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Defect in 3′-phosphoadenosine 5′-phosphosulfate formation in brachymorphic mice

[sulfate adenylyltransferase (ATP sulfurylase)/adenylylsulfate kinase (adenosine phosphosulfate kinase)/chondroitin sulfate proteoglycan]

KAZUYUKI SUGAHARA AND NANCY B. SCHWARTZ*

Departments of Pediatrics and Biochemistry, The Joseph P. Kennedy, Jr., Mental Retardation Research Center, University of Chicago, Chicago, Illinois 60637

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ABSTRACT **Incorporation of $^{35}SO_4^{2-}$ into adenosine 5′-phosphosulfate (APS), 3′-phosphoadenosine 5′-phosphosulfate (PAPS), and chondroitin sulfate was simultaneously assessed with extracts prepared from epiphyseal cartilage of neonatal normal or homozygous brachymorphic mice. Radioactivity measured in APS, PAPS, and chondroitin sulfate of extracts from brachymorphic cartilage was approximately 300%, 9%, and 13% of the normal levels, respectively. Even though more APS accumulated in the mutant cartilage extracts, APS actually synthesized (total $^{35}SO_4^{2-}$ incorporated into APS, PAPS, and macromolecular products) was only 17% of that in the normal. However, of the amount synthesized, 90% and 55% of newly synthesized APS were converted to PAPS by cartilage extracts of normal and brachymorphic mice, respectively. Specific assays for ATP sulfurylase (sulfate adenylyltransferase; ATP:sulfate adenylyltransferase, EC 2.7.7.4) and APS kinase (adenylylsulfate kinase; ATP:adenylylsulfate 3′-phosphotransferase, EC 2.7.1.25) showed that the sulfurylase enzyme activity is reduced to approximately 1/2 and the kinase to approximately 1/14 in brachymorphic mice. These results suggest that the production of an undersulfated proteoglycan in brachymorphic cartilage results from a defective conversion of APS to PAPS.**

Brachymorphism in mice is characterized by disproportionately short stature (1). The cartilage matrix of homozygous recessive brachymorphic mice (*bm/bm*) reacts poorly with stains for sulfated glycosaminoglycans (2). The mutant cartilage matrix contains normal collagen fibrils, but the proteoglycan aggregate granules are smaller than normal and are present in reduced numbers, particularly in the columnar hypertrophic zones of the growth plate (2). The mutant cartilage contains normal levels and types of collagen as well as normal levels of glycosaminoglycans (3). However, both the sulfate content in the cartilage and the incorporation of labeled sulfate is lower in mutant cartilage than in normal cartilage (3). The glycosaminoglycans from the mutant cartilage are found to be less negatively charged than those from normal cartilage on the basis of ion-exchange chromatography and electrophoresis (3). After digestion with chondroitinase, larger amounts of nonsulfated glycosaminoglycan disaccharide were obtained from the mutant cartilage (3). Most recently, it has been demonstrated that comparable amounts of $^{35}SO_4^{2-}$ are incorporated from 3′-phosphoadenosine 5′-phospho[^{35}S]sulfate ([^{35}S]PAPS) into chondroitin sulfate by the normal and mutant cartilage extracts (4). In contrast, when sulfation was initiated from ATP and $^{35}SO_4^{2-}$, a reduction to less than 25% in sulfate incorporation into chondroitin sulfate proteoglycan was observed (4). These results indicate normal levels of the sulfotransferase activities, but defective synthesis of sulfate donor (PAPS) in brachymorphic cartilage. In the present study, incorporation of $^{35}SO_4^{2-}$ into adenosine 5′-phosphosulfate (APS) and PAPS as well as chondroitin sulfate was investigated. Additionally, individual enzyme assays were carried out for the enzymes required for synthesis of APS and PAPS.

MATERIALS AND METHODS

[^{35}S]PAPS [2.2 Ci/mmol (1 Ci = 3.7×10^{10} becquerels)] and carrier-free $H_2{}^{35}SO_4$ (43 Ci/mg) were purchased from New England Nuclear. Other chemicals and enzymes were obtained from the following sources: nonradioactive PAPS and APS (P-L Biochemicals), ATP (Sigma), sodium molybdate, ammonium molybdate, and sodium bisulfite (Baker), inorganic pyrophosphatase (EC 3.6.1.1) from baker's yeast, type III (Sigma), and chondroitinase AC (Miles Laboratories).

A breeding colony was established from homozygous brachymorphic (*bm/bm*) mice obtained from the Jackson Laboratory (Bar Harbor, ME). Cartilage tissue was obtained from 4-day-old homozygous brachymorphic and normal C57BL/6J mice by dissection of distal femoral heads and proximal tibial heads as described (5). Cartilage tissue was homogenized at 4°C in 0.05 M Tris·HCl, pH 8.0, or 0.1 M Tris·HCl, pH 8.5, depending on the experiments. Enzyme assays were performed on 10,000 × *g* supernatant fluids of the homogenates. The concentration of protein was measured by the method of Lowry *et al.* (6).

Incorporation of $^{35}SO_4^{2-}$ into APS, PAPS, and Endogenous Acceptor. Incorporation of $^{35}SO_4^{2-}$ into APS, PAPS, and endogenous acceptor was determined as reported (4). The reaction mixture of 275 μl contained 25 μl of 0.1 M ATP, 10 μl of 10 mM cysteine·HCl, 5 μl each of carrier-free $H_2{}^{35}SO_4$, 1 M $MgCl_2$, and 0.01 M NAD^+, and 225 μl of cartilage extracts in 0.05 M Tris·HCl, pH 8.0. After incubation at 37°C, the reaction was terminated by freezing. An aliquot of the solution was analyzed by paper electrophoresis at a potential of 130 V/cm for 60 min in pyridine/acetate buffer, pH 5.3 (pyridine/acetic acid/water, 5:2:493 vol/vol). Under these conditions, standard APS and PAPS migrated approximately 27 cm and 35 cm, respectively. Radioactive material remaining at the origin was considered to be sulfated endogenous chondroitin sulfate, because essentially all of this material was sensitive to chondroitinase AC (data not shown). Radioactivity was measured for determination of APS, PAPS, and endogenous acceptors by assaying 1-cm segments of the paper in a liquid scintillation counter (Packard model 3385).

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Abbreviations: APS, adenosine 5′-phosphosulfate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; APS kinase, ATP:adenylylsulfate phosphotransferase; ATP sulfurylase, ATP:sulfate adenylyltransferase.

*To whom reprint requests should be addressed.

APS Kinase Assay. APS kinase (adenylylsulfate kinase; ATP:adenylylsulfate phosphotransferase, EC 2.7.1.25) was assayed according to Robbins and Lipmann (7). An assay mixture of 200 μl contained 50 μl of 0.4 M Tris·HCl, pH 8.5, 10 μl each of 0.1 M ATP and 0.02 M $MnCl_2$, 20 μl of 15 mM APS, and 110 μl of cartilage extract in 0.1 M Tris·HCl, pH 8.5. After 60 min of incubation, the reaction was terminated by boiling for 3 min. PAPS formation was determined by the method of Gregory and Lipmann (8). This assay is based on the measurement of liberation of *p*-nitrophenol from its sulfate as determined by a change in absorbance at 400 nm, usually within a small range (0.015–0.065). However, the method is so sensitive and specific for PAPS that the significant differences in APS kinase activity can be determined.

ATP Sulfurylase Assay. ATP sulfurylase (sulfate adenylyltransferase; ATP:sulfate adenylyltransferase, EC 2.7.7.4) was assayed according to Robbins (9) by the measurement of the rate of inorganic phosphate release from ATP in the presence of molybdate and pyrophosphatase. The reaction is based on the fact that molybdate may substitute for sulfate as substrate for ATP-sulfurylase (10). The advantage of this method is that the reaction product, adenosine phosphomolybdate, is unstable and hydrolyzes spontaneously, and therefore, results in no product inhibition. In contrast, the physiological reaction product, APS, strongly inhibits ATP sulfurylase (7). The incubation mixture (300 μl) contained 30 μl each of 1 M Tris·HCl, pH 8.0, 0.1 M ATP, 0.1 M $MgCl_2$, 0.05 M Na_2MoO_4, inorganic pyrophosphatase solution (50 μg/ml), and 150 μl of cartilage extracts in 0.05 M Tris·HCl, pH 8.0. After 80 min of incubation at 37°C, the reaction was terminated by addition of ice-cold 10% trichloroacetic acid. The supernatant fluid was assayed for inorganic phosphate by a modified method of Fiske and SubbaRow (11).

RESULTS

A possible defect in the sulfation pathway during synthesis of PAPS from ATP and $^{35}SO_4^{2-}$ in *bm/bm* cartilage was suggested from the results obtained in the previous study (4). Consequently, the individual steps involved in this pathway that could alter APS or PAPS synthesis were assessed.

[^{35}S]APS, [^{35}S]PAPS, and ^{35}S-labeled endogenous products were simultaneously determined by paper electrophoresis after incubation of cartilage extracts from normal or brachymorphic mice with $H_2{}^{35}SO_4$. After 50 min of incubation, normal cartilage extracts utilized approximately 14.3% of the radioactive sulfate, while brachymorphic cartilage extracts utilized only 2.4%. As shown in Fig. 1, incorporation of $^{35}SO_4^{2-}$ into endogenous acceptors with brachymorphic cartilage extracts attained only 12.6% of the normal level and is consistent with the observations previously reported (4). The accumulated [^{35}S]PAPS in extracts of mutant cartilage was only 8.7% of that in normal extracts, confirming that PAPS synthesis is defective as suggested from the previous study (4). Thus, the decreased incorporation of $^{35}SO_4^{2-}$ into endogenous acceptors is presumably due to the limited supply of [^{35}S]PAPS as discussed below.

In contrast, accumulated [^{35}S]APS was approximately 300% of the normal level. However, because radioactivity incorporated into both PAPS and macromolecular products is derived from that first incorporated into APS, the radioactive sulfate utilized reflects the total APS produced during the incubation. Thus, APS produced in the mutant cartilage extracts was only 17% of that in the normal. However, it is unlikely, as discussed below, that the defect results from decreased APS synthesis rather than the conversion of APS to PAPS.

On the basis of the results shown in Fig. 1, percentage of conversion of APS to PAPS at each assay time was calculated.

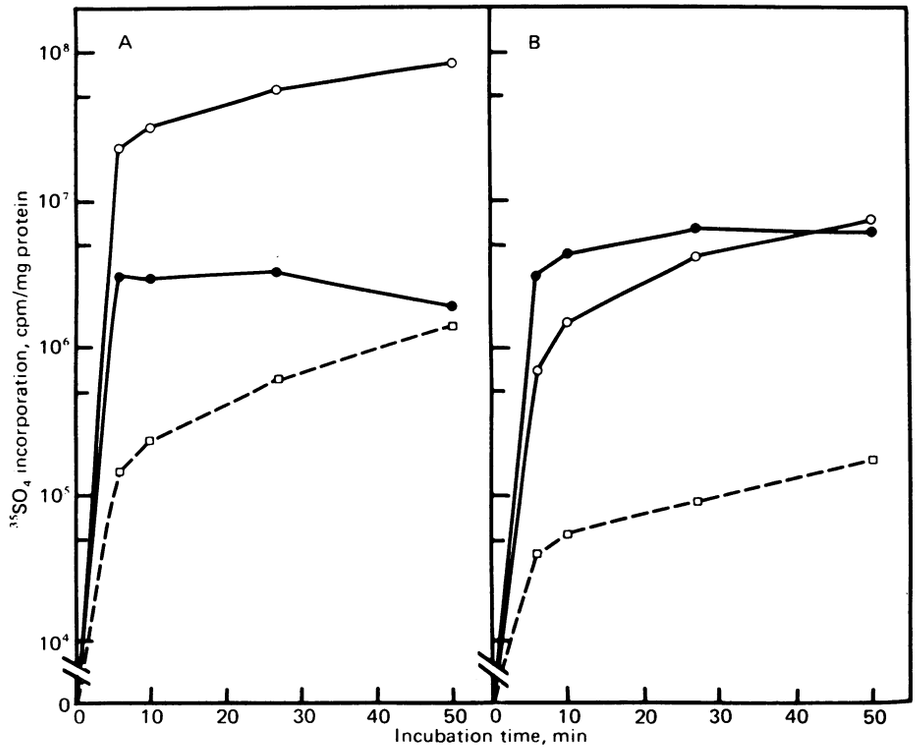

FIG. 1. Incorporation of $^{35}SO_4^{2-}$ into APS, PAPS, and endogenous acceptor. APS (●), PAPS (○), and endogenous sulfated macromolecules (□) were simultaneously determined by paper electrophoresis after incubation of cartilage extracts from normal (*A*) and brachymorphic (*B*) mice with $H_2{}^{35}SO_4$ (1.26×10^8 cpm). The whole mice had been frozen at −20°C for two weeks before use. Approximately 200 μg of enzyme protein was used.

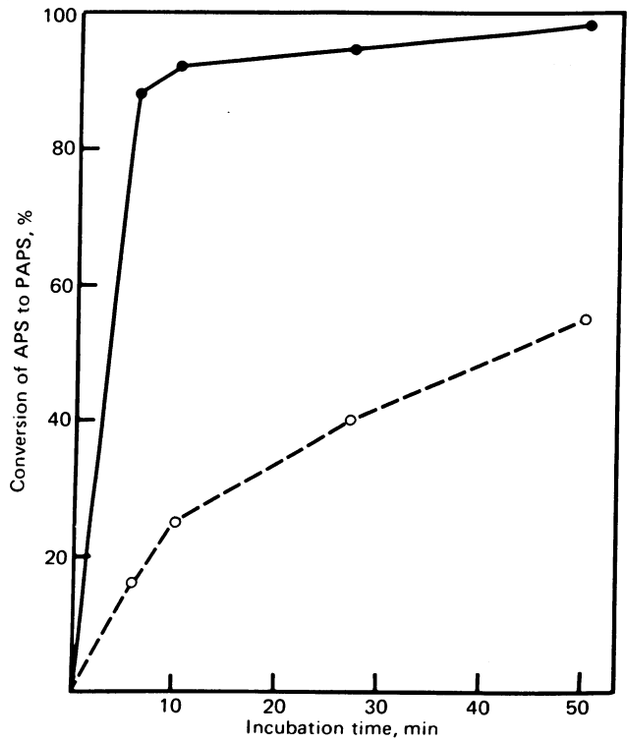

FIG. 2. Percent conversion of APS to PAPS. The values were obtained from the calculations that were made on the basis of the results shown in Fig. 1. ●, Normal mice; ○, *bm/bm* mice.

If one defines the amount of each product in terms of cpm, then the percent conversion may be determined as follows: % conversion of APS to PAPS = 100 × (cpm PAPS + cpm macromolecular product)/(cpm APS + cpm PAPS + cpm macromolecular product). Corrections were not made for the possible degradation of each component. In the case of normal cartilage extracts, the percentage of conversion was found to be 90–95% throughout the reaction period (Fig. 2). In contrast, the mutant cartilage extracts yielded 18, 25, 40, and 55% after incubation of 6, 10, 27 and 50 min, respectively. Thus, the percentage of conversion of APS to PAPS was significantly decreased in the mutant cartilage extracts even though total APS produced was less than 20% of that in the normal extracts. This finding appears to support the defective conversion of APS to PAPS.

In order to clarify which step of PAPS synthesis is defective, the specific enzymes involved were assayed directly. Colorimetric enzyme assays were used for the ATP sulfurylase and APS kinase. APS kinase was assayed in extracts obtained from

Table 1. APS kinase activity in extracts of normal and brachymorphic cartilage

Litter	APS kinase specific activity C57BL/6J	*bm/bm*
1	7.9	0.55
2	11.2	0.75
3	9.5	0.70
Average	9.5 (100)*	0.67 (7)*

Approximately 300 μg of enzyme protein was used for incubation, and enzyme activity is expressed as nmol of PAPS formed per mg of protein per hr at 37°C. Assays were performed on cartilage extracts from three different litters of freshly sacrificed normal (five each) and brachymorphic (six each) mice.
*Percentage of the control value is expressed in parentheses.

Table 2. ATP-sulfurylase activity in extracts of normal and brachymorphic cartilage

Exp.	ATP sulfurylase specific activity C57BL/6J	*bm/bm*
1	3.0 (100)	1.7 (57)
2	3.7 (100)	1.8 (49)

Between 300 and 400 mg of protein was used for incubation, and enzyme activity is expressed as μmol of inorganic phosphate formed per mg of protein per hr at 37°C. Percentage of control values is expressed in parentheses. Experiments 1 and 2 were performed on cartilage extracts from frozen and freshly sacrificed mice, respectively.

three sets each of normal and brachymorphic cartilage prepared from three different litters of freshly sacrificed age-matched mice. The results are shown in Table 1. The differences among the values may be explained by the individual variations and partly by the error of the assay method. The results show a decrease to less than 1/13 in APS kinase in brachymorphic cartilage and are consistent with those obtained from the $^{35}SO_4^{2-}$ incorporation experiments. As shown in Table 2, a decrease to approximately 1/2 in ATP sulfurylase in brachymorphic cartilage extracts was observed with a preparation from frozen as well as from freshly sacrificed mice. This reduction in ATP sulfurylase activity may be a secondary phenomenon, as discussed below. These results demonstrate that the primary defect results from a defect in the enzymic step of PAPS synthesis rather than APS synthesis. However, there still remains a possibility that the defect results from an increased degradation rather than a decreased synthesis of PAPS, because the APS kinase activity was measured by quantitation of newly synthesized PAPS.

DISCUSSION

These results confirm and extend the previous finding of a substantial reduction in sulfation of glycosaminoglycans by extracts of cartilage from brachymorphic mice (3, 4). The defect in the sulfation pathway in the synthesis of PAPS in *bm/bm* cartilage, which was suggested from the previous study (4), has now been localized to a single enzymic step by two different approaches. When the amounts of the three reaction products (APS, PAPS, and chondroitin sulfate) were measured, formation of PAPS and subsequent sulfation of chondroitin sulfate were reduced to less than 15% in mutant cartilage extracts. The conversion of APS to PAPS was nearly quantitative in extracts of normal cartilage, but far less efficient in extracts of brachymorphic mice, suggesting a primary block in the pathway of conversion of APS to PAPS. Presumably because of this reduction in conversion, more APS accumulates in the mutant cartilage extracts. Although more [^{35}S]APS was accumulated in the mutant, this amount was not comparable to the decrease in PAPS synthesized as might be expected if a defect occurred in conversion of APS to PAPS. This is presumably due to a feedback inhibition by APS on its synthesis, because APS is known to strongly inhibit this synthesis (7). In fact, the APS level increased somewhat even though it was utilized far less efficiently in the mutant. In additional experiments, no increased hydrolysis of APS was observed by the mutant when [^{35}S]APS was incubated with cartilage extracts (unpublished observations), suggesting that the reduction in total synthesis of APS does not result from possible degradation.

Percent consumption of newly synthesized PAPS was calculated by the following equation: % consumption of PAPS = 100 × (cpm macromolecular product)/(cpm PAPS + cpm macromolecular product). Less than 6% of newly synthesized PAPS was utilized for sulfation of endogenous acceptor in both

normal and mutant cartilage extracts. In spite of such an apparent excess of PAPS, markedly less radioactivity was incorporated into endogenous acceptors by mutant cartilage extracts (Fig. 1). Presumably, this is not due to lower sulfotransferase activities but rather is due to a decreased PAPS concentration. In the previous study in which comparable levels of sulfotransferase activities were observed in extracts of normal and mutant cartilage (4), the PAPS concentration utilized (9 μM) was more than 1000 times greater than that of PAPS produced by the cartilage extracts in the present study. Concentrations of newly synthesized [^{35}S]PAPS in the experiments shown in Fig. 1 were calculated to be approximately 9 or 0.9 nM in extracts of normal or mutant cartilage, respectively. Therefore, it may be inferred that this 10-fold difference in PAPS concentration at this low level resulted in the difference in sulfation of endogenous chondroitin sulfate, although comparable levels of sulfotransferase activities could be detected in the presence of an excess of PAPS (4).

The defective APS kinase activity was confirmed by measurement of the activities of ATP sulfurylase and APS kinase. The latter enzyme activity was decreased to less than 1/13 in *bm/bm* cartilage, concomitant with the observed reduction of PAPS formation shown in Fig. 1. ATP sulfurylase levels were also consistently found to be lower in mutant tissue than in normals. The reduction in ATP sulfurylase may be a secondary phenomenon due to the inhibition by APS, which presumably accumulates in the mutant tissue. Alternatively, the marked inhibition produced in the PAPS synthetic pathway by the substantial reduction in APS kinase levels may cause a secondary decrease in ATP sulfurylase levels.

The possibility exists that the defect is due to reduced APS kinase activity or the production of an inhibitor. The latter seems unlikely, because in preliminary mixing experiments intermediate activity levels were observed (unpublished observations). It remains to be determined whether there is a reduction in enzyme protein or an abnormal enzyme molecule. Furthermore, it is possible that the apparent decreased APS kinase activity is due to increased degradation of newly synthesized PAPS. However, it is firmly established that the defect in brachymorphic cartilage is due to decreased levels of PAPS synthesis rather than APS synthesis.

PAPS is thought to be an intermediate for synthesis of all naturally occurring sulfated compounds, including sulfated glycoproteins (12), fibronectins (13), sulfatides (14), and glycosaminoglycans. It is, therefore, somewhat surprising that the defect in PAPS synthesis does not result in more severe lesions. However, APS kinase activity was found to be profoundly, but not completely, deficient in brachymorphic mice. Perhaps the PAPS concentration may not be adequate for synthesis of large amounts of highly sulfated proteoglycans as found in cartilage and hence, the primary lesion is expressed as a growth disorder in cartilagenous tissue. In additional experiments, decreased PAPS formation has also been demonstrated in liver extracts of brachymorphic mice, indicating a more widespread distribution of the defective pathway (unpublished observations). However, decreased PAPS formation was not observed in skin extracts, consistent with the previous report of no differences in the electrophoretic properties of glycosaminoglycans from brachymorphic and normal mouse skin (3). These results suggest that there may be two genetically distinct APS kinases.

A defect in biosynthesis of sulfated proteoglycans has thus been found. A disorder in ganglioside biosynthesis in man has been shown to result from a deficient UDP-GalNAc:G_{M3} *N*-acetylgalactosaminyltransferase (15) and represents the only other report of a defect in biosynthesis of a complex carbohydrate-containing macromolecule. The present studies open up a new range of possibilities for study of growth disorders, especially because this defect is primarily expressed in cartilage.

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