Incorporation of N-Acetylmannosamine and N-Acetylglucosamine into Thyroglobulin in Rat Thyroid in Vitro

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FABRIZIO MONACO* AND JACOB ROBBINS

From the Clinical Endocrinology Branch, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

SUMMARY

The incorporation of N-acetylmannosamine ([3H]ManNAc) and N-acetylglucosamine ([3H]GlcNAc and [14C]GlcNAc) has been studied in rat thyroid hemilobes and homogenates. The radioactive label is incorporated into thyroglobulin (TG), [3H]ManNAc showing a time course more rapid than [3H]GlcNAc. [3H]ManNAc is incorporated directly into 19 S thyroglobulin showing no lower molecular weight-labeled precursors of TG either in the soluble or in the solubilized proteins; [3H]GlcNAc is incorporated into 19 S TG and into precursors both in soluble and solubilized proteins. [3H]ManNAc is converted only into N-acetylneuraminic acid (sialic acid). [3H]GlcNAc and [14C]GlcNAc are incorporated mainly as GlcNAc, and an additional small proportion is converted into sialic acid. Both labeled GlcNAc and sialic acid are found in soluble TG but only GlcNAc is found in membrane-bound TG.

[3H]ManNAc is particularly useful to study the last step of carbohydrate incorporation while [3H]GlcNAc is a better tool to study the initiation of carbohydrate incorporation. The presence of the label as sialic acid only in soluble TG suggests that sialic acid is incorporated at the time of TG release from endoplasmic membranes. It may, therefore, serve as an indicator of TG secretion.

Although in recent years there have been many reports on glycoprotein biosynthesis, the process of incorporation of carbohydrates into protein is not yet completely understood. Since the thyroid secretes a glycoprotein, thyroglobulin, this gland can provide a good model for the study of glycoprotein biosynthesis and secretion. TG contains about 10% carbohydrate which has been shown to be present in three distinct units; one has a molecular weight of 1050 and contains mannose and N-acetylglucosamine, the second has a molecular weight of 3200 and contains in addition to N-acetylglucosamine and mannose, galactose, sialic acid (N-acetylneuraminic acid), and fucose (1-3).

Recently a third unit from human TG has been partially characterized; it contains galactosamine and possibly sialic acid (4). It has been suggested that fucose and galactose are incorporated directly into an 18 S protein whereas mannose incorporation has a time course similar to that observed for leucine (5, 6); more recently it has been suggested that mannose is present in two intermediates of TG synthesis, the 6 S and 7 S precursors (7). Since all the labeled carbohydrates used so far are present in the middle of the oligosaccharide chains and are subject to conversion, we wanted to study specifically the first and the last steps of carbohydrate incorporation into TG. N-Acetylmannosamine is the precursor of sialic acid and believed not to be transformed into other sugars (8); N-acetylglucosamine is the first sugar to be incorporated into the polypeptide chain through an N-glycosidic linkage to aspartic acid (8). Neither of these precursors have heretofore been used to study TG synthesis. Glucosamine, reported previously (9, 10) is a less specific precursor since it is incorporated into N-acetylglucosamine, N-acetylgalactosamine, GlcNAc, and sialic acid (11, 12).

MATERIALS AND METHODS

Male Fisher rats, ~250 g weight, were bred in the National Institutes of Health Laboratory Aids Branch and fed Purina laboratory chow.

N-[3H]acetylmannosamine, specific activity 400 mCi per mmole, and N-[3H]acetylglucosamine, specific activity 1.56 Ci per mmole, were purchased from Tracerlab, N-acetyl-d-[1-14C]glucosamine, specific activity 41.7 mCi per mmole, from New England Nuclear, N-acetyl-d mannosamine, N-acetyl-d glucosamine, d-mannose, d-fucose, d-galactose, N-acetylneuraminic acid from Sigma, and N-acetyl-d-galactosamine from Nutritional Biochemicals. Digitonin prepared as previously described (13), was from Fisher. Aquasol, universal liquid scintillation counter mixture, was from New England Nuclear, NCS solubilizer from Amersham-Searle. Earle's solution was provided by the Media Unit of the National Institutes of Health. Neuraminidase of Vibrio cholerae was from Schwarz-Mann.

The rats were lightly anesthetized with ether and then killed by exsanguination; connective tissue was removed from the thyroids which were then immersed in cold (4°C) 0.1 M Tris-HCl.

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1 The abbreviation used is: TG; thyroglobulin.
buffer, pH 7.4. The glands from several animals were cut in quarters, pooled, and pieces totaling ~20 mg were used in each incubation flask. The pooled thyroids were immersed in 2 ml of Earle's solution prewarmed for 20 min with O₂/CO₂ (95:5) and preincubated for 20 min at 37°C in a constant temperature shaking water bath at 120 oscillations per min. After the preincubation, 50 μCi of the labeled carbohydrate was added and incubation was carried out for 1, 3, 6, and 12 hours, gassing the mixture every 15 min with O₂/CO₂. In some experiments the pooled glands (~20 mg) were directly homogenized in 2 ml of Earle's solution, and the homogenate was incubated as above.

After incubation the glands were homogenized in 2 ml of 0.1 M Tris-HCl, 0.25 M sucrose, pH 7.4, in a Potter-Elvehjem homogenizer with a Teflon pestle (clearance 0.003 to 0.005 inch) at 1,100 rpm, three strokes in ice. The homogenate was centrifuged at 105,000 × g for 1 hour at 4°C in a Spinclo L centrifuge rotor 40. The supernatant, S₁, was dialyzed for 2 days at 4°C against four to five changes of 0.1 M Tris-HCl, pH 7.4, buffer. The pellet was rehomogenized in 1 ml of 2% digitonin (13), 10 strokes at 2,400 rpm in ice, and recentrifuged at 105,000 × g for 1 hour at 4°C. Using β3H as label it was shown that up to 71% of the pellet radioactivity was solubilized by this procedure whereas using sodium deoxycholate the recovery was up to 83%; these values are the mean of six experiments for digitonin and of three experiments for deoxycholate. The released labeled proteins examined by sucrose gradient ultracentrifugation showed that more than 90% of the total radioactivity was present in the 19 S TG peak. The amount of nonlabeled protein solubilized from the pellet was frequently low; the protein peak of 19 S TG in sucrose gradient patterns was variable and did not exceed ~20% of the total protein applied on the gradient. No estimation for the recovery of the protein content was carried out.

The supernatant after digitonin treatment, S₁, was dialyzed against 0.1 M Tris-HCl, pH 7.4, buffer for 2 days, and the pellet was dissolved in 0.5 ml of NCS. Aliquots of S₁, S₂, and pellet were counted in a scintillation counter in 15 ml of Aquasol containing 1 ml of distilled water.

Aliquots of soluble (S₁) and solubilized (S₂) proteins were precipitated with 95% ethanol or 10% trichloroacetic acid; the mixture was precipitated with 10% trichloroacetic acid, centrifuged at 3000 × g for 10 min, and the radioactivity in the supernatant was measured in a liquid scintillation counter. More than 92% of the 14C was trichloroacetic acid precipitable.

GlcNAc was released by hydrolyzing labeled 19 S TG, isolated as above, with 4 N HCl for 6 hours in a sealed tube in boiling water. The hydrolysate was then dried under vacuum, dissolved in water, and passed through a small column of Dowex 50-X4 (H⁺ form) (18), eluted with 2 N HCl and the eluate dried at 60°C under vacuum. The material, dissolved in water, was then acetylated as described by O'Brien. To 0.4 ml of the sample, 60 μl of 1 N sodium carbonate and 2 μl of acetic anhydride were added at room temperature. After 5 min Dowex 50 resin (hydrogen form) was added until the pH reached 2.5, then the mixture was centrifuged at 3000 × g for 10 min, and the resulting supernatant was applied on a borate-buffered Whatman No. 3MM paper sheet. High voltage paper electrophoresis was performed with 0.2 M borate buffer, pH 10, at 20 volts per cm for 2½ hours as described (19, 20). Standards (GlcNAc, GalNAc, and ManNAc) were run in parallel on the same sheet. After electrophoresis the markers were located by the silver nitrate method. The portion of the sheet containing the labeled samples was cut into 2-cm segments, extracted, and located as described above.

Sialic acid was determined by the thiobarbituric acid assay of Warren (on samples hydrolyzed in 0.1 N H₂SO₄ for 60 min at 80°C) (21) and by the resorcinol reaction (22) on the 1.4 to 1.8 M ammonium sulfate fractions. Density gradient ultracentrifugation in sucrose was performed as previously described (14). The double antibody technique of immunoprecipitation was done according to Salabé and Robbins (23).

RESULTS

The rate of incorporation of label into thyroid hemilobes and homogenates is shown in Fig. 1. [PH]ManNAc incorporation increased with time reaching a plateau of ~0.5% of the dose at 6 hours after incubation both in hemilobes and in homogenates. [PH]GlcNAc was incorporated at a slower rate, increasing up to 12 hours and reaching at this time about 0.5% of the dose in hemilobes and 0.5% in homogenates. With [PH]GlcNAc but not [PH]ManNAc incorporation into hemilobes was greater than in homogenates at all times studied.

From both compounds was incorporated into the soluble protein (S₁) of hemilobes, increasing to ~40% of the total radioactivity after hydrolysis and paper chromatography was 84 to 95% (mean 90%) of the amount in the protein.

To further identify the labeled product in soluble or solubilized proteins after incubation with N-[PH]acetylmannosamine, 19 S TG isolated by sucrose gradient centrifugation was incubated with neuraminidase from V. cholerae (5 mg of protein per 25 i.u. of enzyme) (17). After the incubation the mixture was precipitated between 1.4 and 1.8 M ammonium sulfate, redissolved, dialyzed, and then centrifuged in a sucrose gradient as described previously. The pattern of the protein showed that the 19 S peak of unlabeled TG was unchanged after neuraminidase treatment although the label had been completely displaced from the 19 S zone. To further establish whether the neuraminidase used had any protease or other glycosidase activity a 19 S TG labeled with [¹⁴C]galactose in vitro was incubated with neuraminidase as above; after the incubation the mixture was precipitated with 10% trichloroacetic acid, centrifuged at 3000 × g for 10 min and the radioactivity in the precipitate and supernatant was measured in a liquid scintillation counter. More than 92% of the ¹⁴C was trichloroacetic acid precipitable.

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FIG. 1. Uptake of N-[3H]acetilmanosamine and N-[3H]acetilglucosamine in rat thyroid hemilobes.

TABLE I
Trichloroacetic acid-precipitable 3H in soluble (S1) and solubilized (S2) fractions labeled with [3H]ManNAc and [3H]GlcNAc in thyroid hemilobes

| Precursor | Time | Trichloroacetic acid-potentiate 3H in soluble fractions (S1) | Trichloroacetic acid-potentiate 3H extracted from particulate fraction with digitonin (S2) |
|-----------|------|----------------------------------------------------------|---------------------------------------------------------------------------------|
| [3H]ManNAc | 1 hour | 27.3 ± 15.4 | 3.2 ± 1.0 |
|           | 3     | 38.5 ± 12.1 | 3.0 ± 2.9 |
|           | 6     | 39.4 ± 11.3 | 2.0 ± 1.1 |
| [3H]GlcNAc | 1     | 29.1 ± 18.6 | 22.3 ± 13.7 |
|           | 3     | 37.4 ± 14.0 | 25.6 ± 14.1 |
|           | 6     | 40.0 ± 12.4 | 38.4 ± 18.1 |
|           | 12    | 41.6 ± 8.7  | 48.2 ± 10.3 |

At the values are the mean ± S.D. of six experiments.

dioactivity by 6 hours (Table I). Whereas 3H from GlcNAc was incorporated to a similar extent into solubilized proteins (S2), the S1 fraction contained negligible radioactivity in hemilobes incubated with [3H]ManNAc.

The soluble proteins after incubation of hemilobes with [3H]-ManNAc and [3H]GlcNAc were examined by sucrose density gradient centrifugation. They showed (Figs. 2 and 3) that the label was present in 19 S thyroglobulin but that most of the radioactivity remained at the top of the gradient at early times. The top 10 fractions collected from these gradients (Figs. 2 and 3, top panels) were pooled, concentrated, and precipitated with 10% trichloroacetic acid or 95% ethanol; 85% of the radioactivity in the case of ManNAc and 35% in the case of GlcNAc was not precipitated, indicating that this amount was not in

Fig. 2. Density gradient ultracentrifugation patterns of the soluble proteins (S1) after incubation of hemilobes (A) or homogenates (B) with N-[3H]acetilmanosamine. Linear sucrose gradient, 10 to 40%, in 0.1 M Tris-HCl pH 7.4; rotor SW 27, small buckets; Spinco L2-65B centrifuge, 26,000 rpm for 20 hours at 20°. The zone labeled 19 S was determined with normal rat TG in the same run. B and T on the abscissa indicate the bottom and top of the tube, respectively.
These findings, however, were inconstant and variable in amount; hence their significance is uncertain. The 27 S peak was seen in about one-third of the analyses, except for hemilobes incubated with [3H]ManNAc where it was seen in one-tenth of the analyses. The 12 S peak was seen in one-half of the analyses of hemilobes incubated with [3H]ManNAc, in one-third of hemilobes incubated with [3H]GlcNAc, and in about one-tenth of analyses of homogenates.

In hemilobes incubated with [3H]GlcNAc the solubilized fraction (S1) contained 12 S, 19 S, and 27 S peaks of radioactivity similar to the S1 fraction, as shown in Fig. 4. In the case of [3H]ManNAc, however, no TG was detected in the S2 fraction at any time.

To investigate the proportion of the labeled protein immunologically related to TG, aliquots of the radioactive soluble and solubilized proteins were subjected to immunoprecipitation. As shown in Table II 80% of the soluble protein labeled with [3H]ManNAc and none of the rather trivial amount of the solubilized protein had the antigenic determinants of TG. The soluble protein labeled with [3H]GlcNAc showed that 74% was immunologically related to TG and the solubilized protein showed 58%.

The 19 S TG recovered from the sucrose gradient of the S1 fraction was pooled, concentrated, and dialyzed. This was hydrolyzed with 0.1 N H2SO4 and also with 4 N HCl in the case of hemilobes incubated with [14C]GlcNAc. Chromatographic and electrophoretic analysis of the hydrolysates is presented in Table III. All of the label from [3H]ManNAc was found in sialic acid. Furthermore, treatment of the thyroglobulin fraction with neuraminidase resulted in complete disappearance of the labeled 19 S peak as determined by sucrose gradient ultra-
TABLE II
Trichloroacetic acid precipitate and specific immunoprecipitate of soluble (S1) and solubilized (S2) protein labeled with [%H]ManNAc and [%H]GlcNAc

| Precursor | Fraction | Sialic acid | GlcNAc |
|-----------|----------|-------------|--------|
| ManNAc    | Soluble proteins (S1) | 86 | 0 |
|           | Solubilized proteins (S2) | 21 | 55 |
| GlcNAc    | Soluble proteins (S1) | 0 | 80 |
|           | Solubilized proteins (S2) | 0 | 80 |

*The values are mean ± S.D. of three experiments (two with hemilobes and one with homogenate) at 1 hour.

DISCUSSION

The results indicate that [%H]ManNAc and [%H]GlcNAc can pass through the thyroid cell membrane and the %H can be incorporated into protein. [%H] from both precursors is also incorporated into protein by thyroid homogenate.

The plateau observed at 6 hours for [%H]ManNAc suggests that the thyroid in vitro is not able to synthesize continuously a glycoprotein with residues which are acceptors for ManNAc. In the case of [%H]GlcNAc, the incorporation rate shows no plateau up to 12 hours of incubation and, in hemilobes, incorporation is greater than for [%H]ManNAc. Therefore, acceptor molecules for this carbohydrate are presumably formed in greater amount in vitro.

The radioactivity present in the fractions of the thyroid extracts which do not sediment in the sucrose gradient is not related to TG since 85% of the radioactivity cannot be precipitated by trichloroacetic acid or ethanol, nor is there any precipitable radioactivity immunochemically related to TG. Presumably these fractions contain oligosaccharides related to the thyroid cell membrane. [%H]ManNAc and [%H]GlcNAc are known to be incorporated into glycoprotein of mouse cell membrane (24). A considerable amount of radioactivity found in the particulate fraction after incubation with [%H]ManNAc is not extractable with digitonin and probably is also unrelated to TG.

Our data show that [%H]ManNAc is converted only into sialic acid in thyroid glycoprotein and is found in 19 S TG. Thus it is a specific detector of the incorporation of sialic acid in TG. Furthermore, the labeled 19 S is found only in the soluble fractions, indicating the addition occurs at the time of TG release from endoplasmic membranes. [%H]GlcNAc is also incorporated into soluble 19 S TG and its specific activity increases with time, the label showing a shift from a membrane-bound form and from slowly sedimenting fractions to the more mature molecule. This is in agreement with the finding of two glycoprotein intermediates in the biosynthesis of TG (7). A portion of [%H]GlcNAc is converted to sialic acid and, consistent with the findings after [%H]ManNAc incorporation, this is found only in the soluble TG.

The labeling experiments are consistent with our finding of a low sialic acid content in thyroglobulin solubilized from cell particles. It has been reported that the carbohydrate content, including sialic acid, of the proteins solubilized from thyroid particles is similar to that from soluble proteins (25). Although these data were from a study on calf thyroid and the procedures used to release proteins from particles and to isolate and partially purify thyroglobulin were different from ours, we have no explanation for the discrepancy between these results and our own on the sialic acid content of the membrane-bound thyroglobulin. Several reports in the literature show that the recovery of solubilized labeled proteins is always high even when different procedures are used but the unlabeled protein recovery can be quite variable (26-28). With our method it is possible to obtain good recovery of [%H]-labeled protein, most of which is 19 S TG. The fact that a significant amount of radioactivity after incubation with [%H]ManNAc is still present in the pellet after treatment with detergent suggests that this label is probably in constituents of the cell membranes that are unrelated to TG synthesis.

Since sialic acid is present in a terminal position in the oligosaccharide chains of thyroglobulin and since we could not detect it in membrane-bound thyroglobulin, it is suggested that sialic acid incorporation may be used as an index for the secretion of this glycoprotein. Its use in studying the secretion of other glycoproteins will be of interest.

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