Biochimica et Biophysica Acta, 480 (1977) 113—121

BBA 68018

ISOLATION OF COLLAGEN GLUCOSYLTRANSFERASE AS A HOMOGENEOUS PROTEIN FROM CHICK EMBRYOS

RAILI MYLLYLÄ, HENRIK ANTTINEN, LEILA RISTELI and KARI I. KIVIRIKKO

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

(Received June 1st, 1976)

Summary

Collagen glucosyltransferase was isolated as a homogeneous protein from chick embryos by a procedure consisting of ammonium sulphate fractionation, two affinity chromatographies and two gel filtrations. The specific activity of the purified enzyme was 32 000 times that of the 15 000 × *g* supernatant of the embryo homogenate, and the enzyme was pure when examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis using three different gel compositions. The molecular weight of the enzyme was about 72 000—78 000 by sodium dodecyl sulphate polyacrylamide gel electrophoresis, the value being dependent on the gel composition. The apparent molecular weight by gel filtration was dependent on the purity and protein concentration. The sedimentation coefficient $S_{20,w}$ was 4.7. The data suggest that the enzyme molecule consists of one polypeptide chain.

Introduction

Collagen contains hydroxylysine-linked monosaccharide and disaccharide units with structures of *O*-β-galactosylhydroxylysine and 2-*O*-α-glucosyl-*O*-β-galactosylhydroxylysine (for reviews, see refs. 1—4). The transfer of glucose from UDPglucose to galactosylhydroxylysyl residues is catalyzed by collagen glucosyltransferase in the presence of Mn^{2+}, which can be partly replaced by certain other bivalent cations [1—8].

Collagen glucosyltransferase was initially purified at a relatively low level from guinea pig skin [5], rat kidney cortex [6], chick embryo cartilage [7] and bovine arterial tissue [9]. More recently, there have been reports of purification of from 2000- to 5000-fold from extract of chick embryo homogenate, either by a procedure consisting of six conventional protein purification steps [8] or by an affinity column procedure on UDPglucose derivate linked to agarose [10] or on collagen linked to agarose [11]. The enzyme had a molecular weight

of about 52 000—54 000 by gel filtration [8], and several other properties of the enzyme and the enzyme reaction were studied [8,12,13]. Complete purification of the enzyme has not, however, been obtained, the molecular weight has been studied only by gel filtration, and nothing is known about the subunit structure of the enzyme.

In the present work collagen glucosyltransferase was isolated as a homogeneous protein from chick embryos by using two affinity column procedures, and attempts were made to determine the molecular weight and subunit structure of the enzyme.

Materials and Methods

Materials

Fertilized eggs of white Leghorn chickens were purchased from Siipikarjanhoitajien liitto r.y. (Hämeenlinna, Finland) and were incubated at 37°C in humidified incubators for 14 or 15 days. Calf skin gelatin substrate [7] and citrate-soluble rat skin collagen [14] were prepared as reported elsewhere. Dialyzable collagen peptides were prepared by digesting 50 g calf skin gelatin with 100 mg of bacterial collagenase (Sigma) at 37°C for 24 h [7], and the dialyzable peptides were ultrafiltrated through a PM 30 membrane in an Amicon ultrafiltration cell and lyophilized.

Uridine diphosphate-D-[^{14}C]glucose (227 Ci/mol) was purchased from New England Nuclear and non-radioactive UDPglucose from Sigma. The radioactive UDPglucose was diluted with the non-labelled compound to final specific activity of 3.3 Ci/mol. Sodium UDPglucuronate was purchased from Sigma, Sepharose 4B and AH-Sepharose 4B from Pharmacia.

UDPglucuronic acid from its carboxyl group was coupled to free amino groups of AH-Sepharose 4B using the carbodiimide method [15] as described in detail elsewhere [10]. This material was termed UDPglucose derivate agarose. Citrate-soluble rat skin collagen was coupled to agarose with cyanogen bromide reaction [11,15,16], and was termed collagen agarose. Both affinity columns were regenerated after use by washing in a beaker with 6 M urea [10,11,16] and then equilibrating with the enzyme buffer (see below).

Purification of collagen glucosyltransferase

All procedures were carried out at 0—4°C and all centrifugations were carried out at 15 000 × g. The standard buffer solution used in chromatography and dialysis steps contained 0.12 M NaCl, 10 mM $MnCl_2$, 50 μM dithiothreitol, 1% glycerol and 50 mM Tris · HCl, pH adjusted to 7.4 at 4°C. This solution was termed "enzyme buffer".

Preparation of ammonium sulphate enzyme. Chick embryos were homogenized and the 15 000 × *g* supernatant of the homogenate was prepared as described previously [8]. The ammonium sulphate fraction (0—45% saturation) of the embryo extract was likewise prepared as described previously [8] except that the dialysis was carried out against the enzyme buffer. The enzyme was stored at −20°C.

Chromatography on collagen agarose column. The ammonium sulphate enzyme was thawed and centrifuged for 30 min, and the supernatant (about 2800

ml) was then passed in a protein concentration of 15—20 mg/ml through the collagen agarose column (2.5 × 18 cm), which was equilibrated with the enzyme buffer. The flow rate was 50—60 ml/h. After the sample had passed through the column, the column was washed with the enzyme buffer until the absorbance at 225 nm was below 0.1. The enzyme was eluted from the column with 15 g of dialyzable collagen peptides in 15 ml of the enzyme buffer with a flow rate of about 40 ml/h, and fractions of 5 ml were collected. All fractions containing the elution peptides (detected by absorbance at 280 nm) were pooled and concentrated to a volume of about 5 ml by ultrafiltration in an Amicon ultrafiltration cell with a PM 30 membrane.

Sephadex G-100 gel filtration I. The enzyme pool was applied to a Sephadex G-100 column (Superfine, Pharmacia). The size of the column was 2.5 × 85 cm, and it was equilibrated and eluted with the enzyme buffer. Fractions of 5 ml were collected, and they were pooled, beginning with the first fraction in the exclusion volume showing an increase in the absorbance at 280 nm and continuing to the last fraction before the elution peptide peak.

Chromatography on UDPglucose derivate agarose column. The pooled enzyme was passed at a flow rate of 5 ml/h through the UDPglucose derivate agarose column (0.5 × 20 cm), which was equilibrated with the enzyme buffer. After the enzyme had passed through the column, it was washed with the enzyme buffer until the absorbance at 225 nm was below 0.1. The enzyme was eluted with 3 g of dialyzable peptides in 3 ml of the enzyme buffer with a flow rate of about 10 ml/h and fractions of 5 ml were collected. The fractions were pooled and concentrated in the same way as those collected after the collagen agarose chromatography.

Sephadex G-100 gel filtration II. The pooled enzyme was applied to the same column as in gel filtration I. Fractions of 5 ml were collected and those having the highest specific activities were pooled, and concentrated to a volume of about 1 ml by ultrafiltration in an Amicon ultrafiltration cell with a PM 10 membrane. This constituted the purified enzyme.

Assays

Collagen glucosyltransferase activity was assayed as described previously [7, 8] except that the specific activity of the UDPglucose was 3.3 Ci/mol and the reaction volume was 100 μl.

One unit of the enzyme activity was defined as the activity required to synthesize an amount of the radioactive product (in dpm) corresponding to 1 μmol in 1 h at 37°C as described previously [8].

The protein content of the enzyme preparation was measured by peptide absorbance at 225 nm using bovine serum albumin as a standard, which gave an absorption coefficient of $E_{225nm}^{1mg/ml} = 7.40$ with a 1-cm light path.

Disc electrophoresis of native enzyme was carried out using either a two-gel system or only one gel, with 7.5% polyacrylamide gels (see ref. 16). Disc electrophoresis of denatured polypeptide chains was performed after reduction and denaturation in the presence of sodium dodecyl sulphate [17]. Experiments were carried out with 10% polyacrylamide gels prepared using the normal amount of cross-linker or 0.5 time the normal amount, and with 5% polyacrylamide gels prepared using 0.5 time the normal amount of cross-linker. The gels

were stained either with Coomassie Brilliant Blue or the periodic acid-Schiff reagent.

The sedimentation coefficient of the enzyme was determined in an MSE Centriscan 75 combination analytical and preparative ultracentrifuge equipped with a photoelectric scanner (280 nm). The rotor speed was 59 000 rev./min., the temperature 23°C and the protein concentration 0.17 mg/ml in a solution containing 0.12 M NaCl, 1% glycerol, 50 μM dithiothreitol and 50 mM Tris · HCl, pH adjusted to 7.4 at +4°C. The sedimentation coefficient was calculated from plots of log r versus time and was corrected to its value in a solvent with the viscosity and density of water at 20°C [18].

Results

Extraction of the enzyme from chick embryos

It was previously reported that the addition of Triton X-100 in a concentration of 0.1% to a solution consisting of 0.2 M NaCl, 50 μM dithiothreitol, and 50 mM Tris · HCl buffer, pH 7.4, doubled the collagen glucosyltransferase activity in the homogenate and the 15 000 × g and 150 000 × g supernatants of the homogenate [8]. Because it is difficult to completely solubilize many glycosyltransferases from the tissues [20], further experiments were carried out to study whether the extraction used previously [8] solubilized the maximal amount of the enzyme activity. The results indicated that an additional increase of about 30% could be obtained in the soluble enzyme activity when the concentration of Triton X-100 was increased to 0.5% and the concentration of NaCl to 0.5 M (Table I). Similar results were found when Nonidet P-40 [19] was used as the detergent (Table I). An increase in the dithiothreitol concentration had no effect on the solubilization. The addition of 2 M urea slightly increased the soluble enzyme activity (Table I). On the basis of these and several additional experiments, it was concluded that the extraction used previously solubilized about 80% of the enzyme activity which can be solubilized under optimal conditions. However, our attempts to purify enzyme preparations extracted with 0.5% detergent and 0.5 M NaCl were complicated by the tendency of some components in such preparations to form precipitates during the first affinity chromatography resulting in greatly reduced flow rates. It was found more advisable, therefore, to use enzyme preparations extracted with 0.1% detergent and 0.2 M NaCl, even though some enzyme was lost in the extraction.

Purification of collagen glucosyltransferase

With the procedure reported in Materials and Methods, an increase of about 32 000-fold in the specific activity of the enzyme as compared to that in the 15 000 × g supernatant of the embryo homogenate was obtained (Table II). When the values were compared to those in the $(NH_4)_2SO_4$ enzyme preparation, the increase in the specific activity was 14 600-fold, and the yield of total activity about 10%. The increase in the specific activity which was found after the collagen agarose column and the first gel filtration was considerably smaller than reported previously [11], because in the present study essentially all fractions which contained protein were pooled after the first gel filtration (see Ma-

TABLE I

EXTRACTION OF COLLAGEN GLUCOSYLTRANSFERASE FROM CHICK EMBRYOS

The chick embryos were homogenized in a cold solution containing 0.2 M NaCl, 50 μM dithiothreitol and 50 mM Tris · HCl, pH adjusted to 7.4 at 4°C (1 ml solution per g of embryo). The homogenate was divided into eight equal portions, and each portion received an addition of 0.5 ml of appropriate stock solution per 1 ml of the portion to make the final concentrations shown in the table. The samples were incubated at 4°C for 1 h with shaking, and were centrifuged at 15 000 × *g* for 30 min. The enzyme activity was assayed in the 15 000 × *g* supernatant and the results are expressed as dpm of glucosylgalactosylhydroxylysine synthesized in an aliquot corresponding to 10 mg wet weight of embryo under standard assay conditions. Each solution contained the compounds shown, and 50 μM dithiothreitol and 50 mM Tris · HCl buffer, pH adjusted to 7.4 at +4°C.

Solution	Glucosyltransferase activity	
	dpm	%
0.1% Triton X-100, 0.2 M NaCl	1110	100
0.5% Triton X-100, 0.2 M NaCl	1200	108
0.1% Triton X-100, 0.5 M NaCl	1180	106
0.5% Triton X-100, 0.5 M NaCl	1440	130
0.5% Nonidet P-40, 0.2 M NaCl	1240	112
0.5% Nonidet P-40, 0.5 M NaCl	1460	131
0.1% Triton X-100, 0.2 M NaCl, 20 mM dithiothreitol	1140	103
0.1% Triton X-100, 0.2 M NaCl, 2 M urea *	1400	126

* Before the assay this sample was dialyzed against the same solution not containing urea.

terials and Methods). Such pooling could be carried out, because the enzyme was separated from the main peak of non-enzyme protein during the second gel filtration.

When the purified collagen glucosyltransferase was stored in a protein concentration of about 0.1 mg/ml, the enzyme lost about one-third of its activity at +4°C in 1 week or at −20°C during several months, provided that the enzyme was not thawed during this period. When the enzyme was concentrated

TABLE II

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

Enzyme fraction	Total protein	Total activity	Recovery	Specific activity	Purification	
					In the procedure	Over the 15 000 × *g* supernatant
	(mg)	(munits)	%	(munits/mg)	(-fold)	(-fold)
$(NH_4)_2SO_4$, 0—45% satn.	54 700	101 000	100	1.84	1	2.16
After collagen agarose column and gel filtration I	27.0	19 200	19	712	387	835
After UDPglucose derivate agarose column and gel filtration II	0.39	10 500	10	26 000	14 600	31 600

above 0.2 mg/ml, the solution became cloudy with a loss of most of the activity.

Purity and molecular weight of the enzyme

Attempts to study the purity of the enzyme by polyacrylamide gel electrophoresis as a native protein were unsuccessful, because most or all of the protein aggregated. A heavy band was seen between the upper and lower gels in the two gel system or a minor band at the top of the gel in the one gel system, the bulk of the protein probably not entering the gel at all in the latter instance.

When the denatured and reduced enzyme was studied by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate [17], with 10% polyacrylamide gel prepared using the normal amount of cross-linker, or 0.5 time the normal amount, or with 5% polyacrylamide gel prepared using 0.5 time the normal amount of cross-linker, only one band was seen in most enzyme preparations. In some enzyme preparations one or two weak additional bands representing far less than 5% of the total protein were seen. In the ordinary experiments the gels were stained with Coomassie Brilliant Blue, but in some experiments the gels were stained with the periodic acid-Schiff reagent. This reagent was found to stain the enzyme protein, and no further bands were seen.

When the mobility of the enzyme band was compared with the mobilities of standard polypeptide chains, a molecular weight of about 72 000 was obtained for the enzyme in 10% polyacrylamide gel prepared using the normal amount of cross-linker (Fig. 1A), whereas a value of about 78 000 was found in 10% gel prepared using 0.5 time the normal amount of cross-linker or in 5% gel likewise prepared with 0.5 time the normal amount of cross-linker (Figs. 1B and 1C). A similar effect of the gel composition was found with two additional enzyme preparations.

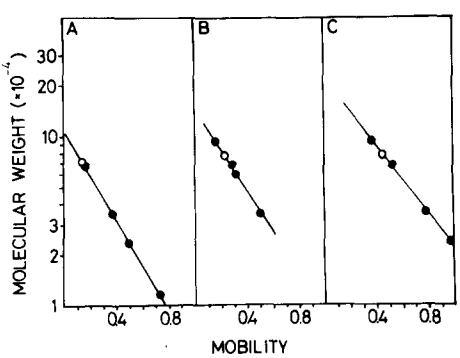

Fig. 1. Determination of the molecular weight of collagen glucosyltransferase by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Experiments were carried out with 10% polyacrylamide gel prepared with the normal amount of cross-linker (A), or 0.5 time the normal amount (B), or with 5% polyacrylamide gel prepared using 0.5 time the normal amount of cross-linker (C). Symbols: relative mobilities of the protein standards (●): phosphorylase *a* (B and C only), bovine serum albumin, catalase (B), pepsin, trypsin (A and C), and cytochrome *c* (A); relative mobility of the enzyme (○).

The molecular weight could not be determined by gel filtration, because the elution position of the enzyme activity was found to be dependent on the purity and protein concentration. A molecular weight of about 52 000—54 000 was previously reported by gel filtration for collagen glucosyltransferase purified by conventional procedures, but in some preparations an additional form was observed, with an elution position corresponding to a molecular weight of about 130 000 [8]. In the present study, the enzyme activity was found in the first gel filtration as a broad peak, a large part being in the exclusion volume of the Sephadex G-100 column. In the second gel filtration the elution position of the enzyme corresponded to a molecular weight of only about 40 000—45 000, even though the same preparations gave a molecular weight of over 70 000 by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Sedimentation experiments in the analytical ultracentrifuge were complicated by aggregation of the protein in concentrations exceeding 0.2 mg/ml. The enzyme could, however, be studied below this concentration. The plot of sedimentation boundary was linear with time and the sedimentation coefficient $S_{20,w}$ was 4.7.

Discussion

Although mammalian tissues contain a large number of different glycosyltransferases, only very few of them have been purified to homogeneity (see ref. 19). This is largely due to their firm attachment to membrane structures of the cells and their tendency to aggregate and lose their activity during the course of the purification. It has been pointed out that the few glycosyltransferases which have been purified are among the more readily solubilized ones [19]. The collagen glucosyltransferase which was isolated in the present study as a homogeneous protein seems to belong to this group of glycosyltransferases, because considerable amounts of its activity can be extracted without any detergent (e.g. refs. 8 and 20). Furthermore, the present data indicated relatively minor additional increases in the soluble enzyme activity when 0.1% Triton X-100 was substituted by a higher concentration of this detergent or Nonidet P-40 in the presence of a high salt concentration or 2 M urea. However, the enzyme showed the other properties common to many glycosyltransferases in that it readily formed aggregates and easily lost its activity.

The specific activity of the purified enzyme, 26 units/mg, is markedly higher than the value of 2.4 units/mg reported for the highly purified enzyme obtained by a procedure consisting of conventional protein purification steps [8]. However, the major band found in the latter enzyme preparation by sodium dodecyl sulphate polyacrylamide gel electrophoresis had identical mobility with the only band in the present enzyme preparations. This suggests that the difference in the degree of purity of the enzyme protein between the two enzyme preparations is considerably smaller than the difference in the specific activities, probably owing to partial inactivation of the enzyme during the six conventional protein purification steps. It is possible that even the present value may not represent the maximal specific activity for the pure enzyme, because the enzyme easily lost activity and because the yield was considerably smaller than expected on the basis of values measured for the enzyme which

did not become bound to the affinity columns and which was lost in pooling of the enzyme fractions.

The molecular weight of the enzyme was about 72 000—78 000 by sodium dodecyl sulphate polyacrylamide gel electrophoresis, the lowest value being found with 10% gels prepared using the normal amount of cross-linker. The sedimentation coefficient $S_{20,w}$ of 4.7 is similar to that of bovine albumin, and likewise suggests a molecular weight of about 70 000, if the enzyme has a similar diffusion coefficient. By contrast, the smallest values found by gel filtration were markedly lower, but this determination was found to be affected by purity of the enzyme and by protein concentration. It thus seems likely that the discrepancy is due to partial adsorption of the enzyme to the gel filtration column resulting in a retarded elution.

The reason for the dependence of the molecular weight on gel composition in the sodium dodecyl sulphate polyacrylamide gel electrophoresis is not known, but it seems probable that the enzyme is a glycoprotein. If such were the case, the apparent molecular weight would be dependent on the gel composition and erroneously high molecular weights would be observed, the higher acrylamide concentrations probably giving more correct values [21,22]. Thus, the value of 72 000 would probably be more correct than the value of 78 000, and even the former value might be slightly too high. The periodic acid-Schiff reagent was found to stain the enzyme protein, this finding being in agreement with the suggestion that the enzyme is a glycoprotein. In addition, it has recently been found that the enzyme has a high affinity to columns containing concanavalin A (Anttinen, H., Myllylä, R. and Kivirikko, K.I., unpublished), which likewise suggests that the enzyme is a glycoprotein.

Because only one protein band was found in the sodium dodecyl sulphate polyacrylamide gel electrophoresis in the presence of mercaptoethanol and because the molecular weight of the enzyme by this method was not smaller than by other methods, it seems probable that the enzyme molecule consists of one polypeptide chain.

Acknowledgements

The work was supported in part by a grant from the Medical Research Council of the Academy of Finland. The authors gratefully acknowledge the expert technical assistance of Miss Raija Leinonen and Mrs. Raija Harju.

References

1 Bornstein, P. (1974) Annu. Rev. Biochem. 43, 567—603
2 Martin, G.R., Byers, P.H. and Piez, K.A. (1975) in Advances in Enzymology (Meister, A., ed.), Vol. 42, pp. 167—191, Interscience Publication, New York
3 Kivirikko, K.I. and Risteli, L. (1976) Med. Biol. 54, 159—186
4 Prockop, D.J., Berg, R.A., Kivirikko, K.I. and Uitto, J. (1976) in Biochemistry of Collagen (Ramachandran, G.N. and Reddi, A.H., eds.), Plenum Publ. Copr., in the press
5 Bosmann, H.B. and Eylar, E.H. (1968) Biochem. Biophys. Res. Commun. 30, 89—94
6 Spiro, R.G. and Spiro, M.J. (1971) J. Biol. Chem. 246, 4899—4909
7 Myllylä, R., Risteli, L. and Kivirikko, K.I. (1975) Eur. J. Biochem. 52, 401—410
8 Myllylä, R., Risteli, L. and Kivirikko, K.I. (1976) Eur. J. Biochem. 61, 59—67
9 Henkel, W. and Buddecke, E. (1975) Hoppe Seyler's Z. Physiol. Chem. 356, 921—928
10 Anttinen, H. and Kivirikko, K.I. (1976) Biochim. Biophys. Acta 429, 750—758

11 Risteli, L., Myllylä, R. and Kivirikko, K.I. (1976) Eur. J. Biochem. 67, 197—202
12 Myllylä, R., Risteli, L. and Kivirikko, K.I. (1975) Eur. J. Biochem. 58, 517—521
13 Myllylä, R. (1976) Eur. J. Biochem., in the press
14 Gallop, P.M. and Seifter, S. (1963) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. 6, pp. 635—641, Academic Press, New York
15 Cuatrecasas, P. and Anfinsen, C.B. (1971) in Methods in Enzymology (Jakoby, W.B., ed.), Vol. 22, pp. 345—385, Academic Press, New York
16 Tuderman, L., Kuutti, E.-R. and Kivirikko, K.I. (1975) Eur. J. Biochem. 52, 9—16
17 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
18 Bowen, T.J. (1971) An Introduction to Ultracentrifugation, p. 171, Wiley-Interscience, London
19 Schwartz, N.B. and Roden, L. (1975) J. Biol. Chem. 250, 5200—5207
20 Spiro, R.G. and Spiro, M.J. (1971) J. Biol. Chem. 246, 4919—4925
21 Segrest, J.P. and Jackson, R.L. (1972) in Methods in Enzymology (Ginsburg, V., ed.), Vol. 28B, pp. 54—63, Academic Press, New York
22 Weber, K. and Osborn, M. (1975) in The Proteins (Neurath, H. and Hill, R.L., eds.), Vol. 1, pp. 179—223, Academic Press, New York