Biosynthesis of Chondroitin Sulfate

PURIFICATION OF GLUCURONOSYL TRANSFERASE II AND USE OF PHOTOOFFINITY LABELING FOR CHARACTERIZATION OF THE ENZYME AS AN 80-kDa PROTEIN*

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A photoaffinity analogue, [β-32P]5-azido-UDP-GlcA, was used to photolabel the enzymes that utilize UDP-GlcA in cartilage microsomes and rat liver microsomes. SDS-polyacrylamide gel electrophoresis analysis of photolabeled cartilage microsomes, which are specialized in chondroitin sulfate synthesis, showed a major radiolabeled band at 80 kDa and other minor radiolabeled bands near 40 and 60 kDa. Rat liver microsomes, which are enriched for enzymes of detoxification by glucuronidation, had a different pattern with multiple major labeled bands near 50–60 and 35 kDa. To determine that the photolabeled 80-kDa protein is the GlcA transferase II, we have purified the enzyme from cartilage microsomes. This membrane-bound enzyme, involved in the transfer of GlcA residues to non-reducing terminal GalNAc residues of the chondroitin polymer, has now been solubilized, stabilized, and then purified greater than 1350-fold by sequential chromatography on Q-Sepharose, heparin-Sepharose, and WGA-agarose. The purified enzyme exhibited a conspicuous silver-stained protein band on SDS-polyacrylamide gel electrophoresis that coincided with the major radiolabeled band of 80 kDa. SDS-polyacrylamide gel analysis of photoaffinity-labeled active fractions from the Q-Sepharose, heparin-Sepharose, and WGA-agarose also indicated only the single radiolabeled band at 80 kDa. Intensity of photolabeling in each of the fractions examined coincided with enzyme activity. The photolabeling of this 80-kDa protein was saturable with the photoprobe and could be inhibited by the addition of UDP-GlcA prior to the addition of the photoprobe. Thus, the photolabeling with [β-32P]5-azido-UDP-GlcA has identified the GlcA transferase II as an 80-kDa protein. The purified enzyme was capable of transferring good amounts of GlcA residues to chondroitin-derived pentasaccharide with negligible transfer to pentasaccharide derived from hyaluronan or heparan.

Proteochondroitin sulfate consists of a wide range of core protein families with covalently attached chondroitin sulfate glycosaminoglycan chains (1). Formation of chondroitin sulfate is initiated by xylosylation of appropriate serine residues in the core protein followed by the addition of two Gal residues, a GlcA residue and a GalNAc residue. The GlcA-Gal-Gal-Xyl portion of the linkage is common to all glycosaminoglycans with the exception of keratan sulfate. Addition of the first GalNAc residue commits the polysaccharide to be a galactosaminoglycan (chondroitin), with subsequent alternating addition of GlcA and GalNAc residues resulting in the formation of the chondroitin polymer (2, 3). Sulfation appears to occur while the polymer is being formed (4). The overall sequence of reactions has been well characterized, with each of the sugar transfers catalyzed by a separate glycosyltransferase (2, 3). Considerable information has been obtained by our laboratory (5–7), as well as others (8, 9), regarding the localization of the individual glycosyl transferases in the Golgi correlating with the established sequence of reactions. The fine structure of chondroitin sulfate appears to be controlled by the membrane topography at the site of synthesis together with the concerted interaction of different glycosyl transferases and sulfotransferases in juxtaposition to the membrane-bound nascent proteoglycan (3).

Biosynthesis of chondroitin sulfate parallels that of heparin/heparan sulfate in many respects. While the only chondroitin sulfate-synthesizing enzymes as yet purified are the xylosyl transferase (10) and chondroitin 6-sulfotransferase (11, 12), seven enzymes participating in synthesis of heparin have been purified (13–19). Moreover, some of these have been shown to be multifunctional, so that a single protein has been shown to contain two separate enzyme activities (15, 18, 20, 21), thus facilitating multiple catalytic events along a single polymer. The presence of such multifunctional proteins in chondroitin sulfate biosynthesis has not yet been investigated due to the lack of purified enzymes.

We and others have previously utilized chick embryo epiphyseal cartilage microsomes together with labeled UDP-GlcA, UDP-GalNAc, and 3′-phosphoadenosine 5′-phosphosulfate to examine chondroitin sulfate synthesis (2, 3, 22). Similar microsomal preparations have been used to transfer [14C]GlcA from UDP-[14C]GlcA to chondroitin oligosaccharides containing non-reducing GalNAc residues (23, 24), thus providing a useful method for measuring the GlcA transferase activity. In addition, these microsomal preparations have been shown to contain GlcA transferase activity for adding GlcA to non-reducing terminal Gal residues such as that found in the Gal-Gal-Xyl linkage region (25, 26). Mixed substrate experiments have indicated that the transfers of [14C]GlcA to the linkage oligosaccharide and to chondroitin oligosaccharides are each catalyzed by separate enzymes (25), which have been termed GlcA transferase I and GlcA transferase II (2).

Attempts to purify and characterize the membrane-associated chondroitin glycosyl transferases have previously not been

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successful due to low copy numbers and an inherent instability after solubilization. We have now been able to stabilize the GlcA transferase II during a 1350-fold purification from chick embryo epiphyseal cartilage microsomes. Silver staining of the purified enzyme preparation demonstrated a prominent band at 80 kDa as well as other minor additional bands. To aid in the purification and identification of the enzyme, we have used a UDP-GlcA analog, \( \beta^{32}P\)-5-N-UDP-GlcA, for a photoaffinity labeling technique that has previously been found to be successful in labeling liver GlcA transferases involved in glucuronidation (27). This analog contains base-substituted azido groups, which upon UV irradiation form highly reactive nitrene intermediates, which in turn form covalent bonds with amino acids at the sugar nucleotide binding site. By this technique, we found that GlcA transferase II is the 80-kDa protein seen on the silver-stained gel.

**EXPERIMENTAL PROCEDURES**

**Materials**

UDP-[1-\( ^{32}P\)]GlcA was purchased from DuPont NEN. Non-radioactive UDP-GlcA, WGA (wheat germ agglutinin)-agarose, and GlcNac were purchased from Sigma. Heparin-Sepharose and Q-Sepharose were purchased from Pharmacia Biotech Inc. Chondroitin and chondroitin pentasaccharide were prepared as described previously (28). Hyaluronan pentasaccharide was prepared by degradation of hyaluronan with Streptomyces hyaluronidase to obtain a hexasaccharide, followed by removal of the unsaturated uronic acid with mercuric acetate (29). Heparan pentasaccharide was obtained from Escherichia coli K5 heparin pentasaccharide (30) by partial degradation with heparitinase to obtain a hexasaccharide, followed by removal of the unsaturated uronic acid with mercuric acetate.

**Assay of GlcA Transferases**

Reaction mixtures contained 0.05 M MES buffer; pH 6.5, 0.015 M MnCl\(_2\), 0.05% Triton X-100; 0.07 mM UDP-[1-\( ^{32}P\)]GlcA (325 mCi/mmol); 5–60 \( \mu \)g of chondroitin pentasaccharide, hyaluronan pentasaccharide, or heparan pentasaccharide; and 12 \( \mu \)l of microsomal fraction or enzyme fraction in a total volume of 15 \( \mu \)l. After a 2-h incubation at 37 °C, the reaction mixtures were spotted on Whatman no. 1 paper and chromatographed in butanol:acetic acid:1 mM NH\(_4\)OH (2:3:1) for 48 h. The origins were eluted with water and counted.

**Purification of GlcA Transferase II**

All operations were carried out at 4 °C unless stated otherwise.

**Step 1: Preparation of Microsomes**—Epiphyseal cartilage from tibias and femurs of 400 17-day-old-chick embryos were suspended in 150 ml of 0.25 M sucrose containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 1 mM N-ethyl maleimide, 0.5 mM EGTA, and 0.1 mM leupeptin, and 0.1 mM leupeptin, and 0.1 mM leupeptin) and homogenized with a Polytron tissue grinder at maximum setting by five 30-s bursts separated by 1 min intervals. The homogenate was centrifuged at 12,000 \( \times \) g for 30 min. The supernatant was saved, and the pellet was rehomogenized as described above after suspension in 100 ml of 0.25 M sucrose containing protease inhibitors. The second homogenate was also centrifuged at 12,000 \( \times \) g, and the supernatant obtained was combined with the first. The pooled 12,000 \( \times \) g supernatant was then centrifuged at 105,000 \( \times \) g for 90 min in a Beckman ultracentrifuge. The pellets obtained were used for further purification.

**Step 2: Solubilization from the Microsomal Pellet**—The microsomal pellets obtained were suspended in buffer A (10 mM HEPES buffer, pH 7.2, containing 10 mM MgCl\(_2\), 2 mM CaCl\(_2\), 1 mM DTT, 20% glycerol, and protease inhibitors). To this suspension an equal volume of buffer B (buffer A containing 2% Triton X-100) was added. The suspension was gently mixed for 60 min and centrifuged at 105,000 \( \times \) g for 90 min. The supernatant was saved, and the pellets were resuspended in buffer A. An equal volume of buffer B containing 2.0 mM NaCl was added. After incubation for 60 min with gentle mixing, the suspension was centrifuged at 105,000 \( \times \) g for 90 min. The supernatant was combined with the first supernatant and used as the solubilized enzyme. By this two-step procedure, we were able to solubilize almost 90% of the GlcA transferase II activity from the membranes.

**Step 3: Chromatography on Q-Sepharose**—After adjusting the NaCl concentration to 0.05 M, the solubilized enzyme was applied on to a 1.5 × 14-cm Q-Sepharose column that was previously equilibrated with buffer C (buffer A containing only 0.02% Triton X-100) plus 0.05 M NaCl. The column was then washed with 10 volumes of the same buffer, and the bound proteins were eluted with a linear NaCl gradient of 0.05–1.0 M in buffer C. Fractions of 3.0 ml were collected and assayed for GlcA transferase II activity. Fractions containing the activity were combined and diluted with buffer C to adjust the NaCl concentration to 0.15 M and used for the next step.

**Step 4: Chromatography on Heparin-Sepharose**—The active enzyme from Step 3 was applied to a heparin-Sepharose column (1.5 × 20 cm) that was equilibrated with buffer C containing 0.15 M NaCl. The column was then washed with five volumes of the same buffer. The bound enzyme was eluted with buffer C containing 0.1 M NaCl. Active fractions were pooled and dialyzed against buffer C containing 0.15 M NaCl.

**Step 5: Chromatography on WGA-Agarose**—The dialyzed enzyme from the previous step was then used for the next step.

At each stage of purification, active fractions were examined by silver staining of 10% SDS gels.

**Photoaffinity Labeling of the Enzyme(s)**

The photoaffinity probe \( \beta^{32}P\)-5-N-UDP-GlcA was synthesized as described previously (31). Mixtures (60 \( \mu \)l) containing 0.2–50 \( \mu \)g of enzyme protein in 0.05 M MES buffer, pH 6.5, 0.015 M MnCl\(_2\), 0.05% Triton X-100, plus 100 \( \mu \)M \( \beta^{32}P\)-5-azido-UDP-GlcA were irradiated at room temperature with a hand-held UV lamp for 90 s. Reaction mixtures that contained heat-inactivated enzyme and reaction mixtures that were not UV-irradiated served as controls. For competition experiments, the appropriate competing ligands were included in the same mixture. In other reactions, chick cartilage microsomes were replaced with rat liver microsomes. Photoaffiliation was terminated by the addition of an equal volume of ice-cold 10% trichloroacetic acid, and the samples were analyzed by SDS-PAGE as described previously (31). Photoincorporation was visualized by autoradiography of the stained SDS-PAGE gels. The relative amount of photoincorporation was determined by densitometric scanning of the autoradiogram.

**RESULTS**

An autoradiogram of chick cartilage microsomes and rat liver microsomes photolabeled with \( \beta^{32}P\)-5-N-UDP-GlcA is shown in Fig. 1. Lane 1, consisting of the addition of the \( ^{32}P \)-labeled probe to cartilage microsomes but without UV ir-
radiation, gave a single radiolabeled band around 72 kDa. This labeling is apparently of a nonspecific nature and is due to the presence of minor contaminants in the probe. Following UV irradiation (lane 2), a much more prominently labeled band at approximately 80 kDa and another band at 40 kDa were seen. This labeling was essentially eliminated when 0.16 mM UDP-GlcA was added before addition of the photoprobe (lane 3). Rat liver microsomes (lane 4), which are enriched for the enzymes of GlcA conjugation for detoxification, demonstrated multiple bands at 50–60 kDa (previously shown to be involved in glucuronidation; Ref. 27) and at 35–37 kDa (previously shown to be a Glc-P-dolichol synthase; Refs. 27, 32, and 33). Additional faint photolabeled bands at approximately 70 kDa and at 80 kDa (the same position as the strongest photolabeled cartilage microsomal band), were also seen. It is of note that cartilage microsomes had no labeled bands near 50–60 kDa, consistent with their inability to transfer [14C]GlcA from UDP-[14C]GlcA to acceptors such as phenolphthalein (detoxification) (23). As with the cartilage microsomes, photoincorporation into rat liver microsomal protein was also inhibited by inclusion of UDP-GlcA during the labeling (lane 5), indicating that the photoprobe was specifically labeling the proteins that utilize UDP-GlcA. All incorporation of label was dependent on UV irradiation.

We then purified the GlcA transferase II activity from the chick cartilage microsomes using chondroitin pentasaccharide as acceptor and photolabeling to provide an additional monitor during the different steps of purification. Approximately 90% of the microsomally bound GlcA transferase II could be solubilized with 1% Triton X-100 and NaCl in the presence of 20% glycerol and 1 mM DTT as outlined under “Experimental Procedures.” Under these conditions the enzyme could be stored for 1 year at −20 °C without any appreciable loss in activity. However, when the glycerol and DTT were omitted, all activity was lost within a few days. Therefore, all purification procedures were carried out in the presence of these ingredients.

Elution of the solubilized GlcA transferase II from a Q-Sepharose column provided two peaks (Fig. 2). The second of these peaks, containing approximately 60% of the GlcA transferase II activity, had no GlcA transferase I activity and was used for further purification. Active enzyme from the Q-Sepharose step was applied to a heparin-Sepharose column and was eluted as a sharp peak with 1.0 M NaCl (Fig. 3). After removal of NaCl by dialysis, the enzyme was mixed with WGA-agarose in a sealed column for 3 h. Under these conditions all the enzyme activity bound to WGA-agarose and was eluted with buffer C to which 0.5 M GlcNAc starting from fraction 25 (Fig. 4). Attempts to purify the enzyme beyond this step were unsuccessful due to the extremely low amounts of protein present and the inability of this enzyme to bind to various affinity ligands such as Sepharose or agarose linked to UDP-GlcA or UDP-hexanolamine, which have been used by others for binding other glycosyl transferases (18, 33–36).

Purification of GlcA transferase II is shown in Table I. Microsomes obtained from the epiphyseal cartilage of 400 chicken embryos were utilized. Measurement of activities in crude homogenates was inconsistent and inaccurate due to interfering activities, so that the purification was calculated from the microsomes, representing less than 1% of the total weight of
Table I

| Purification step            | Total protein* | Total activity | Specific activity | Purification | Recovery |
|------------------------------|----------------|----------------|-------------------|--------------|----------|
| Cartilage homogenate         | 145 mg         | $1^{14}$C cpm $\times 10^6$ | 132 $1^{14}$C cpm $\times 10^6$ | 0.91 | 1 100 |
| Solubilized microsomes       | 62 mg          | 120            | 1.9               | 1 2.1       | 91       |
| Q-Sepharose (Peak II)        | 8.2 mg         | 80             | 9.75              | 11          | 60       |
| Heparin-Sepharose            | 0.35 mg        | 65             | 185               | 203         | 40       |
| WGA-agarose                  | $<0.025$ mg    | 32             | 1230              | >1350       | 24       |

* Determined by the Bio-Rad protein assay.

FIG. 5. SDS-PAGE analysis of GlcA transferase II during different steps of purification. Different fractions during the enzyme purification were subjected to 10% SDS-PAGE under reducing conditions, and the protein bands were visualized by silver staining of the gel. Panel A, lane 1, 100 µg of cartilage microsomes; lane 2, 70 µg of solubilized microsomes. Panel B, pooled fractions (30 µg) containing GlcA transferase II activity after Q-Sepharose chromatography. Panel C, pooled fractions (5 µg) containing GlcA transferase II activity after heparin-Sepharose chromatography. Panel D, fractions from WGA-agarose column. Lane 1, fraction 27; lane 2, fraction 28; lane 3, fraction 29.

FIG. 6. Analysis of photoaffinity-labeled preparations of GlcA transferase II after separation on 10% SDS-PAGE. Aliquots of protein fractions from the different steps in purification were subjected to photoaffinity labeling, separated on 10% SDS-PAGE, and visualized by autoradiography as described under “Experimental Procedures.” Panel A, lane 1, cartilage microsomes; lane 2, solubilized microsomes. Panel B, fractions from Q-Sepharose (Fig. 2). Lane 1, fraction 84; lane 2, fraction 93; lane 3, fraction 99; lane 4, fraction 102. Panel C, fractions from heparin-Sepharose (Fig. 3). Lane 1, fraction 48; lane 2, fraction 51; lane 3, fraction 54. Panel D, fractions from WGA-agarose (Fig. 4). Lane 1, fraction 26; lane 2, fraction 28; lane 3, fraction 30.

FIG. 7. Saturation of GlcA transferase II with UDP-[14C]GlcA and with [β-32P]5-N3UDP-GlcA. Concentrations of UDP-[14C]GlcA from 1.6 µM to 240 µM (○) were assayed for enzyme activity using pentasaccharide as an acceptor as described under “Experimental Procedures.” The same concentrations of [β-32P]5-N3UDP-GlcA (●) were added to enzyme and photoaffinity-labeled as described under “Experimental Procedures.” Values were determined by densitometric scanning of the resulting autoradiograms.

in panel A were no longer present. However, both 80- and 40-kDa labeled bands were seen when fractions from the first Q-Sepharose peak (Fig. 2) were analyzed (data not shown). Intensity of the 80-kDa photolabeled protein was strongest in the fraction that contained the maximum enzyme activity. Enzyme activity in each analyzed fraction from heparin-Sepharose (panel C) and WGA-agarose (panel D) also coincided in intensity of photolabeling with a single band at 80 kDa. There was no labeling observed when the enzyme was heat-inactivated (data not shown).

Pentasaccharide together with varying concentrations of UDP-[14C]GlcA were incubated with pooled fractions from the second peak of the Q-Sepharose column (Fig. 2) as shown in Fig. 7. Varying concentrations of [β-32P]5-N3UDP-GlcA were added to similar enzyme and exposed to UV light as described for photolabeling under “Experimental Procedures.” Results using densitometry to quantitate the bands are shown in Fig. 7. We found that the enzyme was saturable with both UDP-[14C]GlcA and [β-32P]5-N3UDP-GlcA at similar concentrations with an apparent $K_m$ for UDP-GlcA and an apparent $K_p$ for the probe at $5 \times 10^{-5} \text{ M}$.

Purified enzyme eluted from the WGA-agarose column was incubated with UDP-[14C]GlcA plus pentasaccharides prepared from heparan (E. coli K5) and hyaluronan (Table II). There was no incorporation of GlcA into either pentasaccharide, demonstrating that the purified GlcA transferase II was specific for chondroitin synthesis. In contrast, rat liver microsomes had activity in transferring GlcA to the heparan and hyaluronan.
pentasaccharides, as well as to chondroitin pentasaccharide, demonstrating that each pentasaccharide substrate was an acceptor when appropriate enzymes were present.

DISCUSSION

Previous work on chondroitin biosynthesis by chick embryo epiphysseal cartilage microsomes indicated that the glycosyl transferases are firmly bound to the microsomal membranes with nascent proteochondroitin substrate at the site of synthesis (3). In contrast, soluble heparin-synthesizing glycosyl transferases have been found to be present in good quantity in commercial serum sources (18). We were unable to find such an alternative source for soluble GlcA transferase II. Recently, it was reported that the GlcA transferase II activity in serum was developmentally regulated with the activity being the highest at the mid prenatal stage in chicken and bovine sera and at the late prenatal stage in rat serum (37). The activity declined rapidly after this stage in all three species. It is possible that the commercial serum sources that we examined were at later developmental stages so that the GlcA transferase II activity had already declined.

Although GlcA transferase II has previously been solubilized from microsomes using an alkali detergent method or Nonidet P-40 and KCl (38), we found that such activity was lost rapidly, precluding further purification. Therefore, we have used Triton X-100 and NaCl in the presence of 20% glycerol and DTT to solubilize and stabilize the enzyme activity. With this modification, the purified enzyme stored at −20 °C retained full activity for at least 1 year.

We were able to monitor protein content in the purification through the heparin-Sepharose step (Fig. 3, Table I). However as mentioned previously, protein content after the final WGA-agarose step (Fig. 4) was too low to provide an accurate measurement of purification. Therefore, we believe that the purification is actually higher than 1350-fold. Attempts to purify the enzyme beyond this step were unsuccessful, but photoaffinity labeling provided an alternative approach. Similar photoaffinity labeling has been useful in characterizing other difficult to purify glycosyl transferases (32, 33). The co-match of the intensity of the photolabeled protein (Fig. 6) with the peaks of enzyme activity eluted from Q-Sepharose, heparin-Sepharose, and WGA-agarose (Figs. 2–4) and the incapability of the purified enzyme to transfer [14C]GlcA to pentasaccharides other than chondroitin pentasaccharide (Table II), indicate that the 80-kDa protein is the GlcA transferase II. Moreover, the $K_m$ value for UDP-GlcA and the $K_p$ for the photoprobe were essentially identical (Fig. 7), demonstrating that the photoprobe can substitute for UDP-GlcA with approximately the same efficiency of binding at the substrate binding site. A similar apparent $K_m$ for UDP-GlcA was previously reported with intact microsomes (39). It was of particular interest that the $K_m$ for UDP-GlcA did not change with solubilization, suggesting that transport of UDP-GlcA into the lumen of these microsomes was not a limiting factor in biosynthesis.

The 1350-fold purified GlcA transferase II had no GlcA transferase I activity (data not presented), thus confirming that the two activities are independent. However, it is possible that the lower photolabeled band seen at 40 kDa in the chondroitin-synthesizing cartilage microsomes (Fig. 1) may represent the GlcA transferase I.

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