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Sanfilippo disease type D: Deficiency of *N*-acetylglucosamine-6-sulfate sulfatase required for heparan sulfate degradation

(mucopolysaccharidosis/keratan sulfate/lysosomes)

HANS KRESSE, EDUARD PASCHKE, KURT VON FIGURA, WALTER GILBERG, AND WALBURGA FUCHS

Institute of Physiological Chemistry, Waldeyerstrasse 15, D-4400 Münster, Federal Republic of Germany

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ABSTRACT **Skin fibroblasts from two patients who had symptoms of the Sanfilippo syndrome (mucopolysaccharidosis III) accumulated excessive amounts of heparan sulfate and were unable to release sulfate from *N*-acetylglucosamine-6-sulfate linkages in heparan sulfate-derived oligosaccharides. Keratan sulfate-derived oligosaccharides bearing the same residue at the nonreducing end and *p*-nitrophenyl-6-sulfo-2-acetamido-2-deoxy-β-D-glucopyranoside were degraded normally. Kinetic differences between the sulfatase activities of normal fibroblasts were found. These observations suggest that *N*-acetylglucosamine-6-sulfate sulfatase activities degrading heparan sulfate and keratan sulfate, respectively, can be distinguished. It is the activity directed toward heparan sulfate that is deficient in these patients; we propose that this deficiency causes Sanfilippo disease type D.**

The Sanfilippo syndrome (mucopolysaccharidosis III) belongs to the group of inborn errors of mucopolysaccharide catabolism. Typically, patients who have this syndrome are mentally more severely retarded than patients who have other types of mucopolysaccharidoses but have rather mild visceral and skeletal abnormalities (1). Biochemically, the Sanfilippo syndrome can be defined as a group of inherited diseases in which the primary defect leads exclusively to impaired degradation of heparan sulfate and heparin. Lysosomal enzymes known thus far to act only on these glycosaminoglycans or their fragments are 2-deoxyglucoside-2-sulfamate sulfatase (EC 3.10.1.1), α-*N*-acetylglucosaminidase (EC 3.2.1.50), and acetyl-CoA:α-glucosaminide *N*-acetyltransferase. The inactivity of one of these three enzymes causes Sanfilippo disease types A (2–4), B (5, 6), and C (7), respectively. In addition, partial degradation of heparan sulfate by mammalian endoglycosidases has been shown to occur (8–10).

Defects of enzymes that hydrolyze linkages common to both heparan sulfate and dermatan sulfate typically produce more striking skeletal and connective tissue involvement than is seen with defects that involve degradation of heparan sulfate alone. Inactivity of α-L-iduronidase (EC 3.2.1.76) occurs in Hurler, Scheie, and Hurler–Scheie diseases (11, 12), inactivity of iduronide-2-sulfate sulfatase occurs in Hunter disease (13, 14), and inactivity of β-glucuronidase (EC 3.2.1.31) occurs in β-glucuronidase deficiency disease (15, 16).

The 6-sulfated *N*-acetylglucosamine residues present in heparan sulfate and heparin are also constituents of keratan sulfate. Defective degradation of keratan sulfate is the biochemical marker of the Morquio syndrome. DiFerrante *et al.* have described a patient who had a phenotype that appeared to be a composite of manifestations of the Morquio and Sanfilippo syndromes and excreted excessive amounts of heparan sulfate and keratan sulfate in the urine. His fibroblasts were unable to desulfate *N*-acetylglucosamine-6-sulfate and the corresponding sugar alcohol (17, 18). It was therefore suggested that *N*-acetylglucosamine-6-sulfate sulfatase is involved in the catabolism of both types of macromolecules.

In this paper, we describe a new disease, tentatively designated Sanfilippo disease type D, that is characterized by the clinical features of the Sanfilippo syndrome, excessive excretion of heparan sulfate, and the inability to release inorganic sulfate from *N*-acetylglucosamine-6-sulfate residues of heparan sulfate-derived oligosaccharides. A normal capability to act on keratan sulfate fragments bearing the same residue at the nonreducing end was found, suggesting that the requirements for enzymatic hydrolysis of *N*-acetylglucosamine-6-sulfate linkages are different for heparan sulfate and keratan sulfate.

MATERIALS AND METHODS

β-*N*-Acetyl-D-hexosaminidase B (2-acetamido-2-deoxy-β-D-hexoside acetamidodeoxyhexohydrolase, EC 3.2.1.52), kindly provided by T. Ludolph of this institute, was purified from human liver by affinity chromatography (19) and isoelectric focusing. α-*N*-Acetylglucosaminidase was prepared from human urine (20).

Preparation of Substrates. *O*-α-D-6-Sulfo-2-acetamido-2-deoxyglucosyl-(1 → 4)-*O*-uronosyl-(1 → 4)-2,5-anhydro-D-[1-^{3}H]mannitol (GlcNAc(6S)-UA-[1-^{3}H]aMan-ol), provided by R. Basner of this institute (21), was prepared from heparan sulfate. *O*-β-D-6-Sulfo-2-acetamido-2-deoxyglucosyl-(1 → 3)-D-[1-^{3}H]galactitol (GlcNAc(6S)-[1-^{3}H]Gal-ol) and *O*-β-D-6-sulfo-2-acetamido-2-deoxyglucosyl-(1 → 3)-*O*-β-D-6-sulfogalactosyl-(1 → 4)-*O*-β-D-6-sulfo-2-acetamido-2-deoxyglucosyl-(1 → 3)-D-[1-^{3}H]galactitol (GlcNAc(6S)-Gal(6S)-GlcNAc(6S)-[1-^{3}H]Gal-ol), were prepared from corneal keratan sulfate by digestion with *endo*-β-D-galactosidase (a generous gift from S. Suzuki, Nagoya, Japan), gel filtration of the digest on a Sephadex G-50 column, and isolation of the monosulfated disaccharide and the trisulfated tetrasaccharide by preparative paper electrophoresis as described (22). The saccharides were reduced with NaB[^{3}H]$_4$ (New England Nu-

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Abbreviations: GlcNAc(6S)-UA-[1-^{3}H]aMan-ol, *O*-α-D-6-sulfo-2-acetamido-2-deoxyglucosyl-(1 → 4)-*O*-uronosyl-(1 → 4)-2,5-anhydro-D-[1-^{3}H]mannitol; GlcNAc(6S)-[1-^{3}H]Gal-ol, *O*-β-D-6-sulfo-2-acetamido-2-deoxyglucosyl-(1 → 3)-D-[1-^{3}H]galactitol; GlcNAc(6S)-Gal(6S)-GlcNAc(6S)-[1-^{3}H]Gal-ol, *O*-β-D-6-sulfo-2-acetamido-2-deoxyglucosyl-(1 → 3)-*O*-β-D-6-sulfogalactosyl-(1 → 4)-*O*-β-D-6-sulfo-2-acetamido-2-deoxyglucosyl-(1 → 3)-D-[1-^{3}H]galactitol; *p*-NP-GlcNAc(6S), *p*-nitrophenyl-6-sulfo-2-acetamido-2-deoxy-β-D-glucopyranoside.

clear) according to ref. 23, and the reduction products were purified on a Dowex AG 1-X2 (200–400 mesh) column by applying a linear NaCl gradient. The disaccharide had a specific radioactivity of 2.4 Ci/mol (1 Ci = 3.7×10^{10} becquerels), and the tetrasaccharide had a specific radioactivity of 3.4 Ci/mol. *p*-Nitrophenyl-6-sulfo-2-acetamido-2-deoxy-β-D-glucopyranoside [*p*-NP-GlcNAc(6S)] was synthesized from the unsulfated compound (Koch-Light) by a direct sulfation procedure (24). Purification was by preparative high-voltage electrophoresis (22) and chromatography on Dowex 50 W-X8 (Na^+ form) to yield a product containing 97% of the theoretical content of sulfate and glucosamine. The esterification of the primary hydroxyl group was verified by proton nuclear magnetic resonance spectroscopy.

Cell Culture. Fibroblasts from the skin of normal individuals or patients affected with mucopolysaccharidoses were maintained in culture as described (25). The first Sanfilippo type D cell line (R.M.) was obtained from a 7-year-old boy of East Indian origin, by the courtesy of P. Leedham, Birmingham, England. The patient is mentally retarded and has characteristic behavioral disturbances. The second cell line (F.P.), provided by P. Durand, Genoa, Italy, was grown from a 4-year-old girl from Sardinia. As the first patient, she shows coarse facies and hirsutism, but she is not mentally retarded. Both patients excrete excessive amounts of heparan sulfate in their urine. Cell lines GM 2243, designated as "glucosamine-6-sulfate sulfatase deficiency," and GM 2407, multiple sulfatase deficiency, were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). For *N*-acetylglucosamine-6-sulfate sulfatase assays, the cells from a confluent flask (75 cm^2) were suspended in 0.5 ml 0.01 M Tris·HCl, pH 7.2/2 mM NaCl and homogenized by ultrasonication.

Assay of *N*-Acetylglucosamine-6-sulfate Sulfatases. When GlcNAc(6S)-UA-[1-^{3}H]aMan-ol was used as substrate, the incubation mixture contained 5 μl of fibroblast homogenate (12–30 μg of cell protein) and 220 pmol of substrate (11,000 cpm) in 50 mM NaOAc, pH 4.0/0.02% NaN_3/0.0015% bovine serum albumin in a final volume of 10 μl. Incubation was for 3–6 hr at 37°C. The rate of product formation was determined as described (21), except that the composition of the assay mixture differed from the protocol because higher acetate and chloride concentrations were found to inhibit the fibroblast enzyme.

When GlcNAc(6S)-Gal(6S)-GlcNAc(6S)-[1-^{3}H]Gal-ol was used as substrate, the incubation mixture contained 3–6 μg of cell protein, mixed for enzyme stabilization with a 4-fold excess of heat-inactivated protein (20 min, 70°C) prepared from the same cell homogenate, 1.6 nmol of substrate (5500 cpm) and 15 mM sodium formate (pH 3.5) in a final volume of 30 μl. After 10 min at 25°C, the reaction was quenched by adding 500 μl of 1 mM NaOH, and the mixture was loaded onto a 1.5-ml Dowex 1-X2 (200–400 mesh) column. The disulfated product was eluted with 8 ml of 0.6 M NaCl, and the remaining substrate was eluted with 5 ml of 1.5 M NaCl. The fractions were mixed with 2 vol Instagel (Packard) and then assayed for radioactivity. In the third sulfatase assay, 25–60 μg of protein and 4.2 nmol of GlcNAc(6S)-[1-^{3}H]Gal-ol (10,000 cpm) were incubated in 16.6 mM sodium formate, pH 4.0/0.013% NaN_3 in a final volume of 30 μl for 6 hr at 37°C. The mixture was then transferred onto a 0.6-ml Dowex 1-X2 column. Unsulfated material was eluted with 5 ml of H_2O, and substrate was eluted with 5 ml of 0.6 M NaCl. Radioactivity measurements were done as above. In all cases, controls were prepared by separately incubating enzyme and substrate at the appropriate pH. They were mixed after addition of the quenching agent. Blank values accounted for 1.5% of the added radioactivity in the case of the heparan sulfate-derived trisaccharide and for 2% and 0.1% of it in the cases of the keratan sulfate-derived tetrasaccharide and disaccharide, respectively. The radioactivity appearing as the reaction product of normal enzymes was ≈500 cpm when the disaccharide served as substrate and ≈1000 cpm in the cases of the other two substrates.

The hydrolysis of *p*-NP-GlcNAc(6S) was followed by incubation of 15–30 μg of cell protein with 1 μmol of substrate in 50 mM NaOAc, pH 4.0/0.01% bovine serum albumin in a final volume of 200 μl for 3–20 min at 37°C. The reaction was stopped by boiling. β-*N*-Acetylhexosaminidase (150 milliunits) in 10 μl of 10 mM sodium phosphate, pH 6.0/0.15 M NaCl was then added, the incubation was continued for 30 min, and 1.0 ml of 0.4 M glycine/NaOH buffer (pH 10.4) was added. One μmol of product, diluted as stated in the assay, would have an absorbance of 15.3 at 405 nm.

Analytical Methods. Lysosomal enzymes—α-L-iduronidase (11), L-iduronide-2-sulfate sulfatase (26), 2-deoxyglucoside-2-sulfamate sulfatase (3), α-*N*-acetylglucosaminidase (20), acetyl-CoA:α-glucosaminide *N*-acetyltransferase (7), *N*-acetylgalactosamine-6-sulfate sulfatase (26), β-galactosidase (20) (EC 3.2.1.23), arylsulfatases A and B (27) (EC 3.1.6.1), β-glucuronidase (20), and β-*N*-acetylglucosaminidase (20)—were measured as described. Glucosamine, galactose, sulfate, and protein were determined as described (28).

Turnover studies of intracellular [^{35}S]mucopolysaccharides and the characterization of the storage material were performed as described (29, 30).

RESULTS

Storage of Heparan Sulfate. The accumulation of [^{35}S]-mucopolysaccharide was investigated in cell line R.M. The cells accumulated excessive amounts of [^{35}S]macromolecules intracellularly. The extent of storage was in the range observed for Sanfilippo fibroblasts of various genotypes (Fig. 1). More than 99% of the [^{35}S]mucopolysaccharide extracted from the cells after a 24-hr chase was resistant to the action of chondroitin

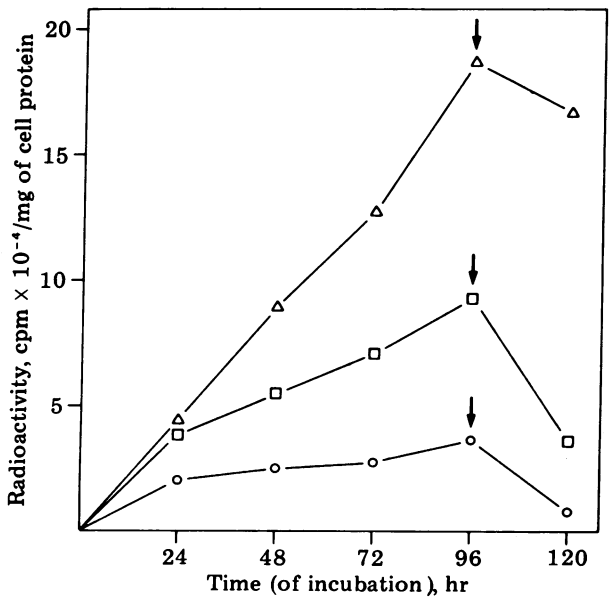

FIG. 1. Accumulation and decay of [^{35}S]sulfated glycosaminoglycans in human skin fibroblasts. Fibroblasts were from patient R.M. (□), from a patient who had Hurler disease (△), and from a normal individual (○). Arrows indicate the time at which the radioactive medium was replaced by a nonradioactive one containing 2 mM Na_2SO_4.

ABC lyase. On a calibrated Sephadex G-50 column, ≈30% and 45% of the stored [^{35}S]mucopolysaccharide were eluted in the void volume of the column and with a mean K_{av} of 0.39, respectively. Both molecular species were extensively degraded by nitrous acid treatment at pH 1.5. Between 1% and 2% appeared as sulfated monosaccharide after nitrous acid degradation at pH 4.5. These findings characterize the storage material as heparan sulfate.

The Enzyme Defect. With the exception of *N*-acetylglucosamine-6-sulfate sulfatase, all exoenzymes known to be involved in the degradation of heparan sulfate (sulfamate sulfatase, α-*N*-acetylglucosaminidase, acetyl-CoA:α-glucosaminide *N*-acetyltransferase, α-L-iduronidase, β-glucuronidase, and iduronide-2-sulfate sulfatase) as well as other glycosaminoglycan-degrading hydrolases (β-*N*-acetylhexosaminidase, arylsulfatase B, *N*-acetylgalactosamine-6-sulfate sulfatase, β-galactosidase) were present at normal levels in the fibroblast homogenates from patients R.M. and F.P. The low molecular weight of the storage material suggests a normal endoglycosidic breakdown of heparan sulfate (10).

When the heparan sulfate-derived oligosaccharide GlcNAc(6S)-UA-[1-^{3}H]aMan-ol was used as substrate, a pronounced sulfatase deficiency was noted. At most, 3% of the mean normal activities were found for both cell lines at all pH values tested (Fig. 2). Mixing experiments excluded the presence of excessive amounts of an endogenous sulfatase inhibitor. The inactivity of this sulfatase should be mirrored by the presence of *N*-acetylglucosamine-6-sulfate or glucosamine-6-sulfate (or both) residues at the nonreducing end of the heparan sulfate chains. Low-molecular-weight heparan sulfate from the fibroblasts of patient R.M., which was labeled in the sulfate and the glucosamine moieties, was partially desulfated by a homogenate of Sanfilippo B fibroblasts (Fig. 3*B*), whereas *N*-acetyl-glucosamine was liberated only after addition of purified α-*N*-acetylglucosaminidase (Fig. 3*D*). Thus, sulfated *N*-acetylglucosamine residues were found in the terminal position of the storage material.

Surprisingly, however, the fibroblasts from both patients

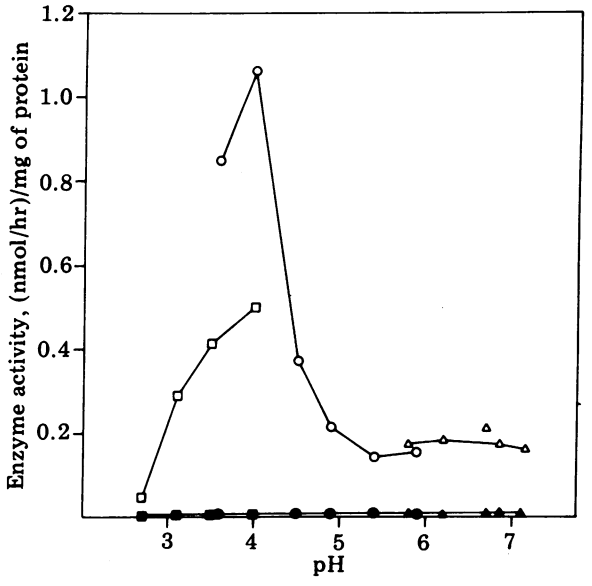

FIG. 2. pH dependence of *N*-acetylglucosamine-6-sulfate sulfatase acting on GlcNAc(6S)-UA-[1-^{3}H]aMan-ol. Fibroblast homogenates from a normal individual (open symbols) and from patient R.M. (closed symbols) were incubated in 50 mM sodium formate (□, ■), 50 mM sodium acetate (○, ●), and 50 mM Tris-maleate·HCl (△, ▲) buffers.

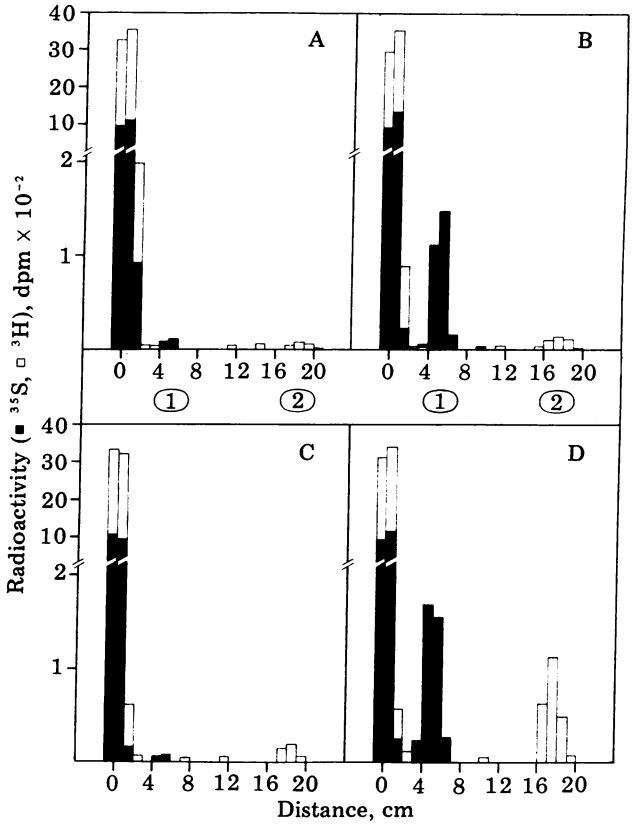

FIG. 3. Evidence for presence of sulfated *N*-acetylglucosamine residues at the nonreducing end of heparan sulfate of cell line R.M. [^{35}S]sulfate- and [6-^{3}H]glucosamine-labeled storage material from this cell line was incubated either with R.M. fibroblasts (*A*), Sanfilippo B fibroblasts (*B*), R.M. fibroblasts plus α-*N*-acetylglucosaminidase (*C*), or Sanfilippo B fibroblasts plus α-*N*-acetylglucosaminidase (*D*). R.M. fibroblasts grown to confluency in a 75 cm^2 flask were incubated for 3 days with $^{35}SO_4$ (15 μCi/ml) and [6-^{3}H]glucosamine (15 μCi/ml; specific activity 38 Ci/mmol). Medium was then replaced by chase medium containing 2 mM Na_2SO_4 and incubation was continued for 24 hr. From the cell extract, heparan sulfate was obtained by chromatography on Ecteola (31) followed by digestion with chondroitin ABC lyase (30) and chromatography on a Sephadex G-50 column in a $NaDodSO_4$-containing buffer (32). Material that had served as substrate was eluted with a mean K_{av} of 0.39 and precipitated by using ethanol with glycogen as carrier. The incubation was performed with 100 μg of cell protein in 50 mM NaOAc buffer, pH 4.0/0.02% NaN_3 at a final volume of 45 μl for 24 hr at 37°C. After boiling, 4 milliunits of α-*N*-acetylglucosaminidase in 25 μl of 10 mM sodium phosphate buffer (pH 6.0) or buffer alone was added. Incubation was continued for 8 hr. The digest was spotted on Whatman 3 MM paper and separated by using 1-butanol/1 M NH_3/HOAc (2:1:3 by volume). Paper segments (1 cm) were eluted with 4 ml of H_2O before radioactivity determinations. Standards were inorganic sulfate ① and *N*-acetylglucosamine ②.

hydrolyzed *N*-acetylglucosamine-6-sulfate linkages at the terminal positions of the keratan sulfate-derived disaccharide and tetrasaccharide to a normal extent (Table 1). Mutant and normal fibroblasts exhibited the same pH optimum found for the trisulfated tetrasaccharide. A synthetic substrate, *p*-NP-GlcNAc(6S), could also be desulfated, as shown by the susceptibility of the reaction product to β-*N*-acetylhexosaminidase action. The enzyme defect, therefore, appears to be restricted to *N*-acetylglucosamine-6-sulfate linkages in heparan sulfate.

In fibroblasts having multiple sulfatase deficiency, the activity toward GlcNAc(6S)-UA-[1-^{3}H]aMan-ol was reduced to a greater extent than those toward the other substrates, whereas

Table 1. *N*-Acetylglucosamine-6-sulfate sulfatase activity against a variety of substrates in homogenates of cultured skin fibroblasts

Subject	Enzyme activity, (nmol product/mg of cell protein)/hr			
	GlcNAc(6S)-UA-[1-^{3}H]aMan-ol*	GlcNAc(6S)-[1-^{3}H]Gal-ol*	GlcNAc(6S)-Gal(6S)-GlcNAc(6S)-[1-^{3}H]Gal-ol†	p-NP-GlcNAc(6S)*
Control (n = 5)	0.33 (0.15–0.55)	0.28 (0.09–0.40)	120 (40–230)	1200 (800–2400)
Sanfilippo D				
R.M.	0.01	0.25	113	1300
F.P.	0.01	0.27	144	1500
GM 2243	0.47	0.19	92	960
Hunter	0.47	0.23	71	1100
Sanfilippo A	0.97	0.36	167	2500
Morquio A	0.24	0.31	120	1500
Maroteaux–Lamy	0.65	0.33	157	1200
Metachromatic leukodystrophy	0.88	0.22	170	2700
Mucolipidosis II	0.07	0.04	15	240
Multiple sulfatase deficiency	0.01	0.14	58	660

* At 37°C.
† At 25°C.

in mucolipidosis II fibroblasts, all the sulfatase activities were reduced to a similar extent. A homogenate of cell line GM 2243, designated by the Human Genetic Mutant Cell Repository as glucosamine-6-sulfate sulfatase deficiency, showed sulfatase activities in the normal range when tested with the substrates used in this study. It was found, however, to contain unmeasurably low sulfamate sulfatase activity, suggesting that the donor of the cells suffered from Sanfilippo disease type A.

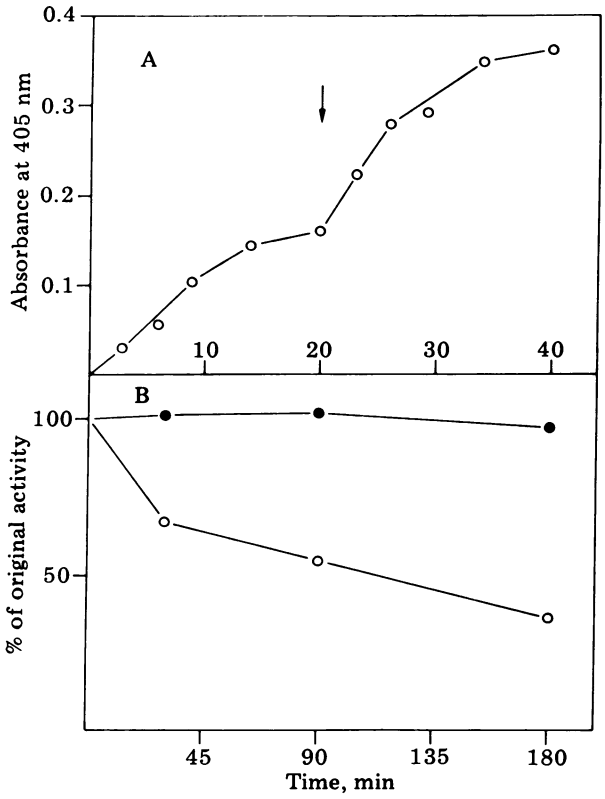

FIG. 4. Time dependence and thermal stability of *N*-acetylglucosamine-6-sulfate sulfatases. (*A*) *p*-NP-GlcNAc(6S) and enzyme (control cell extract) were incubated under standard assay conditions. At the time indicated by the arrow, the amount of enzyme present was doubled by addition of cell extract that had been preincubated at pH 4.0 for 20 min. Corrections were made for blanks and volume changes. (*B*) A homogenate of normal fibroblasts was preincubated at 37°C in 15 mM sodium formate buffer (pH 3.5) for the time indicated. Activity toward GlcNAc(6S)-UA-[1-^{3}H]aMan-ol (●) and GlcNAc(6S)-Gal(6S)-GlcNAc(6S)-[1-^{3}H]Gal-ol (○) was determined as described.

Kinetic Differences Between *N*-Acetylglucosamine-6-sulfate Sulfatase Activities. The sulfatase acting on GlcNAc(6S)-Gal(6S)-GlcNAc(6S)-[1-^{3}H]Gal-ol and *p*-NP-GlcNAc(6S) showed an anomalous kinetic behavior. The initial enzyme activity was proportional to the amount of enzyme protein, but there was no proportionality with time. A preincubation experiment excluded enzyme inactivation and substrate depletion as sole causes of the anomalous kinetics (Fig. 4*A*). Inorganic sulfate had no influence on enzyme activity when added in an amount equimolar to the desulfated product. Short-term incubations could not be performed for the sulfatase acting on GlcNAc(6S)-UA-[1-^{3}H]aMan-ol. Extrapolation of the activity curve to zero time indicated linearity of the reaction. The keratan sulfate-degrading sulfatase was more thermolabile than the heparan sulfate-hydrolyzing sulfatase (Fig. 4*B*).

DISCUSSION

The biochemical findings described in this paper refer to two patients who exhibited typical clinical symptoms of the Sanfilippo syndrome and excreted excessive amounts of heparan sulfate in the urine but had no keratosulfaturia (D. Patrick and P. Durand, personal communications). None of the enzymes responsible for the occurrence of the biochemical subtypes A, B, and C of the Sanfilippo syndrome were affected. Therefore, the patients were tentatively classified as suffering from Sanfilippo disease type D.

The impaired degradation of GlcNAc(6S)-UA-[1-^{3}H]aMan-ol and the enzymatic analysis of the storage material led to the conclusion that the enzyme defect in Sanfilippo D fibroblasts concerns a *N*-acetylglucosamine-6-sulfate sulfatase required for heparan sulfate degradation. The breakdown of keratan sulfate-derived oligosaccharides with *N*-acetylglucosamine-6-sulfate residues at the nonreducing end was normal. The *N*-acetylglucosamine-6-sulfate residues that were not degradable by the patients' fibroblasts are characterized by an α-glycosidic linkage to a uronic acid. Substances susceptible to desulfation had the amino sugar β-glycosidically linked to 6-sulfated galactose, galactitol, or an aromatic aglycon.

Genetic evidence had led to the assumption that a single *N*-acetylglucosamine-6-sulfate sulfatase participates in the degradation of heparan sulfate and keratan sulfate (17, 18). The following findings, however, suggest that the activities directed toward heparan sulfate and toward keratan sulfate are distinct enzymatic properties: (*i*) isolated deficiency of heparan sul-

fate-degrading sulfatase, (*ii*) different residual activities in multiple sulfatase deficiency disorder fibroblasts, (*iii*) anomalous kinetics of the keratan sulfate-degrading enzyme, and (*iv*) differences in the thermostability of the two types of activity. These observations would be compatible with the assumption of two different *N*-acetylglucosamine-6-sulfate sulfatase proteins. Our results, however, do not rule out the possibilities that both enzymes share a common subunit, that both activities reside in the same enzyme protein, or that Sanfilippo disease type D is caused by the deficiency of yet another component required for expression of heparan sulfate-degrading sulfatase activity.

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