ACTA CHEMICA SCANDINAVICA 17 (1963) 1057–1061

Studies on a Matrix for the Biosynthesis of Collagen

OLLE SNELLMAN

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

Investigations performed on reticulin tissue have shown that it contains a glycoprotein, nucleotide peptides of collagen as well as collagen itself. The peptides are carboxyl-activated esters and the nucleotide is guanosine triphosphate. Collagen, glycoprotein and nucleotide peptides build up a highly ordered complex where the collagen and the glycoprotein are the template on which the nucleotide peptides are laid down.

In the preceding paper [1] it was shown that reticulin contained a glycoprotein of unusual structure. In this paper the reticulin has been further investigated. It seems to consist of collagen, glycoprotein and nucleotide peptides of collagen, building a highly ordered system for the extra cellular step in the biosynthesis of collagen.

EXPERIMENTAL

Urea extracts of reticulin. The preparation of the reticulin used in all the investigations here described was the same as in the previous paper.

500 mg reticulin were dissolved in 20 ml of a urea buffer containing 8 M urea and 0.1 M phosphate buffer pH 6.8. Up to 80 % of the material dissolved rapidly in the buffer to give a slightly yellow quite clear solution. The insoluble material contained collagen. When the solution was dialysed against water a heavy precipitate was formed long before the urea buffer had dialysed out. The glycoprotein precipitated here together with collagen and some of the peptides. The precipitate could be dissolved again in the urea buffer.

Chromatography of the extract. The directly dissolved material was chromatographed in an IRC 50 column equilibrated with the same buffer. The same urea buffer was used as eluant. Assays have been carried out on the effluent taken up in a fraction collector using the procedure described by Lowry *et al.*[2] for proteins and the orcinol method [3] for hexoses.

For comparison collagen from rat tail tendon was dissolved in the urea buffer and chromatographed. One component was obtained, coming out soon after the hold up volume (Fig. 1).

When the solution of reticulin was chromatographed determinations with the orcinol method showed one quite well defined component. The assays of protein in the fractions did not show a well defined component, as in the case of tendon collagen. Diagrams such as that in Fig. 2 were obtained, showing material coming out over a great range of frac-

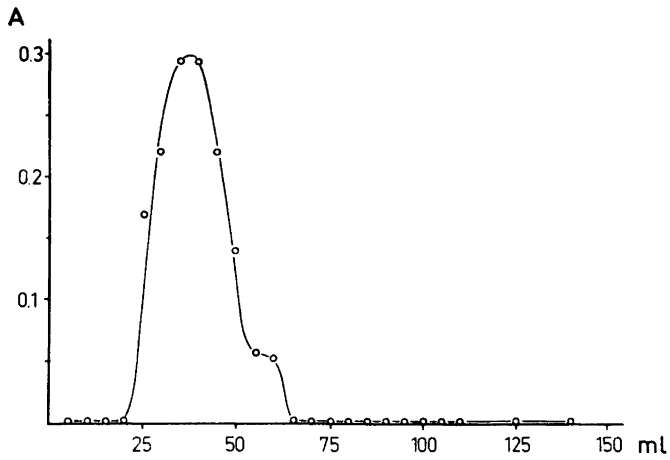

Fig. 1. A chromatograph of rat tail tendon collagen in 8 M urea — 0.1 M phosphate buffer pH 6.0.

tions. Fractions from different ranges were collected and dialysed. About half of the material dialysed out. Urea was added to the material remaining in the bag and the solution was chromatographed again. In this case only one component was obtained, coming out as tendon collagen (Fig. 3). The same result was obtained from which ever part of the original curve the fractions were collected.

The part dialysed out was concentrated and run again. It contained a large quantity of peptides which were not resolved with this chromatographic technic.

Extracts of reticulin in sodium acetate solutions. As the urea buffer solutions were somewhat difficult to handle it was found better to use 0.5 M sodium acetate as an extraction solution. The peptides and the glycoprotein dissolved easily in this solution and the collagen more slowly. The peptides did not seem to be connected with the collagen in these extracts.

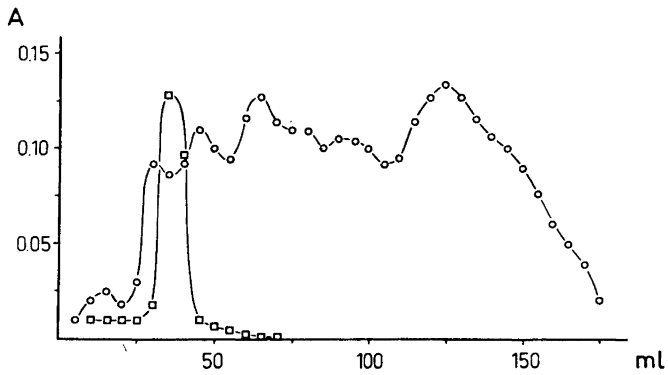

Fig. 2. A chromatograph of reticulin dissolved in 8 M urea — 0.1 M phosphate buffer pH 6.0. O Protein values. □ Hexose determinations.

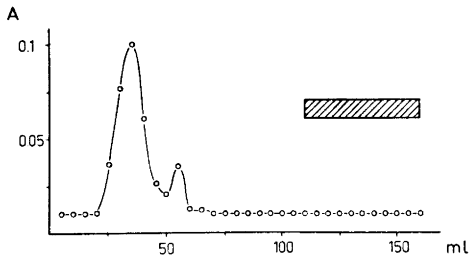

Fig. 3. The rechromatography of eluate from the previous chromatogram after dialysis. The same buffer used as before. The eluate is taken from the range indicated by the barrel.

All the peptides were dialysable, leaving only glycoprotein and collagen in the retentate. Fig. 4 demonstrates a chromatographic analysis of the bag content when urea buffer has been added showing that no peptides were left. The glycoprotein was isolated here. It had the same end-groups as given in the previous paper and did not react with pepsin.

The dialysable peptides. The dialysate outside the bag was made slightly acid. It was put into a dialysis bag and an air stream was blown on to it to concentrate it by evaporation. It contained a large amount of nucleotides. The ultra violet spectrum obtained at several pH's coincided with that of guanosine.

The peptides were filtered through a Sephadex G50 column. The guanosine derivative followed the peptides, which come out somewhat retarded as one component. It seemed, therefore, that the nucleotides were bound to the peptides. This was checked by means of the hydroxamate test according to Lipmann and Turtle [4]. A reaction was obtained at pH 7.0 and a very faint one at pH 6.5. This is to be expected from a carboxyl activated peptide esterified at 2′ or 3′ on the ribose of the nucleotide (Weiss and Zachau [5]).

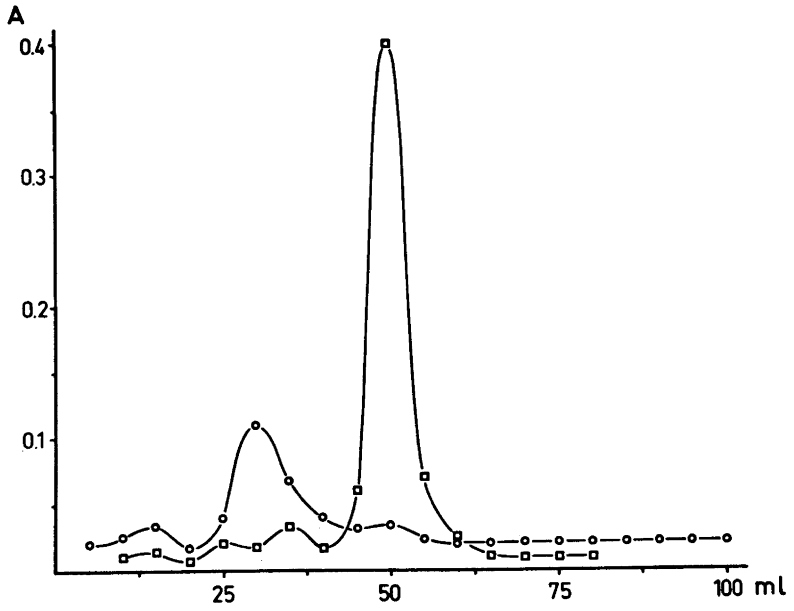

Fig. 4. The chromatography of a dialysed sodium acetate extract in 8 M urea — 0.1 M phosphate buffer pH 6.0. O Protein determinations. □ Hexose determinations.

Labile phosphate has been determined according to Fiske and Subbarow [6]. The values have been related to the ultra violet absorption at 260 mμ of the solutions. If the latter is referred to the molar absorbtion of guanosine a ratio UV/P = 2.0 is found showing that the nucleotide must be guanosine triphosphate.

Some preliminary experiments have been done to see if it was possible to separate the nucleotide peptide mixture on a DEAE column. The dialysate was concentrated and gel filtrated through a Sephadex column in order to remove salts. The salt free dialysate was put on the DEAE-cellulose column and eluted with ammonium carbonate solutions. The absorption at 260 mμ of the effluent was measured. About 32 % of the material went straight through, 52 % came out between 0.01 and 0.02 M carbonate and 16 % was obtained as 13 well defined separate components in the range 0.03 to 0.10 M ammonium carbonate. Here 12 components were of the same size and the remaining component contained twice the amount.

It ought to be possible to separate the peptides and experiments are under way to do this. It can already be concluded that the peptides occur in molar ratios. The number of peptides has been estimated to be 85 if counted in molar ratios.

The total amount of phosphate in 100 mg reticulin was 20 μmole, determined according to King [7]. Assays of the neutral hexoses as well as of N-acetyl glucosamine in the reticulin both indicated that there were 25 mg glycoprotein in 100 mg reticulin. Thus there are 6.7 μmoles nucleotides and 2.8 μmoles glycoprotein in 100 mg reticulin.

DISCUSSION

It seems that a complex exists between collagen, nucleotide peptides and glycoprotein in the reticulin. It can be assumed that about 20 % fibrous collagen occurs in the reticulin as so much collagen remains undissolved. Assuming this it can be calculated from the phosphate content that 2 moles of collagen are associated with 85 peptides. (The molecular weight of collagen is assumed to be 340 000 according to Boedtker and Doty [8]). This fits rather well with the number calculated from the chromatography experiments where a figure of 85 peptides was obtained. Thus in the complex there should be 1 mole collagen and 1 mole split up into about 85 different peptides. In the reticulin there are for 3.0 moles nucleotide peptide 1.2 moles glycoprotein. This is only a little more than would be expected if 3 peptides were bound to every glycoprotein molecule. It might be that a small part of the glycoprotein is bound to two collagen molecules. It can be assumed that a complex occurs which is built up of 1 mole collagen, about 29 moles glycoprotein and about 85 moles of different guanosine triphosphate peptides of collagen.

This complex is bound together partly with hydrogen bonds and partly with polar groups.

Regarding the glycoprotein it seems that this is bound with hydrogen bonds. This can be seen from the chromatography in urea buffer where a breakdown of the hydrogen bonds in the complex occurs and the glycoprotein behaves as if it was free. It can be pointed out that the NH-groups in N-acetyl glucosamine are especially apt to form hydrogen bonds. It can thus be expected that to each of the three pairs of oligosaccharide chains in the glycoprotein one of the three chains in the collagen molecule is bound. In the preceding paper about the glycoprotein it was mentioned that peptides with alanine and proline were especially firmly bound to the glycoprotein. It is therefore to be expected that the glycoprotein is bound in the regions where few polar groups occur, *i.e.* in the interband parts. In these regions, where many iminogroups

occur, the chain cannot form intramolecular hydrogen bonds and thus there are free CO-groups which can form hydrogen bonds with the glycoprotein.

In the polar regions the nucleotide peptides will be bound to the collagen chains through the phosphate groups. Schmitt *et al.*[9] have investigated how collagen precipitates from solutions using electron microscopy. When ATP was added a new form precipitated, the so-called SLS-type, which consisted of collagen molecules arranged perfectly side by side so that the same groups in different molecules were ordered on the same line perpendicular to the axes of the molecules. ITP was found to cause the same pattern, but not ADP or AMP. It seemed that there were pecularities in the structure which gave rise to this type of aggregation. Gross[10] found that the collagen bound 12 % ATP, *i.e.* one mole collagen will bind 86 moles ATP. They have not investigated the behaviour of GTP but it can be expected that it behaves as ATP and ITP, especially as it seems that nucleotide peptides and ATP are bound in equimolar quantities. The place on the collagen molecule towards which a peptide is directed is determined by the sequence so that a peptide is fixed on a spot which has the same sequence as itself.

The complex is thus highly ordered with the nucleotide peptides lying along the collagen chains with the same amino acid sequence through the phosphate groups and the glycoprotein.

Here is a matrix ready for the biosynthesis of collagen. In the common biosynthesis of proteins GTP is necessary for the step in which an activated amino acid moves from the soluble RNA to its place in the sequence. At the same time a SH-dependent coupling enzyme must be present. This enzyme is so sparely occurring in the reticulin tissue that scarcely any biosynthesis occurs. In a forthcoming paper experiments will be described showing that by adding the enzyme and ascorbic acid biosynthesis can be performed in reticulin.

Acknowledgements. The author is indebted to Dr. B. Sylvén for his kind interest and encouragement. The investigations was supported by grants from the *Swedish Natural Science Research Council* and the *Swedish Cancer Society*.

REFERENCES

1. Snellman, O. *Acta Chem. Scand.* **17** (1963) 1049.
2. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, J. *J. Biol. Chem.* **193** (1951) 265.
3. Winzler, R. *Methods of Biochemical Analysis.* Interscience Publ., New York, **2** (1954) 279.
4. Lipmann, F. and Turtle, L. C. *J. Biol. Chem.* **161** (1945) 415.
5. Weiss, S. B. and Zachau, H. G. *Proc. 4th Intern. Congr. Biochem.* Pergamon Press, London, **8** (1958) 217.
6. Fiske, C. H. and Subbarow, J. *J. Biol. Chem.* **66** (1925) 375.
7. King, E. J. *Biochem. J.* **26** (1932) 292.
8. Boedtker, H. and Doty, P. *J. Am. Chem. Soc.* **78** (1956) 4267.
9. Schmitt, F. O. and Hodge, A. J. *Leder* **11** (1960) 74.
10. Gross, J. *J. Biophys. Biochem. Cytol.* **2** (1956) 261.

Received September 24, 1962.