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AFFINITY CHROMATOGRAPHY OF COLLAGEN GLUCOSYLTRANSFERASE ON A UDP-GLUCOSE DERIVATIVE COUPLED TO AGAROSE

HENRIK ANTTINEN and KARI I. KIVIRIKKO

Department of Medical Biochemistry, University of Oulu, Oulu (Finland)

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Summary

UDP-glucuronic acid from the carboxyl group was coupled to agarose via a six-carbon atom spacer, and columns prepared from this material were used in an affinity chromatography of collagen glucosyltransferase. The enzyme was found to have a high affinity for such columns in the presence of Mn^{2+} in the buffer, whereas a considerably lower affinity was noted in the absence of such ions. The enzyme could be eluted from the column with either EDTA, UDP-glucose, or small peptides prepared from collagen, the peptides being the most effective eluting agent. After elution the enzyme was separated from the peptides by gel filtration. With this procedure a collagen glucosyltransferase purification of about 3000-fold was obtained from extract of chick embryos by relatively simple steps.

Collagen galactosyltransferase was found to have no affinity for the column, suggesting that the binding was not only due to the UDP moiety, but that the uronic acid derivate of glucose also contributed to its specificity.

Introduction

Collagens from interstitial tissues and from basement membranes contain hydroxylysine-linked disaccharide units with a structure of 2-*O*-α-glucopyranosyl-*O*-β-galactopyranosylhydroxylysine (for review, see refs. 1—6). The transfer of glucose from UDP-glucose to galactosylhydroxylysyl residues is catalyzed by collagen glucosyltransferase in the presence of Mn^{2+} or certain other bivalent cations [1—11].

Collagen glucosyltransferase was initially studied at a relatively low level of purification from guinea pig skin [7], rat kidney cortex [8], chick embryo cartilage [9], and bovine arterial tissue [10]. Very recently, an over 2000-fold purification of the enzyme from whole chick embryos was reported, involving a

procedure consisting of six conventional protein purification steps [11]. The enzyme was found to have a molecular weight of about 52 000—54 000 by gel filtration, and several other properties of the enzyme were also studied [11, 12]. Complete purification of the enzyme has not been obtained, however.

In the present work, affinity chromatography is performed on collagen glucosyltransferase using a column prepared by coupling UDP-glucuronic acid from the carboxyl group to agarose via a 6-carbon atom spacer. The enzyme was found to have a high affinity to this column, and it was possible to obtain collagen glucosyltransferase purification of about 3000-fold from extract of chick embryos by relatively simple steps.

Materials and Methods

Materials

AH-Sepharose 4B was purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden), and sodium UDP-glucuronate from Sigma Chemicals Co. (St. Louis, Mo.). Dialyzable peptides were prepared from bovine Achilles tendon collagen by collagenase digestion [9]. Glucosylgalactosylhydroxylysine was prepared and purified from sponge collagen [13], and then converted to galactosylhydroxylysine by hydrolysis with 0.05 M H_2SO_4 at 100° C for 24 h [14]. The resulting galactosylhydroxylysine was further purified by gel filtration on Sephadex G-15. The final preparations of galactosylhydroxylysine and glucosylgalactosylhydroxylysine were over 95% pure when examined with an amino acid analyzer. The sources of the other materials were the same as specified elsewhere [11,12].

Coupling of UDP-glucuronic acid to agarose

UDP-glucuronic acid from the carboxyl group was coupled to free amino groups of AH-Sepharose 4B using the carbodiimide method [15]. The AH-Sepharose 4B comprises Sepharose 4B to which 1,6-diaminohexane has been coupled. This material was allowed to swell for 6 h in 0.5 M NaCl, then washed, first with large volumes of 0.5 M NaCl, and then with large volumes of distilled water. Finally, 10 ml of packed gel was suspended in distilled water to give a volume of 20 ml. 300 mg of sodium UDP-glucuronate were dissolved in 1 ml of distilled water, and this solution was added to the gel. The pH was adjusted to 4.7 by adding 0.2 M and 0.05 M HCl, and then 800 mg of 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide was added in 3 ml of distilled water over a period of 15 min. The pH was kept between 4.7 and 4.8 during this period, the pH control being continued for 1 h. The reaction was allowed to proceed at room temperature with gentle agitation for 24 h, after which the mixture was cooled to 4° C and washed with 1 l of 1 M NaCl. The gel was then stored in 1 M NaCl at 4° C until used.

Affinity chromatography of collagen glucosyltransferase

All procedures were carried out at 0—4° C. Chick embryos were homogenized and the 15 000 × *g* supernatant of the homogenate prepared as described previously [11]. The $(NH_4)_2SO_4$ fraction (0—45% saturation) of the embryo extract was likewise prepared as described previously [11], except that the final dialysis

was carried out against a solution (termed "enzyme buffer") containing 0.12 M NaCl, 1% glycerol, 10 mM $MnCl_2$, 50 μM dithiothreitol and 50 mM Tris · HCl buffer, adjusted to pH 7.4 at 4°C. The enzyme was stored at -20°C in aliquots of about 150 ml.

An aliquot of the $(NH_4)_2SO_4$ fraction was thawed and centrifuged at 15 000 × *g* for 30 min. The protein concentration was adjusted to 20 mg/ml with the enzyme buffer, and an aliquot of up to 150 ml was passed at a flow rate of about 5 ml/h through the affinity column, which had a bed volume of 10 ml and was equilibrated with the enzyme buffer. In some experiments, the bed volume of the column was only 2 ml, and the flow rate was then about 2—3 ml/h. After the sample had passed through the column, the 10-ml column was washed first with 60 ml of the enzyme buffer, and then with 100—150 ml of the same buffer but with an NaCl concentration of 1 M.

The enzyme was eluted from the 10-ml column with 3 g dialyzable collagen peptides in 4 ml of enzyme buffer at a flow rate of about 3 ml/h. The column was subsequently eluted with enzyme buffer without the peptides. After the experiment the column was regenerated by passing 100 ml of 6 M urea it, and then equilibrated with the enzyme buffer.

The absorbance of the fractions eluted with the peptides was measured at 230 nm, and the fractions were pooled, beginning with the first fraction showing any increase in absorbance, and continuing until the last fraction after the peptide peak which still had an absorbance above 2.0. The pooled fractions were concentrated by ultrafiltration in an Amicon ultrafiltration cell with a PM-10 membrane to about 2 ml. The cell was washed twice with 0.5 ml of the enzyme buffer, and these washings were pooled with the enzyme.

The enzyme concentrate was then applied to a Sephadex G-150 column (Pharmacia) of size 2.5 × 85 cm. The column was equilibrated and eluted with a solution containing 0.15 M NaCl, 1% glycerol, 50 μM dithiothreitol and 20 mM Tris · HCl buffer, adjusted to pH 7.4 at 4°C as reported earlier [11]. Fractions of 5 ml were collected, and the fractions containing most of the enzyme activity were pooled and concentrated in an Amicon ultrafiltration cell with a PM-10 membrane to a volume of about 1 ml.

Assays

Collagen glucosyltransferase activity was assayed in a final volume of 200 μl containing 0.001—2 mg/ml enzyme protein, depending on purity, 30 mg/ml calf skin gelatin substrate, or 2 mg/ml citrate-soluble rat skin collagen substrate, 60 μM UDP-glucose (12 Ci/mol), 10 mM $MnCl_2$, 0.02—0.1 M NaCl, 1 mM dithiothreitol and 50 mM Tris · HCl buffer adjusted to pH 7.4 at 20°C [9,11]. The gelatin or collagen substrate was heated to 60°C for 10 min and rapidly cooled to 0°C immediately before addition to the incubation mixture [12]. The samples were incubated at 37°C for 45 min, and the product assayed as reported previously [9,11]. One unit of enzyme activity was defined as the amount of enzyme required to synthesize $2.66 \cdot 10^7$ dpm (corresponding to 1 μmol) glucosylgalactosylhydroxylysine in 1 h at 37°C under conditions in which the concentrations of the reactants are those indicated above and in which a saturating concentration of denatured purified citrate-soluble rat skin collagen is used as a substrate [11].

Collagen galactosyltransferase activity was assayed as reported previously [9]. The protein content of the enzyme preparations was measured by peptide absorbance at 225 or 230 nm using bovine serum albumin as a standard. Disc electrophoresis was carried out using 7.5% polyacrylamide gels at 4°C with 42.5 mM Tris · HCl and 46.3 mM glycine buffer adjusted to pH 9.6 at 4°C as the upper buffer, and 120 mM Tris · HCl buffer adjusted to pH 8.8 at 4°C as the lower buffer. The gels were stained with Coomassie brilliant blue.

Results

Binding of collagen glucosyltransferase to the affinity column

The binding of collagen glucosyltransferase to the affinity column was tested with a column having a bed volume of 2 ml. A chick embryo $(NH_4)_2SO_4$ fraction with a protein concentration of 20 mg/ml was passed through this column at a flow rate of 2—3 ml/h, and fractions of 3 ml were collected. Enzyme activity was assayed in aliquots of the $(NH_4)_2SO_4$ enzyme passed into the column and of the fractions from the column. The enzyme activity of the fractions from the column in ml was expressed as a percentage of that of the original $(NH_4)_2SO_4$ enzyme.

Essentially all the protein in the $(NH_4)_2SO_4$ enzyme, but only about 10% of the enzyme activity was recovered in the column effluent suggesting that about 90% of the collagen glucosyltransferase activity of the $(NH_4)_2SO_4$ enzyme had been bound to the column (Fig. 1, closed symbols). The results further suggested that it would be possible to pass at least 30 ml of $(NH_4)_2SO_4$ enzyme, corresponding to 600 mg protein, through a 2-ml column without exceeding the capacity of the column.

In order to study whether the presence of Mn^{2+} in the enzyme buffer was a necessary condition for binding, experiments were carried out which were identical to the above except that the $(NH_4)_2SO_4$ enzyme was dialyzed against a buffer not containing Mn^{2+} and the column was likewise equilibrated with a

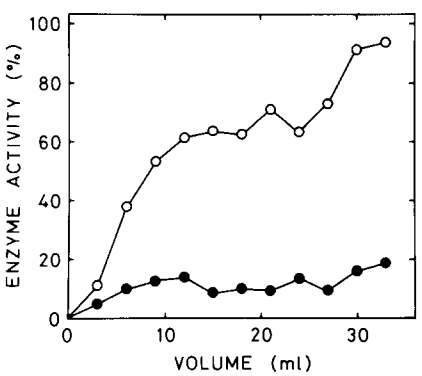

Fig. 1. Collagen glucosyltransferase activity in the effluent of a 2-ml affinity column after passing a chick embryo $(NH_4)_2SO_4$ fraction with a protein concentration 20 mg/ml through the column. The enzyme activity of the column effluent in ml is expressed as a percentage of that of the original $(NH_4)_2SO_4$ fraction. The experiments were carried out in the presence (●) and absence (○) of Mn^{2+} in the buffer as described in the text.

Mn^{2+}-free buffer. A partial binding of collagen glucosyltransferase to the affinity column was found in these experiments (Fig. 1, open symbols), but of a markedly lower degree than that observed in the presence of Mn^{2+}.

Elution of collagen glucosyltransferase from the affinity column

Several methods were tested for eluting the enzyme from the affinity column. No significant elution was observed when the NaCl concentration of the enzyme buffer was increased to 1 M, indicating that it was not an ion-exchange process which was mainly responsible for binding the enzyme. Small amounts of enzyme could be eluted with 25% saturated $(NH_4)_2SO_4$ solution, but the recovery and specific activity of the enzyme were very low. Since the presence of Mn^{2+} was required for optimal binding, elution with buffers containing 20 mM EDTA and no Mn^{2+} was tried. Significant amounts of enzyme could be eluted with such buffers, but large volumes were required, as a consequence of which the enzyme was eluted as a dilute solution and rapidly lost part of its activity. Experiments on the elution of collagen glucosyltransferase with enzyme buffer containing 10 mg per ml of UDP-glucose indicated that up to 30% of the enzyme activity applied to the column could be eluted. Recovery was variable, however, and the specific activity of the final enzyme preparations was lower than that obtained with the procedure reported below.

Small peptides prepared by collagenase digestion of collagen have previously been reported to serve as substrates for collagen glucosyltransferase [8,9,12], although they are less effective substrates than collagen itself [8,12]. Since it seemed possible to separate the enzyme easily from small peptides after the elution step, dialyzable peptides prepared by collagenase digestion of bovine Achilles tendon collagen [9,12] were tested for elution. This proved to be the most satisfactory method for eluting the enzyme, the best yields of enzyme activity being obtained with enzyme buffer containing 0.6—1 g/ml of the peptides (Fig. 2). As significant amounts of protein but virtually no enzyme were

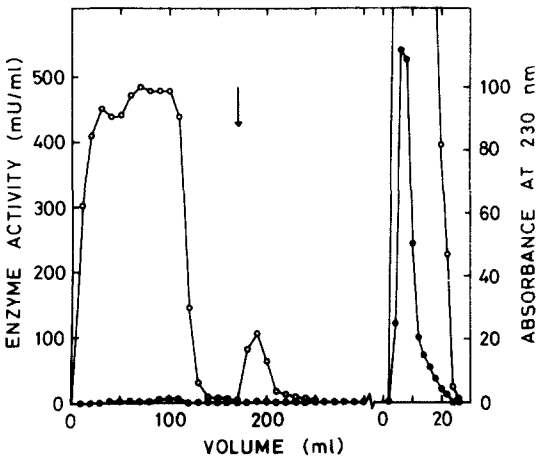

Fig. 2. Affinity chromatography of collagen glucosyltransferase on a 10-ml affinity column, and elution of the enzyme with dialyzable peptides prepared by collagenase digestion of collagen. Conditions were as described in the text. The arrow indicates the change to a buffer containing 1 M NaCl. ●, enzyme activity; ○, absorbance.

eluted with 1 M NaCl, the column was eluted in the final procedure with enzyme buffer containing 1 M NaCl prior to elution with the peptides (Fig. 2).

Free galactosylhydroxylysine has also been reported to act as a substrate for collagen glucosyltransferase [8]. Consequently, free galactosylhydroxylysine was prepared from sponge collagen as described in Materials and this compound was tested for eluting the enzyme. Since bovine Achilles tendon collagen contains only about 0.2% galactosylhydroxylysine by weight [16], this compound was tested at lower concentrations than the peptides. No significant elution of the enzyme was observed with enzyme buffer containing 10 mg/ml of galactosylhydroxylysine, which corresponds to an acceptor concentration about 10 times higher than that which gave high yields of enzyme in elution with the peptides. As the preparation and purification of large amounts of galactosylhydroxylysine is considerably more tedious than the preparation of the peptides, no tests were made at higher concentrations of galactosylhydroxylysine. Similarly, no significant elution of the enzyme was observed with 10 mg/ml of free glucosylgalactosylhydroxylysine, the product of reaction with free galactosylhydroxylysine.

Separation of collagen glucosyltransferase from the elution peptides

The enzyme became separated from some of the peptides when the enzyme pool obtained from the elution step was concentrated by ultrafiltration, and the remaining peptides were separated by gel filtration on a Sephadex G-150 column (Fig. 3). The enzyme activity was eluted before the peptides, and calibration of the Sephadex G-150 column with standard proteins indicated that the elution position corresponded to a molecular weight of about 52 000—54 000, as reported earlier for collagen glucosyltransferase after a purification of over 2000-fold by conventional protein purification steps [11].

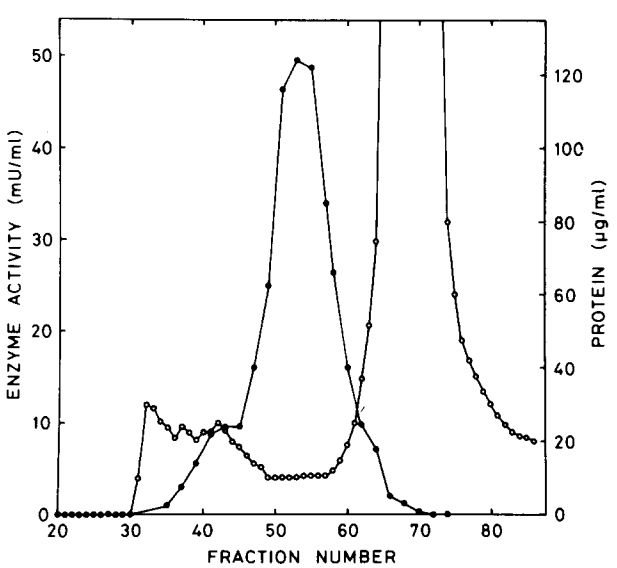

Fig. 3. Separation of collagen glucosyltransferase from the elution peptides by gel filtration on a Sephadex G-150 column. Conditions as described in the text. Fractions Nos. 50—56 were pooled, and constituted the purified enzyme. ●, enzyme activity; ○, protein.

TABLE I

PURIFICATION OF COLLAGEN GLUCOSYLTRANSFERASE FROM EXTRACT OF CHICK EMBRYOS BY THE AFFINITY CHROMATOGRAPHY PROCEDURE

Enzyme fraction	Total protein (mg)	Total activity (munits)	Recovery (%)	Specific activity (munits/mg)	Purification (-fold)
15 000 × *g* supernatant	8430	7520	100	0.89	1
$(NH_4)_2SO_4$ enzyme	2120	4750	63	2.24	2.5
After gel filtration	0.39	1080	14	2770	3110

The specific activity of the final enzyme pool was about 3000 times greater than in the 15 000 × *g* supernatant of the embryo homogenate, and the recovery of the enzyme activity was about 14% (Table I). If the specific activity and recovery are compared with those in the $(NH_4)_2SO_4$ fraction, the degree of purification obtained is about 1200-fold and the recovery about 23%. In spite of the relatively high degree of purification obtained, polyacrylamide gel electrophoresis showed the presence of two major bands and two minor bands, indicating that the enzyme was not entirely pure.

Affinity of collagen galactosyltransferase to the column

The binding of collagen galactosyltransferase to the affinity column was tested with a 2-ml column by carrying out similar experiments as for collagen glucosyltransferase. No significant binding was observed (Fig. 4), and the enzyme activity in fractions from the column varied between 85 and 110% in three experiments. The small decreases in enzyme activity seen in Fig. 4 are probably due to slight inactivation of the collagen galactosyltransferase during chromatography.

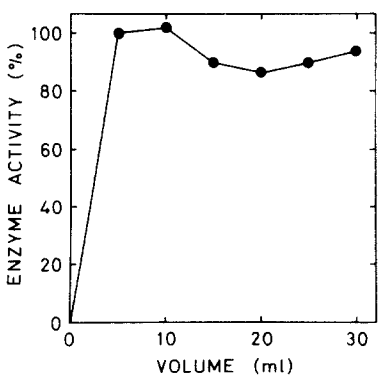

Fig. 4. Collagen galactosyltransferase activity in the effluent of a 2-ml affinity column after passing a chick embryo $(NH_4)_2SO_4$ fraction with a protein concentration 20 mg/ml through the column. The enzyme activity is expressed as described in Fig. 1.

Discussion

Although collagen glucosyltransferase does not catalyze the transfer of glucuronic acid to collagen, it seemed possible that the enzyme may have an affinity to UDP-glucuronic acid once the free carboxyl group has been bound in a coupling reaction. The availability of agarose derivates containing free amino groups made it easy to couple UDP-glucuronic acid to agarose using the carbodiimide method [15]. Since several studies have demonstrated that it may be important to have the critical groups sufficiently distant from the solid matrix to minimize steric interference during the affinity chromatography [15], AH-Sepharose 4B was used in the coupling reaction. In this material, the spacer group comprises a 6-carbon atom chain with a free amino group.

Collagen glucosyltransferase was found to have a high affinity for the column. The binding was markedly enhanced by the presence of Mn^{2+}, which suggests the requirement of an enzyme-Mn^{2+} for the binding to occur. This finding is in agreement with a recent study on the protection of collagen glucosyltransferase by the substrate and the co-substrate against inhibition with sulphydryl reagents [11]. The results [11] likewise suggest the requirement of an enzyme-Mn^{2+} for the binding of the substrate and the co-substrate.

Barker et al. [17] studied the properties of UDP-agarose, without the sugar moiety, for the purification of UDP-galactose: *N*-acetylglucosamine galactosyltransferase from bovine milk. Their work suggested that the major binding energy of UDP-galactose was contributed by the UDP moiety. They further demonstrated that glycogen synthetase also showed an affinity for the UDP-agarose column. Our column differs from the UDP-agarose column by virtue of the presence of the sugar derivate. This seems to increase the specificity of the binding, as collagen galactosyltransferase was found to have no affinity for this column. The K_m values for UDP-glucose and UDP-galactose in their reactions with the corresponding collagen glycosyltransferase are similar [9], and binding of both transferases could thus be expected if the only factor determining the binding were the UDP moiety.

Several methods could be used in the elution of the affinity column. The best results were obtained by using dialyzable peptides prepared by collagenase digestion of collagen. This method has the advantage that the specificity of the procedure is probably further increased, the binding being based on an affinity for the co-substrate, and the elution on an affinity for the substrate.

With the procedure reported in this study, a collagen glucosyltransferase purification of about 3000-fold was obtained from extract of chick embryos. This degree of purification is only slightly higher than that recently reported for a procedure consisting of six conventional protein purification steps [11]. The present procedure is markedly simpler to carry out, however, and the total enzyme activity recovery is also much higher. It now seems possible to carry out experiments for final purification of the enzyme by combining the affinity column procedure either with another affinity column based on a different binding or with appropriate steps from the previous procedure [11], which is based on conventional protein purification methods.

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