetyl tryptophan (0.02 to 0.05 M).
F_o : fluorescence intensity without quencher ; F : fluorescence intensity with quencher ; $\Delta F = F_o - F$; C : total concentration of —— amino acid derivatives (M x 10) or of ▬▬ wheat germ agglutinin (M x 10^4).

DISCUSSION

The binding of *O*-(4-methylumbelliferyl)-glycosides (MUF-glycosides) to cancanavalin A (1, 13), to wheat germ agglutinin (2,3) and to *Griffonia simplicifolia* I (6) induce a large decrease of the ligand fluorescence. Several authors have attempted to correlate the relative fluorescence quantum yield of the complex to the polarity of the ligand environment (1, 3, 4-7). The data presented above make this interpretation questionnable.

Although in some low dielectric constant solvents such as dioxane (3) the fluorescence quantum yield of MUF-glycoside is ten fold lower than in water, the data shown in figure 1 clearly demonstrate that a low polarity environment

cannot account *per se* for the quenching observed upon MUF-glycoside binding to the lectins.

Indeed, the solvent quenching is more likely related to specific properties of the solvent molecules than to a bulk property such as the solvent dielectric constant . On the contrary the fluorescence quenching of MUF-GlcNAc observed in the presence of *N*-acetyl-L-tryptophan, indolacetate, L-tyrosine methylester or dithiodipropionate could be due to the formation of a charge transfer complex with the aromatic (or disulfide) groups, the coumarin acting as an electron acceptor (14). The other amino acids tested as well as a dipeptide glycylglycine, urea and guanidinium chloride do not quench the MUF-glycoside fluorescence. So, no one of these amino acids or side chain analogues can be accounted for the quenching induced by wheat germ agglutinin. As shown in figure 2, the extent of MUF-GlcNAc fluorescence quenching by wheat germ agglutinin is similar to that obtained in the presence of tryptophan, tyrosine derivatives or cystine analogues.

According to previous studies using sugar inhibition of agglutination, the binding site of wheat germ agglutinin comprises three subsites (15). One subsite (B) binds the acetamido group (16, 17) as shown by nuclear magnetic resonance studies, and contains tyrosine residues (Tyr-71 and Tyr-29) (18). The vicinal subsite (C) might contain a tryptophan residue as shown by phosphorescence studies of wheat germ agglutinin thiomercury-glycoside interactions (19). Cristallographic results show two types of sites : a tryptophan residue (21_{II}) present in the secondary sites is located at 8 Å from the second / third GlcNAc (GlcNAc)$_3$; a tryptophan residue close to the second GlcNAc of (GlcNAc)$_2$ is possible in position 61_{II} in the primary sites (18). Wheat germ lectin is exceptionally rich in half cystine (16 disulfide bonds per protomer). It is therefore tempting to interpret the quenching of the fluorescence of *O*-(4-methylumbelliferyl)-*N*-acetyl-β-D-glucosaminide induced upon binding to wheat germ agglutinin as a specific interaction of the coumarin aglycone with an aromatic side chain of tyrosine and/or tryptophan residues and/or with a disulfide bond.

In the case of the concanavalin A which contains no disulfide bond, the presence of two tyrosyl residues (Tyr-12 and Tyr-100) is clearly shown in the

carbohydrate binding region according to the 3D structure (20). The proximity of these tyrosine residues and the 4-methylumbelliferyl group of MUF-α-D-mannopyranoside and the observed quenching of the MUF-glycoside fluorescence upon binding to concanavalin A are consitent with the data reported above.

Unlike concanavalin A and wheat germ agglutinin, lysozyme increased the fluorescence of MUF-glycoside upon binding (8). According to the 3D structure of lysozyme (21) the 4-methylumbelliferyl group of MUF-tri-*N*-acetylchitotrioside and of MUF-tetra-*N*-acetylchitotetraoside should be located in the subsites D and E, respectively (4, 5), where there is neither aromatic nor disulfide groups but carboxylate (Asp-52), carboxamide (glu-35 and glu-57) and aliphatic (Leu-56) groups (21).

On the basis of the present study, the fluorescence quenching of MUF-glycoside upon binding to concanavalin A and wheat germ agglutinin is related to the presence of aromatic (Trp or Tyr) groups or sulfur containinggroups in the binding site and the absence of quenching of MUF-glycoside upon binding to lysozyme is in agreement with the absence of such groups in the related subsites. So, the quenching of MUF-glycoside fluorescence upon binding to a lectin or an enzyme is not related to a low dielectric environment but is related to interactions of the MUF group with aromatic amino acids (Tyr or Trp) or sulfur amino acids.

ACKNOWLEDGEMENTS

This work was supported in part by grants form ACC 77.7 0252 and ACC 78.7.1086 from Délégation Générale à la Recherche Scientifique et Technique.

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