Elevated chondroitin 6-sulfotransferase activity in fetal bovine serum

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Received 7 February 1985

Assay conditions for chondroitin sulfotransferase in serum were established using a rapid and simple paper disk method. Sulfotransferase activities towards endogenous serum glycosaminoglycans and exogenously added chondroitin were determined for fetal, newborn and adult bovine sera. The results indicate that 3′-phosphoadenosine 5′-phosphosulfate (PAPS); chondroitin 6-sulfotransferase activity in fetal calf serum is several times that in newborn or adult bovine sera. The enzyme transfers sulfate onto position 6 of internal nonsulfated galactosamine units of endogenous and exogenous chondroitin.

Sulfation *Chondroitin sulfate* *Sulfotransferase*

1. INTRODUCTION

Defective sulfation of chondroitin sulfate results in growth defect [1,2]. Enhanced degradation of sulfate donor, PAPS, causes undersulfation of chondroitin sulfate in Lowe's syndrome and the patients exhibit severe pathological conditions [3]. These observations suggest the biological importance of the sulfation of glycosaminoglycans (GAGs) and other macromolecules. Biosynthetic mechanisms of sulfation of macromolecules including GAGs have only been poorly understood. In the course of the investigation of sulfation reactions in various mammalian tissues, it was found that a considerable amount of ^{35}S radioactivity is incorporated into chondroitinase-sensitive materials upon incubation of fetal calf serum (FCS) with ^{35}S-labeled PAPS. [^{35}S]Sulfate incorporation was markedly higher with FCS than with adult bovine serum. This paper reports the elevated chondroitin 6-sulfotransferase activity found in FCS.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; ΔDi-0S, 2-acetamide-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; ΔDi-4S, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulfo-D-galactose

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2. MATERIALS AND METHODS

2.1. *Materials*

[^{35}S]PAPS (2.2 Ci/mmol) was purchased from New England Nuclear. Nonradioactive PAPS was from Sigma. 2,3-Dimercaptopropan-1-ol (DMP) was obtained from Wako, Osaka, Japan. Pronase P was from Kaken Kagaku, Tokyo. Chondroitinase AC, ΔDi-OS, ΔDi-4S, ΔDi-6S, and GAG preparations were from Seikagaku Kogyo, Tokyo. Fetal and newborn calf sera, and horse serum were obtained from Gibco. Adult bovine and human sera were prepared from fresh blood supplied by a local slaughterhouse and donated by volunteers, respectively.

2.2. *Methods*

The paper disk method developed for the assay of serum glycosyltransferases [4] was applied to the sulfotransferase assays. This method is advan-

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tageous for multiple assays over the trichloroacetic acid precipitation/centrifugation or paper chromatographic methods [5]. The standard incubation conditions were determined so as to maximize the total [^{35}S]sulfate incorporation into acid-insoluble endogenous acceptors using FCS. The Zn^{2+}-specific chelator DMP [6] as well as EDTA were utilized to protect PAPS from enzymatic breakdown. The pH optimum was in the range of 7–8. The apparent K_m for PAPS was 7.4 μM. The following two methods were thus established depending upon the sulfate acceptors. Dilution of sera was made with buffer I (10 mM Hepes-NaOH, pH 7.4) if necessary.

Assay 1 was designed to determine the sulfotransferase activities towards both endogenous acceptors and heated FCS added exogenously. Each incubation mixture contained 20 μl (800 μg protein) serum, 10 μl buffer II (see below), 10 μl of 60 μM [^{35}S]PAPS ($3–8 \times 10^5$ cpm), and 20 μl buffer I (for the assay without exogenous acceptors) or 20 μl (800 μg protein) of heated FCS which had been treated at 56°C for 3 h. The heat treatment completely abolished the sulfotransferase activities of FCS. Buffer II contained 30 mM EDTA, 60 mM $MgCl_2$, 30 mM DMP in 60 mM Hepes-NaOH, pH 7.4. Following incubation at 37°C for 30–90 min, incorporation of [^{35}S]sulfate into acceptors was determined by the paper disk method. Aliquots of the reaction mixture were spotted onto paper disks, which were sequentially washed in 10% trichloroacetic acid, ethanol/ether (2:1, v/v) and ether as reported [4], and counted in a liquid scintillation counter.

Assay 2 was designed to determine the sulfotransferase activities towards exogenously added chondroitin. The incubation mixture contained 20 μl (40 μg protein) serum, 10 μl buffer II, 10 μl of 60 μM [^{35}S]PAPS ($1–3 \times 10^5$ cpm), and 20 μl of 6 mg/ml chondroitin. The K_m for chondroitin (sodium salt) was 0.17 mg/ml. Determination of transfer of [^{35}S]sulfate to exogenous chondroitin was made in a modified paper disk method where washing of the paper disks was carried out in *n*-butyric acid/0.5 M ammonia (5:3, v/v) (solvent I) instead of 10% trichloroacetic acid. Sulfate transfer to exogenous chondroitin was proportional to the amount of serum protein and to the incubation period until approx. 20% of PAPS was consumed.

Isolation of GAGs labeled with [^{35}S]sulfate were carried out as follows. FCS was incubated overnight as in Assays 1 and 2 with [^{35}S]PAPS (3.2×10^6 cpm) and then GAGs were isolated. The assay 1 incubation mixture was treated with 0.5 M NaOH at 4°C for 18 h, neutralized with 1 M HCl, and digested with pronase [7]. Following 5% trichloroacetic acid treatment of the pronase digest, the trichloroacetic acid-soluble fraction was treated with ether to extract trichloroacetic acid, mixed with carrier GAGs and chromatographed on a column (1.0×56 cm) of Sephadex G-50 fine with 0.05 M pyridine-acetate buffer (pH 5.3) as reported [7]. The excluded fractions containing GAGs (3.98×10^5 cpm) were evaporated to dryness and digested with chondroitinase as described [7]. The assay 2 incubation mixture was treated with 5% trichloroacetic acid. The trichloroacetic acid-soluble fraction was chromatographed on Sephadex G-50 as above. The excluded fractions containing GAGs (2.02×10^6 cpm) were isolated and subjected to chondroitinase digestion. The trichloroacetic acid-insoluble fraction contained a negligible amount of labeled GAGs. Exogenous chondroitin inhibited sulfate incorporation into endogenous acceptors by 90%.

Radioactivity was determined by liquid scintillation counting. Protein was determined by the method of Lowry et al. [8].

3. RESULTS AND DISCUSSION

Upon incubation of FCS with [^{35}S]PAPS under the established conditions, sulfate incorporation into endogenous acceptors was proportional to the incubation period and to the amount of serum protein in the range of 600–1200 μg. With less than 600 μg of protein the linearity was not obtained, presumably because the concentrations of endogenous acceptors and/or other endogenous requirements were too low. In the appropriate range of protein concentration, [^{35}S]sulfate transfer to endogenous acceptors was determined for various sera. The results indicate that FCS has several times the activity of sera from newborn calves or adult animals (fig.1). Although most of the serum samples were supplied in a frozen state, adult bovine sera and human sera were prepared from fresh blood. Upon repeated freeze-thawing no loss of activity of FCS was observed. Thus, the lower activity of sulfate transfer in sera from newborn or

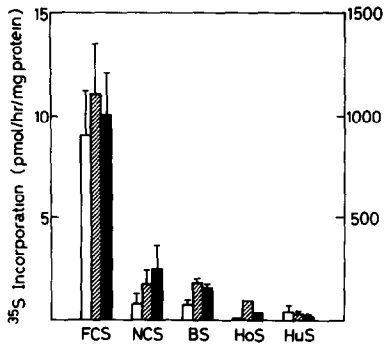

Fig.1. Sulfotransferase activities in various sera. The assays were carried out as described in section 2. Open, hatched, and solid columns indicate activities with endogenous acceptors, exogenous heated FCS and exogenous chondroitin, respectively. Each column and each vertical bar represent the mean and the standard deviation, respectively. The left ordinate shows the activities with endogenous acceptors and heated FCS while the right ordinate shows those with chondroitin. FCS, fetal calf serum; NCS, newborn calf serum; BS, adult bovine serum; HoS, adult horse serum; HuS, adult human serum. Eight FCS, 4 NCS, 3 BS, 1 HoS, and 3 HuS samples were assayed.

adult animals is not due to inactivation of sulfotransferases or acceptors during storage.

In the next experiments each incubation was carried out with heated FCS exogenously added. In its presence [^{35}S]sulfate incorporation into an acid-insoluble fraction was proportional to the amount of serum protein tested and to the incubation period. Under these conditions FCS still showed higher activities than sera from newborn calves or adult animals (fig.1), suggesting that the higher sulfate incorporation observed with FCS was attributed to higher sulfotransferase activities rather than higher concentrations of endogenous acceptors or activators.

Characterization of the endogenous ^{35}S-labeled products obtained from the incubation of FCS with [^{35}S]PAPS indicated that 93% of the ^{35}S-labeled macromolecules was sensitive to chondroitinase AC and that 77 and 11% of the produced radioactive oligosaccharides were ΔDi-6S and ΔDi-4S, respectively. These results indicate that the higher incorporation of sulfate with FCS is due most likely to a higher enzymatic activity of the sulfotransferase which catalyzes sulfate transfer onto the 6 position of internal nonsulfated galactosamine residues of endogenous chondroitin (or chondroitin sulfate) proteoglycans. The sulfotransferase assay was then carried out with exogenous chondroitin as a sulfate acceptor. As shown in fig.1, with chondroitin as an acceptor each serum showed 30–100-fold the sulfotransferase activity detected with heated FCS (note the scale). The results confirmed the substantial difference in the sulfotransferase activity between FCS and sera from newborn or adult animals. Sulfated products obtained from the incubation of FCS with [^{35}S]-PAPS and exogenous chondroitin were analyzed. ^{35}S-labeled products were recovered by extraction with 5% trichloroacetic acid followed by gel filtration on Sephadex G-50. Under the conditions used 65% of the ^{35}S radioactivity added to the reaction was found in this fraction (see section 2). The isolated radioactive material was completely digested by chondroitinase AC as judged by gel filtration (not shown). Upon paper chromatography of the oligosaccharide fraction 96 and 3% of the radioactivity were recovered in ΔDi-6S and ΔDi-4S, respectively (fig.2). No detectable oversulfated disaccharide was observed. These results indicate that the observed sulfate transfer measured with exogenous chondroitin as an acceptor was also predominantly

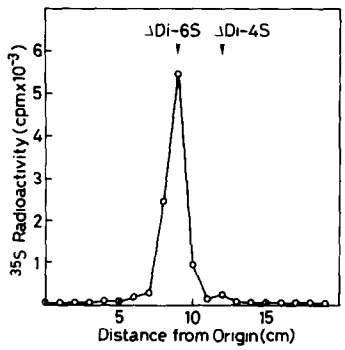

Fig.2. Descending paper chromatography of the chondroitinase digest. Isolation of ^{35}S-labeled GAGs and preparation of the chondroitinase AC digest were described in section 2. An aliquot of the oligosaccharide fraction (9700 cpm) was mixed with 0.2 μmol each of ΔDi-4S and ΔDi-6S, and chromatographed on Toyo No51A paper in solvent I for 36 h. The carrier disaccharides were localized under the UV-lamp. For determination of radioactivity the paper was cut into 1 cm-wide strips and counted in a liquid scintillation counter.

onto position 6 of internal nonsulfated galactosamine units of chondroitin, and that the 6-sulfotransferase activity is indeed higher in FCS than in sera from newborn or adult animals.

Proteoglycans or GAGs are thought to be involved in morphogenesis and development of many tissues [9]. Developmental changes in chondroitin sulfate isomers have been reported for some tissues on the basis of GAG analyses. However, alterations of sulfotransferase activities supporting such changes have not been reported. Rather it has been indicated that degree of sulfation can be regulated in some instances by the availability of PAPS [1,3,10] or by the environmental sulfate concentrations [11]. The present studies demonstrate elevated chondroitin 6-sulfotransferase activity in fetal calf serum, which may reflect developmentally controlled biosynthesis of chondroitin sulfate in some unlocated tissue. Predominant serum GAGs are low sulfated or fully sulfated chondroitin 4-sulfate, and chondroitin 6-sulfate, if any, is only a minor component [12]. It is unlikely that the 6-sulfotransferase functions in blood since the PAPS level therein is below the limit of detection. Rather it possibly functions in the cell or tissue from which it originates. It is of interest to investigate the origin of the enzyme and a possibility of elevation of the enzyme activity with oncogenesis.

ACKNOWLEDGEMENTS

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan. The authors thank Ms Hayuru Sakai for her excellent secretarial assistance.

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