

A Glycoprotein from Reticulin Tissue

OLLE SNELLMAN

From the Cancer Research Division of Radiumhemmet, Karolinska Institutet, Stockholm 60, Sweden

A glycoprotein has been obtained from reticulin tissue. The structure of the glycoprotein is two peptide chains connected with bridges of oligosaccharides. Three pairs of oligosaccharides occur in the molecule. One of each pair contains galactose and three N-acetyl glucosamines and the other contains mannose and three N-acetyl glucosamines. The first one is bound to the peptide chains with ester bonds and the latter with ether bonds. A tentative picture of the structure is given.

While investigating methods for the extraction of collagen from skin Bowes, Elliot and Moss¹ observed that a glycoprotein went into solution together with the collagen. Consden² has also observed that a glycoprotein is present in human subcutaneous tissue. There seems to have been no isolation of a definite glycoprotein, in either case.

An investigation of a glycoprotein occurring in skin as well as in other kinds of reticular tissue is reported here.

METHODS

Column electrophoresis has been performed in an electrophoresis apparatus (type LKB) using cellulose powder as supporting medium. A 0.05 M phosphate buffer pH 6.8 has been used. The sulphuric acid-cysteine method of Dische³ was used for the detection of carbohydrate in the effluents from the column.

Determinations of hexose contents were made with the orcinol method⁴ and N-acetyl glucosamine was determined according to Reising, Strominger and Leloir⁵. For assays of carbohydrate the substance was hydrolysed in 2 M H₂SO₄ at 100°C for 12 h. In order to recognize the different carbohydrates in the hydrolysates paper electrophoresis was performed in borate buffer at pH 8.6 as well as paper chromatography according to Bayly *et al.*⁶ The ratio between the different carbohydrates was determined on a Dowex 1 column in the borate cycle according to Khym and Zill⁷. The collected fractions (15 ml) were evaporated to dryness on a water bath and then dissolved in 1 ml water. Carbohydrates were determined in these solutions by the anthrone method.

For qualitative amino acid analysis determinations paper chromatography using the system butanol-acetic acid-water and phenol has been used. In some cases the ratio between different amino acid was determined by cutting out the spots where the amino

acids were thought to be, extracting and determining the ninhydrin values. N-Terminals have been determined according to Sanger and C-terminals using carboxypeptidase, both as described in Ref.⁸ Aminopeptidase has been used for elucidating the C-terminal sequences.

Preparation of the reticulin tissue. In the first experiments skin from rats was used. However, as skin is a rather complex tissue, in later work reticulin from spleen and lymphatic nodes was used, because it yielded clear reticulin tissue preparations more easily.

900 g spleen from cow was cut in thin slices and gently rubbed under water until all parenchymal cells had disappeared and only a white-yellow network of reticulin remained. About 90 g wet weight was obtained. It was cut in small pieces and left in acetone for some days, then being dried in air. 40 g acetone treated material was obtained. An extraction was made in a Soxhlet's apparatus with a mixture of benzene and acetone (1:1). The substance was milled in a mortar, yielding 15 g of powder. This material was used to obtain the glycoprotein, and will be referred to as reticulin.

Preparation of the glycoprotein. 1 g of the reticulin tissue was suspended in 400 ml of a 0.2 M KCl-HCl buffer at pH 2.0. 10 mg pepsin was added and the solution was incubated for three days at 37°C. After that time only a smaller residue remained amounting to about 1 % of the original material. This solution was first dialysed against running tap water for 1 day and then against distilled water for 1 day. It was evaporated to about 20 ml, shaken with phenol and dialysed again. After that it was concentrated to a small volume and dried over tablets of NaOH. About 200 mg of a slightly yellow powder was obtained.

The same procedure was performed with the same result and about the same yield with lymphatic nodes. Rat skins were milled in dry ice and defatted in the same way first extracted with 0.05 M phosphate buffer, pH 6.8 for 24 h. The material was separated from the solution and washed in water before treatment with the pepsin solution.

Collagen from rat tail tendon was also treated in the same manner, but no glycoprotein was obtained.

The glycoprotein dissolved easily in water and seemed to be soluble in all proportions. It was precipitated first with about 80 % acetone. Even at high concentrations in water the viscosity was low. The glycoprotein gave a positive reaction with the periodic acid-Schiff test, similar to reticulin.

Column electrophoresis showed one component, which moved only very slightly towards the acid side (Fig. 1).

The ultra violet absorption spectrum showed a maximum at 225 $m\mu$. At longer wavelengths the absorption was low. The absorption band at 225 $m\mu$ might be due to the richly occurring amide groups.

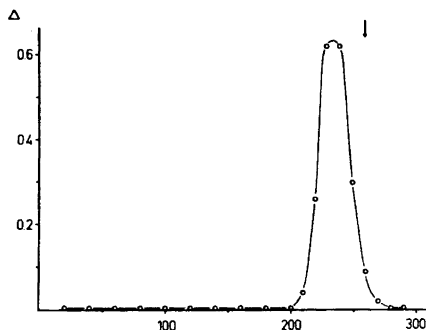


Fig. 1. Column electrophoresis of the glycoprotein in 0.05 M phosphate buffer pH 6.8. Time 24 h. Ordinate: Dische reaction (414–380) $m\mu$. The arrow indicates starting point.

ANALYSES

Carbohydrates. In a hydrolysate of the glycoprotein the following carbohydrates were identified: mannose, galactose and glucosamine. They were identified both by paper electrophoresis and by paper chromatography. Using an ion exchanger according to Khym and Zill the following ratios were obtained: mannose to galactose, 1:1, mannose and galactose to glucosamine, 1:3. The amount of hexose in the glycoprotein was determined by the orcinol method to be 12 % and by the method of Reissig *et al.* the amount of N-acetylglucosamine was found to be 44.5 %.

Amino acids. Hydrochloride acid was used to hydrolyse the substance, a large excess being required on account of the great amount of carbohydrates present, in other case the amino acids are partly destroyed. The following amino acids have been recognized: Asp, Glu, Thr, Lys, Val, Phe, Leu. Paper chromatography could be used as a purity index. Peptides adhered very strongly to the glycoprotein and were difficult to remove. These peptides contained proline and alanine and if these amino acids were absent the substances could be considered as pure.

The N-terminals were aspartic acid and glutamic acid, occurring in equal parts. The only amino acid split off by carboxypeptidase was glutamine.

Assuming that there were two N-terminals in the molecule a value of 9000 was found for the molecular weight as determined from the N-terminal content.

The effect of papain on the glycoprotein. During some experiments it was observed that the solution containing the glycoprotein darkened and became orange colored when incubated with papain (Worthington). It was assumed that this change depended on the carbohydrate moiety and therefore this part was investigated.

A glycoprotein solution was incubated with a small amount of crystallized papain at 25°C for 12 h. Column electrophoresis was made on the solution. Negative values were obtained with the cysteine reaction for carbohydrates (Fig. 2). The substance was electrophoretically inhomogeneous and the greater part of it was much more negatively charged than before. The solution was

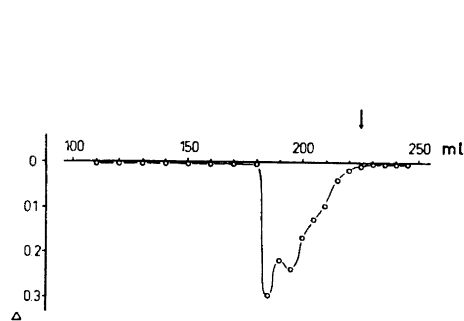


Fig. 2. Column electrophoresis of papain digested glycoprotein. Data see Fig. 1.

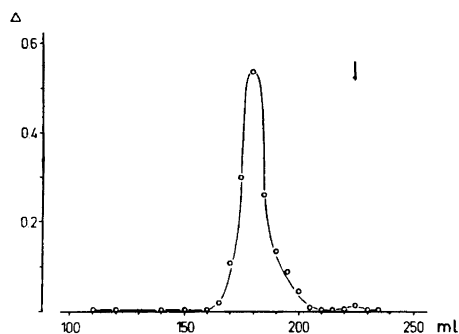


Fig. 3. Column electrophoresis of papain incubated glycoprotein which had been hydrolysed in dilute acetic acid and dialysed. Data see Fig. 1.

dialysed after treatment with a 10 % solution of acetic acid at 105°C for 12 h. An analysis showed that half of the carbohydrate content had dialysed out and the other part remained in the bag.

The dialysate contained glucose and another carbohydrate derivative which was responsible for the colour. This derivative changed to glucosamine on hydrolysis in 7.6 M HCl. Probably it is an anhydroderivative of N-acetyl glucosamine. Further investigations on that point have not been done. At the reaction galactose evidently has been inverted to glucose. The ratio between the carbohydrates was 1:3 as determined by the method of Khym and Zill.

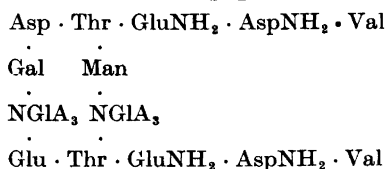
The retentate consisted now of an electrophoretically homogeneous substance much more acid than before (Fig. 3). This protein was hydrolysed and the carbohydrates determined. It contained mannose and glucosamine in the ratio 1:3.

The experiments showed that two kinds of oligosaccharides occur in the glycoprotein: one kind which is bound by ester bonds to the peptide chains and contains galactose and N-acetyl glucosamine in the ratio 1:3 and another ether bound containing mannose and N-acetylated glucosamine in the ratio 1:3.

The action of elastase on the glycoprotein. Elastase is able to split the glycoprotein. 100 mg glycoprotein were incubated in 10 ml water with 10 µg elastase at 37°C for 12 h. The solution was dialysed. The dialysis water was evaporated yielding 27 mg of dry material. The carbohydrate dialysed out was one third of the total. Column electrophoresis showed one slightly acid component.

Analyses of the dialysable component showed that it contained the carbohydrates: galactose, mannose and glucosamine, and the following amino acids: Asp, Glu, Thr, Val. The C-terminal was valine. Determinations with carboxypeptidase showed the following sequence: GluNH₂·AspNH₂·Val(OH) and no further amino acids because carbohydrate bonds prevented further action. The part not digested was purified. It contained all the carbohydrates and the amino acids Asp, Glu, Thr in the relation 1:1:1.8.

Thus it seems that the N-terminal peptide has the structure



The retentate from the dialysis contained one component which was electrophoretically homogeneous. It contained the amino acids: Asp, Glu, Thr, Lys, Phe, Leu. Thus valine was now missing. The N-terminals were Phe and Leu in equal parts. After extracting them as DNP-derivatives the residue lacked these amino acids.

Polyaminopeptidase split off Phe, Leu, GluNH₂, AspNH₂ and some Glu and no further amino acids. The sequences immediately following after the two valines are



followed by amino acids bound to carbohydrates.

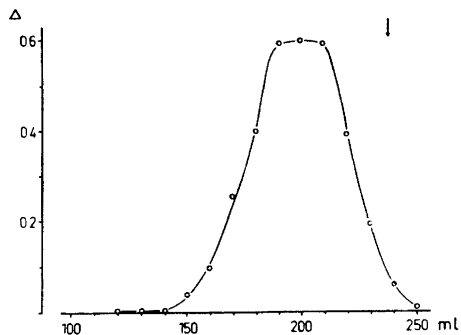


Fig. 4. Column electrophoresis of lysozyme incubated glycoprotein. Ordinate: ninhydrin reaction. Time 48 h.

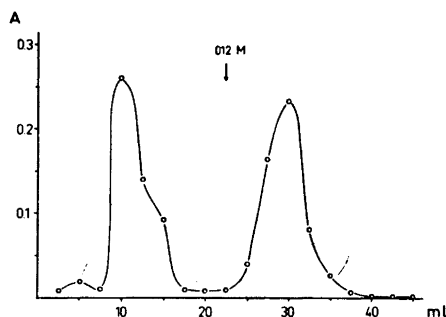


Fig. 5. Ion exchange chromatography on Dowex 2 of glycoprotein digested with lysozyme. Ninhydrin reaction.

Cleavage with lysozyme. From the experiments it could be concluded that the two peptides in the glycoprotein were connected by oligosaccharides containing three N-acetylated glucosamines in series. Therefore it was decided to cleave the carbohydrate chains with lysozyme.

Lysozyme is a β -glucosaminidase which acts on N-acetylated glucosamines in oligosaccharides, hydrolyzing β -glycoside bonds connecting N-acetyl glucosamine with other components. Preferentially it seems to hydrolyse 1,4-bonds. Takadiastase, which contains a β -glucosaminidase, has also been used.

50 mg glycoprotein were digested with 0.2 mg lysozyme for 18 h at 37°C. The treatment rendered all of the glycoprotein dialysable. The compound hardly moved during column electrophoresis, only a broadening of the zone being obtained (Fig. 4). Obviously the ester bonds were intact and therefore no new electrical charges had appeared.

On an anion exchange column (Dowex 2) it was, however, possible to separate two components (Fig. 5). One component (A) which came out immediately and another (B) which appeared in 0.12 M acetic acid.

All the carbohydrates came out with the component A, and B was free of carbohydrates. Component A contained the amino acid phenylalanine which was missing in B, which contained leucine instead. The N-terminal group of A was aspartic acid and that of B glutamic acid.

It seemed that the β -glucosaminidase split the glycoside bonds binding N-acetyl glucosamine to threonine, but did not split the ester bonds. On the ion exchanger, however, this was done. The ester bonds between N-acetyl glucosamine and glutamic acid were evidently very labile in this case. As the B chain was free from carbohydrates it was connected only to N-acetyl glucosamine residues. The neutral hexoses remained on the chain A where all connections should be with hexoses.

GluNH₂·AspNH₂ and stopped there. Thus Asp Thr seem to be bound to hexoses. It can be assumed that the sequence of A₁ is GluNH₂·AspNH₂·Asp·Thr·GluNH₂.

A₂ contained Asp, Glu, Thr, Val, Phe, Lys and hexoses. Carboxypeptidase gave the sequence GluNH₂·AspNH₂·Asp·Lys(OH) and no further amino acid, because carbohydrates prevented further action.

From these studies it follows that the last part of chain A should be GluNH₂·AspNH₂·Asp·Lys·GluNH₂·AspNH₂·Asp·Thr·GluNH₂, and that of chain B

GluNH₂·AspNH₂·Asp·Lys·GluNH₂·AspNH₂·Glu·Thr·GluNH₂.

Here the pair Asp·Thr in chain A and Glu·Thr in chain B are bound to oligosaccharides in the manner as can be seen in Fig. 6.

The sequence of the carbohydrate bound amino acids in the middle part has not been determined but it can be assumed that they do not differ from the other bonds of the same type as they contain the same amino acids. The structure can therefore tentatively be given as in Fig. 6.

DISCUSSION

The glycoprotein obtained occurs in skin, lymphatic nodes and spleen but not in matured collagen. It belongs to the reticulin. This tissue shows a strong aldose reaction. The glycoprotein gives the periodic acid Schiff reaction (PAS) as reticulin does. It is therefore likely that the origin of the reaction is the same in both cases.

This preparation method did not show whether or not the glycoprotein was a fragment of a larger molecule. However, indications from studies with other preparation methods for the glycoprotein to be described in another forthcoming paper, show that it is not a fragment.

The tentative structure of the glycoprotein given agrees fairly well with the molecular weight found. The types of bonds between the carbohydrates have not been determined directly. However, the enzymes splitting the bonds split only β -glycoside bonds so these must occur. Lysozyme splits 1,4-glycoside bonds. Such bonds can also be expected from the unusual inversion observed of galactose to glucose, as well as from the ease with which the ester bonds are split. The ester bond connecting N-acetyl glucosamine with glutamic acid is especially easily cleaved, often with the loss of N-acetyl glucosamine. This may give rise to many difficulties when preparing and investigating the substance. If a partial cleavage occurs an electrophoretically inhomogeneous substance is obtained. Many proteolytic enzymes will act as esterases and split the bond.

The particular effect of papain on the glycoprotein is as yet unexplained and is not sufficiently studied to be discussed thoroughly, but it may be that the carbohydrates changed to an anhydro form.

The peculiarities in the structure of the glycoprotein point definitively towards a special function. Reticulin can be considered as a tissue in a very low state of differentiation and probably the glycoprotein has something to do with the formation of collagen. It occurs in reticulin but not in the matured collagen. It seems to be tightly bound to collagen with hydrogen bonds. The substance offers many possibilities for such bonds, which probably occur in

those regions of collagen which are rich in proline and alanine. These amino acids are difficult to remove from the glycoprotein.

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