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COVALENT LABELLING OF SPECIFIC MEMBRANE CARBOHYDRATE RESIDUES WITH FLUORESCENT PROBES *

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Summary

Two new fluorescent labelling techniques are described: one specific for sialic acid residues and a second specific for galactose and some of its derivatives. Using either technique it is possible to label covalently the desired carbohydrate residue with any one of a large variety of fluorescent probes. When the sialic acid labelling procedure is applied to human erythrocyte membranes, only the glycophorin species are labelled. However, when the galactose-directed labelling scheme is applied, fluorescence is also observed on membrane lipid components and on band 3. For each technique, the fluorescent labelling pattern is shown to reflect the distribution of the respective sugar component on the erythrocyte membrane. Thus, these techniques should provide both selectivity and versatility in the fluorescent labelling of specific carbohydrate residues in highly heterogeneous biological systems.

Introduction

The usefulness of fluorescence spectroscopy in obtaining molecular information on any heterogeneous system depends heavily on the investigator's ability to define the location of the reporter molecule. Thus, changes in the fluorescence lifetime or quantum yield of a membrane bound probe can signal a conformational change [1—3], but the value of such an observation is limited unless the specific binding site of the fluorophore can be identified. Similarly, Förster energy transfer measurements can be used as a 'spectroscopic ruler' to evaluate distances on a molecular level only if the locations of the donor and

* A preliminary report of part of this study has been presented [19].

acceptor molecules are well characterized [4—6]. Fluorescence depolarization [7—9], microscopic polarity [10—12], and photobleaching studies [13—15] also increase significantly in value when the precise location of the fluorophore is well known. Clearly, if fluorescence spectroscopy is to be applied to the study of such highly heterogeneous systems as a ribosome or a biological membrane, techniques must be devised whereby specific, well-defined sites can be labelled with fluorescent molecules.

In this communication we describe two fluorescence labelling techniques, one which is specific for sialic acid residues and a second which is specific for galactose and some of its derivatives. Both labelling techniques take advantage of well characterized procedures for the selective oxidation of these specific sugar residues [16—18]. Thus, where galactose or sialic acid residues are confined to a single protein species, it should be possible to label specifically this protein species, regardless of the heterogeneity of the biological system.

Experimental procedures

Fresh human blood was obtained from the Central Indiana Regional Blood Bank with citrate/dextrose solution added and was used before it was officially outdated. Fluorescein amine (isomer II), fluorescein amine (isomer I), dansyl cadaverine, ethidium bromide, and galactose oxidase were obtained from Sigma Chemical Co. Other reagents used were: 2-aminoanthracene, 3-aminofluoranthene and 9-aminoacridine (Aldrich Chemical Co.); dansyl hydrazine (Pierce Chemical Co.); galactose oxidase (Worthington Biochemical Corp.); sodium borohydride (Alfa Division); and sodium metaperiodate (Mallinckrodt Chem. Co.). Steady-state fluorescence measurements and all excitation and emission spectra were obtained in the ratio mode on a Perkin Elmer MPF-44A fluorescence spectrophotometer. Before conducting any fluorescence experiment the membranes were extensively diluted to avoid any inner filter effects. Further dilution had no effect on the fluorescence measurements.

Labelling of sialic acid residues. Erythrocytes were separated from whole blood by washing in isotonic phosphate buffer, pH 7.4. The packed erythrocytes were stirred with an equal volume of 2 mM sodium metaperiodate in an ice bath for 20 min to selectively oxidize the external sialic acid residues on the membrane, as described by Gahmberg and Andersson [16]. Incubation at higher temperatures or for longer periods may permit the transport of the metaperiodate into the erythrocyte cytoplasm where additional labelling may occur [16]. The unreacted metaperiodate was subsequently removed by washing the cells in cold isotonic phosphate buffer containing 0.1 M glycerol. The washed cells were then allowed to react with an equal volume of the appropriate fluorescent primary amine, presumably by formation of a Schiff's base with the newly formed carbonyls on the sialic acid residues of the membrane. The reaction was carried out at 37°C for 2 h in a shaker bath, and a 2 mM concentration of the added fluorophore (introduced in the same phosphate buffer) was usually found to be sufficient for maximum membrane labelling. Following the above incubation, the cells were pelleted by centrifugation and the Schiff's base linkage was permanently reduced by treating the erythrocytes with an equal volume of 50 mM $NaBH_4$ in isotonic phosphate buffer, pH 8, at 22°C for 30

min. The cells were then washed extensively in isotonic phosphate buffer, pH 7.4, containing 1% bovine serum albumin in order to remove the unreacted fluorescent amine. Hemoglobin-free membranes were prepared from these cells according to the method of Dodge et al. [20], and then dialyzed against isotonic phosphate buffer, pH 7.4, to remove the final traces of any unreacted fluorophore.

Labelling of membrane galactose residues. Washed whole erythrocytes were mixed with 2 vols. of isotonic phosphate buffer, pH 7.0, containing 10 μg/ml of galactose oxidase [16]. The mixture was incubated at 37°C in a shaker bath for 3 h and afterwards the galactose oxidase was removed by washing three times in isotonic phosphate buffer. The cells were then reacted with the appropriate fluorescent amine and treated in all subsequent steps as described above.

Identification of fluorescent bands on polyacrylamide disc gels. Polyacrylamide gel electrophoresis of labelled erythrocyte membranes was carried out in 0.2% sodium dodecyl sulfate (SDS) on 13 cm long disc gels [21], where approximately 0.1 mg membrane was loaded on each gel. The gels were fixed and stained with either Coomassie brilliant blue or periodic acid-Schiff staining reagents, according to the procedures of Fairbanks et al. [22]. The location of the fluorescent bands on unstained and unfixed gels was determined by slicing the gels into 1.5 mm slices and extracting the protein from each slice by incubating the gel slice for 30 min at 37°C in 2 ml of 50 mM bicarbonate and 0.05% SDS solution in a shaker bath. The fluorescence of each fraction was then monitored on a Perkin Elmer MPF-44A fluorescence spectrophotometer. Since solubilized gel slices containing no protein showed a broad fluorescence emission near 425 nm, it was often necessary to dialyze each solubilized gel fraction to remove this background fluorescence. This dialysis step, however, was unnecessary if fluorescent probes with emission maxima (λ_{em}) at longer wavelengths were chosen; e.g., fluorescein amine λ_{em}, 520 nm.

Results and Discussion

The strategy behind both the sialic acid and the galactose residue labelling procedures was to oxidize selectively the appropriate carbohydrate residues to form the reactive carbonyls on the membrane. The sialic acids were selectively oxidized with metaperiodate, while the oxidation of galactose residues was catalyzed by galactose oxidase. The newly formed carbonyls were then reacted with the primary amino function of a fluorescent amine, and the resulting Schiff's base was permanently fixed by reduction with sodium borohydridide.

While this technique allows a tremendous variety in the choice and design of fluorescent probes for membrane labelling, two important restrictions do apply. First, the pK_a of the primary amine must be near or below the pH of the buffer bathing the cells, so that the amine will be present in its reactive, unprotonated form. This restriction practically eliminates any aliphatic amines from consideration, but it also selects aryl amines (pK_a 3—5) as likely candidates. Thus, we observed no reaction when dansyl cadaverine, a primary aliphatic amine, was added to metaperiodate treated cells, while addition of aryl amines such as fluorescein amine (isomer II) and 9-aminoacridine produced extensive labelling.

A second restriction concerns the solubility of the fluorescent amines in the aqueous reaction buffer. If the fluorescent probe used in the labelling procedure was very hydrophobic, e.g., 2-aminoanthracene, 3-aminofluoranthene, and so forth, then the extent of covalent labelling was found to be diminished. This arose presumably because the probe partitioned heavily into the membrane, away from the oxidized sugar residues. The highly hydrophobic fluorophores were also undesirable because the unreacted molecules were generally difficult to remove from the membrane. We monitored the removal of the noncovalently bound probe molecules by conducting the entire labelling scheme on control erythrocytes which had not been oxidized with either metaperiodate or galactose oxidase. Whenever fluorescence was still observed in these control membranes, it was assumed that removal of the unbound probe was also incomplete in the oxidized membranes. This was found to be the case for 1- and 2-aminoanthracene, 3-aminofluoranthene, dansyl hydrazine, and fluorescein amine (isomer I).

With these restrictions in mind it is still possible to choose from a large variety of commercially available fluorescent amines, e.g., fluorescein amine (isomer II), 9-aminoacridine, ethidium bromide, rhodamine 110, and so forth, and to select a probe with the fluorescence lifetime and spectral characteristics best suited for each particular experiment. These latter probes readily react with oxidized sugar residues and any noncovalently bound molecules are easily removed from the membrane.

Fig. 1 shows the excitation and emission spectra of erythrocyte membranes labelled with fluorescein amine (isomer II) according to the metaperiodate oxidation procedure, where the label resides solely on the glycophorin species in the membrane (see below). The emission maximum is at 520 nm and the major excitation maxima are at 470 nm and approx. 504 nm. Both the excitation and the emission spectra closely resemble the spectra of free fluorescein amine in ethanol, but differ significantly from fluorescein amine's excitation and emission spectra in water. This may imply that the fluorescein amine site is slightly protected from the aqueous buffer.

The specific carbohydrate labelling sites on the erythrocyte membrane were determined by correlating the positions of the fluorescein amine fluorescence with the positions of the protein bands on sodium dodecyl sulfate polyacrylamide disc gels. Fig. 2 shows the alignment on 7% gels of the fluorescein amine fluorescence with the periodic acid-Schiff staining bands. It can be seen that all glycophorin species (glycophorin A, monomer and dimer, and glycophorin B, monomer and dimer) are equally labelled, suggesting that the labelling technique does not discriminate among the various membrane sialic acid sites. Furthermore, no labelled lipid is observed near the tracking dye, indicating that the probe resides exclusively on the higher molecular weight membrane components.

Because of the large number of Coomassie blue staining bands in the red cell membrane, we observed invariably some overlap between the fluorescein amine peaks and one or more Coomassie blue staining bands. Thus, the possibility that polypeptides other than glycophorin were also labelled by the metaperiodate oxidation technique could not be completely excluded on the basis of a single comparison of gel banding patterns. However, by changing the percent

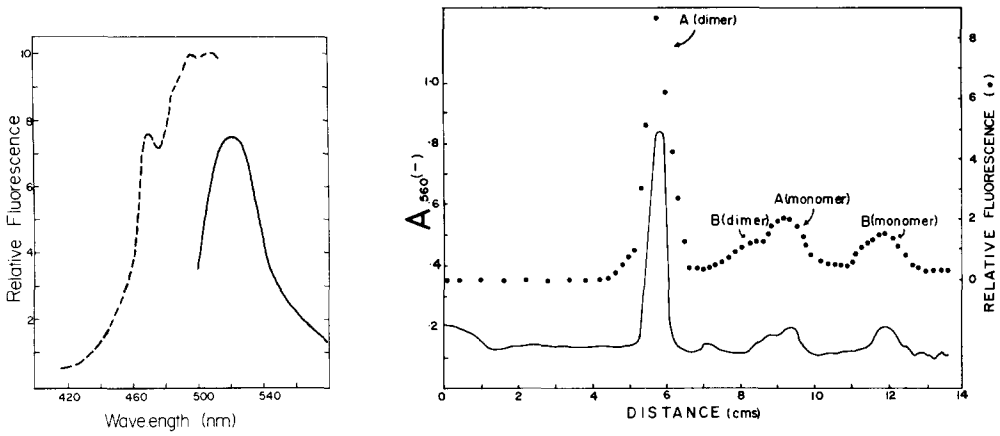

Fig. 1. The uncorrected excitation (- - - - - -) and emission (———) spectra of erythrocyte membranes labelled with fluorescein amine isomer II, by the sialic acid oxidation technique. The excitation and emission slits were 6 nm. The emission wavelength for the excitation spectrum was 520 nm and the excitation wavelength for the emission spectrum was 470 nm.

Fig. 2. Correlation of the positions of the fluorescein amine isomer II fluorescence (• •) with the positions of the periodic acid-Schiff staining bands (———) of the erythrocyte membrane on 7% polyacrylamide disc gels. The membranes were labelled according to the sialic acid labelling procedure, and approx. 0.1 mg of membrane was applied to each gel.

acrylamide in the gel system, it was possible to alter the relative migrations of the glycoproteins with respect to the Coomassie blue staining bands. Using this procedure, e.g. by varying the acrylamide concentration between 5% and 10%, we observed that the fluorescein amine fluorescence invariably correlated with the periodic acid-Schiff staining bands, and that neither band 3 nor any other Coomassie blue staining band was labelled.

This important conclusion was further supported by following the purification of glycophorin [23,24], using the fluorescein amine labels as a marker. At each step of the purification scheme we monitored both the fluorescence in the discarded fraction and also in the glycophorin containing fraction. The membrane fluorescence was always observed to partition with the glycophorin, and only negligible amounts of fluorescence were carried over into the discarded fractions. The final product contained no cysteine (indicating the absence of the major Coomassie blue staining bands of the red cell membrane), and had a specific fluorescence roughly 15 times that of the original source membranes.

The galactose oxidase labelling procedure yielded membranes which differed in one major respect from the membranes labelled by the sialic acid oxidation procedure: three additional membrane components were labelled by the galactose oxidase technique. Thus, in addition to the major sialoglycoproteins of the red cell membrane, we observed that band 3, a very high molecular weight species, and a very low molecular weight component of the membrane were also labelled (Fig. 3). The low molecular weight component is probably a sialoglycolipid, since it migrates near the tracking dye and is stained by the periodic acid-Schiff reagent. The high molecular weight component at the gel origin, however, is more perplexing, since it shows up as a periodic acid-Schiff staining

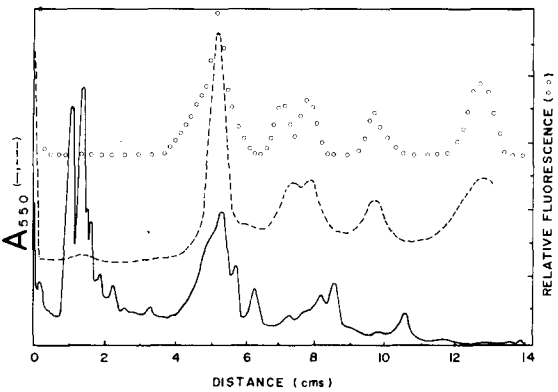

Fig. 3. Correlation of the positions of the fluorescein amine isomer II fluorescence (○ ○) with the positions of the periodic acid-Schiff staining bands (- - - - - -) and the positions of the Coomassie blue staining bands (———) of the erythrocyte membrane on 5.6% polyacrylamide disc gels. The membranes were labelled according to the galactose labelling procedure, and approx. 0.1 mg of membranes were applied to each gel.

band on polyacrylamide disc gels. In view of the fact that no periodic acid-Schiff staining band is normally observed at this position, it would seem that the galactose oxidation procedure may initiate a glycophorin cross-linking reaction, which does not normally occur in unmodified membranes.

In conclusion, the apparent differences in the specificity of the galactose oxidase and the metaperiodate labelling procedures probably derive solely from the differences in the distribution of the galactose and sialic acid residues among the various membrane components. Thus, while sialic acid is apparently confined to the erythrocyte glycophorins [16,25], galactose and *N*-acetylgalactosamine are also found on band 3 [16,26,27] and on numerous glycolipids [16,25]. It would, therefore, appear that the two fluorescence labelling techniques are highly specific for their target carbohydrate residues, and that any heterogeneity in membrane labelling must reflect the heterogeneity in carbohydrate location. In cases where either sialic acid or galactose residues are confined to a single membrane component, well-defined fluorescence studies with a large variety of fluorescent probes can now be designed.

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