Studies on the Chemical and Enzymatic Modification of Glycoproteins

A GENERAL METHOD FOR THE TRITIATION OF SIALIC ACID-CONTAINING GLYCOPROTEINS

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SUMMARY

A general method is described for the radioactive labeling of proteins containing terminal sialic acid in the carbohydrate moieties. Quantitative conversion of these residues to a radioactive 7-carbon analogue of sialic acid was achieved by sequential periodate oxidation and tritiated borohydride reduction. Evidence is presented to establish the identity of the radioactive derivative as 5-acetamido-3,5-dideoxy-D-arabino-2-heptulosonic acid and to indicate it as the sole site of tritium incorporation in the carbohydrate chain. Upon injection into rats, labeled preparations of ceruloplasmin and orosomucoid exhibited a normal half-life in the plasma and proved suitable for metabolic studies. The data indicate that the 7-carbon sialic acid analogue mimics sialic acid with respect to hydrolysis by neuraminidase and in regulating survival of glycoproteins in the circulation.

MATERIALS AND METHODS

N-Acetylneuraminic acid and Clostridium perfringens neuraminidase (1.5 units per mg) were purchased from Calbiochem and Worthington, respectively. Sodium 131I, carrier-free, in 0.1 N NaOH, was obtained from New England Nuclear and potassium borohydride-T (324 mCi per mmole) was obtained from Amersham-Searle Corporation, Des Plaines, Illinois. The former was measured in a Tracerlab Deepwell Auto-Gamma Counter and tritium was assayed in a Packard Tri-Carb scintillation spectrometer utilizing a Triton X-100-toluene mixture.

Crystalline human ceruloplasmin was generously provided by Anatol Morell, Albert Einstein Medical College. Orosomucoid was prepared by the method of Whitehead and Sammons (7) modified for larger quantities. To 13 liters of human plasma, obtained from the National Institutes of Health Blood Bank, were added 130,000 NIH units of topical thrombin (Parke-Davis) and the mixture incubated for 1 to 2 hours at 37° prior to storage overnight at 4°. The resulting clot was removed by centrifugation and the serum dialyzed at 4° against 5 volumes of 0.05 M sodium acetate buffer, pH 4.3. After 1 day, the dialysate fluid was changed and the dialysis continued for an additional 24 hours. A flocculent precipitate was removed by centrifugation and the supernatant fluid was charged onto a column of diethylaminoethyl-cellulose, 10 X 27 cm (Whatman microgranular DE 52), that had been equilibrated with the above buffer. The column was then washed with the equilibrating buffer until the optical density at 280 nm fell to 0.075. A linear gradient was then arranged with a mixing chamber containing 4 liters of the above buffer connected to a reservoir containing an equal volume of 0.10 M sodium acetate buffer, pH 4.3. After 1 day, the dialysate fluid was changed and the dialysis continued for an additional 24 hours. A flocculent precipitate was removed by centrifugation and the supernatant fluid was charged onto a column of diethylaminoethyl-cellulose, 10 X 27 cm (Whatman microgranular DE 52), that had been equilibrated with the above buffer. The column was then washed with the equilibrating buffer until the optical density at 280 nm fell to 0.075. A linear gradient was then arranged with a mixing chamber containing 4 liters of the above buffer connected to a reservoir containing an equal volume of 0.10 M sodium acetate buffer, pH 4.0. The single large peak was prepared by the method of Whitehead and Sammons (7) modified for larger quantities. To 13 liters of human plasma, obtained from the National Institutes of Health Blood Bank, were added 130,000 NIH units of topical thrombin (Parke-Davis) and the mixture incubated for 1 to 2 hours at 37° prior to storage overnight at 4°. The resulting clot was removed by centrifugation and the serum dialyzed at 4° against 5 volumes of 0.05 M sodium acetate buffer, pH 4.3. After 1 day, the dialysate fluid was changed and the dialysis continued for an additional 24 hours. A flocculent precipitate was removed by centrifugation and the supernatant fluid was charged onto a column of diethylaminoethyl-cellulose, 10 X 27 cm (Whatman microgranular DE 52), that had been equilibrated with the above buffer. The column was then washed with the equilibrating buffer until the optical density at 280 nm fell to 0.075. A linear gradient was then arranged with a mixing chamber containing 4 liters of the above buffer connected to a reservoir containing an equal volume of 0.10 M sodium acetate buffer, pH 4.0. The single large peak
emerging toward the end of the gradient was pooled and 50 g of ammonium sulfate per 100 ml of eluate were added. The precipitate was removed by centrifugation and an additional 18 g of ammonium sulfate was added for each 100 ml of the supernatant fluid. This precipitate was dissolved in a minimal amount of water and dialyzed exhaustively against water prior to recovery by lyophilization; 7.4 g of a white, soluble powder was obtained.

Periodate consumption was assayed by iodometric titration (8) and formaldehyde by the chromotrophic acid procedure (9). Sialic acid was measured with thiobarbituric acid (10) or resorcinol (11), and fucose by the cysteine-sulfuric acid assay of Dische (12).

Descending paper chromatographic separations were carried out at room temperature for 15 to 17 hours. The solvent systems employed were: Solvent A, n-butylacetate-acetic acid-water (3:2:1); Solvent B, n-butyl alcohol-pyridine-1 N HCl (5:3:2); Solvent C, n-butyl alcohol-n-propyl alcohol-0.1 N HCl (1:2:1); Solvent D, n-butyl alcohol-acetic acid-water (4:1:5).

**Experimental Procedures**

Preparation of [AcNeu7]Ceruloplasmin—To 3.7 ml of a solution containing 36.4 mg of recrystallized human ceruloplasmin (2.7 μmoles of sialic acid) in Buffer A (0.1 M sodium chloride, pH 5.6, containing 0.15 M sodium chloride) were added 2.4 ml of 0.012 M NaI04. After incubation at 0°C for 10 min, the oxidation was stopped by the addition of an excess of ethylene glycol and the solution was dialyzed overnight at 4°C against 0.05 M sodium phosphate, pH 7.4, containing 0.15 M sodium chloride.

To the cold oxidized ceruloplasmin solution was added 1 mg of trinitiated KBH4 dissolved in 0.2 ml of 0.01 M NaOH. After mixing, the solution was warmed to room temperature and the reduction allowed to continue for 30 min with stirring. To insure completeness of reduction, 3 mg of nonradioactive KBH4 were then added and the incubation continued for another 30 min. Excess borohydride was removed by dialysis against Buffer A for several hours prior to exhaustive dialysis against 0.05 M sodium phosphate, pH 7.4, containing 0.15 M sodium chloride. A yield of 31.7 mg of [AcNeu7]ceruloplasmin was obtained with a specific activity of 4.25 × 10^6 dpm per mg.

When the above procedure was repeated with omission of periodate, the resulting control preparation was determined to have a specific activity of 0.35 × 10^6 dpm per mg. A similar blank value for nonspecific incorporation of tritium into ceruloplasmin has been reported previously (1). In both cases, the ratio of At61:At62 (0.038) was unchanged. Concentration was calculated on the basis of the absorption coefficient, At60°C 13.05 (1).

Preparation of [AcNeu7]Orosomucoid—The oxidation and subsequent reduction of orosomucoid was accomplished under the same conditions as ceruloplasmin except that the amount of material used was adjusted to maintain a comparable ratio between periodate and sialic acid. Thus, to 18.6 ml of a Buffer A solution containing 41 mg of orosomucoid (15.8 μmoles of sialic acid), 12.3 ml of 0.012 M NaI04 were added and the oxidation carried out as described above. After reduction and dialysis against Buffer A, and subsequent dialysis against water, the preparation was lyophilized to yield 38.5 mg of product.

The abbreviations used are: AcNeu, N-acetylatedaemic acid; AcNeu, N-acetylneuraminic acid.

At the end of the oxidation step, 14.1 moles of formaldehyde had been released and 28.9 moles of periodate had been consumed per mole of orosomucoid. These ratios were calculated on a dry weight basis and an assumed molecular weight of 40,000 (13). The specific activity of the final product was 11.5 × 10^6 dpm per mg. The specific activity of the control orosomucoid, prepared in the absence of periodate, was 0.55 × 10^6 dpm per mg.

Preparation of Partially Desialylated [AcNeu7]Ceruloplasmin—An alternate preparation of tritiated ceruloplasmin, 6.1 mg (4.06 × 10^6 dpm per mg) was treated with 0.47 unit of C. perfringens neuraminidase in 4.0 ml of sodium acetate-phosphate buffer, pH 5.6, for 50 min at 26°C. At this point, 45% of the tritium remained in the supernatant fraction after precipitation of an aliquot of the protein with cold 5% trichloroacetic acid. The neuraminidase was removed by affinity chromatography employing Sepharose 4B to which had been coupled rabbit anti-C. perfringens neuraminidase-globulin (14). The column, 1.4 × 7.4 cm, was developed with 0.01 M sodium phosphate, pH 7.4, containing 0.85% sodium chloride. These fractions containing both the tritium and the blue color of ceruloplasmin were pooled, dialyzed overnight against the developing buffer, and concentrated by ultrafiltration in Schleicher and Schuell collodion bags. The product, 3.5 mg (2.14 × 10^6 dpm per mg) of partially desialylated [AcNeu7]ceruloplasmin had lost 47% of its original tritium content.

Preparation of 131I-Orosomucoid—The chemical iodination of orosomucoid with 131I was carried out as described previously (15). The final preparation had an estimated specific activity of 2.7 × 10^6 cpm per μg. In excess of 69% of the total counts had been released and 28.9 moles of periodate had been consumed.

Preparation of Trinitiated -Carbon Derivative of Sialic Acid—The crystalline β-methylglycoside of AcNeu7 was synthesized essentially as described by Yu and Ledeen (4) except that the reduction step was carried out with trinitiated borohydride. The resulting heptulosonic acid derivative was dissolved in water to give a 1.3% solution and hydrolyzed at 100°C for 60 min. The slight discoloration arising during the heating step was removed by filtration through a small (1 to 2 mg) pad of charcoal. Upon lyophilization of the colorless filtrate, the theoretical yield of AcNeu7 was obtained with a specific activity of 1.8 × 10^6 dpm per mg.

**Results**

Extensive periodate oxidation of glycoproteins results in destruction of the terminal, nonreducing residues as well as those pyranosidically linked neutral sugars within the oligosaccharide chain in which carbon 3 is unsubstituted. This procedure has found application in studies on structure determination and is the basis of the widely used Smith degradation (16).

Locus of Tritium Incorporation in Protein—For the purposes of the present study, conditions were sought whereby maximal oxidation of the sialic acid residues could be achieved with little or no destruction elsewhere in the molecule. Under optimal conditions, 2 moles of periodate are consumed and 1 mole of formaldehyde is released per mole of sialic acid present in the intact molecule. Attainment of these conditions is illustrated by the limited periodate treatment of orosomucoid in which oxidation of the sialic acid residues was essentially completed within 10 min (Fig. 2). The oxidation of ceruloplasmin (not shown) was similarly completed within the same time period, as
judged by maximal formaldehyde formation. Here, however, the yield of formaldehyde leveled off at 8 to 9 moles per mole of ceruloplasmin. This value is artificially low and results from interference by ceruloplasmin in the chromotropic acid assay. When a correction is applied, by the use of an internal standard, the release of formaldehyde approaches 12 moles per mole of protein. Periodate consumption is also more difficult to determine in the presence of ceruloplasmin because of a larger than normal scatter in the end point of the iodometric titration. However, after 10 to 15 min, approximately 24 moles of periodate were consumed per mole of protein. Since the sialic acid content of ceruloplasmin was determined to be 12 moles per mole, based upon an assumed molecular weight of 160,000 (17), it is apparent that periodate oxidized all of the sialic acid residues and little else.

Sialic acid is readily cleaved from the native glycoproteins by mild acid hydrolysis or exposure to C. perfringens neuraminidase and it was presumed that the 7-carbon sialic acid derivative would be similarly sensitive to both acid hydrolysis and to enzymatic cleavage. Consequently, the radioactive products were hydrolyzed for 1 hour at 80°C in 0.1 N H2SO4 and precipitated by the addition of 3 volumes of ethanol-chloroform (9:1 v/v). The results, summarized in Table I, indicate that more than 90% of the tritium of both proteins was released into the supernatant fraction. The 4 to 5% of the original radioactivity remaining in the pellet compares closely with the nonspecific incorporation occurring in the absence of periodate oxidation (see "Experimental Procedures"). Upon treatment with neuraminidase, approximately two-thirds of the radioactivity was susceptible to enzymatic hydrolysis. These yields are minimal since no attempt was made to drive the enzymatic reaction to completion by increasing the amount of neuraminidase or by extending the time of incubation.

The tritiated material released from the protein by both enzymatic and acidic hydrolysis was subjected to paper chromatography in several solvent systems and the papers were developed with AgNO3 (18) and thiobarbituric acid (19). A characteristic thioarbituric acid-positive reaction product was seen between AcNeu7 and N-acetylneuraminic acid, and reactive towards thiobarbituric acid, was presumed to be a trace amount of the 8-carbon analogue of sialic acid and was not further investigated. No additional thioarbituric acid reactive area was detectable. The reaction products isolated after either acidic or enzymatic hydrolysis of both proteins were indistinguishable.

Minimal destruction of carbohydrate constituents elsewhere in the molecule was evidenced by the negligible amounts of radioactivity located in the areas corresponding to typical degradation products such as glycerol, erythritol, and threitol (Fig. 3). Since the terminal, nonreducing fucosyl residues are highly susceptible to destruction by periodate oxidation, the fucose content of ceruloplasmin was determined directly (12); more than 90% of the material present in the control was demonstrable in the oxidized and reduced protein.

The migration, in several solvent systems, of the radioactive product relative to that of sialic acid is summarized in Table II. The major radioactive peak cochromatographed with synthetic AcNeu7 and gave a positive reaction when sprayed with the

Table I

| Protein        | Fraction examined | Tritium recovered |
|----------------|-------------------|------------------|
|                | Acid hydrolysis   | Enzymatic hydrolysis |
|                | %                 | %                |
| Orosomucoid    | Supernatant       | 85               | 78               |
|                | Pellet            | 5                | 15               |
| Ceruloplasmin  | Supernatant       | 81               | 58               |
|                | Pellet            | 4                | 26               |
Fig. 3. Paper chromatographic tracing of the radioactive reaction product. Tritiated orosomucoid was acid hydrolyzed and the soluble radioactive product was recovered, as described in the legend to Table I. An aliquot was chromatographed overnight in n-butylacetate-acetic acid-water (3:2:1) and a radioactive tracing was made on a Vanguard autoscanner.

Table II

| Solvent system employed* | Migration relative to sialic acid | Synthetic AcNeu7 | Isolated reaction productb |
|-------------------------|----------------------------------|------------------|---------------------------|
| A                       | 1.36                             | 1.36             |
| B                       | 1.38                             | 1.43             |
| C                       | 1.28                             | 1.30             |
| Dc                      | 1.13                             | 1.15             |

* See "Methods and Materials."

† The reaction product was recovered after acid hydrolysis of tritiated orosomucoid, as described in the legend to Table I.

‡ This chromatogram was allowed to develop for 24 hours before removing from the solvent tank.

Table III

Comparison of calorimetric yields of isolated reaction product with that of authentic standards

| Colorimetric assay                | Origin of isolated reaction product |
|-----------------------------------|-------------------------------------|
|                                   | AcNeu7     | AcNeu7* | Orosomucoid* | Ceruloplasmin* |
| Thiobarbituric acid               | 56,000     | 30,600  | 4,08         |
| Resorcinol                        | 6,500      | 7,200   |              |
| Thiobarbituric acid to resorcinol | 8.56       | 4.23    | 4.08         | 4.40         |

* The reaction products were isolated from the modified glycoproteins by acid hydrolysis, as described in the legend to Table I.

† The thiobarbituric acid assay was carried out as described by Warren (10) with the exception that the periodate oxidation was conducted at 37° and the chromogen was extracted into 2.5 ml of cyclohexanone.

‡ The resorcinol assay (11) was scaled to one-half volume.

thiobarbituric acid reagent. In no case was it possible to detect residual, intact sialic acid.

Colorimetric analysis of the isolated reaction product indicated a marked loss of sensitivity in the thiobarbituric acid assay and a somewhat augmented reaction in the resorcinol assay. In order to utilize these differences for quantitative purposes, a direct comparison of the reactivity of synthetic AcNeu7 and intact sialic acid was undertaken. The initial erratic results observed with AcNeu7 in the thiobarbituric acid assay as described by Warren (10) were shown to result from incomplete periodate oxidation. Increasing the temperature of the oxidation step from ambient to 37° resulted in an augmented and more stable color yield for AcNeu7. This modification diminished the sensitivity of intact sialic acid by approximately 20%. In the resorcinol assay, the absorption maximum of both synthetic AcNeu7 and the isolated reaction product was shifted to a higher wave length and was essentially flat between 590 and 620 nm. All values recorded for this assay, however, were determined at 580 nm where absorption was maximum for sialic acid (11).

Table III lists the ratio of extinction coefficients of AcNeu7 and sialic acid in the two color assays. Comparable examination of the radioactive products obtained by acid hydrolysis of the modified glycoproteins yielded a ratio closely paralleling that of authentic AcNeu7 thereby providing additional evidence in support of the identification of the reaction product.

Serum Circulation Time of Labeled Protein—The previously indicated correlation between serum survival time and sialic acid content revealed that desialylated ceruloplasmin was cleared from the circulation within 10 to 15 min after injection, in marked contrast to the survival time of the fully sialylated protein (2). With the subsequent indication that removal of only two of the sialic acid residues of ceruloplasmin was sufficient to effect this rapid disappearance from the circulation (20), it became possible to test this observation on the sialic acid-modified protein. The characteristic pattern of short time survival curves resulting from the injection of intact and partially desialylated preparations of [AcNeu7]ceruloplasmin, shown in Fig. 4, duplicate closely the results seen earlier with the 6Cu-labeled protein (20). Further confirmation of the similarity between authentic ceruloplasmin and the tritiated [AcNeu7]ceruloplasmin derivative, with respect to their circulation time in the bloodstream, is illustrated in Fig. 5. Upon injection of the latter into a female albino rat, the half-life in the circulation was found to be 25 hours, in close agreement with the reported value of 27 hours for 6Cu-labeled ceruloplasmin (20).

In contradistinction to ceruloplasmin, orosomucoid represents a group of proteins for which no specific labeling procedure...
is available. Consequently, when radioactive material is required, such proteins are iodinated chemically despite the frequent incidence of damage to the physical and biological properties of the molecule. In contrast to the complete disappearance of asialo-orosomucoid from the circulation within minutes after injection (3), the short and long time survival curves of the tritiated orosomucoid derivative are shown in Figs 4 and 5, respectively. In the latter figure, the half-life of human \[\text{[AcNeu']}\]orosomucoid in the rat was estimated to be 22 hours. Under comparable conditions, the half-life of \(^{131}\text{I}\)-orosomucoid appeared to be slightly less, averaging 18 hours.

**DISCUSSION**

Current methods for the preparation of radioactively labeled proteins have been limited largely to the introduction of iodine atoms into the aromatic residues of the polypeptide chain. This procedure, in addition to its lack of specificity, frequently results in damage to the protein molecule and uncertain effect upon biological activity. The present method makes available a simple and supplementary technique suitable for metabolic studies on circulating proteins, the majority of which are sialic acid-terminated glycoproteins. Moreover, in addition to the serum proteins, this procedure has been successfully extended to include the gonadotrophic hormones; continuing studies indicate that essentially full retention of hormonal activity accompanies the incorporation of tritium into the modified sialic acid residues of human chorionic gonadotrophin and follicle-stimulating hormone.

In the design of this method, care was exercised to define conditions such that maximal oxidation of the sialyl residues was achieved with minimal, or no, oxidation elsewhere in the molecule. The complete destruction of sialic acid, the stoichiometry of periodate consumption with formaldehyde production and the recovery of a single radioactive product, identified as \[\text{AcNeu'}\], all attest to the essential achievement of this goal. However, it is important to note that exposure to longer periods of periodate oxidation at room temperature, followed by borohydride reduction, may result in marked destruction of the underlying carbohydrate chain of the glycoproteins with possibly untoward effects. The normal half-life of the modified proteins described here stands in contrast to the striking consequences attendant upon removal of sialic acid (2, 20).

In an earlier study on ceruloplasmin, the rapid clearance from the circulation was shown to be correlated with the presence of intact terminal, galactosyl residues since alteration of the latter with galactose oxidase, or removal with \(\beta\)-galactosidase, diminished the rapid hepatic accumulation of the asialoprotein and resulted in an increased circulation time approaching normality (2). The present results extend these observations with the indication that intact sialic acid is not essential for survival; the heptulosonic acid analogue is equally effective. Significantly, the latter derivative is also cleaved by neuraminidase and the resulting desialylated protein is rapidly cleared from the circulation.

Finally it should be emphasized that the present method of labeling, when combined with the previously described incorporation of tritium into the terminal galactose of the asialoprotein (1), permits a direct examination of the metabolic sequela specifically related to the removal of sialic acid.

*Manuscript in preparation.*
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