Biochem. J. (1985) **229**, 269–272
Printed in Great Britain

Protein-bound glycogen is linked to tyrosine residues

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(*Received 6 March 1984/16 April 1985; accepted 26 April 1985*)

Tyrosine–glycogen obtained from retina proteoglycogen by exhaustive proteolytic digestion was radiolabelled with ^{125}I. The ^{125}I-labelled tyrosine–glycogen was degraded by amylolytic digestion to a very small radioactive product, which was identified as iodotyrosine by h.p.l.c. The amylolytic mixture used released glucose and maltose that were α-linked to the phenolic hydroxy group of *p*-nitrophenol. No free iodotyrosine was found before or after the intact [^{125}I]iodotyrosine–glycogen was subjected to two cycles of the Edman degradation procedure. The linkage between protein and glycogen was alkali-stable. Therefore it is concluded that the protein-bound glycogen was *O*-glycosidically linked to the phenolic hydroxy group of tyrosine. The amino acid has not been heretofore found to be involved in the linkage of carbohydrates to proteins.

Evidence for the glycoprotein nature of glycogen in retina has previously been described suggesting that at least part of glycogen occurs as proteoglycogen (Aon & Curtino, 1984). Strong physical and chemical dissociative conditions failed to release the protein moiety from the polysaccharide. However, conclusive proof of the glycoprotein nature of retina glycogen required the isolation of a glycopeptide and the characterization of the amino acid(s) involved in the linkage between protein and carbohydrate (Rodén & Horowitz, 1978).

In the present work we describe that tyrosine–glycogen was released from proteoglycogen after exhaustive proteolytic digestion. Evidence for the glycosidic linkage of glucan to the phenolic hydroxy group of tyrosine is presented. This is a novel amino acid implicated in the linkage of carbohydrates to proteins.

Materials and methods

Materials

Glucoamylase from *Rhizopus genus* mould (9.6 units/mg of protein), α-amylase (di-isopropyl phosphorofluoridate-treated, type 1A; 1000 units/mg of protein), papain (type IV; 23 units/mg of solid), concanavalin A–Sepharose 4B, monoiodotyrosine and di-iodotyrosine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pronase (grade B; 45 units/mg of solid) was from Calbiochem (Los Angeles, CA, U.S.A.). Bio-Gel P-2 was from Bio-Rad Laboratories (Richmond, CA, U.S.A.). $Na^{125}I$ (carrier-free; 8.5Ci/mg) was from New England Nuclear (Boston, MA, U.S.A.).

Preparation of ^{125}I-labelled tyrosine–glycogen

Tyrosine–glycogen was obtained by digestion of retina proteoglycogen with papain and Pronase as described previously (Aon & Curtino, 1984) and passage through a Sepharose CL-4B column equilibrated and eluted with 60mM-NH_4HCO_3. The tyrosine–glycogen fraction, which was eluted near the void volume, was precipitated twice with ethanol, iodinated with $Na^{125}I$ by the method of Cuatrecasas (1973) and passed again through the Sepharose column in order to remove $Na^{125}I$. Radioactive fractions, which were co-eluted with glycogen near the void volume, were pooled, the mixture was concentrated under vacuum, and the ^{125}I-labelled tyrosine–glycogen was washed twice by precipitation with ethanol and redissolved in water.

Amylolytic digestion

The iodinated tyrosine–glycogen (0.2mg of glyogen; 3.5×10^6 c.p.m.) was incubated in a final volume of 0.25ml with a mixture of α-amylase (4 units) and glucoamylase (0.7 unit) in 10mM-phosphate buffer, pH7.0, at room temperature for 16h. Under these conditions the amylolytic mixture was able to hydrolyse *p*-nitrophenyl α-

glucoside and *p*-nitrophenyl α-maltoside. No proteolytic activity was detected in either α-amylase or glucoamylase preparations (Aon & Curtino, 1984). The anylolytic digest was passed through a Sephadex G-25 column, and the radioactive material was further purified by passage through a column of dowex 50W X8 (H^+ form), washed with water and eluted with 7M-NH_3.

Manual Edman degradation procedure

For manual Edman degradation (Tarr, 1977), ^{125}I-labelled tyrosine–glycogen (3×10^4 c.p.m.; 50 μg of glycogen) in 100 μl of pyridine/water (1:1, v/v) containing 12.5% (v/v) trimethylamine was subjected to coupling with phenyl isothiocyanate (10 μl) at 50°C for 3 min. The solution was washed three times with benzene (200 μl) and dried under vacuum. The cleavage was carried out with trifluoroacetic acid (25 μl) at 50°C for 6 min. After drying, the residue was redissolved in water (50 μl) and extracted three times with 200 μl of benzene/ethyl acetate (1:2, v/v), and the radioactivity of the combined extracts was quantified. When conversion was carried out, the benzene/ethyl acetate extracts were dried, 100 μl of 1M-HCl in methanol was added to each and the mixtures were incubated at 50°C for 10 min. Each solution was dried, mixed with the phenylthiohydantoin derivatives of monoiodotyrosine and di-iodotyrosine (standards) and analysed by h.p.l.c. An internal standard of [^{3}H]serine was used in each of the degradation cycles. Where indicated, the phenylthiocarbamoyl derivatives of iodotyrosine formed after the coupling reaction were extracted into benzene/ethyl acetate without prior cleavage. The recovery of the phenylthiocarbamoyl derivatives in benzene/ethyl acetate was not affected when [^{125}I]monoiodotyrosine was mixed with glycogen

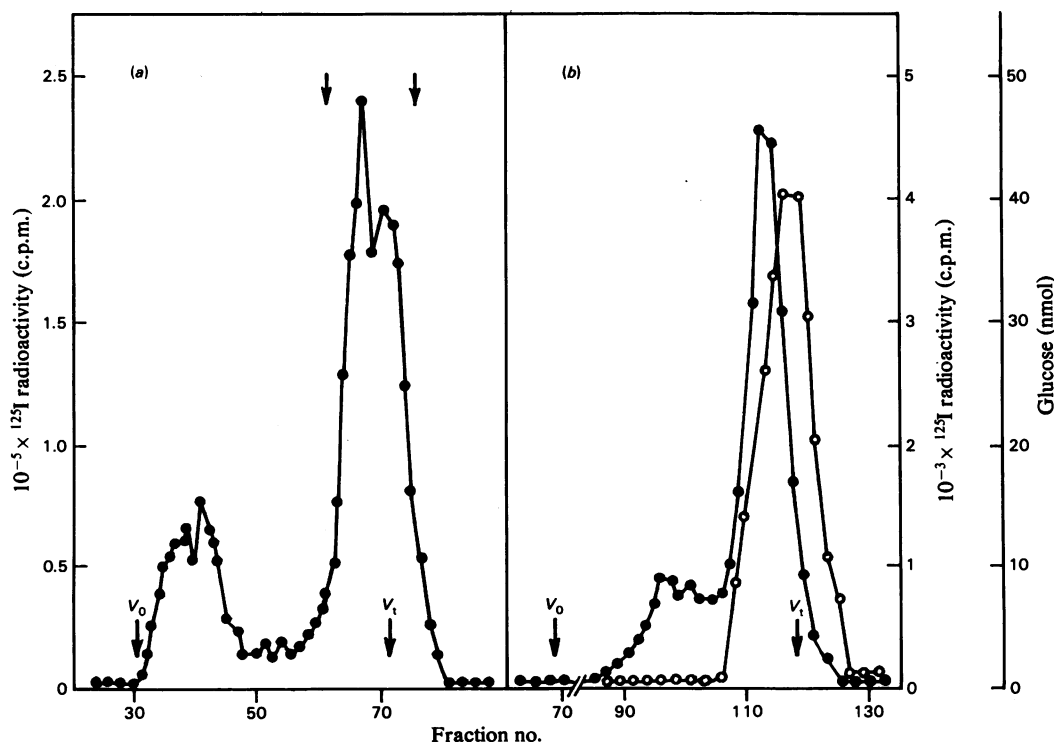

Fig. 1. *Elution profiles on Sephadex G-25 and Bio-Gel P-2 of the ^{125}I-labelled tyrosine–glycogen after treatment with α-amylase and glucoamylase*

The amylolytic digestion was carried out as described in the Materials and methods section. (*a*) The Sephadex G-25 column (1 cm × 36 cm) was equilibrated and eluted with 0.1M-NH_3. Fractions (0.25 ml) were collected and monitored for radioactivity. (*b*) A portion of the small radioactive product (2.5×10^4 c.p.m.) isolated from the Sephadex G-25 column (*a*, pooled fractions between arrows) and further purified by cation-exchange chromatography (see the Materials and methods section) was mixed with glucose (45 μg) and passed through a Bio-Gel P-2 column (0.8 cm × 100 cm) equilibrated and eluted with 0.1M-NH_4HCO_3 (adjusted to pH 9.6 with aq. NH_3). Fractions (0.33 ml) were collected and monitored for radioactivity (●) and glucose content (anthrone reaction) (○). V_0 and V_t are the excluded volume and the totally included volume respectively.

(50 μg) before being subjected to the coupling reaction.

H.p.l.c. analysis and radioactivity measurement

Reverse-phase h.p.l.c. analysis was carried out as described by Gates *et al.* (1979) on a 5 μm-particle-size Ultrasphere ODS column (4.6 mm × 250 mm). Radioactivity was measured as described previously (Aon & Curtino, 1984).

Results and discussion

Tyrosine–glycogen was obtained from proteoglycogen by digestion with papain/Pronase and radiolabelled by iodination with [^{125}I]iodide (see the Materials and methods section). Treatment of the ^{125}I-labelled tyrosine–glycogen with a mixture of α-amylase and glucoamylase resulted in a decrease in its molecular size from 1000 kDa to a size small enough to be completely included in a Sephadex G-25 column (Fig. 1*a*). About 70% of the Sephadex G-25-included material was eluted from a Bio-Gel P-2 column only slightly ahead of free glucose in partially overlapping peaks (Fig. 1*b*).

When the ^{125}I-labelled tyrosine–glycogen was subjected to affinity chromatography on concanavalin A-Sepharose as described by Finne & Krusius (1982), all the labelled material was bound to the lectin. One-half of the total label was eluted with α-methyl glucoside, and the balance was irreversibly bound, as was a commercial sample of glycogen. About 65% of the total label was not retained by the concanavalin A-Sepharose column when the ^{125}I-labelled tyrosine–glycogen was subjected to amylolysis as described in Fig. 1 (results not shown). These results are consistent with the conclusion that the bulk of the labelled material after amylolysis might be glucose-free [^{125}I]iodotyrosine, because of its failure to bind to concanavalin A and its very small size. Further analysis confirmed this conclusion.

The small radioactive material eluted from Sephadex G-25 was further purified by cation-exchange chromatography and treated with phenyl isothiocyanate, and the coupling-reaction mixture was washed and extracted with benzene/ethyl acetate, with omission of the cleavage step (see the Materials and methods section). About 60% of the

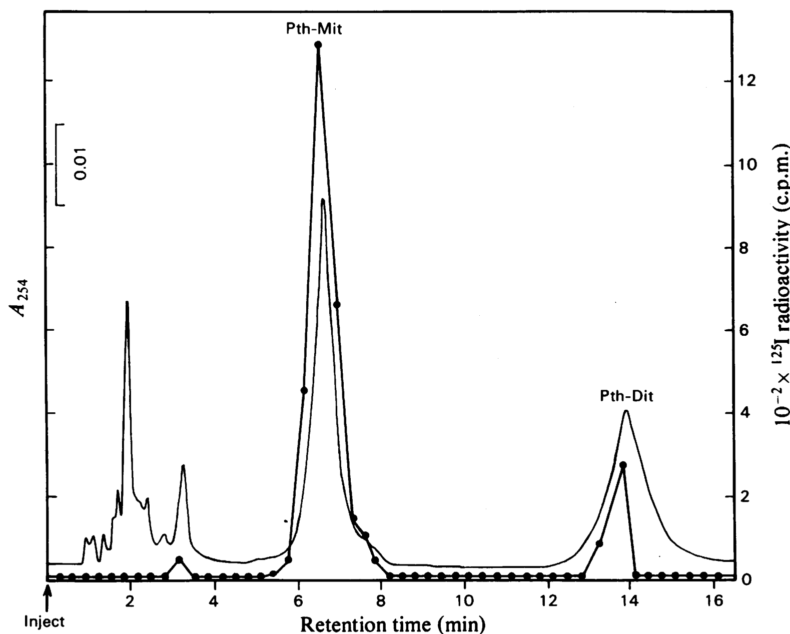

Fig. 2. *Reverse-phase h.p.l.c. analysis of the labelled phenylthiohydantoin derivatives obtained from the small radioactive product after amylolysis of the iodinated tyrosine–glycogen*

The iodinated product isolated and purified as described in Fig. 1 (3×10^4 c.p.m.) was subjected to coupling with phenyl isothiocyanate, washing, extraction (without prior cleavage) and conversion (see the Materials and methods section). A portion (3.5×10^3 c.p.m.) of the labelled phenylthiohydantoin derivative obtained was mixed with the phenylthiohydantoin derivatives of monoiodotyrosine and di-iodotyrosine (Pth-Mit and Pth-Dit respectively) (standards) in a total volume of 20 μl and analysed by h.p.l.c. as indicated in the Materials and methods section. Absorbance was monitored at 254 nm (——). Samples were collected at 18 s intervals and monitored for radioactivity (●—●).

total label was recovered in the upper, organic-solvent, phase, converted into the phenylthiohydantoin derivative and analysed by h.p.l.c. All of the radioactivity was co-eluted with standards of phenylthiohydantoin derivatives of iodotyrosines, the main derivative found being that of monoiodotyrosine (Fig. 2). When the ^{125}I-labelled tyrosine–glycogen was treated with phenyl isothiocyanate under the conditions described above, no radioactivity was recovered in the organic-solvent extract. Also, no radioactivity was extracted in benzene/ethyl acetate after the ^{125}I-labelled tyrosine–glycogen was subjected to two cycles of the Edman degradation procedure. This behaviour of the iodinated tyrosine–glycogen in the Edman degradation was similar to that of glycopeptides having glycosylated *N*-terminal residues (Tomita *et al.*, 1978).

Thus a tyrosine residue bearing the glucan chain was obtained from proteoglycogen after exhaustive digestion with papain and Pronase. The linkage between tyrosine and carbohydrate was alkali-stable (0.1 M-NaOH/0.1 M-$NaBH_4$ at room temperature for 24h) (Aon & Curtino, 1984), and was hydrolysed by an amylolytic mixture that under the same conditions released glucose and maltose from *p*-nitrophenyl α-glucoside and *p*-nitrophenyl α-maltoside respectively. Therefore the possibility of an ester linkage involving the carboxy group of tyrosine is ruled out, and it is concluded that tyrosine was glycosidically linked to glucan through the phenolic hydroxy group.

The failure for a photocolorimetric detection of iodine consumption when an ether derivative of tyrosine instead of tyrosine was subjected to iodination in an iodine/iodide solution supports the concept of the phenolate anion being the tyrosine species that is iodinated (Mayberry *et al.*, 1965). However, we were able to detect the formation of iodotyrosine in tyrosine–glycogen by iodination with Na^{125}I of high specific radioactivity in the presence of chloramine-T and h.p.l.c. of the [^{125}I]iodotyrosine phenylthiohydantoin derivative. Thus the *O*-glycosylated tyrosine residue was iodinated through the much less reactive undissociated phenyl group (Berliner, 1951) to an extent enough to be detected by monitoring of radioactivity.

New insight into storage-polysaccharide biosynthesis could be gained from the present studies.

This research was supported in part by grants from Consejo Nacional de Investigaciones Científicas y Técnicas, Consejo de Investigaciones Científicas y Tecnológicas de la Provincia de Córdoba and Secretaría de Estado de Ciencia y Tecnología.

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