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Fluorescence Spectrometry in Studies of Carbohydrate-Protein Interactions

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Fluorometric spectroscopy is a powerful tool for investigating the interaction between a carbohydrate ligand and binding proteins. The measurement is done *in situ* and thus circumventing the need for separation of bound ligand from the free ligand. The source of the fluorophore can be intrinsic, *i.e.*, the tryptophan in the protein, or extrinsic (contained in the ligand). Techniques for assessing the affinity used are measurement on fluorescence intensity change, lifetime, polarization anisotropy, and energy transfer. The last technique can also be used to study conformational structures of glycopeptides. It is also useful in designing substrates for endo-type enzymes which allow continuous monitoring of the reaction.

Key words: energy transfer, conformation, glycopeptides, substrates.

The value of fluorescence spectrometry in biochemical and biological research is well recognized (*1, 2*). In the investigation of carbohydrate-protein interactions, fluorescence spectrometry has proven to be a very powerful tool. The intention of this article is to concisely review different methodology of fluorescence spectrometry as applied to the studies on carbohydrate-protein interactions.

An interaction between two molecules can be investigated from many different angles, but the most important information to be obtained is the strength of interaction. The strength of interaction or affinity is often expressed as either *association constant* or *dissociation constant*.[2] Many methods for the measurement of association or dissociation constants require physical separation of the bound form of one of the reactans, usually the carbohydrate ligand, from the free unbound form. Such methods, although straightforward in principle, can result in a less accurate assessment of the interaction, because during the time required for separation of the bound from the free ligand, some dissociation of the complex into its components can take place, especially if the affinity is weak (*3*). Most ideally, the strength of interaction should be measured without perturbing the equilibrium. Fluorescence spectrometry accomplishes this goal.

Fluorescence data can be obtained in the form of changes in fluorescence of binding protein or ligands, or as changes in polarization or anisotropy. These topics will be discussed separately.

Changes in protein fluorescence upon binding of ligands

A. Change in Fluorescence upon Binding of Non-Fluorescent Carbohydrates—Practically all proteins that bind carbohydrates, including immunoglobulins, contain tryptophan, whose innate fluorescence can change upon binding of carbohydrates. Since the fluorescence of tryptophan is influenced by its microenvironment, it is easy to understand that any changes (conformational or otherwise) which affect the environment of tryptophan would cause changes in its fluorescence properties. An example based on the actual experimental data (*4*) is shown in Fig. 1A to illustrate the fluorescence change upon ligand binding. The changes may be an increase or a decrease in intensity, with (*5*) or without change (*6*) of the spectral shape or the peak position of maximum intensity. Although the change in tryptophan fluorescence sometimes is interpreted as "involvement of tryptophan in the binding," it may not be the consequence of the direct participation of tryptophan in binding.

In practice, the maximum change in the fluorescence intensity (ΔF_{max}) can be obtained either visually by plotting the ΔF values against the concentration of ligand, or it can be more accurately obtained by plotting ΔF/[Free] *vs.* ΔF as shown in Fig. 1B. Since the maximum change in fluorescence, ΔF_{max}, can be brought about when all the available binding sites are occupied by the ligand, and if it can be assumed that fractional occupation of the available sites results in a linearly proportional change in the fluorescence, then the fraction of the sites occupied ($\bar{v}$) can be easily calculated. Thus, a Scatchard plot ($\bar{v}$/[Free] *vs.* $\bar{v}$) can be constructed from such data to obtain the K_a from the slope of the plot, because

$$\bar{v}/[\text{Free}] = K_a - \bar{v}K_a$$

A general procedure for such measurement of the fluorescence change when protein binds a ligand has been described (*4*). Detailed thermodynamic analysis of anti-galactan monoclonal antibody was performed by this method using the data obtained at different temperatures (*7*). Although Scatchard plot is commonly used for analysis of

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[2] Although "affinity constant" or "binding constant" is occasionally used to designate the notion of association constant, it is preferable to use "association" constant to denote the equilibrium constant for the reaction A+B⇌AB.

physical constants of binding, computer-assisted non-linear regression using programs such as LIGAND (*8*) or NONLIN (*9*) may be more preferable. An example of using NONLIN to fit the fluorescence titration curves of Gal repressor binding Gal is shown in Fig. 2A (*6*). It is noteworthy that the affinity determination was accomplished despite the fact that the maximum fluorescence increase upon binding of Gal amounted to less than 8% of that before addition of Gal (Fig. 2B).

In one of the earliest examples, the fluorescence changes of hen's egg lysozyme as it binds *N*-acetyl-glucosamine (GlcNAc) or *N*-acetyl-muramic acid (MurNAc) and their derivatives and oligosaccharides (*10*) were used to determine the association constants (*11*). Although the relative magnitude of the change in fluorescence intensity was not large, the method allowed the deciphering of the individual subsites (A-E). When wheat germ agglutinin binds chitooligosaccharides, similar fluorescence changes were observed, and the magnitudes of change were much larger than those observed for lysozyme (*10*).

Another early example is the studies on antibody-antigen reactions by Jolley and Glaudemans (*12*). The detailed analysis of interaction of anti β-(1-6)-galactan antibodies with a series of deoxyfluoro derivatives was also reported (*13*). Although the binding of the fluoro-derivatives was much weaker than the natural antigen, they were able to determine the binding affinity (K_a) as low as 10^2 M^{-1}.

A good example of greater efficacy of fluorescence spectrometry compared to other methods is given by the measurement of binding by *Bandeiraea simplicifolia* isolectins. Association constants for the binding of methyl α-D-galactopyranoside (Me α-D-Gal) and methyl 2-acetamido-2-deoxy-α-D-galactopyranoside (Me α-D-GalNAc) to three isolectins (A_4, A_2B_2, B_4) were determined by equilibrium dialysis and fluorescence enhancement measurements. Equilibrium dialysis measurement indicated that although isomeric tetramers, A_4, A_2B_2, and B_4, have approximately the same K_a for Me α-D-Gal ($1.6\text{-}2.1\times10^4$ M^{-1}) for Me α-D-Gal, A_4 and A_2B_2, bound Me α-D-GalNAc with stronger affinities, $K_a = 1.87\times10^5$ M^{-1} and $K_a = 1.19\times10^5$ M^{-1}, respectively, while binding by B_4 was too weak to be measured. The presence of four additional very weak Me α-D-GalNAc binding sites for B_4 ($K_a = 1.26\times10^2$ M^{-1}) was, however, detected by measurement of fluorescence enhancement (*14*).

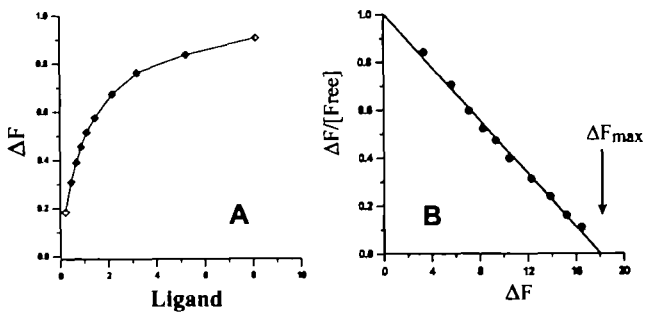

Fig. 1. **An example of changes in fluorescence as a protein binds a specific carbohydrate ligand.** A: Apparent changes in fluorescence. The value of ΔF is determined by plotting ΔF/Free ligand *vs.* ΔF (*4*).

B. Fluorescence Change after Binding of Fluorescent or Colored Carbohydrates—When a carbohydrate ligand itself contains a fluorescent probe, the fluorescence of the ligand may change upon binding. This can be caused either due to the change in the microenvironment of the fluorescent probe or as the result of energy transfer (which will be discussed later). The former can occur even when there is no tryptophan in the protein or when the tryptophan in the protein is not affected. Some examples are given below.

The direction and the magnitude of the fluorescence changes vary depending on the nature of interaction. The fluorescence of 4-methylumbelliferyl α-D-mannopyranoside (MU α-Man) is totally quenched upon binding to concanavalin A (Con A), but Con A fluorescence was not affected by binding of ligands (*15*). The binding of 4-methylumbelliferyl galactosides (MU α/β-Gal) by winged bean (*Psophocarpus tetragonolobus*) basic agglutinin (WBA I) was examined by extrinsic fluorescence titration and stopped-flow spectrofluorometry (*16*). Upon binding to WBA I, the fluorescence of MU α-Gal decreased in intensity, accompanied by a decrease in UV absorbance with a blue shift, and a decrease in fluorescence excited-state lifetimes. In contrast, fluorescence of MU β-Gal increased concomitantly with the UV-absorbance which was accompanied by a red shift, and an increase in fluorescence excited-state lifetimes, suggesting that the MU group of α- and β-Gal experiences polar and non-polar microenvironment, respectively, upon binding to WBA I.

The nature and the position of the substituent on a sugar, including its aglycon, affects association constant (see

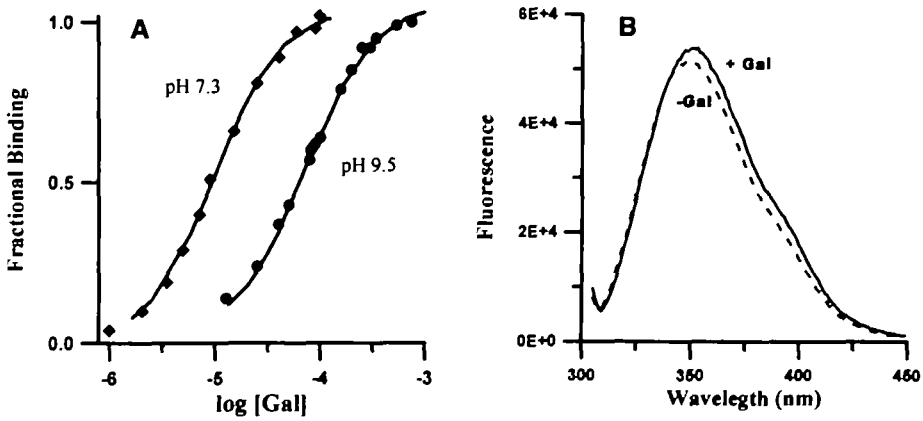

Fig. 2. **The effect of pH on Gal binding by GalR.** A: Fractional binding *vs.* log[Gal]. B: Changes in the emission spectra before and after addition of saturating amount of Gal.

above) as well as changes in fluorescence properties. *Erythrina cristagalli* agglutinin, a dimeric lectin (*17*), was shown to bind both MU β-D-Gal and MU β-D-GalNAc by equilibrium dialysis. However, the changes in extrinsic fluorescence in this case was too small to be useful for quantification. However, upon binding of *N*-dansyl-D-galactosamine to the lectin, there was a fivefold increase in fluorescence intensity of this ligand, and was thus used to study the binding of carbohydrates with the *E. crystagalli* lectin. The greater association constant for *N*-dansyl-D-galactosamine was caused by a very favorable entropic factor contributed by the dansyl group without affecting the innate carbohydrate-protein interaction (*17*).

Polarization and anisotropy

A small molecule moves and rotates freely and very rapidly in solution, so that a fluorescent probe attached to it will be free of directionality (*i.e.*, isotropic). However, when such a molecule is bound to a large molecule which is relatively slow in rotation and tumbling, then the fluorescent probe on the small molecule can acquire directionality (*i.e.*, anisotropic) and is said to be "polarized." Polarization (P) is defined as

$$P=(I_{\parallel}-I_{\perp})/(I_{\parallel}+I_{\perp})$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities observed parallel and perpendicular, respectively, to an arbitrary axis (p. 559 in Ref. *18*).

The extent of such polarization is related to tightness of the binding, and therefore measurement of polarization anisotropy can lead to determination of association constant (*19, 20*). Polarization anisotropy experiments revealed that 2 binding sites per ricin (RCA-60) B chain and 4 binding sites per dimeric agglutinin (RCA-120) (*21*). These results are in good agreement with the equilibrium dialysis results.

Recently fluorescence polarization was used to determine the binding of Lewis^x oligosaccharides by L-selectin. Different Lewis^x glycosides containing a fluorescein group at the terminal position of aglycon were employed (*22*). In this work, r (anisotropy) was determined by the following relationship:

$$r=(V_V-GV_H)/(V_V+2GV_H)$$

where V_V and V_H, respectively, represents vertically and horizontally detected fluorescence when excited in the vertical direction, and $G=H_V/H_H$ where H_V and H_H represent, respectively, vertical and horizontal detection under horizontal excitation. It was found that in order to produce anisotropy, the fluorescein group must be directly attached to the glycosyl residue, as the presence of an ethylene group results in enough flexibility of the fluoresceinyl group to dissipate the anisotropy.

Fluorescence energy transfer

Fluorescence energy transfer (FRET) has been called "molecular ruler" (*1*) capable of measuring the distances in the range of 0.5-10 nm and is thus complementary to nOe in nuclear magnetic resonance. Although nOe is a powerful tool for the conformational analysis of carbohydrates (*23-25*), it is most suitable in the range of 0.1-0.5 nm. For the measurement of distances longer than *ca.* 0.5 nm, one can only determine the distances by stepwise sequential measurement of nOe. FRET, on the other hand, can directly measure the distance ranging from 0.5 to 10 nm, which is often the range most frequently encountered in confor-

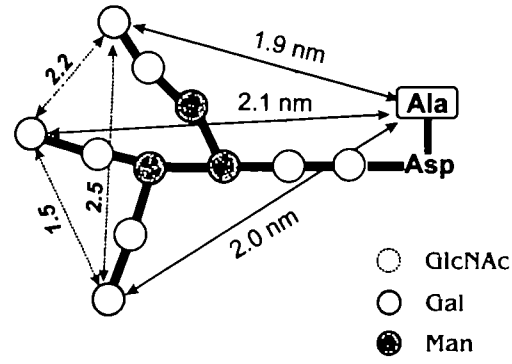

Fig. 4. **Diagram of Ala-Gal distances obtained from fluorescence energy transfer experiments.** The inter-Gal distances (*25*) are also shown (in nm) for comparison.

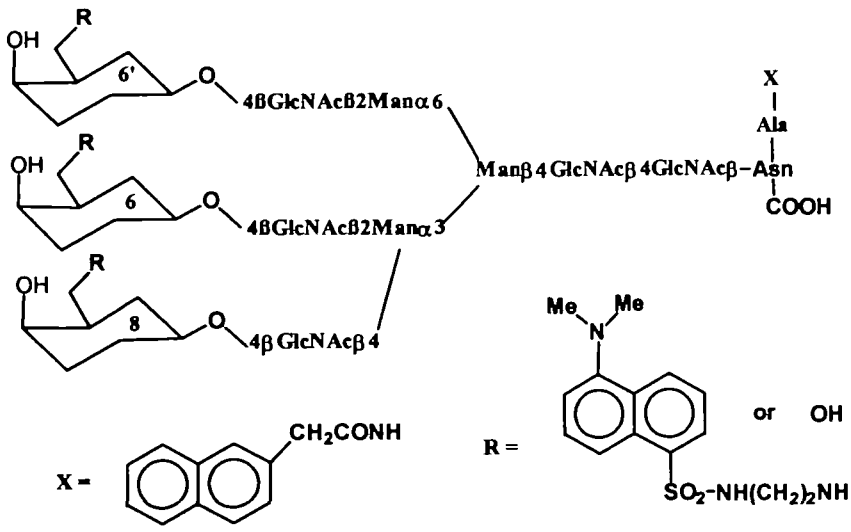

Fig. 3. **Bi-fluorescently labeled triantennary glycopeptide for conformational analyses.**

mational studies of branched oligosaccharides in glycoconjugates. FRET is governed by the Förster's equation as shown below:

$$E = R^6/(R^6 + r^6)$$

where E is the efficiency of energy transfer, r is the distance between the donor and the acceptor, and R is the Förster constant which has to be determined for each specific pair of fluorescent donor-acceptor and the specific experimental conditions (*26*). It is clear from the equation that the efficiency of energy transfer is quite sensitive to the distance.

For FRET to occur efficiently, the donor emission peak and the acceptor excitation peak must overlap as much as possible. A list of effective pairs of donor-acceptor has been compiled (*27*). Each donor-acceptor pair has its unique range of distances, so that the choice of a pair should be as carefully made before the actual fluorescence labeling.[3]

As mentioned above, some examples of fluorescence enhancement of a fluorescent ligand upon binding to protein is caused by FRET between tryptophan in the protein and an acceptor in the ligand (*e.g.*, a dansyl group).

A. Use of FRET for the Studies of Oligosaccharide Conformation—It is important to understand conformational structures of carbohydrate ligands which are recognized by proteins. FRET can be a valuable tool in investigating such conformational problems. For example, the inter-Gal distances in a classic triantennary oligosaccharide or glycopeptide are in the range of 1.5-3.5 nm (*24, 25*), and is therefore more suited for measurement by FRET than by nOe. Similarly, the distance between the peptide portion of a triantennary glycopeptide and the individual terminal Gal residues is in the range where FRET is more direct and efficient than other techniques.

For example, Rice *et al.* (*28, 29*) modified a triantennary glycopeptide from asialofetuin with naphthyl group (N-terminal Ala) and dansyl group (Gal residues) (Fig. 3) and found the static distance between the naphthyl (Nap) group on the N-terminal Ala and the dansyl group (Dan) on the terminal Gal to be 1.9, 2.0, and 2.1 nm as shown in Fig. 4. Measurement of the life-time of decay in such a system can reveal further information on the conformational structures. By using such a technique, the above study (*30*) showed that one of the three branches in the triantennary structure was relatively inflexible but the other two can have two discrete distances—corresponding to their extended (longer distance) and folded (shorter distance) conformations. More amazingly, however, when the unlabeled branches were enzymatically trimmed down to the branching Man, thus creating a bi-fluorescently labeled linear structures, each of the linear structures derived from the three branches showed a single conformation of the extended type (*31*). This can be interpreted to mean that the presence of a neighboring branch is causing the branch to assume the folded conformation, at least part of the time (Fig. 6).

We developed a method of labeling two different chains of monosialylated biantennary glycopeptide as shown in Fig. 5 (*32*). The FRET studies on biantennary glycopeptides, using the same pair of fluorescent donor and acceptor as was used in the studies of triantennary glycopeptide showed that the antenna 6′ was bimodal, but mostly in the extended form, and the antenna 6 was of a single broad distribution (*33, 34*) (Fig. 6). When there is a Fuc attached

[3] There is a dichotomy in this process. In reality, one will have to use an educated guess as to which pair is the best, since the distance that is to be measured is not known yet. Since the effort of attaching fluorescent probes on the carbohydrate groups can be considerable (*32, 60*), choosing a correct pair is of paramount importance.

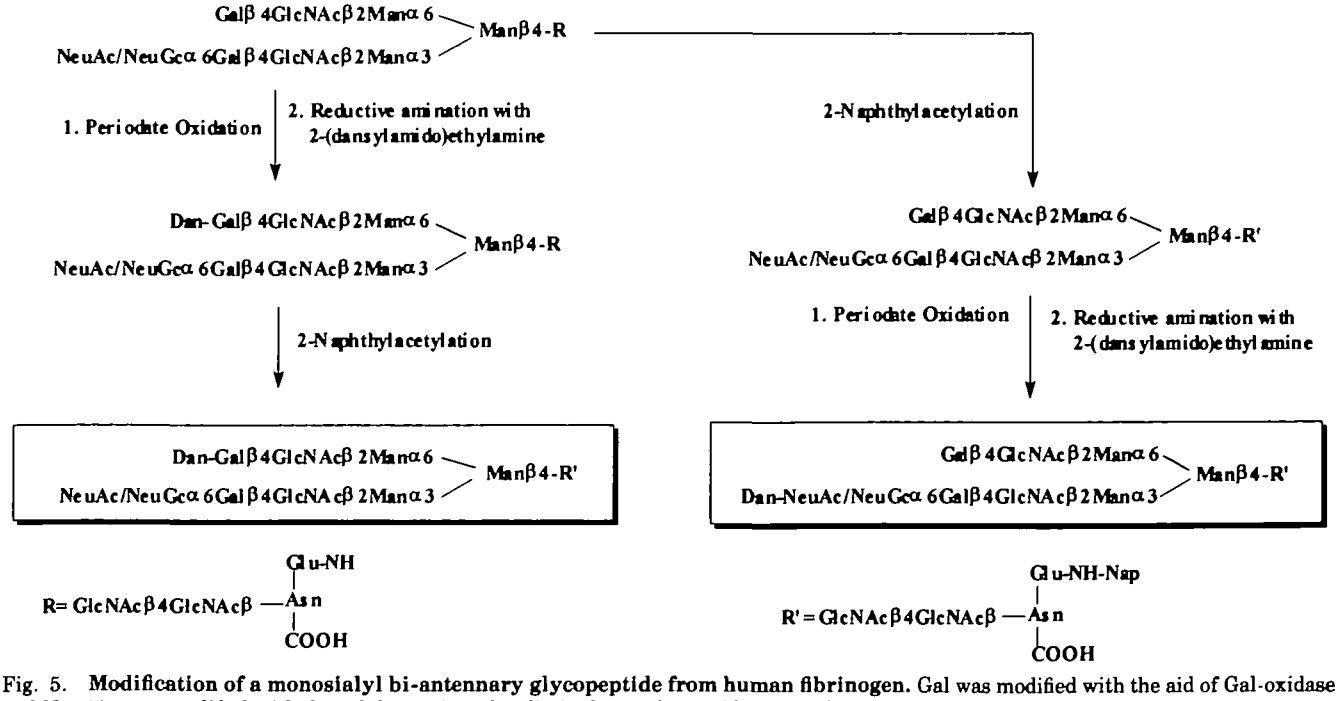

Fig. 5. **Modification of a monosialyl bi-antennary glycopeptide from human fibrinogen.** Gal was modified with the aid of Gal-oxidase and Neu5Ac was modified with dansyl derivative after limited periodate oxidation (*33*).

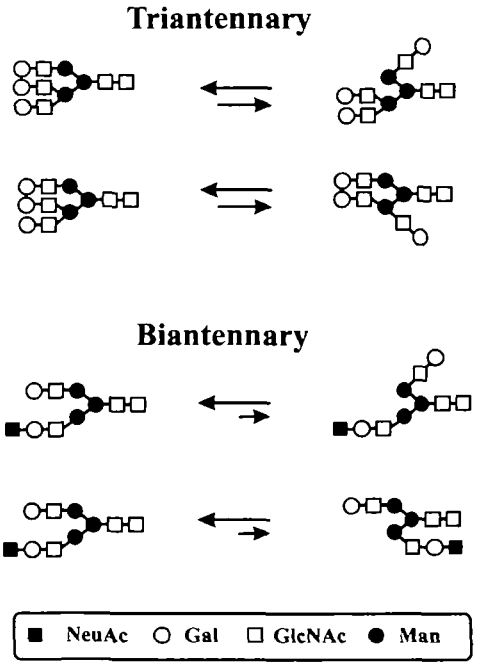

Fig. 6. **Branch flexibility of tri and biantennary glycopeptides.**

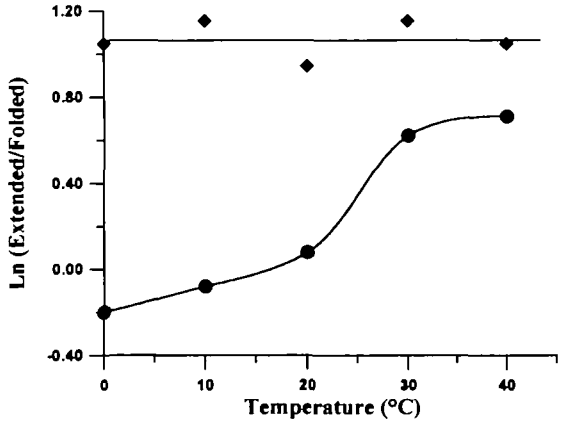

Fig. 7. **Changes of conformational distribution of fucosylated biantennary glycopeptide as a function of temperature (*35*).**

to the 6-position of GlcNAc at the root, orientation of the Manα(1-6)-linked branch is dramatically altered. This branch showed an extended/folded ratio of *ca.* 3 between 0-40°C in the presence of the Fuc, but the ratio decreased drastically as the temperature is raised (*35*). This relationship is diagrammed in Fig. 7.

FRET was used to measure conformational changes of a peptide when it acquires Asn-linked oligosaccharides (*36*). Attachment of carbohydrate at Asn clearly affects the peptide to adopt a more compact set of conformational ensemble, as measured by the distance between N- and C-termini.

B. FRET for Measurement of Enzymatic Activity—There is an obvious potential in applying FRET for the activity assay of endo-type carbohydrases and related enzymes. A bi-fluorescently labeled glycopeptide (Fig. 3),

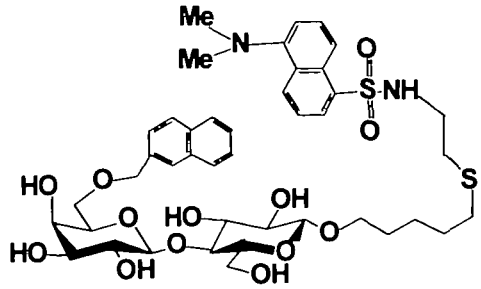

Fig. 8. **A bi-fluorescently labeled neoglycolipid useful for measurement of ceramide glycanase activity (*39, 40*).**

neoglycolipid (Fig. 8) or oligosaccharide substrates (*37, 38*) can be constructed to cause FRET[4] the fluoro/chromophores. Upon hydrolytic cleavage of such substrates, the fluorescence donor and acceptor will be no longer on the same molecule and FRET will cease to occur. This is the principle of the FRET-based enzyme assay. The advantage of such a substrate, in addition to its sensitivity, is that it permits continuous monitoring of the progress of the reaction in a single vessel.

Matsuoka *et al.* (*39, 40*) demonstrated that a lactoside containing a dansyl group at the terminal position of aglycon and a naphthyl group on the 6-position of the Gal (Fig. 8) can be hydrolyzed by ceramide glycanase (CGase) from American leech (*41*). Using this substrate, CGase assay can be performed more sensitively, and the parameters of enzyme kinetics can be obtained by continuous monitoring. Although either increase in the donor (naphthyl group) emission or decrease in the acceptor (dansyl) emission can be measured, the former provided better results.

Similarly, glycopeptides modified in the same fashion as those used for conformational studies of glycopeptides (*28, 30, 34, 42*) were found to be useful for assay of glycoamidases (*33*). Essentially the same principle was used in the design of substrates for α-amylases (*37, 38*), albeit different combinations of fluorescent probes were used.

Affinity electrophoresis

Affinity capillary electrophoresis (ACE) has been used to determine binding affinity of many systems (*43*), including carbohydrate-protein interactions. The mobility of a carbohydrate-binding protein may change upon binding of carbohydrate ligand—due to change in its charge, conformation, or molecular size, or combination thereof. When the carbohydrate ligands must be labeled for detection, a fluorescent group is most frequently used for higher sensitivity.

In a recent example of ACE for carbohydrate-binding protein, binding of fluoresceine-labeled carbohydrate ligands to Concanavalin-A was measured to obtain binding affinity (*44*). Although carbohydrate-protein interactions had been investigated by ACE by measuring UV-absorption (*45, 46*), use of fluorescence-labeled carbohydrate ligands in these instances greatly enhanced sensitivity. This is an advantage when high ligand concentration must

[4] Some of these substrates function on the principle of fluorescence quenching. In a broad sense, quenching of fluorescence can be considered as FRET.

be avoided (*44*).

Affinity electrophoresis is not limited to capillary zone electrophoresis. Gel slab electrophoresis on agarose or polyacrylamide with imbedded Concanavaline A has been used for analyses of oligosaccharides (*47*). Application of this principle to a 2-D protocol was recently described (*48*).

When the labeled ligands are also negatively or positively charged, binding of such ligands may cause more pronounced change in protein mobility. The study of the binding of anionic carbohydrate by human serum amyloid P component by use of capillary zone electrophoresis (*45*) is such an example, although UV-absorption rather than fluorescence was used.

Glycofluoropolymer

A glycopolymer usually refers to synthetic polymer containing well defined carbohydrate groups. Interactions between glycopolymers and carbohydrate-binding proteins will often manifest a "glycoside cluster effect" (*49*). Copolymerization of a fluorophore monomer, *N*-2-propenyl-(5-dimethylamino)-1-naphthalene sulfonamide, a Gal-containing monomers (or other glycosides), and acrylamide resulted in a water-soluble glycopolymer, which showed $e_{ex} = 340$ nm and $e_{em} = 448$ and 528 nm. When this glycopolymer carrying galactose residues was saturated with *Ricinus communis* lectin (RCA60), its fluorescence intensities of the polymer decreased by 20 and 14%, respectively (without changes in the shape of the spectra) (*50*). The apparent K_a for binding of this glycofluoropolymer by RCA60 is $10^8\ M^{-1}$. Presumably, such a water soluble glycopolymer would have a greater flexibility and offer better accessibility of Gal residues for carbohydrate-binding proteins and thus provides better affinity for binding. With further refinement of the system, glycofluoropolymer may find many more interesting applications.

Preparationof fluorescent carbohydrate derivatives

Preparation of Fluorescent Carbohydrate Derivatives—The simplest fluorescent carbohydrate derivatives that one can prepare are glycosides containing fluorescent aglycons. As mentioned above, MU glycosides, favorite substrates for exo-glycosidase assays, are also suitable fluorescent derivatives for investigation of carbohydrate-protein interactions, and MU glycosides of a wide variety of sugars are commercially available. Alternatively, ω-aminoalkyl glycosides (*51*) can be modified with fluorescent probes. The presence of methylene groups in the aglycon allows a considerable degree of free rotation of the fluorescent probe, which can be advantageous in avoiding anisotropy. An access to fluorescent probes *via* allyl glycosides, *i.e.*, by ozonolysis of the double bond, and reductive amination of the generated aldehyde with 7-amino-4-methylcoumarin has been reported (*52*).

Amino sugars (*e.g.*, glucosamine, galactosamine) can be modified at their amino groups with fluorescent probes. For example, galactosamine modified with dansyl group has been extremely valuable in studies of binding mode of a plant lectins (*5, 17, 50, 53-55*). Glycosylamines of most common sugars, which can be prepared easily now (*56*), are readily modified with fluorescent probes (*22, 57, 58*). Interestingly, a glycosylamine is produced fleetingly as the primary product of glycoamidase action on a glycopeptide, and a successful trapping of the glycosylamine generated by glycopeptidase F with phenylisothiocyanate and naphthylisocyanate to form respectively a UV- and fluorescent probe has been reported (*57*).

Galactose oxidase is a useful tool for modification of natural glycopeptides or oligosaccharides, if terminal Gal or GalNAc residues are available (*59*). The hydroxymethyl group of Gal/GalNAc is converted to aldehydo group which can then be modified with fluorescent probe by reductive amination (*60*). An aldehydo group can also be generated in sialylated oligosaccharide, glycopeptide, glycolipid, or glycoprotein by periodate oxidation of the exocyclic chain of sialic acids without affecting other sugars (*61*). Alternatively, fluorescently modified sialic acids can be incorporated into oligosaccharides or glycopeptides by transglycosylation (*61*) or by sialyl transferase after conversion of the modified sialic acid into CMP-derivative (*62, 63*).

Chemical methods generally applicable for attaching fluorescent probes at the non-reducing termini have been described (*37, 38*). Glycopeptides may be modified with fluorescent probes at either the N-terminus (*28, 34*) or possibly at the C-terminus (*64*).

Other considerations

The advantage of using fluorometry in studies of carbohydrate-protein interactions is that the binding can be studied at equilibrium without physical separation of the bound complex from the free ligand and the protein. The disadvantage may be that almost all fluorescent probes are hydrophobic and much bulkier than radioisotopes. It would be prudent to establish that the modification of a ligand with fluorescent probe does not perturb the nature of protein-ligand interaction.

Fluorescently labeled carbohydrate ligands can be used in the same way as radiolabeled ligands. The sensitivity of fluorometry may not be quite as high as those by means of radiometry, but specifically designed dye and laser combination offers an amazing degree of sensitivity (*65*).

An interesting example of changes in fluorescence of anthrylboronic acid upon binding to polyols has been reported (*66*). This type of reagent may find valuable applications in glycobiology in future.

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