Partial Purification and Characterization of Collagen Galactosyltransferase from Chick Embryos

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Collagen galactosyltransferase was purified 50–150-fold from chick-embryo extract. The tissue homogenate was prepared in the presence of Triton X-100, since the addition of the detergent doubled the enzyme activity in the homogenate and the extract. Three species of the enzyme activity with different molecular weights were recovered on gel filtration, the mol.wts. being about 450000, 200000 and 50000. Collagen galactosyltransferase activity was strongly inhibited by *p*-mercuribenzoate, and stimulated by the addition of dithiothreitol to the incubation system. Studies on substrate requirements indicated that denatured citrate-soluble collagen is a more effective substrate than gelatinized insoluble collagen, as judged from their K_m values. Experiments on three peptide fractions prepared from citrate-soluble collagen indicated that a fraction with an average mol.wt. of 500–600 contained peptides large enough to meet a minimum requirement for interaction with the enzyme. However, longer peptides were clearly better substrates. When native and heat-denatured citrate-soluble collagens were compared as substrates, practically no synthesis of galactosylhydroxylysine was found with native collagen. This finding suggests that the triple-helical conformation of collagen prevents the galactosylation of hydroxylysine residues.

Collagen contains hydroxylysine-linked monosaccharide and disaccharide units with structures of *O*- β -D-galactosylhydroxylysine and 2-*O*- α -glucosyl-*O*- β -D-galactosylhydroxylysine (Butler & Cunningham, 1966; Spiro, 1967, 1969). The transfer of galactose from UDP-galactose to hydroxylysine residues is catalysed by collagen galactosyltransferase in the presence of Mn^{2+} , which can be partly replaced by certain other bivalent cations (M. J. Spiro & R. G. Spiro, 1971; Myllylä *et al.*, 1975a; for reviews on collagen biosynthesis see Grant & Prockop, 1972a,b,c; Bornstein, 1974; Kivirikko, 1974; Prockop *et al.*, 1976).

Relatively little is known about collagen galactosyltransferase and the galactosylation reaction. The presence of this enzyme activity has been demonstrated in a number of connective tissues (R. G. Spiro & M. J. Spiro, 1971), and some characteristics have been studied at a relatively low degree of purification from guinea-pig skin (Bosmann & Eylar, 1968), rat kidney cortex (M. J. Spiro & R. G. Spiro, 1971) and chick-embryo cartilage (Myllylä *et al.*, 1975a). Studies with collagen galactosyltransferase from guinea-pig skin suggested that free hydroxylysine could act as an acceptor (Bosmann & Eylar, 1968), but this finding was not confirmed in studies with the enzyme from rat kidney cortex and chick-embryo cartilage (M. J. Spiro & R. G. Spiro, 1971; Myllylä

et al., 1975a). Galactose-free small peptides prepared by Pronase and collagenase digestion of glomerular basement-membrane collagen were not galactosylated by the rat kidney-cortex enzyme (M. J. Spiro & R. G. Spiro, 1971), but diffusible peptides prepared by collagenase digestion of tendon collagen did act as substrates for the enzyme from chick-embryo cartilage (Myllylä *et al.*, 1975a). Collagen galactosyltransferase catalyses the additional galactosylation of denatured collagens from various sources *in vitro* (M. J. Spiro & R. G. Spiro, 1971; Myllylä *et al.*, 1975a). The transferase from rat kidney cortex was also reported to galactosylate native collagen (M. J. Spiro & R. G. Spiro, 1971), but the reaction was carried out for 2 h at 37°C, which is very close to the denaturation temperature of collagen, and no kinetic measurements were carried out. Recent studies on collagen glucosyltransferase have shown that this enzyme does not act on native collagen (Myllylä *et al.*, 1975b). Thus the effect of the collagen conformation on the galactosylation reaction seemed to require additional study.

In the present work, collagen galactosyltransferase was partially purified from homogenate of whole chick embryos, and this enzyme preparation was used for the further characterization of the transferase with special emphasis on its substrate requirements.

Experimental

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.

Collagen, soluble in 0.075M-sodium citrate buffer, pH 3.7, was prepared from the skin of normal and lathyrus rats (Gallop & Seifter, 1963), and gelatinized insoluble collagen from the skin of normal calves (Myllylä *et al.*, 1975a). Diffusible peptides were prepared by digesting 1g of citrate-soluble rat skin collagen with 1 mg of highly purified bacterial collagenase (Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.) at 37°C for 24h, and freeze-drying the diffusible peptides (Myllylä *et al.*, 1975a).

UDP-D-[¹⁴C]galactose (274 Ci/mol) and UDP-D-[¹⁴C]glucose (227 Ci/mol) were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.), and non-radioactive UDP-galactose and UDP-glucose from Sigma. The radioactive UDP-glycosides were diluted with the non-labelled compounds to final specific radioactivities of 10 Ci/mol for UDP-galactose and 3.3 Ci/mol for UDP-glucose.

Purification of collagen galactosyltransferase

All procedures were carried out at 0–4°C, and samples were stored at 0–4°C without freezing between the steps. All centrifugations were carried out at 15000g.

Preparation of the initial extract. A total of 300 14-day chick embryos were homogenized in batches of 30 embryos in a solution of 0.2M-NaCl, 0.1% Triton X-100, 50 µM-dithiothreitol and 20mM-Tris/HCl buffer, adjusted to pH 7.4 at 4°C (1 ml of solution per g of embryo) by using a Waring blender at full speed, twice for 30s with a 1 min interval. The homogenate was left with occasional stirring for about 1 h, and was then centrifuged for 30 min.

Ammonium sulphate fractionation I. Solid (NH₄)₂SO₄ was slowly stirred into the supernatant fraction to a final concentration of 20% saturation. After centrifugation for 20 min the pellet was discarded, and solid (NH₄)₂SO₄ was added to the supernatant to a final concentration of 60% saturation. The pellet obtained by centrifugation for 20 min was dissolved in 0.2M-NaCl/1% glycerol/50 µM-dithiothreitol/20mM-Tris/HCl buffer, adjusted to pH 7.4 at 4°C. The preparation was dialysed for 4 and 12 h against two separate 16-litre volumes of this solution, and centrifuged to remove a small amount of insoluble material.

Calcium phosphate gel fractionation. A suspension of calcium phosphate gel (Calbiochem, London W.1, U.K.) diluted with distilled water to a concentration

of 30 mg/ml was added to the (NH₄)₂SO₄ fraction I, in a protein concentration of 30 mg/ml. The ratio was generally 90 mg of solid/100 mg of protein, but this was varied depending on the adsorption capacity of the gel preparation. The mixture was stirred for 15 min, and the gel then removed by 10 min centrifugation. The pellet was eluted successively with 2-litre solutions containing 0.15M-NaCl and 50 µM-dithiothreitol, adjusted to pH 7.4 at 4°C, and increasing concentrations of potassium phosphate as follows: (a) 0.02M; (b) 0.06M; (c) 0.09M; (d) 0.15M; (e) 0.18M. At each step the pellet was homogenized in the solution with a Teflon-and-glass homogenizer and eluted for 20 min with stirring, followed by centrifugation for 10 min. Solid (NH₄)₂SO₄ was slowly stirred into the eluates to a final concentration of 60% saturation, and the pellets, obtained after centrifugation for 20 min, were dissolved in 0.05M-NaCl/1% glycerol/50 µM-dithiothreitol/20mM-Tris/HCl buffer, adjusted to pH 7.4 at 4°C. They were then dialysed for 4 and 12 h against two separate 10-litre volumes of this solution, and centrifuged to remove a small amount of insoluble material. Two or three of the eluates having the highest specific activity were combined and purified further.

DEAE-cellulose chromatography I. The enzyme from the calcium phosphate gel was applied to a column (7 cm × 50 cm) of DEAE-cellulose (DE 23; Whatman Biochemicals, Maidstone, Kent, U.K.), equilibrated with 0.07M-NaCl/1% glycerol/50 µM-dithiothreitol/20mM-Tris/HCl buffer, adjusted to pH 7.4 at 4°C. The column was eluted at a flow rate of 300 ml/h, first with 300 ml of the equilibrating buffer, and then with the same solution with its NaCl concentration increasing stepwise as follows: 0.10M, 300 ml; 0.12M, 450 ml; 0.14M, 450 ml; 0.16M, 450 ml; 0.18M, 450 ml; 0.20M, 400 ml; 0.25M, 2000 ml. Fractions having at least one-third of the specific activity of the fraction with the highest specific activity were pooled. Usually most of the enzyme activity was recovered in the eluates containing 0.12M- and 0.14M-NaCl.

Ammonium sulphate fractionation II. The pool of enzyme after purification on DEAE-cellulose was precipitated by stirring solid (NH₄)₂SO₄ into the solution to a final concentration of 60% saturation. The pellet was then eluted successively with solutions containing (NH₄)₂SO₄ as follows: (a) 50% saturation, (b) 40%, (c) 30%, (d) 20% and (e) 10%, all in 0.2M-NaCl/50 µM-dithiothreitol/20mM-Tris/HCl buffer, adjusted to pH 7.4 at 4°C. In elution (a), 300 ml was used, and elsewhere 200 ml. Solid (NH₄)₂SO₄ was stirred into the eluates to a final concentration of 60% saturation, and the pellets obtained by centrifugation were dissolved in 30–40 ml of 0.15M-NaCl/1% glycerol/50 µM-dithiothreitol/20mM-Tris/HCl buffer, pH adjusted to 7.4 at 4°C. They were then dialysed for 4 and 12 h

against two separate 10-litre volumes of this solution. Two or three of the eluates with the highest specific activity, usually those having 20 or 30% saturation of $(\text{NH}_4)_2\text{SO}_4$, were pooled.

DEAE-cellulose chromatography II. The enzyme after $(\text{NH}_4)_2\text{SO}_4$ fractionation II was diluted with 1% glycerol/50 μM -dithiothreitol/20 mM-Tris/HCl buffer, adjusted to pH 7.4 at 4°C, to a final NaCl concentration of 0.05 M, and applied to a column (5 cm $\times$ 50 cm) of DEAE-cellulose (DE 23, Whatman), equilibrated with 0.05 M-NaCl/1% glycerol/50 μM -dithiothreitol/20 mM-Tris/HCl buffer, pH adjusted to 7.4 at 4°C. The column was eluted at a flow rate of 300 ml/h with the same solution, with a stepwise increase in NaCl concentration: 0.07 M, 200 ml; 0.10 M, 200 ml; 0.12 M, 300 ml; 0.14 M, 300 ml; 0.16 M, 300 ml; 0.18 M, 300 ml; 0.20 M, 200 ml; 0.25 M, 1000 ml. Fractions having at least 80% of the specific activity of the fraction with the highest specific activity were pooled and concentrated to a volume of about 9.5 ml by ultrafiltration in an Amicon ultrafiltration cell (Amicon Corp., Lexington, MA, U.S.A.) with a PM-30 membrane.

Ultragel AcA 34 gel filtration. The enzyme pool after DEAE-cellulose chromatography II was applied to a column (2.5 cm $\times$ 85 cm) of Ultragel AcA 34 (LKB-Produkter AB, Bromma, Sweden), which was equilibrated and eluted with 0.15 M-NaCl/1% glycerol/50 μM -dithiothreitol/20 mM-Tris/HCl buffer, adjusted to pH 7.4 at 4°C. Fractions (5 ml) were collected, and those containing most of the enzyme activity were pooled and ultrafiltered (see above) to a volume of 2 ml. This constituted the purified enzyme.

Assay of collagen galactosyltransferase activity

Enzyme activity was assayed in a final volume of 100 μl containing 0.16–13 mg of enzyme protein/ml, depending on purity, 40 mg of calf skin gelatin substrate/ml, or 3 mg of citrate-soluble rat skin collagen substrate/ml, 60 μM -UDP-[^{14}C]galactose (10 Ci/mol), 10 mM-MnCl₂, 0.02–0.1 M-NaCl, 50 mM-Tris/HCl buffer, pH adjusted to 7.4 at 20°C [a slight modification of that used by Myllylä *et al.* (1975a)] and 1 mM-dithiothreitol. The gelatin substrate and the citrate-soluble rat skin collagen were heated to 60°C for 10 min and rapidly cooled to 0°C immediately before addition to the incubation mixture (Myllylä *et al.*, 1975b). The samples were incubated at 37°C for 45 min, and the reaction was stopped by adding 2 ml of 1% phosphotungstic acid in 0.5 M-HCl. The reaction product was assayed as reported previously (Myllylä *et al.*, 1975a), and the radioactivity of the [^{14}C]galactosylhydroxylysine determined. When the enzyme reaction was carried out with collagen galactosyltransferase purified through the first DEAE-cellulose chromatography

or further steps, no radioactivity was detected on paper electrophoresis (Myllylä *et al.*, 1975a) in any other peak than that of the galactosylhydroxylysine. Thus paper electrophoresis was used only for reactions carried out with less purified enzymes.

When the diffusible collagen peptides were examined as substrates, the reaction product was assayed by using an amino acid analyser (Myllylä *et al.*, 1975a), since the peptides could not be precipitated by the phosphotungstic acid reagent.

Definition of units of enzyme activity

One unit of enzyme activity is defined here as that amount of enzyme required to synthesize 2.22×10^7 d.p.m. of galactosylhydroxylysine, corresponding to 1 μmol , in 1 h at 37°C with the reactant concentrations indicated above and a saturating concentration of denatured purified citrate-soluble rat skin collagen as substrate. Thus 1 munit of enzyme activity was the amount required to synthesize 2.22×10^4 d.p.m. of galactosylhydroxylysine.

Most of the assays were carried out with 40 mg of calf skin gelatin substrate/ml (Myllylä *et al.*, 1975a), and the values corrected to a saturating concentration of citrate-soluble collagen, as described previously for collagen glucosyltransferase (Myllylä *et al.*, 1976).

Other assays

Collagen glucosyltransferase activity was assayed as reported previously (Myllylä *et al.*, 1975a, 1976). The protein content of the enzyme preparations was measured by peptide absorbance at 225 nm by using bovine serum albumin as a standard, which gave an absorption coefficient of $E_{225\text{nm}}^{1\text{mg/ml}} = 7.40$ with a 1 cm light-path.

Results

Partial purification of collagen galactosyltransferase

To study the effect of detergents on the extraction of collagen galactosyltransferase activity, chick embryos were homogenized as described in Table 1, and a portion of the homogenate was incubated with 0.1% Triton X-100 at 4°C for 1 h. Treatment of the homogenate with this detergent doubled the enzyme activity in the homogenate and supernatants, whereas no significant change was observed in the distribution of the measurable enzyme activity between the soluble fractions (Table 1).

In the initial experiments collagen galactosyltransferase had been partially purified from chick-embryo cartilage by steps that were basically similar to those reported in the present study (Myllylä *et al.*, 1975a). This approach was limited

Table 1. *Effect of Triton X-100 on collagen galactosyltransferase activity in the homogenate and homogenate fractions of chick embryos*

Chick embryos were homogenized in a cold solution consisting of 0.2M-NaCl, 50 μ M-dithiothreitol and 50mM-Tris/HCl buffer, adjusted to pH 7.4 at 4°C (2 ml of solution per g of embryo). The homogenate was divided into two equal portions, and Triton X-100 was added to one to a final concentration of 0.1%. Both samples were incubated at 4°C for 1 h with occasional shaking, and were then centrifuged at 15000g for 30 min. The supernatants were further centrifuged at 150000g for 45 min, and the enzyme activity was assayed in samples of the homogenates and the 15000g and 150000g supernatants, each sample corresponding to 20mg wet weight of embryo. The results are expressed as d.p.m. of galactosylhydroxylysine synthesized under standard assay conditions.

Fraction	-Triton		+Triton		(+Triton/-Triton) ×100
	(d.p.m.)	(% of value in homogenate)	(d.p.m.)	(% of value in homogenate)	
Homogenate	3500	100	6960	100	199
15000g supernatant	3370	96	6700	96	199
150000g supernatant	2910	83	5990	85	206

Table 2. *Partial purification of collagen galactosyltransferase from extract of chick embryos*

For definition of units of enzyme activity see the Experimental section.

Enzyme fraction	Total protein (mg)	Total activity (munits)	Recovery (%)	Specific activity (munits/mg)	Purification (-fold)
15000g supernatant	48600	40800	100	0.84	1
(NH ₄) ₂ SO ₄ I	32200	37700	92	1.17	1.4
Calcium phosphate gel	5220	17400	43	3.34	4
DEAE-cellulose I	1370	12500	31	9.15	11
(NH ₄) ₂ SO ₄ II	590	8750	22	14.9	18
DEAE-cellulose II	34.7	1660	4	47.8	57
Ultrogel AcA 34	13.8	771	2	56.0*	67

* Fraction with highest specific activity: 74.0munits/mg; purification 88-fold.

by the difficulties involved in preparing large amounts of the original material. Our subsequent experiments indicated that the transferase activity in the extract of whole chick embryos was about half (or even slightly more) of that obtained from extract of chick-embryo cartilage, so that since it was easy to prepare the former in large quantities, the present work was carried out with transferase from whole chick embryos.

Considerable difficulties were encountered in the purification of the enzyme activity. This was in part due to the tendency of the enzyme to aggregate, and hence in all fractionation steps multiple forms were present, which interfered with a clear-cut separation. In addition, the enzyme was very unstable, and activity losses were noted during all the steps. With the procedure reported here, collagen galactosyltransferase was purified about 50–150-fold from extract of chick-embryo homogenate, the recovery ranging from about 1 to 3%. The purification of one typical preparation is shown in Table 2, the degree of purification obtained being 67-fold with a 2% recovery, and the highest

specific activity in a single gel-filtration fraction corresponding to an 88-fold purification.

In the final gel filtration of the preparation shown in Table 2 two major peaks of collagen galactosyltransferase activity were recovered (Fig. 1). Calibration of the column with standard proteins [Blue Dextran, thyroglobulin (mol.wt. 660000), alcohol dehydrogenase (mol.wt. 150000), bovine serum albumin (mol.wt. 68000), trypsin (mol.wt. 23300) and cytochrome *c* (mol.wt. 11700)] indicated that the mol.wts. corresponded to about 450000 and 200000 for globular proteins. However, an additional minor peak was noted, the mol.wt. of which was about 50000. When a portion of this enzyme preparation was stored frozen and then re-examined in the same column, over 90% of the enzyme had been inactivated, and almost all the remaining enzyme activity was recovered in the position corresponding to the minor peak. The presence of this minor peak was also noted in other enzyme preparations.

The final enzyme preparations were entirely free of collagen glucosyltransferase activity.

Fig. 1. Gel filtration of partially purified collagen galactosyltransferase on an Ultrogel Aca 34 column as the last step in the purification procedure

Conditions were as described in the Experimental section. Fractions nos. 35–42 and 48–50 were pooled and constituted the purified enzyme. ●, Enzyme activity; —, protein.

Fig. 2. Inhibition of collagen galactosyltransferase activity by various concentrations of *p*-mercuribenzoate

The enzyme was preincubated with *p*-mercuribenzoate at 37°C for 20 min, and the reaction was then carried out for 45 min with the same concentration of *p*-mercuribenzoate. No dithiothreitol was added to the incubation mixture.

Effect of thiol reagents on collagen galactosyltransferase activity

The activity of collagen galactosyltransferase was strongly inhibited by *p*-mercuribenzoate (Fig. 2); 50% inhibition was observed with about a 7 μM concentration of the reagent.

In view of this, experiments were carried out

Fig. 3. Double-reciprocal plots of substrate concentration and initial velocity for the galactosylation of denatured citrate-soluble collagen from lathyrctic rats (○) and of gelatinized insoluble calf skin collagen (●)

The values shown on the abscissa are reciprocal values of the substrate concentration in g/l and those on the ordinate are reciprocal values of the velocity in $10^{-4} \times$ radioactivity (d.p.m.) of the product formed in 45 min. To facilitate visual comparison between the two substrates, both plots are shown in the same Figure, but the actual K_m value for gelatin was determined from plots drawn on a different scale.

to study whether the addition of dithiothreitol to the standard incubation system, not treated with *p*-mercuribenzoate, stimulated the enzyme activity. An increase in enzyme activity of about 20–30% was found in the presence of 0.5–5 mM-dithiothreitol.

Comparison of denatured collagens and small peptides as substrates for the enzyme

Collagen galactosyltransferase from rat kidney cortex (M. J. Spiro & R. G. Spiro, 1971) and chick-embryo cartilage (Myllylä *et al.*, 1975a) has been reported to catalyse the additional galactosylation of denatured collagens *in vitro*. In the present experiments citrate-soluble collagens from the skin of normal and lathyrctic rats and gelatinized insoluble calf skin collagen were compared as substrates for the enzyme. The K_m values for the two citrate-soluble collagens ranged from about 2 to 4 g/l in various experiments, whereas that for gelatinized insoluble calf skin collagen ranged from about 35 to 70 g/l (for a typical experiment, see Fig. 3). The V_{max} for the gelatinized insoluble collagen was more variable, depending on the particular gelatin preparation, and in some experiments it was slightly less than that for the citrate-soluble collagens, whereas in others it was somewhat larger (Fig. 3).

Fig. 4. Gel filtration on a Sephadex G-25 column of diffusible peptides prepared by collagenase digestion of citrate-soluble rat skin collagen

(a) A sample containing 500mg of peptides in 1ml of 0.1M-NaCl was applied to a column (2.5cm×35cm) of Sephadex G-25, which was equilibrated and eluted at 22°C with 0.1M-NaCl. The void volume of the column was 72.5ml. Fractions (1ml) were collected and their E_{280} was measured. The fractions were pooled, forming three new fractions (I, II and III) as indicated by vertical thin lines. (b) Rechromatography of 2mg portions of fractions I (○), II (△) and III (●) on the same column. E_{225} was measured. Calibration of the column with standard proteins indicated that the elution positions correspond to molecular weights of about 3700, 1200 and 500–600 respectively.

To study the effect of the peptide chain length of the substrate on its interaction with the enzyme, a sample of a citrate-soluble collagen preparation was treated with collagenase as described under 'Materials'. On termination of this treatment part of the collagen was still in the form of non-diffusible large polypeptides, and the diffusible material consisted of a mixture of peptides of varying sizes, as shown by gel filtration on a

Table 3. Comparison of various peptide fractions as substrates for collagen galactosyltransferase

The peptides were prepared and fractionated as described in the text and Fig. 4, and tested as substrates for the reaction at two concentrations. The reaction was carried out in duplicate, and each value represents the mean of the two values.

Substrate	Substrate concentration (g/l)	Galactosyl-hydroxylysine formed (d.p.m.)
Citrate-soluble collagen	0.4	460
Fraction I	0.4	310
	2.0	1060
Fraction II	0.4	280
	2.0	960
Fraction III	0.4	60
	2.0	250

Sephadex G-25 column (Fig. 4a). This mixture was divided into three fractions as shown in Fig. 4(a), and these were freeze-dried. Samples of the fractions were rechromatographed on the same column (Fig. 4b). Calibration of the column [Blue Dextran, CoA (mol.wt. 767), glutathione (mol.wt. 303), L-proline (mol.wt. 115)] indicated that the average mol.wt. of fraction I was about 3700, that of fraction II about 1200 and that of fraction III about 500–600. Further samples of the fractions were hydrolysed with 6M-HCl at 120°C for 18h, evaporated to dryness and assayed in a Jeol JLC-5AH automatic amino acid analyser. As the original peptide fractions contained some salt, the peptide content of the fractions was calculated from their hydroxyproline content, assuming that this value was similar in all fractions to that in collagen. The molar ratio of hydroxylysine to hydroxyproline was about 0.09 in all three fractions.

When the three fractions were examined as substrates for collagen galactosyltransferase at two concentrations, by far the lowest rate of galactosyl-hydroxylysine formation was found with fraction III (Table 3). The rate observed with fraction II was about 4.3-fold (when calculated as an average of the values obtained at the two peptide concentrations) and that with fraction I about 4.7-fold compared with that obtained with fraction III. However, even the rate observed with fraction I was about 30% lower than that observed with the citrate-soluble collagen from which the peptides were prepared.

Comparison of native and heat-denatured citrate-soluble collagens as substrates for collagen galactosyltransferase

In order to study the effect of collagen conformation on the reaction with galactosyltransferase, native and heat-denatured citrate-soluble collagens were compared as substrates. Since the denaturation

Fig. 5. Comparison of native (○) and denatured (●) citrate-soluble collagen as substrates for collagen galactosyltransferase

The reaction was carried out as described in the Experimental section, except that the incubation temperature was 30°C. The substrate concentration in (a) and (b) was 0.4 mg/ml. The reaction was studied as a function of time (a), enzyme concentration (b), and substrate concentration (c).

temperature of citrate-soluble collagen at neutral pH is only about 38°C (Rauterberg & Kühn, 1968), these experiments were carried out at 30°C to ensure that the native collagen remained in its triple-helix conformation. As the solubility of native collagen at neutral pH is rather low, and since native collagen gradually begins to precipitate as fibrils after incubation at 37°C for some 30–50 min (see Siegel, 1974), the highest substrate concentration used was 0.4 mg/ml. Consequently, a slight non-linearity was noted in the time curve and enzyme-concentration curve of the reaction with denatured collagen as a substrate.

A clear difference was found between native and denatured collagen as substrates when the reaction was studied as a function of time, enzyme concentration or substrate concentration (Figs. 5a–5c), and very little galactosylhydroxylysine synthesis occurred with the native collagen.

Discussion

The purification of collagen galactosyltransferase was found to be a more difficult problem than that of collagen glucosyltransferase, which had recently been purified about 2000–3000-fold from chick-embryo extract both by a procedure consisting of conventional protein-purification steps (Myllylä *et al.*, 1976) and by an affinity-column procedure (Anttinen & Kivirikko, 1976). As discussed by Schwartz & Rodén (1975), unusual difficulties are often encountered in the purification of glycosyltransferases. These difficulties include an often firm association with cell membranes, a tendency for solubilized and partially purified preparations to aggregate, with consequent loss of activity, and a dependence of enzymic activity on lipids to the extent that complete inactivation may occur in their absence

(Schwartz & Rodén, 1975). Many of these problems were apparent during the present study. The enzyme activity in the chick-embryo homogenate and in the supernatants could be doubled by treatment with Triton X-100, suggesting that the enzyme in cells is associated with membrane structures in such a way that no reaction can be obtained in the assay procedure. During the purification procedure the enzyme showed a marked tendency to lose its activity. Accordingly, it was possible to obtain only a relatively small increase in the specific activity of the enzyme during purification. As a result of the inactivation, it seems likely that the degree of purification of the enzyme protein was greater than that of the enzyme activity.

In the final gel filtration three species of the enzyme activity with different molecular weights were recovered, the mol.wts. being about 450 000, 200 000 and 50 000. Since other glycosyltransferases show a tendency to aggregate (Schwartz & Rodén, 1975), it is possible that at least the highest value represents an aggregate of either the enzyme alone or the enzyme with some other proteins.

Experiments on the effect of thiol reagents on collagen galactosyltransferase activity suggested that free thiol groups are present in the active centre of the enzyme. These findings are very similar to those reported on collagen glucosyltransferase activity (Myllylä *et al.*, 1976).

Studies on substrate requirements of the enzyme indicated that denatured citrate-soluble collagen is clearly a more effective substrate than gelatinized insoluble collagen, as judged from their K_m values. The reason for this difference is not known, but it may be partly due to loss of some hydroxylysine residues in the process of cross-linking in collagen and partly due to hydrolytic changes which take place during the gelatinization process

(Veis, 1967). For reasons which are not apparent, the K_m for gelatinized insoluble calf skin collagen found in the present experiments, 35–70 g/l, is lower than the value of 140 g/l reported previously in studies with enzyme from chick-embryo cartilage (Myllylä *et al.*, 1975a).

Previous studies on the three other enzymes catalysing intracellular post-translational modifications in collagen biosynthesis, namely proline hydroxylase, lysine hydroxylase and collagen glucosyltransferase, have indicated that they all act more readily on substrates having longer polypeptide chains (see Cardinale & Udenfriend, 1974; Prockop *et al.*, 1976; Myllylä *et al.*, 1975b). The present and previous data on collagen galactosyltransferase indicate that this enzyme is similar in this respect. The enzyme does not act on free hydroxylysine (M. J. Spiro & R. G. Spiro, 1971; Myllylä *et al.*, 1975a) or on small carbohydrate-free peptides prepared by collagenase and Pronase digestion of glomerular basement-membrane collagen (M. J. Spiro & R. G. Spiro, 1971). The present results indicated that a peptide fraction having an average mol.wt. of 500–600 contained peptides large enough to meet a minimum requirement for the interaction with the enzyme, but longer peptides were clearly better substrates. It is noteworthy that even the peptide fraction with an average mol.wt. of 3700 was not as good a substrate as denatured citrate-soluble collagen consisting of polypeptide chains with a mol.wt. of 95000.

Experiments in which native and denatured collagens were compared as substrates for the enzyme indicated that the substrate conformation is an important determining factor for the reaction. Practically no synthesis of galactosylhydroxylysine was found with native collagen as substrate, and thus the triple-helix conformation seems to prevent galactosylation of the hydroxylysine residues. The data do not indicate whether the triple-helix conformation prevents galactosylation of all hydroxylysine residues in the newly synthesized collagen polypeptide chains, because the experiments were carried out on the additional galactosylation of citrate-soluble collagen, and some of the hydroxylysine residues in this collagen had already been galactosylated *in vivo* (Spiro, 1969). However, the extent of galactosylation of hydroxylysine residues in several other collagens, such as the type II collagen of cartilage (Miller, 1971) and the type IV collagen of basement membranes (Spiro, 1969; Kefalides, 1973), is much higher than that of the type I collagen of rat skin. Accordingly, in the biosynthesis of these collagens, at least, the galactosylation reactions must probably occur before triple-helix formation in the polypeptide chains.

The triple helix of collagen has previously been

found to prevent the hydroxylation of proline (Rhoads *et al.*, 1971; Berg & Prockop, 1973; Murphy & Rosenbloom, 1973) and lysine (Kivirikko *et al.*, 1973; Ryhänen & Kivirikko, 1974) residues and the glucosylation of galactosylhydroxylysine residues (Myllylä *et al.*, 1975b), and thus all four enzymes that are presently known to catalyse intracellular post-translational modifications in collagen biosynthesis seem to have a similar substrate-conformation requirement.

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