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DIFFERENT PROPERTIES OF RESIDUAL N-ACETYLGALACTOSAMINE-6-SULFATE SULFATASE IN FIBROBLASTS FROM PATIENTS WITH MILD AND SEVERE FORMS OF MORQUIO DISEASE TYPE A

Josef Glössl, Pierre Maroteaux, Paola Di Natale, and Hans Kresse

Institute of Physiological Chemistry, University of Münster, D-4400 Münster, Federal Republic of Germany (J.G., H.K.)

Hopital des Enfants Malades, F-75730 Paris, Cedex 15, France (P.M.)

2nd Institute of Biochemistry, 2nd Medical School, University of Naples, I-80131 Naples, Italy (P. Di N.)

Correspondence should be addressed to:

Dr. Josef Glössl, Physiologisch-Chemisches Institut der WWU, Waldeyerstr. 15, D-4400 Münster, FRG

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SUMMARY

The properties of N-acetylgalactosamine-6-sulfate (GalNAc-6-S) sulfatase (EC 3.1.6.?) in normal fibroblasts were compared with those in fibroblasts from patients with different phenotypic expressions of Morquio disease type A (mucopolysaccharidosis IV A). The two patients exhibiting a mild variant of the disorder could be distinguished from the classical form of the disease by the absence of gross dysmorphic features of the face, absence of neurological defects, less reduced body height, and by less pronounced radiological abnormalities.

Using a disulfated trisaccharide prepared from chondroitin-6-sulfate as substrate of GalNAc-6-S sulfatase, the enzyme from normal fibroblasts exhibited a sharp pH optimum at pH 4.0 when assayed in 0.02 M sodium acetate buffer. At this pH the normal range of activity was 13-67 nmole/hour and mg cell protein. The fibroblast enzyme activity of severely affected individuals was about 1 nmole/hour and mg protein with the highest residual activity at pH 3.0 (0.02 M sodium formate buffer). The enzyme of one of the mild variants was maximally active at pH 4.0 with a value of approximately 0.8 nmole/hour and mg protein, whereas that of the other one gave a pH activity curve which was a composite of the latter two profiles. At the respective pH optima the residual activities did not differ significantly. The mutant enzymes had with 15 µmole/l the same Michaelis constant as the normal GalNAc-6-S sulfatase but they were all more thermolabile than their normal counterpart. Complementation after fusion of fibroblasts from different types of patients did not occur.

SPECULATION

The phenotypic expression of Morquio disease type A might depend on the residual activity of GalNAc-6-S sulfatase at the pH which is physiologically reached in lysosomes. This pH is most likely above the pH optimum of the sulfatase. In spite of similar residual activities among the patients that mutation which causes a shift of the pH profile of the enzyme to the more acidic side would lead to a more impaired degradation of storage material. This might cause more severe clinical symptoms than a mutation which does not affect the pH optimum of the enzyme.

Additionally, our results might provide a method to prognosticate the course of the disease.

INTRODUCTION

The Morquio syndrome (mucopolysaccharidosis IV) is a rare inherited disorder of mucopolysaccharide catabolism. It differs from other mucopolysaccharidoses by clinical symptoms such as disproportionate dwarfism, and spondyloepiphysial dysplasia. Affected individuals develop corneal clouding and are of normal intelligence (13, 20). The biochemical hallmark of the Morquio syndrome is an inadequate degradation of keratan sulfate. In most cases the patients excrete elevated amounts of keratan sulfate with their urine (18) and store that polysaccharide in cartilage (19) and liver (14).

Like the Sanfilippo syndrome (mucopolysaccharidosis III), the Morquio syndrome is biochemically heterogenous. The apparently more frequent type A is due to the inactivity of N-acetylgalactosamine-6-sulfate (GalNAc-6-S) sulfatase (4, 10, 12, 19). This sulfatase hydrolyzes sulfate ester bonds present at the nonreducing end of chondroitin-6-sulfate oligosaccharides (7, 10) and might also act on galactose-6-sulfate linkages in keratan sulfate (4). The latter assumption, however, awaits final proof by testing appropriate keratan sulfate-derived oligosaccharides as substrates. Type B has been ascribed to a partial deficiency of acid β-galactosidase (1, 8, 16, 23), an enzyme required for keratan sulfate degradation too (8, 24).

Since a more profound deficiency of β-galactosidase would lead to the clinical picture of G_{M1}-gangliosidosis (15) instead of the Morquio syndrome, it was not surprising that the patients known so far to suffer from Morquio disease type B exhibit rather mild clinical symptoms. These include less reduced body height, almost absent coarsening of facial features and relatively late onset of the first clinical signs (8). In the clinical literature the term "Dale-variant" has been used to describe such patients with a mild course of the disease (20). We report here that GalNAc-6-S sulfatase deficiency may also be associated with a mild clinical variant of the Morquio syndrome. The properties of the residual sulfatase activity can be related to the severity of the disorder.

CLINICAL DESCRIPTION

de S., the first patient considered as suffering from a mild variant of the Morquio syndrome, is a 29 year old female of French origin. She was the only offspring of a nonconsangineous couple. Her disease became obvious at the age of six, but shortness of the trunk and waddling gait were noted at two and four years, respectively. At the age of eleven skeletal radiography gave the following findings: flattened vertebral bodies with tongue-like anterior projections but without hypoplasia of the 12th dorsal and 1st lumbar vertebra; narrow ilia and wide fossae acetabulae; small and flat femoral heads without coxa valga; small and deformed epiphyses of the long and carpal bones; slightly cone-shaped metacarpals.

The present physical examination reveals a female, 136 cm high, with normal facial appearance. Her chest is prominent with pectus carinatum. There is no hepatomegaly. The limbs show moderate genu valgum, pedes plani and limited flexion of hip and knee. Slit-lamp examination reveals a diffuse corneal haze which was not visible at the age of eleven. The neurological examination gave normal results. The long bones are now deformed and the femoral heads dislocated. Qualitative analysis of urinary mucopolysaccharides demonstrated excessive excretion of keratan sulfate. The patient delivered recently a normal baby.

B.B., the second patient with a variant form of the Morquio syndrome, is a 5 year old girl of Italian ancestry who was born from healthy, unrelated parents. Physical examination at birth revealed bilateral mild cubitus valgus, incomplete extension of the knees, enlarged heads of long bones, and flat feet with metatarsus varus and hallux valgus. Her growth was considered normal up to an age of 2 6/12 years. At that time barrel chest and dorsal kyphosis were noted. The radiological examination showed spondyloepiphysial dysplasia consistent with the diagnosis of a variant type of the Morquio syndrome. At the age of four her height was 95 cm (approximately 5th percentile).

The patient appears presently as a pleasant girl with a hight of 101 cm, with very mild prognathism, but without dysmorphic features of the face. She has a very mild barrel chest, mild knock knees, mild metatarsus varus bilaterally, and mild generalized hirsutism. Audiometrical and neurological examinations gave normal results. There is no evidence of corneal clouding. The skeletal radiographic survey shows features of dysostosis multiplex as ovoid shape of the lower dorsal vertebral bodies, incomplete formation of the acetabular roofs, and disturbed ossification of the epiphyses of radius and ulna. The patient excreted excessive amounts of keratan sulfate as determined by qualitative analysis.

Patients with the severe form of Morquio syndrome represented the full clinical and radiological picture of the disorder.

METHODS

Fibroblast cultures from the patients and from normal individuals were obtained from skin biopsies and further propagated as described previously (2, 25). Cell hybridization was induced by treating confluent cultures for 1 minute at room temperature with 50 % (w/w) polyethylene glycol (mol.weight 1400-1600) in Hanks' Balanced Salt Solution according to ref. 3. Between 35 % and 40 % bi- or multinuclear cells were obtained. For enzyme activity determinations cultures grown to confluency in a 25 cm^2 Falcon plastic flask were harvested by trypsinization. The cell pellet was washed twice with 0.15 M NaCl, suspended in 0.2 ml water and homogenized by 10 cycles of freezing and thawing.

The preparation of 6-sulfo-N-acetylgalactosamine-glucuronic acid-6-sulfo-N-acetyl-[1-^{3}H]galactosaminitol as substrate of GalNAc-6-S sulfatase had been described (6). The standard reaction mixture contained in a final volume of 60 µl the following: 0.46 nmol substrate (about 8000 cpm), 1.2 µmol sodium acetate or sodium formate buffer of the respective pH, 18 µg bovine serum albumin (BSA), and 2-3 µg cell protein in case of normal cells and 20-30 µg in case of Morquio fibroblasts, respectively. After 30 min. (normal cells) or 2 h (Morquio fibroblasts) at 37° C, the reaction was quenched by addition of 0.5 ml cold water and the mixture immediately transferred to a 0.6 ml column of unused Dowex 1 x 2, 200-400 mesh, Cl form. Monosulfated product was eluted with 5 ml 0.4 M NaCl, and remaining substrate with 5 ml 0.9 M NaCl. Radioactivity was determined after addition of 10 ml Instagel (Packard). Enzyme activity was calculated from the percentage of radioactivity appearing in the 0.4 M NaCl fraction minus blank value (about 1.6 %).

Other lysosomal enzymes (7) and protein (11) were quantitated as quoted earlier.

RESULTS

Optimal conditions for the measurement of GalNAc-6-S sulfatase activity in fibroblasts were reevaluated since the enzyme purified from human placenta showed different pH optima which depended on the acetate buffer concentration and the stage of purification (7). Reducing the acetate buffer concentration from 0.1 M to 0.02 M and including 0.03 % (w/v) bovine serum albumin in the assay mixture led to a shift of the pH optimum of the enzyme of normal fibroblasts from pH 4.8 (6) to pH 4.0 and at the latter pH to a 40-fold increase of enzyme activity (Tab. I). The previously published protocol (6) therefore has to be replaced.

Under the improved assay conditions the activity in skin fibroblasts from normal individuals was 13-67 nmoles of substrate hydrolyzed per hour and milligram cell protein.

The GalNAc-6-S sulfatase of patients with mild and severe forms of the Morquio syndrome hydrolyzed between 0.2 and 0.8 nmoles per hour and mg cell protein at pH 4.0. The activities of β-galactosidase, β-N-acetylhexosaminidase and N-acetylglucosamine-6-sulfate sulfatase were within normal limits. These results establish the diagnosis of Morquio disease type A.

GalNAc-6-S sulfatase of normal fibroblasts exhibited a sharp pH optimum at pH 4.0 (Fig. 1A), whereas fibroblast homogenates from patients with the severe form of the disorder had the highest residual sulfatase activity between pH 2.6 and pH 3.2. The enzyme of patient de S., however, was again most active at pH 4.0 (Fig. 1B).

Two pH optima at pH 3.0 and pH 4.0 were obtained upon testing cell extracts from patient B.B. (Fig. 1B). At the respective pH optima, significant differences among the residual activities of the mutant enzymes were not observed.

Normal and mutant enzymes had the same affinity towards the trisaccharide substrate. A K_m value of 15 µmol/l was found for the normal sulfatase and for the enzyme from the patients with mild and severe expression of the disease. These measurements were done at pH 4.0 (Fig. 2), and in the case of the latter enzyme additionally at pH 3.0 (result not shown).

Mutant GalNAc-6-S sulfatases were more thermolabile than the enzyme of normal fibroblasts (Fig. 3).

Hybridization of the different Morquio A cell lines failed to result in a measurable increase of GalNAc-6-S sulfatase activity. Enzyme activity was determined during a period of up to 14 days after fusion. Since at least 35 % binuclear cells were obtained, complementation would have easily been detected. Fusion between normal and Morquio A fibroblasts, however, resulted in an increase of enzyme activity by 15-32 % over the mean activity of the respective parental cells.

DISCUSSION

The present report describes for the first time a mild clinical variant of the Morquio syndrome where GalNAc-6-S sulfatase deficiency had been established by specific enzyme assay. It thus appears that a mild form of this disorder (Dale variant) can either be due to GalNAc-6-S sulfatase or to β-galactosidase deficiency.

The following observations suggest that the severe and the mild form (as represented by patient de S.) of Morquio disease type A are caused by different allelic mutations at the GalNAc-6-S sulfatase locus: (i) fusion of fibroblasts from patients with different phenotypes did not result in complementation of the defect, and (ii) the patients could be distinguished by the pH activity profile of the residual GalNAc-6-S sulfatase.

Under optimal conditions, V_{max} and K_m of the mutant enzymes seemed to be similar. This statement, however, is subject to methodological limitations since 7 % of the terminal sulfate groups of the substrate used are esterified with the C-4 instead with the C-6 hydroxyl group of N-acetylgalactosamine (6). Using UDP-N-acetylgalactosamine-4-sulfate as substrate, sulfate release by fibroblasts was optimal at pH 3.3 (5). Therefore it seemed possible that at pH 3.0, which is the pH optimum of GalNAc-6-S sulfatase from severely affected patients, the true residual activity is hidden by simultaneous N-acetylgalactosamine-4-sulfate sulfatase action. Though this cannot rigorously be excluded, it appears to be less likely since more than 10 % of the substrate could be hydrolyzed at pH 3.0 by Morquio fibroblasts, the reaction was linear with time, and the Michaelis constant was similar regardless of which cell line was used as enzyme source.

The existence of different phenotypical expressions of GalNAc-6-S sulfatase deficiency, in spite of similar residual activities, could possibly be explained by taking into account the intralysosomal pH value. Though this pH is not exactly known it might not be lower than pH 4.7 (17) or only one pH unit below that of the cytosol (9). Whatever is considered as correct the intralysosomal pH should be higher than the pH optima of GalNAc-6-S sulfatase. It seems therefore conceivable that a mutant causing a shift of the pH profile to the more acidic side exhibits *in vivo* a lower actual activity than a mutant which has the same pH optimum as the normal enzyme. Caution, however, is required in interpreting the results since our *in vitro* measurements cannot reflect the *in vivo* situation.

Fibroblast extracts of patient B.B. exhibited a pH activity profile of GalNAc-6-S sulfatase which could be considered as a combination of the curves obtained for the enzymes from patient de S. and from severly affected patients, respectively. Whether this patient will develop a phenotype of intermediate severity and therefore represents a genetic compound of the mutations leading either to severe or to mild Morquio disease type A or whether her disorder is caused by another allelic mutation cannot be decided at the present time. It remains also to be seen whether the differences in mutant GalNAc-6-S sulfatases can be used to prognosticate the course of the disease.

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25. Informed consent was obtained from all subjects in this study.

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TABLE I

Measurement of N-acetylgalactosamine-6-sulfate sulfatase activity in normal fibroblast homogenates.

Buffer	Activity of N-acetylgalactosamine-6-sulfate sulfatase[+]
0.1 M sodium acetate buffer, pH 4.0	0.2
pH 4.8	2.5
0.02 M sodium acetate buffer, pH 4.0	14
pH 4.8	6
0.02 M sodium acetate buffer, 0.03 % BSA	
pH 4.0	100
pH 4.8	39

[+]Results are expressed as percent of maximum activity.

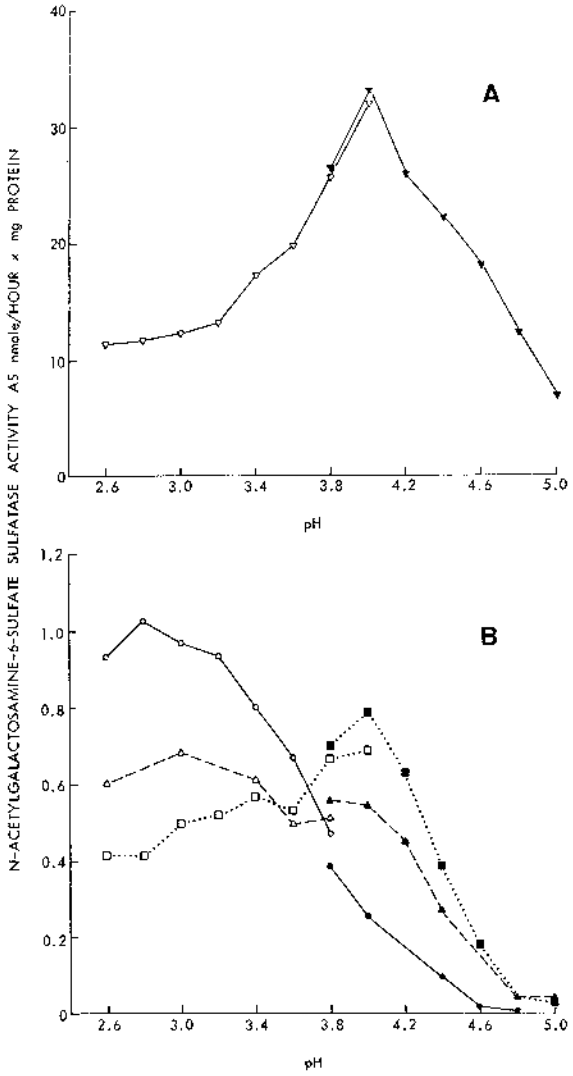

Figure 1:

pH activity profile of GalNAc-6-S sulfatase of normal (▽, ▼) fibroblasts (A) and of fibroblasts from patients de S. (□, ■) and B.B. (△, ▲), and from a patient with severe phenotypic expression of Morquio disease type A (○, ●) (B). Open symbols represent measurement of enzyme activity in 20 mM sodium formate buffer and closed symbols in 20 mM sodium acetate buffer, respectively. The pH values of the buffers added were adjusted to give the pH values indicated for the incubation mixtures.

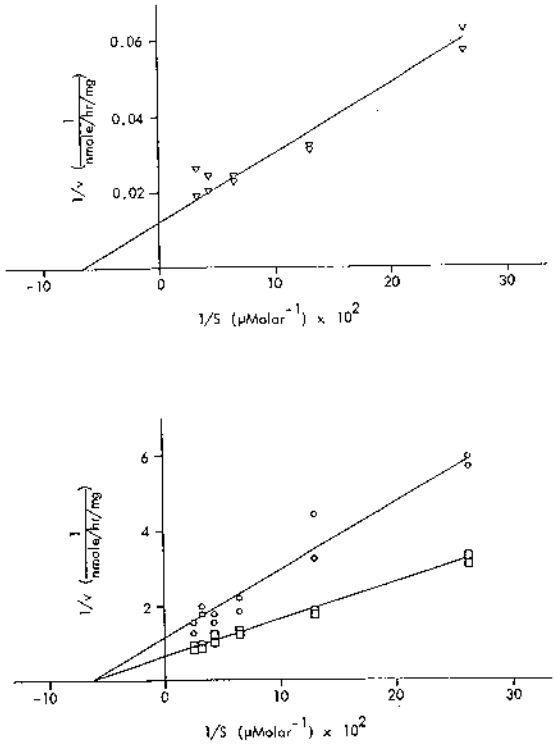

Figure 2:

Determination of K_m and V_{max} of GalNAc-6-S sulfatase. GalNAc-6-S sulfatase activity of normal fibroblasts (▽) and of fibroblasts from patients with mild form (de S., □) and severe form (○) of Morquio disease type A was measured in 20 mM sodium acetate buffer, pH 4.0.

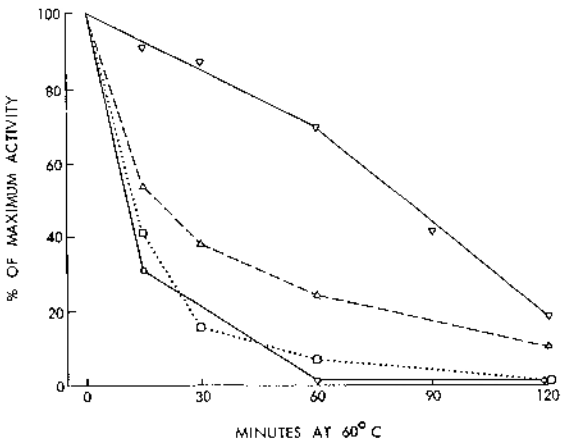

Figure 3:

Heat stability of GalNAc-6-S sulfatase of normal fibroblasts (▽) and of fibroblasts from patients de S. (□), B.B. (△) and from a patient with severe phenotypic expression of Morquio disease type A (○). GalNAc-6-S sulfatase activity was measured at pH 4.0 after preincubation of fibroblast homogenates (1 mg protein/ml H_2O) at 60°C for the times indicated.

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