Characterization of the proteoglycogen fraction non-extractable from retina by trichloroacetic acid

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The trichloroacetic acid-insoluble 1,4- α -glucan fraction from bovine retina was purified and characterized. It is a proteoglycogen fraction containing a 42 kDa protein moiety similar in size to the protein moiety of the trichloroacetic acid-soluble proteoglycogen fraction. The apparent weight-average M_r of acid-insoluble and acid-soluble proteoglycogens are 4.7×10^5 and 7.0×10^5 respectively. The present results support suggestions from earlier studies indicating that acid-insoluble proteoglycogen is the precursor of the acid-soluble form.

INTRODUCTION

Glycogen covalently bound to protein as a single proteoglycogen molecule was demonstrated in the trichloroacetic acid extract from a retina membrane preparation (Aon & Curtino, 1984). Glycogen is bound to the protein through the phenolic hydroxy group of tyrosine residues (Aon & Curtino, 1985). Another 1,4- α -glucan fraction is not extracted by the acid from the membrane preparation. This glucan fraction incorporated [¹⁴C]-glucose from UDP-[¹⁴C]glucose *in vitro* at a higher rate than the acid-soluble fraction (Miozzo *et al.*, 1986), and a chase with UDP-glucose suggested that it could be the precursor of proteoglycogen (Curtino *et al.*, 1979; Aon & Curtino, 1984). However, the acid-insoluble glucan had not been previously characterized, and it was not known whether it was also bound to protein.

It has been recently shown (Rodriguez & Steven, 1988) that a 40 kDa protein present in the glycogen of rabbit heart and liver and a 42 kDa protein found in high-speed supernatants of heart, liver, muscle and retina cross-reacted with antibodies raised against rabbit muscle glycogenin. Glycogenin is the name given to a 37 kDa protein which is bound to muscle glycogen (Whelan, 1986).

The present paper describes, for the first time, the purification and characterization of a 1,4- α -glucan non-extractable from tissue by trichloroacetic acid. The acid-insoluble fraction of retina is a proteoglycogen fraction with a weight-average M_r lower than that of the acid-soluble fraction. The protein moiety of both proteoglycogens is a 42 kDa polypeptide. Our results, together with related investigations (Pitcher *et al.*, 1987; Rodriguez & Steven, 1988), give support to the hypothesis (Krisman, 1972) that glycogen is synthesized on to a protein molecule.

MATERIALS AND METHODS

Trypsin [treated with tosyl-L-phenylalanylchloromethane ('TPCK'); type XIII], α -amylase (di-isopropyl

phosphorofluoridate-treated, type 1A; 1000 units/mg of protein), glucoamylase from the mould *Rhizopus* (9.6 units/mg of solid), Tos-Lys-CH₂Cl, PMSF, leupeptin, pepstatin A, Sepharose CL-4B, dextrans and protein molecular-mass standards were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Na¹²⁵I (carrier-free; 17.4 Ci/mg) was from the Comision Nacional de Energia Atomica (Buenos Aires, Argentina).

Preparation of retina membranes

Membranes from bovine retina were prepared as described previously (Aon & Curtino, 1984), except that retinas were homogenized in the presence of 50 μ M-Tos-Lys-CH₂Cl and 50 μ M-PMSF and the membrane suspension was stored at -70°C .

Isolation of acid-soluble and acid-insoluble proteoglycogens

All steps were carried out at $1-2^\circ\text{C}$ unless otherwise noted. The membrane suspension (2.5 ml; 68 mg of protein; 1.3 and 4.3 mg of acid-insoluble and acid-soluble glycogen respectively) was extracted by mixing with $\frac{2}{3}$ vol. of 25% (w/v) trichloroacetic acid and centrifuged at 500 *g* for 5 min. For isolation of the acid-soluble proteoglycogen fraction, the supernatant was centrifuged at 4000 *g* for 10 min before mixing with 2 vol. of ethanol. Proteoglycogen was collected by centrifugation at 4000 *g* for 15 min, dissolved in 1% KCl, and precipitated by addition of ethanol. The precipitate was dissolved in 0.1% SDS, the solution was heated at 100°C for 3 min, cooled in ice, made 10% in trichloroacetic acid and centrifuged at 4000 *g* for 10 min. The supernatant was mixed with 2 vol. of ethanol, centrifuged at 4000 *g* for 15 min and proteoglycogen was washed twice by addition of ethanol to the precipitate which had been redissolved in 0.5% KCl. The proteoglycogen fraction was dissolved in 0.2 ml of 0.1 M-sodium phosphate buffer, pH 7.5, and stored at -70°C . The purified fraction contained 1.5 μ g of protein/100 μ g of glycogen, and the total amount recovered was 4 mg of glycogen, as measured by the anthrone or the iodine reaction.

Abbreviations used: Tos-Lys-CH₂Cl, tosyl-L-lysylchloromethane ('TLCK'); PMSF, phenylmethanesulphonyl fluoride.

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For the isolation of the acid-insoluble proteoglycan, the membranes precipitated by trichloroacetic acid were washed twice by resuspending the precipitate in 10% acid with the aid of sonication. The washed precipitate was dissolved by addition of 0.2 M-sodium phosphate buffer (pH 8)/0.2 M-disodium phosphate/12% SDS (1:3:4, by vol.) and heated at 100°C for 3 min. The weight ratio of detergent to protein was 10. After addition of 10 vol. of water the proteoglycan fraction was collected by centrifugation at 235 000 *g* for 3 h at 15°C. The upper 26 ml of the total 27 ml supernatant was slowly siphoned off, and the 1 ml supernatant at the bottom, which contained 79–86% of the total acid-insoluble proteoglycan fraction, was mixed with 26 ml of 0.1% SDS and centrifuged at 235 000 *g* for 3 h. The 1 ml supernatant at the bottom was washed by centrifugation as above and finally cooled, made 10% in trichloroacetic acid and centrifuged at 4000 *g* for 10 min. The precipitated protein was discarded, and the proteoglycan fraction present in the supernatant was precipitated with ethanol, washed and dissolved in buffer as indicated for the acid-soluble proteoglycan. The purified fraction contained 3 µg of protein/100 µg of glycogen, and the total amount recovered was 0.56–0.74 mg of glycogen. As indicated for the acid-soluble fraction, the quantification of acid-insoluble polysaccharide by the anthrone reaction gave a result similar to that obtained by the iodine reaction.

Preparation of ¹²⁵I-labelled proteoglycan

Proteoglycan (0.5–0.9 mg in glycogen) was iodinated with [¹²⁵I]iodide (0.5 mCi) as described previously (Aon & Curtino, 1985). After iodination the labelled proteoglycan was precipitated and washed twice with ethanol as indicated above.

Amylolytic treatment

Glucoamylase (2 mg of solid) was resuspended in 1 ml of water and briefly sonicated. After centrifugation, 0.15 ml of supernatant was mixed with 65 µg of α-amylase in a total vol of 1 ml. Digestions were carried out with the indicated amount of this amylolytic mixture at room temperature for 48 h in the presence of 1 mM-Tos-Lys-CH₂Cl, 50 µM-PMSF and 20 µg/ml each of leupeptin and pepstatin. The amylolytic mixture hydrolysed the *O*-glycosidic linkage between tyrosine residues and glycogen (Aon & Curtino, 1985).

SDS/polyacrylamide-gel electrophoresis

Electrophoresis was carried out as described elsewhere (Laemmli, 1970) on 12.5% (w/v) acrylamide resolving gels with a 3% stacking gel.

Analytical methods

Glycogen and protein were measured as described before (Aon & Curtino, 1984).

RESULTS AND DISCUSSION

The 1,4-α-glucan fraction non-extracted by trichloroacetic acid from the retina membrane preparation was isolated by high-speed centrifugation after solubilization of the acid-insoluble residue with detergent (see the Materials and methods section). This fraction gives a iodine-glucan-complex spectrum similar to that of glycogen (result not shown) and constitutes about a quarter

of the total glycogen present in the retina membrane preparation.

When the trichloroacetic acid-soluble and -insoluble fractions were subjected to SDS/polyacrylamide-gel electrophoresis, glycogen moved through the 3% stacking gel. A broad band was seen in this zone by autoradiography of the ¹²⁵I-labelled proteoglycan fractions (Fig. 1a).

Glycogen-bound protein was characterized after amyloysis of the polysaccharide moiety with an amylolytic mixture that also hydrolysed the linkage between tyrosine residues and glycogen (see the Materials and methods section). After amylolytic digestion (Fig. 2a) the protein disappeared from the stacking gel in both fractions and entered in the 12.5% acrylamide gel as a band of weight-average *M_r* 42 000. Thus the acid-insoluble 1,4-α-glucan was a proteoglycan fraction containing a protein moiety similar in size to that of the acid-soluble proteoglycan fraction.

In the acid-insoluble fraction amyloysis did not shift completely the position of a sharp band seen on the top of the resolving gel. This could indicate that this proteoglycan was amylolysed to a lesser extent. However, no band was seen on the top of the resolving gel after amyloysis of the proteoglycan fraction preincubated with trypsin under conditions in which the bulk of the 42 kDa protein was not digested (Fig. 1b). It was concluded that the acid-insoluble proteoglycan fraction was contaminated with high-*M_r* protein(s), which was (were) digested by the preincubation with trypsin. The size of proteoglycans was determined by gel filtration on Sepharose CL-4B of the ¹²⁵I-labelled fractions pre-

Fig. 1. SDS/polyacrylamide-gel electrophoresis of the ¹²⁵I-labelled protein of acid-soluble and acid-insoluble proteoglycans

Autoradiograph of the acid-soluble (A) and acid-insoluble (B) proteoglycan fractions run in 12.5% gels before (–) and after (+) amyloysis. (a) The ¹²⁵I-labelled proteoglycan fractions (50 µg of glycogen; 340 000 c.p.m.) were incubated with 2 µl of the amylolytic mixture in a total volume of 50 µl as indicated in the Materials and methods section. (b) Before amyloysis each of the ¹²⁵I-labelled proteoglycan fractions [50 µg of glycogen (2.25 µg of protein) and 1 × 10⁶ c.p.m. in A and 142 µg of glycogen (4.3 µg of protein) and 1 × 10⁶ c.p.m. in B] was incubated at 37°C for 18 h with 90 ng of trypsin in a total volume of 60 µl, and digestion was stopped with 1 mM-Tos-Lys-CH₂Cl. Aliquots (340 000 c.p.m.) were amylolysed as in (a).

Fig. 2. Elution profiles on Sepharose CL-4B of ^{125}I -labelled proteoglycan fractions after trypsin treatment

The ^{125}I -labelled proteoglycan fractions soluble (—) and insoluble (---) in trichloroacetic acid were digested with trypsin as in Fig. 1(b). An aliquot (1.1×10^6 c.p.m.) of each digested proteoglycan was passed through the column (1 cm $\times$ 100 cm), eluted with 60 mM- NH_4HCO_3 and fractions (0.32 ml each) were collected and monitored for radioactivity. The elution positions of dextrans of weight-average M_r 5×10^5 , 1.5×10^5 and 0.68×10^5 are indicated (D_5 , $D_{1.5}$ and $D_{0.68}$).

incubated with trypsin under the conditions shown in Fig. 1(b). The acid-insoluble and acid-soluble proteoglycans had a weight-average M_r of 4.7×10^5 and 7.0×10^5 respectively (Fig. 2). These values were similar to those obtained by chromatography of the proteoglycan fractions labelled before purification by incubation of the retina preparation with $2 \mu\text{M}$ -UDP- ^{14}C glucose at 2°C for 6 min (weight-average M_r of 4.5×10^5 and 7.2×10^5 respectively; results not shown). The treatment with trypsin before chromatography avoided interferences in the chromatogram caused by the iodinated high- M_r protein(s) present in the acid-insoluble fraction. The labelled peptides released by trypsin were eluted near the total volume of the column. The weight-average M_r of acid-soluble proteoglycan from retina is smaller than that from other tissues, which ranges between 4×10^6 to greater than 500×10^6 (Calder & Geddes, 1985). However, this comparison must be made with caution, since the bovine eyes were immersed in ice 3–5 min after death, and some of the rapid post-mortem changes which are usually avoided by freezing in liquid nitrogen might have occurred. Therefore the size of retina proteoglycans is described in terms of apparent M_r . The results described in the present work add chemical evidence to earlier studies (Curtino *et al.*, 1979; Aon & Curtino, 1984; Miozzo *et al.*, 1986) which led to the conclusion that the trichloroacetic acid-insoluble fraction is the precursor of acid-soluble proteoglycan. It should be noted that the acid-insoluble proteoglycan becomes soluble in trichloroacetic acid after the acid-insoluble residue is solubilized in SDS and proteoglycan washed with detergent by high-speed centrifugation (see the Materials and methods section). How-

ever, treatment of the membrane suspension with 1 M-NaCl has no effect on the amount of proteoglycan recovered in the acid-insoluble residue. Thus the non-acid-extractability of the insoluble proteoglycan appears to be related to hydrophobic interactions with protein(s) or membrane(s). An enlargement of the polysaccharide moiety by incorporation of glucose from UDP-glucose may result in the dissociation of proteoglycan to the acid-extractable form.

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