STUDIES ON THE BIOSYNTHESIS OF THE CHONDROITIN SULFATE-PROTEIN LINKAGE REGION

Torsten Helting and Lennart Rodén

Departments of Pediatrics and Biochemistry, the La Rabida-University of Chicago Institute, and the Joseph P. Kennedy, Jr. Mental Retardation Research Center, University of Chicago, Chicago, Illinois 60637

Received May 3, 1968

The carbohydrate-protein linkage region of chondroitin 4-sulfate (Rodén and Smith, 1966) and heparin (Lindahl, 1966) has the following structure:[1]

$$\text{GlcUA} \xrightarrow{\beta 1,3} \text{Gal} \xrightarrow{\beta 1,3} \text{Gal} \xrightarrow{\beta 1,4} \text{Xyl},$$

with xylose O-glycosidically bound to serine.

Studies on the biosynthesis of the linkage region have shown that xylose and galactose may be transferred from UDP-xylose and UDP-galactose to endogenous acceptors in several tissue systems (Grebner et al., 1966; Robinson et al., 1966). Xylosylserine and galactosylxylitol were isolated from the reaction products, indicating that the incorporation represented the formation of the first two monosaccharide units of the polysaccharide chain (Reactions I and II, respectively).

This paper presents evidence for the addition of the second galactose residue of the linkage region (Reaction III) to an endogenous acceptor as well as to $\text{Gal} \xrightarrow{\beta 1,4} \text{Xyl}$, using the embryonic chick cartilage system of Robinson et al. (1966).

The biosynthesis of the chondroitin sulfate chain proper has previously been investigated and it has been shown that glucuronic acid and N-acetyl-galactosamine are transferred from the respective uridine nucleotide sugars to oligosaccharide acceptors containing the alternate sugar of the repeating disaccharide unit at the non-reducing end (Telser et al., 1966). The formation

[1] The following abbreviations are used: GlcUA, glucuronic acid; $\text{Gal} \xrightarrow{\beta 1,4} \text{Xyl}$, 4-O-β-D-galactosyl-D-xylose; $\text{Gal} \xrightarrow{\beta 1,3} \text{Gal}$, 3-O-β-D-galactosyl-D-galactose; $\text{Gal} \xrightarrow{\beta 1,3} \text{Gal} \xrightarrow{\beta 1,4} \text{Xyl}$, 3-O-β-D-galactosyl-4-O-β-D-galactosyl-D-xylose; $\text{GlcUA} \xrightarrow{\beta 1,3} \text{Gal} \xrightarrow{\beta 1,3} \text{Gal} \xrightarrow{\beta 1,4} \text{Xyl}$, 3-O-β-D-glucuronosyl-3-O-β-D-galactosyl-4-O-β-D-galactosyl-D-xylose.

of the first glucuronic acid unit of the polysaccharide chain is of particular interest, since it is linked to galactose rather than to N-acetylgalactosamine. Evidence for the occurrence of this reaction (Reaction IV) in the chick cartilage system will also be reported in this communication.

Experimental and results.

Incorporation of galactose-^{14}C into endogenous acceptor.

Epiphyses from the femurs and tibias of 60-65 13 days old chick embryos were homogenized in a Potter-Elvehjem homogenizer in 8 ml of a buffer of the following composition: Tris-acetate, 50 mM; $MnCl_2$, 3 mM; KCl, 70 mM; Na_2 EDTA, 1 mM, cysteine·HCl, 6 mM. After centrifugation of the homogenate at 10 000 g for 10 minutes at 2°, the supernatant liquid was stored for 30 days or less at -20°. Preparations derived from 24 dozen embryos were combined and centrifuged at 300 000 g for 60 minutes. Incubation of the pellet with UDP-galactose-^{14}C under the conditions described in the legend to Fig. 1 resulted in incorporation of 11% of the added radioactivity into TCA-precipitable material A neutral oligosaccharide fraction was isolated by acid hydrolysis followed by deionization; paper electrophoresis of this fraction in borate buffer yielded several radioactive peaks (Fig. 1a), including components migrating as Gal $\xrightarrow{\beta 1,4}$ Xyl, Gal $\xrightarrow{\beta 1,3}$ Gal and Gal $\xrightarrow{\beta 1,3}$ Gal $\xrightarrow{\beta 1,4}$ Xyl. These fractions were eluted with 0.05 M HOAc, passed through a column (1x4 cm) of Dowex 50-X8 (H^+ form, 200-400 mesh) and separately chromatographed on paper in ethyl acetate: acetic acid: water, 3:1:1 (solvent A). It is seen from Fig. 1b-c that this procedure led to the isolation of three fractions with R_f values identical to those of Gal $\xrightarrow{\beta 1,4}$ Xyl (yield: 13 000 cpm, 33 μμmoles), Gal $\xrightarrow{\beta 1,3}$ Gal (yield: 386 cpm, 1.0 μμmole) and Gal $\xrightarrow{\beta 1,3}$ Gal $\xrightarrow{\beta 1,4}$ Xyl (yield: 326 cpm, 0.8 μμmoles).

Digestion of all three fractions with β-galactosidase yielded galactose as the only radioactive product, as shown by paper chromatography of the digest in ethyl acetate: pyridine: water, 8:2:1 (solvent B). Further characterization of the presumed Gal $\xrightarrow{\beta 1,3}$ Gal was obtained by lead tetraacetate oxidation. In this reaction, Gal $\xrightarrow{\beta 1,3}$ Gal characteristically yields 2-O-β-D-galactosyl-D-lyxose (Rodén and Smith, 1966). Oxidation of a sample containing 290 cpm gave a product with an electrophoretic mobility identical to that of 2-O-β-D-galactosyl-D-lyxose. After elution of this material with 0.05 M HOAc, the eluate was passed over Dowex 50 and repeatedly concen-

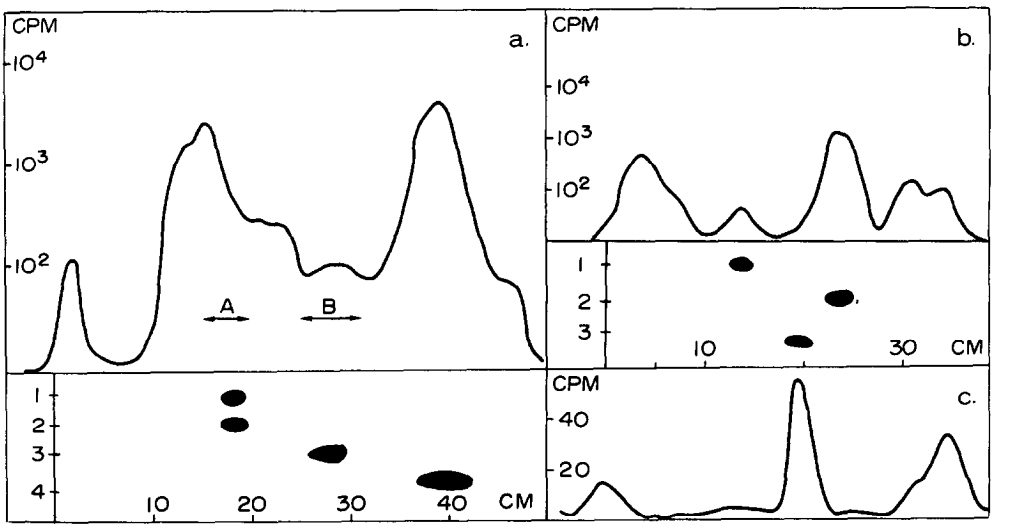

Fig. 1. Transfer of galactose from UDP-galactose-^{14}C to an endogenous acceptor. The complete system contained Tris buffer, pH 6.0, of the composition described in the text; UDP-galactose-^{14}C (0.5 μC; 276 mC/mmole); UDP-xylose (1 μmole); and the 300 000 g pellet derived from 24 dozen chick embryos in a final volume of 1.5 ml. After incubation for 2 hours at 37°, one volume of 10% TCA was added. The precipitate was collected by centrifugation at 20 000 g for 10 minutes and washed 4 times with 5% TCA, twice with ethanol: ether (1:1) and once with ether. Yield: 54 mg. A small aliquot was dissolved in 0.1 ml of 1 M KOH at 100°; after dilution to 1.0 ml, a 0.6-ml sample was mixed with 8.6 ml of toluene and 6.0 ml of ethanol and the radioactivity determined in a Tri Carb liquid scintillation counter. Specific activity: 21 000 cpm/mg protein. After solubilization of the TCA precipitate (8×10^5 cpm) by digestion with papain at 60° for 12 hours (0.3 mg of enzyme in 1 ml of a 0.1 M acetate buffer, pH 5.5, containing 0.005 M EDTA and 0.005 M cysteine), the digest was hydrolyzed at pH 1.5 for 5 hours at 100°. The solution was adjusted to pH 5.0 with NaOH and passed through columns (2 x 4 cm) of Dowex 1-X2 (acetate form, 200-400 mesh) and Dowex 50-X8 (H^+ form, 200-400 mesh). The effluent (1.5×10^5 cpm) was concentrated to dryness with several additions of methanol, dissolved in 0.1 ml of water and subjected to electrophoresis on Whatman No. 3MM paper (0.04 M borate buffer, pH 9.2, 35 volts/cm, 4 hours). Fig. 1a shows the distribution of radioactivity as determined by a Packard radiochromatogram strip scanner, Model 7200. The sections indicated were eluted and the eluates gave the patterns shown in Fig. 1b (from section A) and Fig. 1c (from section B) after chromatography in solvent A.
(1), Gal $\xrightarrow{\beta 1,3}$ Gal $\xrightarrow{\beta 1,4}$ Xyl; (2), Gal $\xrightarrow{\beta 1,4}$ Xyl; (3), Gal $\xrightarrow{\beta 1,3}$ Gal; (4), Galactose.

---

trated to dryness with methanol. The oxidation product was then digested with β-galactosidase and, after desalting by electrophoresis, galactose (33 cpm) and lyxose (22 cpm) were demonstrated in the digest by paper chromatography in solvent B. These findings provide evidence for the identity of

the unknown compound with Gal $\xrightarrow{\beta 1,3}$ Gal and for the occurrence of Reaction III in the chick cartilage system.

Transfer of galactose from UDP-galactose-^{14}C to Gal $\xrightarrow{\beta 1,4}$ Xyl.

The modest yields of Gal $\xrightarrow{\beta 1,3}$ Gal and Gal $\xrightarrow{\beta 1,3}$ Gal $\xrightarrow{\beta 1,4}$ Xyl which were obtained in the experiments with endogenous acceptor prompted further studies with exogenous substrates. Incubation of the 10 000 g supernatant liquid of the cartilage homogenate with UDP-galactose-^{14}C in the presence of added Gal $\xrightarrow{\beta 1,4}$ Xyl gave a product (yield: 25 000 cpm, 63 μμmoles) which had the same mobility as Gal $\xrightarrow{\beta 1,3}$ Gal $\xrightarrow{\beta 1,4}$ Xyl in paper chromatography (solvent A) and in electrophoresis in borate buffer (Fig. 2). This compound was not formed in controls without Gal $\xrightarrow{\beta 1,4}$ Xyl. On chromatography on Sephadex G-10, the compound emerged at the same effluent volume as Gal $\xrightarrow{\beta 1,3}$ Gal $\xrightarrow{\beta 1,4}$ Xyl. After digestion with β-galactosidase all the radioactivity migrated as galactose (paper chromatography, solvent B). Partial acid hydrolysis (pH 1.5; 3 hours; 100°) of a sample containing 15 000 cpm

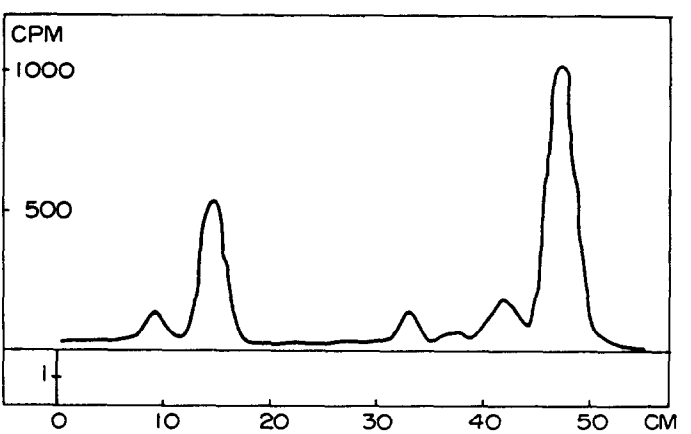

Fig. 2. Formation of Gal $\xrightarrow{\beta 1,3}$ Gal $\xrightarrow{\beta 1,4}$ Xyl from Gal $\xrightarrow{\beta 1,4}$ Xyl and UDP-galactose-^{14}C. The complete system contained 0.5 ml of the 10 000 g supernatant liquid of the cartilage homogenate (pH 5.6), 2.8 μmoles of Gal $\xrightarrow{\beta 1,4}$ Xyl and 0.5 μC of UDP-galactose-^{14}C (276 mC/mmole). The reaction was terminated by the addition of 3 volumes of ethanol. The ethanol supernatant was deionized and a sample was subjected to paper electrophoresis in borate buffer (35 volts/cm; 7 hours). The compound migrating as Gal $\xrightarrow{\beta 1,3}$ Gal $\xrightarrow{\beta 1,4}$ Xyl was eluted with 0.05 M HOAc; the eluate was passed over Dowex 50 and concentrated to dryness with several additions of methanol. Yield: 25 000 cpm, 63 μμmoles.
(1), Gal $\xrightarrow{\beta 1,3}$ Gal $\xrightarrow{\beta 1,4}$ Xyl.

yielded Gal $\xrightarrow{\beta 1,3}$ Gal (715 cpm) and lead tetraacetate oxidation of this hydrolytic fragment resulted in the formation of 2-O-β-D-galactosyl-D-lyxose, as evidenced by paper electrophoresis and paper chromatography.

Transfer of glucuronic acid from UDP-glucuronic acid-^{14}C to Gal $\xrightarrow{\beta 1,3}$ Gal $\xrightarrow{\beta 1,4}$ Xyl.

Evidence for Reaction IV in the chick cartilage system was obtained by an experiment similar to the one described above. Gal $\xrightarrow{\beta 1,3}$ Gal $\xrightarrow{\beta 1,4}$ Xyl (1.6 μmoles) was used as acceptor for the transfer of glucuronic acid from UDP-glucuronic acid-^{14}C (0.5 μC; 125 mC/mmole) under experimental conditions otherwise similar to those described in Fig. 2. A compound was formed (yield: 8 000 cpm, 45 μμmoles) which migrated to the same position as GlcUA $\xrightarrow{\beta 1,3}$ Gal $\xrightarrow{\beta 1,3}$ Gal $\xrightarrow{\beta 1,4}$ Xyl in electrophoresis in pyridine-acetate buffer, pH 5.3, in borate buffer and in paper chromatography (solvent A). This compound was absent in controls without added Gal $\xrightarrow{\beta 1,3}$ Gal $\xrightarrow{\beta 1,4}$ Xyl. Digestion with β-glucuronidase released all the radioactivity as free glucuronic acid. Acid hydrolysis (0.5 M H_2SO_4; 100° for 3 hours) gave a product which migrated as 3-O-β-D-glucuronosyl-D-galactose in electrophoresis at pH 5.3 and in paper chromatography in solvent A. Lead tetraacetate oxidation of this material gave a compound with the same electrophoretic and chromatographic mobility as 2-O-β-D-glucuronosyl-D-lyxose, indicating the formation of a 1,3-linkage between glucuronic acid and the terminal galactose residue of the acceptor trisaccharide.

Discussion

The findings described above together with earlier studies (Robinson et al., 1966), provide evidence for the biosynthesis of the two galactose residues (Reactions II and III) in the protein-carbohydrate linkage region of chondroitin 4-sulfate. Since the galactose acceptors in the two reactions are different, i.e., xylose in Reaction II and galactose in Reaction III, the question arises whether the two reactions are catalyzed by the same or two different enzymes. Likewise, the glucuronosyltransferase involved in the addition of glucuronic acid to galactose (Reaction IV) may possibly differ from the enzyme that catalyzes the transfer of glucuronic acid to N-acetylgalactosamine residues to form the remainder of the polysaccharide chain.

Acknowledgement

This work was supported by USPHS grant AM-05996 and by grants from the American Heart Association and the Chicago Heart Association. We are indebted to Dr. A. Dorfman for helpful discussions.

References

Grebner, E.E., Hall, C.W., and Neufeld, E.F., Biochem. Biophys. Res. Commun., 22, 672 (1966).

Lindahl, U., Arkiv Kemi, 26, 101 (1966).

Robinson, H.C., Telser, A., and Dorfman, A., Proc. Nat. Acad. Sci., 56, 1859 (1966).

Roden, L., and Smith, R., J. Biol. Chem., 241, 5949 (1966).

Telser, A., Robinson, H.C., and Dorfman, A., Arch. Biochem. Biophys., 116, 458 (1966).