Carbohydrate Research, 75 (1979) 11–16

SPIN-LABELLING OF SIALIC ACID IN SOLUBLE AND CELL-SURFACE GLYCOPROTEINS

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(Received November 21st, 1978; accepted for publication, January 9th, 1979)

ABSTRACT

A new, mild method is described for spin-labelling sialic acid residues *in situ*. The procedure involves the formation of C-1 sialamides and has been applied to a serum glycoprotein, a mucin, tissue sections from human colon, and erythrocyte membrane components. The selectivity of the method and its possible applicability to other types of labelling are discussed.

INTRODUCTION

As understanding of the importance of carbohydrates in determining biological specificity has advanced, so the development of new methods for labelling the carbohydrate-containing components of biological materials has become important. Thus, sialic acid, found as the terminal unit of oligosaccharide components of glycoproteins and glycolipids, has been shown to be responsible for such properties of these materials as the circulatory lifetime of plasma glycoproteins[1], the net negative charge of the erythrocyte membrane[2], and its calcium ion-binding capacity[3]. In addition, there is evidence to suggest that sialic acid plays a role in malignancy, since desialylated tumour cells, used alone and in combination with other materials to immunize mice, can cause remission of small tumours[4].

We now describe a new method for spin-labelling sialic acid, with the further objective of studying the effects of various binding processes upon its mobility and environment. The chemistry involved may be readily adapted to other types of experiments, for example, fluorescent or radiolabelling, and tagging of protein amine groups with a similar variety of labels. The method involves the formation, in the presence of a water-soluble coupling reagent, of an amide linkage between CO_2H-1 of sialic acid and an amine group in the label. A useful complement is thus added to the already widespread method[5] of oxidation followed by borohydride reduction at C-7 or C-8. We describe spin-labelling of a mucin and a plasma glycoprotein, some control materials lacking carbohydrate carboxylate groups, tissue sections, and human erythrocytes.

MATERIALS AND METHODS

Materials. — 4-Amino-2,2,6,6-tetramethylpiperidin-1-oxyl (Eastman–Kodak), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) (Sigma), calf fetuin (Type IV, Sigma), *Vibrio cholera* neuraminidase (EC 3.2.1.18) (Behringwerke, 500 U/ml), deoxyribonucleic acid (DNA), and bovine and human serum albumins (BSA and HSA; all A grade, Calbiochem) were used without further purification. Bovine submaxillary mucin (BSM, Boehringer Mannheim Corp.) was dissolved in water, and the solution was filtered through a centriflo membrane (Amicon Corp., retention limit 5×10^4 daltons). The reconstituted retentate was lyophilized, then treated with 0.1M potassium hydroxide (30 min at room temperature), and neutralized with 50mM sulphuric acid, to remove *O*-acetyl substituents[6]. Fetuin contains no *O*-acetyl groups[7].

Sections (5 μm) from human colon were mounted on cover slips (9 × 29 mm) and all manipulations performed thereon. *O*-Deacetylation was accomplished by using 0.5% potassium hydroxide in 70% ethanol for 30 min at room temperature. Human erythrocytes were either from fresh or recently outdated blood; in the latter case, prior to spin-labelling, they were incubated in excess of rejuvenating solution[8] for 1 h at 37°. After lysing the cells by suspension in distilled water, membranes were isolated by centrifugation at 20,000 r.p.m. for 30 min.

Assays. — Sialic acid was assayed by a combination of two methods: the thiobarbituric acid (TBA) assay, for free sialic acid only[9], and the periodate–resorcinol assay, which can be adapted either for ketosidically bound sialic acid only (PRBA), or for both free and bound material (PRT)[10].

Desialylation. — Desialylation was accomplished either by acid hydrolysis or treatment with neuraminidase, and the process was monitored by using the assay procedures. Acid hydrolysis of glycoproteins was performed with 25mM H_2SO_4 at 80° for 1 h, the products from which were found to behave identically with those from neuraminidase digestion. Digestion was performed in the presence of the enzyme (100 U/ml) incubated for 24 h at 37° in acetate buffer (pH 5.5) containing 0.1% of calcium chloride[11], after which an equal quantity of enzyme was added and incubation continued for a further 24 h. Tissue sections were treated with 50mM sulphuric acid at 80° for 1 h. Erythrocytes were incubated for 1 h at 37° with neuraminidase (10 U/ml) in 0.154M sodium chloride–0.01M sodium acetate (pH 5.5) containing calcium ions (mM).

Electrophoresis. — Electrophoresis was performed on Sepraphore III cellulose acetate strips in Tris-barbital–sodium barbital buffer (pH 8.6) for 35 min at 300 V, and the strips were stained by using Alcian Blue.

Labelling procedure. — Labelling was carried out at room temperature by using a 100-fold molar excess of coupling reagent (EDC) and spin-label at pH values between 4.5 and 6.0, and was generally complete after 3 h. Erythrocytes were treated at pH 6.0 for 1 h in 0.154M sodium chloride. Residual label and coupling reagent were removed by dialysis or membrane filtration, except in the case of the tissue sections, which

were washed with several changes of water and with 0.5M sodium chloride in 0.1M sodium acetate (pH 4.0), and the erythrocytes, which were removed by centrifugation and resuspended several times in 0.15M saline.

Spectroscopy. — E.p.r. (electron paramagnetic resonance) spectra were obtained at room temperature on a Varian E3 instrument, by using solutions that typically contained 5 mg of glycoprotein/ml. Power levels were less than 20 mW, no saturation being observed, and at 1G modulation. A flat cell was used for packed cells and solutions, while slides with mounted tissue sections were placed on a cylindrical Teflon insert (10 mm in diameter) having a centrally located cutaway (30 × 10 mm). Single integration was performed on the first derivative spectrum, and the absorption mode peaks were weighed, as a means of assaying total numbers of spins. Correlation times were calculated by using a previously described method[12].

RESULTS AND DISCUSSION

The effectiveness of the method was initially tested by using bovine submaxillary mucin, whose main prosthetic group[13] is a disaccharide comprising 2-acetamido-2-deoxy-D-galactose and terminal, non-reducing sialic acid; the latter constituted ~8.5% of the dry weight. The e.p.r. spectrum of the labelled material is shown in Fig. 1A, and, as expected, the rate of rotational reorientation of the label is reduced by attachment to the macromolecule. Fig. 1B shows the spectrum of BSM treated under the same conditions following desialylation with acid, indicating a rather small amount (~1%) of labelling of non-sialic carboxylates. Labelling at C-1 would be expected to render a sialic acid residue resistant to *Vibrio cholera* neuraminidase[14], and it was found that the spectrum remained unchanged after treatment of the labelled BSM with the enzyme and removal of products of low molecular weight. Assays, although not quantitatively reliable in the presence of modified sialic acids, confirmed that a greater proportion were resistant to the enzyme in the spin-labelled glycoprotein than in a control treated only with coupling reagent. Electrophoresis on cellulose acetate suggested that most of the carboxylate groups were "masked", as the spin-labelled BSM failed to migrate from the origin; it should be noted, however, that EDC may react with a carboxylate to give a stable *N*-acylurea[15] which will not react further with amine to the sialamide.

Similar experiments were performed with fetuin, whose oligosaccharide chains are fewer in number but more complex; the asparagine-linked units[16] contain 14 monosaccharide residues and 3 branches, terminating with sialic acid, and the serine-linked unit[17] is a tetrasaccharide containing two sialic acid residues. Sialic acid constituted 8.0% of the glycoprotein by weight. The spectrum of labelled fetuin is shown in Fig. 1C, and that of asialofetuin, treated in similar fashion, in Fig. 1D. In this case, a very significant background signal is present. Although the PRT assay indicated that 3% of the sialic acid was still present in the acid-treated fetuin, integration of the e.p.r. signal showed that only a small fraction (~1%) of the spins present could be accounted for by it, even assuming 100% efficiency of labelling. About half

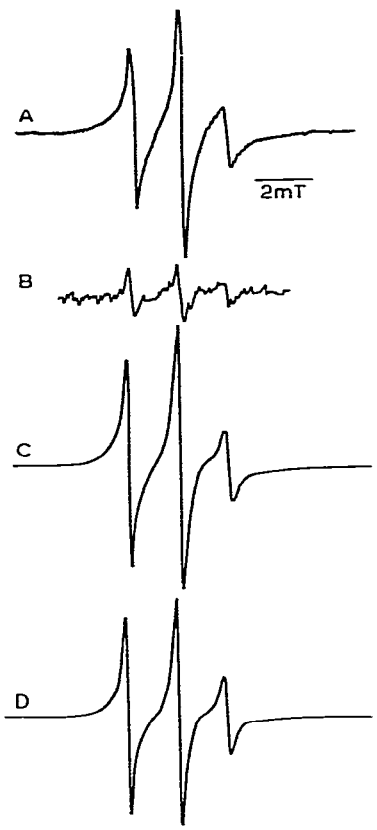

Fig. 1 (left). E.p.r. spectra of glycoproteins treated with spin-label and coupling reagent: A, BSM; B, asialo-BSM; C, fetuin; D, asialo-fetuin.

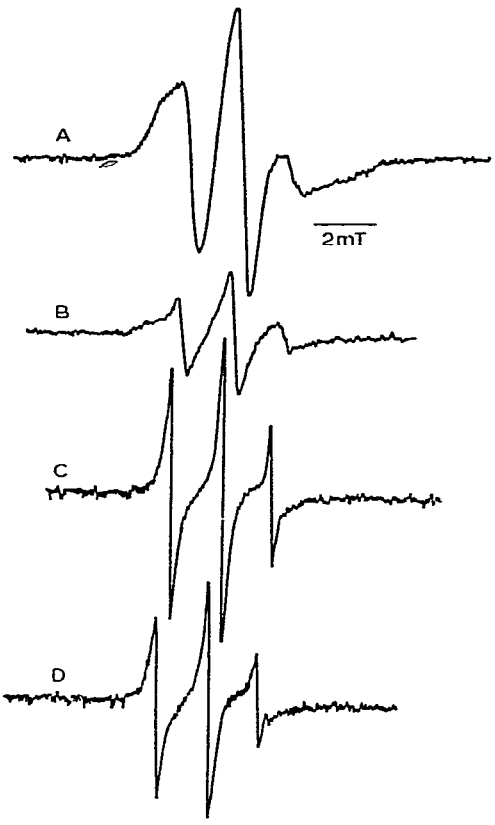

Fig. 2 (right). E.p.r. spectra of materials treated with spin-label and coupling reagent: A, 5-μm section of human colon; B, desialylated section; C, human erythrocytes; D, desialylated erythrocytes.

as many spins were present in the labelled asialofetuin as in the native material. The integrated spectrum of the native material could be accounted for by assuming that 18% of the sialic acid residues were labelled (or some smaller proportion together with other "background" carboxylates), so that although sialic acid is labelled, the process is clearly not as exclusive as that for BSM. The "background" signal can best be accounted for by protein carboxylate side-chains; about twice as many aspartic and glutamic acid residues (per weight of protein) occur in fetuin (17%)[18] as in BSM (9%)[19] (some, however, present as Asn and Gln), and the former also has slightly fewer sialic acid residues. A decrease in the reaction pH to 3 did not enhance the selectivity, but resulted simply in a lower yield.

Similar experiments were then performed on materials known to contain anionic groups of non-carbohydrate origin: bovine and human serum albumins and calf thymus DNA. All of these materials, treated to the same labelling procedure, displayed substantial signals, indicating that the method, as presently employed,

fails to distinguish between phosphates, amino-acid side-chain carboxylates, and sialic acid carboxylates. In the case of BSM, the relative absence of amino dicarboxylic acids leads to the observed specificity of labelling. However, ι-carrageenan was not labelled under the same conditions, indicating that half-sulphate esters present in mucins or connective-tissue polysaccharide would not contribute a background signal.

The calculated correlation times for labelled fetuin, BSM, BSA, and HSA have similar values ($1–2 \times 10^{-9}$sec), despite the variation in molecular weights, indicating that the spin-label is able to reorient at least partially independently of the protein. Of more interest is the fact that the position of the label (whether on a carbohydrate prosthetic group of short or long length or attached to an amino-acid functional group) appears not to affect the correlation time (τ for asialofetuin $\simeq$ τ for native fetuin; τ for BSA and HSA $\simeq$ τ for native fetuin).

The labelling method was sufficiently mild to be applicable to both tissue sections and human erythrocytes. The spectrum of a formaldehyde-fixed, 5-μm section of human colon mounted on a cover slip is shown in Fig. 2A, and Fig. 2B shows that of a similar section labelled after saponification and acid hydrolysis to remove sialic acid both from membranes and from soluble glycoproteins. The background signal in this case could have arisen from connective-tissue uronic acids as well as amino-acid carboxylates and phosphates.

Unfixed human erythrocytes, treated under rather milder conditions (as described in the methods section), gave the spectrum shown in Fig. 2C. Prior treatment with neuraminidase, followed by the same labelling procedure, gave the large "background" signal shown in Fig. 2D and presumably attributable to lipid head-group phosphates and amino acid side-chain carboxylates. Cell lysis was minimal under the reaction conditions. Addition[20] of $K_3Fe(CN)_6$ to a concentration of 0.3M caused the signal to be broadened beyond the detection limit, indicating the labels to be present exclusively on the outer surface of the cells; when the membranes were separated, the signal was found therein, and none was present in the supernatant. The labelled cells give a more mobile spectrum than the other systems studied, again suggesting the involvement of head-group phosphates, whereas the fixed tissue section gives rise to the broadest spectrum of the series. The majority of sialic acid residues are expected to be present in mucin rather than in cell-surface components (an average section contains relatively little of these); the effect of fixation on tissue sections and mucous gels is a subject of further investigation.

ACKNOWLEDGMENTS

The authors thank Dr. F. G. Herring for the design of the Teflon spectrometer-insert, and C. W. Ramey for helpful discussions. This work was supported by a research grant (to C.F.A.C., L.D.H., and P.E.R.) from the Natural, Applied and Health Sciences Grants Committee of U.B.C. J.D.A. is the recipient of an I. W. Killam predoctoral fellowship.

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