BIOSYNTHESIS OF CHONDROITIN SULFATE: IMMUNOPRECIPITATION OF INTERACTING XYLOSYLTRANSFERASE AND GALACTOSYLTRANSFERASE

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1. Introduction

The chondroitin sulfate chains of cartilage proteoglycan are synthesized on the membranes of the endoplasmic reticulum by the concerted action of six distinct glycosyltransferases [1]. Experiments that demonstrated an enzyme–enzyme interaction between the glycosyltransferases that catalyze the first two consecutive transfer reactions in the synthesis of chondroitin sulfate chains have been reported [2]. It was observed that an affinity matrix designed to adsorb xylosyltransferase[1] was also capable of binding UDP-galactose:xylose galactosyltransferase[1], following equilibration with xylosyltransferase [2]. In the present work, immunochemical evidence was obtained which further confirms that a specific interaction occurs between these two glycosyltransferases.

2. Materials and methods

2.1. *Chemicals and enzymes*

[^{14}C] UDP-Xylose (252 mCi per mmole) and [^{14}C] UDP-galactose (270 mCi per mmole) were purchased from New England Nuclear. Core protein from bovine cartilage proteoglycan (PGSD)[2] was prepared by Smith degradation as described by Baker et al. [3]. Catalase (beef liver) and lactate dehydrogenase (beef heart) were obtained from Worthington.

2.2. *Enzyme assays*

Xylosyltransferase was assayed by procedures which have been described in detail [4]. Briefly, incubation mixtures contained 0.2 mg of PGSD, 0.4 μmole of $MnCl_2$, 0.75 μmole of KCl, 1.5 nmoles of [^{14}C] UDP-xylose (specific activity approximately 30 μCi per μmole) and approximately 5 μg of enzyme protein in a total volume of 0.075 ml. After incubating the mixture for 60 min at 37°C, 0.25 mg of bovine serum albumin and 0.20 ml of 10% trichloroacetic acid-4% phosphotungstic acid were added. Precipitated protein was recovered by centrifugation, washed twice with 5% trichloroacetic acid and redissolved in 0.1 ml of 1.0 M NaOH for liquid scintillation counting.

Galactosyltransferase was assayed by incubating 2 μmoles of xylose, 1.5 nmoles of [^{14}C] UDP-galactose (34.5 μCi per μmole), 1 μmole of $MnCl_2$, and approximately 15 μg of enzyme protein in a total volume of 0.075 ml. After 60 min at 37°C the reaction was terminated by heating the tubes at 100°C for 2 min. The product (4-*O*-β-D-galactosyl-D-xylose) was separated and quantitated as previously described [4]. Alternatively, this enzyme was assayed with 0.4 μmole of *O*-β-D-xylosyl-L-serine as substrate instead of D-xylose [4]. Following incubation for 60 min at 37°C, the product (4-*O*-β-D-galactosyl-D-xylosyl-L-serine) was isolated by Dowex column chromatography as described [5].

Lactate dehydrogenase was assayed as described [6] by measuring the rate of decrease in absorbancy at 340

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[1] Xylosyltransferase refers to UDP-D-xylose: core protein xylosyltransferase and galactosyltransferase refers to UDP-D-galactose: D-xylose galactosyltransferase.

[2] *Abbreviations*: MES, 2-(*N*-morpholino) ethanesulfonic acid; PGSD, Smith-degraded chondroitin sulfate proteoglycan; LDH, lactate dehydrogenase.

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nm as NADH was oxidized. A unit is defined as the amount of enzyme activity which causes an initial rate of oxidation of one micromole of NADH per minute under standard conditions at 25°C.

Catalase was assayed essentially as described [7] by spectrophotometric measurement of the disappearance of peroxide at 240 nm. One unit is defined as the amount of enzyme activity which decomposes one micromole of hydrogen peroxide per minute under standard conditions at 25°C.

2.3. *Enzyme preparation*

Xylosyltransferase was purified approximately 4000-fold to apparent homogeneity from an embryonic chick cartilage homogenate [8]. The purification procedure includes differential centrifugation, ammonium sulfate precipitation and Sephadex gel filtration as previously described [5], followed by affinity chromatography on a matrix of PGSD-Sepharose [8]. The specific activity of the preparation used was approximately 9×10^6 cpm of product formed per mg protein.

Galactosyltransferase was obtained from the particulate fraction of an embryonic chick cartilage homogenate. The enzyme was solubilized by detergent-salt treatment and partially purified by gel filtration (approx. 40-fold) as described [9]. Prior to incubation with antiserum, the galactosyltransferase was diluted with an equal volume of 0.05 M MES buffer, pH 6.5, containing 0.25 M KCl[3]. The specific activity of the preparation used in these studies was 5.2×10^5 cpm of product formed per mg protein.

2.4. *Immunological procedures*

Antiserum to purified chick xylosyltransferase was prepared in rabbits [10]. Briefly, immunization was performed by injecting purified xylosyltransferase (approximately 0.3 mg) mixed with Freund's complete adjuvant subcutaneously every third week. The serum was obtained from the blood of the immunized ani-

[3] The following standard buffer solution was used throughout these experiments: 0.05 M MES, pH 6.5, containing 0.012 M $MgCl_2$, 0.003 $MnCl_2$ and 0.25 M KCl.

Table 1
Xylosyltransferase and galactosyltransferase activities following immunoprecipitation

Sample	Radioactivity incorporated into product, cpm			
	Xylosyltransferase		Galactosyltransferase	
	Precipitate	Supernatant	Precipitate	Supernatant
1. Xylosyltransferase		34,979		
2. Xylosyltransferase + pre-immune serum	768 (1%)	20,600		
3. Xylosyltransferase + immune serum	10 300 (92%)	914		
4. Galactosyltransferase		394		8758
5. Galactosyltransferase + immune serum			609 (9%)	5252
6. Xylosyltransferase + galactosyltransferase+ immune serum	14 300 (95%)	673	2954 (80%)	860
7. Immune serum		303		206

As described in Materials and methods, antixylosyltransferase antibody was added to purified xylosyltransferase in the absence or presence of partially purified galactosyltransferase, and the mixture was incubated for 12 hr at 4°C. After centrifugation, the immunoprecipitate was resuspended in 0.05 M MES buffer, pH 6.5, containing 0.25 M KCl, and the supernatant fluid and precipitate were assayed separately for enzyme activity under standard assay conditions. The three-component systems were set up in duplicate for analysis of both xylosyltransferase and galactosyltransferase activities. Numbers in parentheses indicate amount of total recovered activity present in the immunoprecipitate.

mal one week after the last injection. Immunodiffusion was performed as described by Ouchterlony [11]. Precipitations were carried out by incubating 50 μl of unfractionated immune serum with 50 μl of purified xylosyltransferase or other enzymes for 12 hr at 4°C. (A control incubation mixture of pre-immune rabbit serum and xylosyltransferase did not form a precipitate.) After centrifuging the incubation mixture at 500 *g* for 10 min, the precipitate was resuspended in 50 μl of 0.05 M MES buffer, pH 6.5, containing 0.25 M KCl, and both supernatant fluid and precipitate were assayed separately for enzyme activity. The concentrations of the four enzymes measured in this study were negligible in immune serum.

3. Results

As demonstrated previously, immune serum against purified xylosyltransferase gave single precipitin bands by immunodiffusion and immunoelectrophoresis [8]. When precipitin reactions were performed in solution, a total recovery of only 30–50% of the original xylosyltransferase activity was obtained; however, of the total recovered activity, greater than 90% of the xylosyltransferase activity was found in the immunoprecipitate (table 1). Pre-immune serum, while not forming an immunoprecipitate with xylosyltransferase, still caused a loss of enzyme activity (table 1) which may indicate that rabbit serum contains a substance inhibitory to the xylosyltransferase reaction. An additional loss of xylosyltransferase activity following precipitation with immune serum may be due to inaccessibility of enzyme to the protein substrate because of the particulate nature of the enzyme-antibody complex.

When a mixture of galactosyltransferase and xylosyltransferase was incubated with immune serum, both enzyme activities were found in the resulting precipitate, whereas the immune serum did not precipitate galactosyltransferase in the absence of xylosyltransferase (table 1). Although 80% of the total galacto-

Table 2
Enzyme activities following immunoprecipitation

	Enzyme activity (cpm incorporated into product or units/mg protein)			
	Xylosyltansferase		Catalase, LDH or galactosyltransferase	
	Precipitate	Supernatant	Precipitate	Supernatant
1. Catalase				2.37
2. Catalase activity in immune serum				0.016
3. catalase + immune serum			0.073 (5%)	1.51
4. Catalase + xylosyltransferase + immune serum	17 171 (99%)	150	0.061 (4%)	1.55
5. Lactate dehydrogenase (LDH)				110.0
6. LDH activity in immune serum				2.8
7. LDH + immune serum			1.4 (2%)	96.5
8. LDH + xylosyltransferase + immune serum	13 872 (97%)	489	3.5 (3%)	106.0
9. Galactosyltransferase + xylosyltransferase + immune serum	12 500 (95%)	580	3186 (78%)	900

Incubation conditions and assays of both supernatants and immunoprecipitates were identical to those described in table 1. Activities of xylosyltransferase and galactosyltransferase are expressed as cpm of radioactivity incorporated into product under standard conditions, while catalase and lactate dehydrogenase activities are expressed as units per mg of protein. Numbers in parenthesis refer to amount of total recovered activity of each enzyme which is present in the immunoprecipitate.

syltransferase activity was found in the immunoprecipitate, only 50% of the original activity was recovered.

Under comparable conditions, two other enzymes, catalase and lactate dehydrogenase, did not precipitate along with the xylosyltransferase-antibody complex (table 2).

4. Discussion

These findings suggest that an association between embryonic chick cartilage xylosyltransferase and galactosyltransferase can occur when these two enzymes are mixed in solution. Both enzymes may then coprecipitate following the addition of an antiserum to xylosyltransferase. This interaction is at least partially specific since two other enzymes, catalase and lactate dehydrogenase, did not coprecipitate with xylosyltransferase under similar conditions.

A number of observations indicate that the ionic milieu is important to the behavior of xylosyltransferase. The enzyme is extracted from tissues by buffers with a high salt concentration; while a decrease in ionic strength results in reversible aggregation [5,8,9]. However, since the precipitation of xylosyltransferase and galactosyltransferase by anti-xylosyltransferase occurred in buffer of high salt concentration, an ionic association between these two enzymes was probably not involved. Similar conclusions were drawn previously when an affinity matrix was used as an artificial membrane to demonstrate the interaction between these two enzymes [2]. In this instance, the binding between the two glycosyltransferases was not dissociated by salt alone but rather required detergent at elevated ionic strength. We have also observed, using a solubilized glycosyltransferase preparation containing 0.5% Nonidet P-40, that most of the xylosyltransferase (70%) was precipitated by immune sera, while only 6% of the galactosyltransferase was coprecipitated (unpublished). These findings suggested that the interaction between xylosyltransferase and galactosyltransferase may involve binding between hydrophobic regions of the two enzyme molecules.

An interaction between the first two glycosyltransferases which catalyze consecutive reactions in the biosynthesis of chondroitin sulfate has now been demonstrated by two independent means. Whether such enzyme-enzyme interactions participate in the organization of a multi-enzyme glycosyltransferase complex in vivo remains to be established. Mechanistically, interactions involving all of the glycosyltransferases would be advantageous to the system by increasing the catalytic efficiency of the overall reaction. Alternatively, it is also possible that the xylosyltransferase–galactosyltransferase interaction is unique in the chondroitin sulfate reaction sequence. The association–dissociation of these two enzymes, controlled in part by the hydrophobicity of the environment, may play an important role in the regulation of chondroitin sulfate biosynthesis at the site of chain initiation.

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References

[1] Rodén, L. (1970) in: Metabolic Conjugation and Metabolic Hydrolysis (Fishman, W. H., ed.), Vol. II, p. 345. Academic Press, New York.
[2] Schwartz, N. B., Rodén, L. and Dorfman, A. (1974) Biochem. Biophys. Res. Commun. 56, 717.
[3] Baker, J. R., Rodén, L. and Stoolmiller, A. C. (1972) J. Biol. Chem. 247, 3838.
[4] Rodén, L., Baker, J. R., Helting, T., Schwartz, N. B., Stoolmiller, A. C., Yamagata, S. and Yamagata, T. (1973) Methods in Enzymology 28, 638.
[5] Stoolmiller, A. C., Horwitz, A. L. and Dorfman, A. (1972) J. Biol. Chem. 247, 3525.
[6] Kornberg, A. (1955) Methods in Enzymology 1, 441.
[7] Beers, R. F., Jr. and Sizer, I. W. (1952). J. Biol. Chem. 195, 133.
[8] Schwartz, N. B. and Rodén, L. (1974). Carbohyd. Res., in press.
[9] Schwartz, N. B. and Rodén, L., J. Biol. Chem., submitted.
[10] Darcy, D. A. (1966) in: Analytical Methods of Protein Chemistry (Alexander, P. and Lundgren, H. P., eds.), Vol. 4, p. 104. Pergamon Press, New York.
[11] Ouchterlony, O. (1958). Progr. Allergy 5, 1.