

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

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SUMMARY

The properties of N-acetylgalactosamine-6-sulfate
(GalNAc-6-S) sulfatase (EC 3.1.6.7) 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 compo-
site of the latter two profiles. At the respective pH optima the
residual activities did not differ significantly. The mutant
enzymes had with 15 μ mole/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 prog-
nosticate 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 spondyloepiphyseal 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 heterogeneous. The apparent-
ly more frequent type A is due to the inactivity of N-acetyl-
galactosamine-6-sulfate (GalNAc-6-S) sulfatase (4, 10, 12, 19).
This sulfatase hydrolyzes sulfate ester bonds present at the non-
reducing 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 GM₁-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 nonconsanguineous
couple. Her disease became obvious at the age of six, but short-
ness 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 muco-
polysaccharides 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 spondyloepiphyseal 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
height of 101 cm, with very mild prognathism, but without dys-
morphic 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 represen-
ted the full clinical and radiological picture of the disorder.

METHODS

Fibroblast cultures from the patients and from normal indi-
viduals 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 cm²
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-[1-³H]galactosaminol as substrate of
GalNAc-6-S sulfatase had been described (6). The standard reaction
mixture contained in a final volume of 60 μ l the following:
0.46 nmol substrate (about 8000 cpm), 1.2 μ mol sodium acetate or
sodium formate buffer of the respective pH, 18 μ g bovine serum
albumin (BSA), and 2-3 μ g cell protein in case of normal cells
and 20-30 μ g 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 per-
centage of radioactivity appearing in the 0.4 M NaCl fraction
minus blank value (about 1.6 %).

Other lysosomal enzymes (7) and protein (11) were quanti-
tated as quoted earlier.

RESULTS

Optimal conditions for the measurement of GalNAc-6-S sulfa-
tase 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 enzymes 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 μ mol/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 severely 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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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.

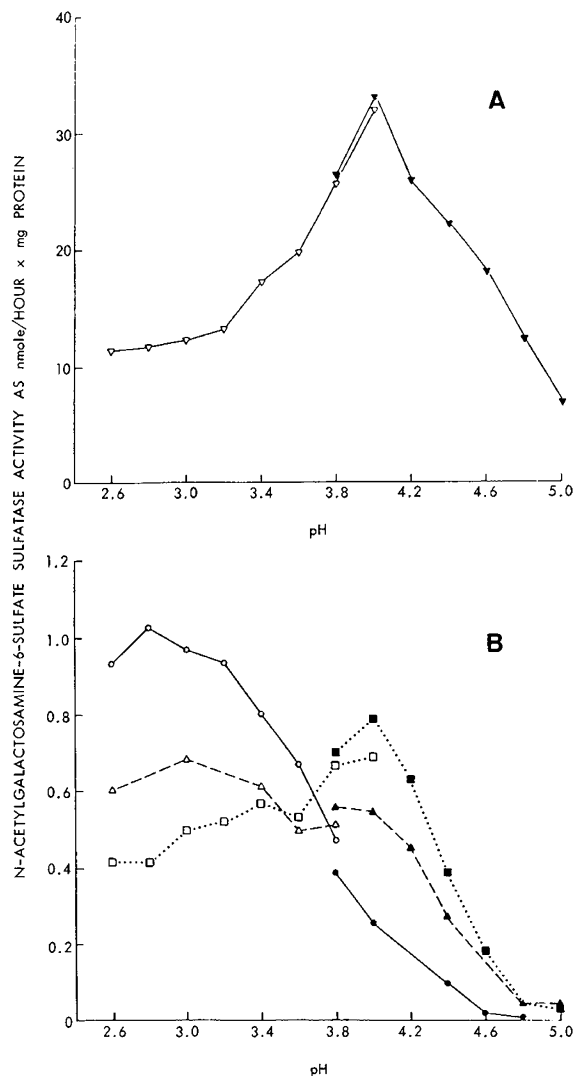


Figure 1:

pH activity profile of GalNAc-6-S sulfatase of normal (∇ , \blacktriangledown) fibroblasts (A) and of fibroblasts from patients de S. (\square , \blacksquare) and B.B. (Δ , \blacktriangle), and from a patient with severe phenotypic expression of Morquio disease type A (\circ , \bullet) (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.

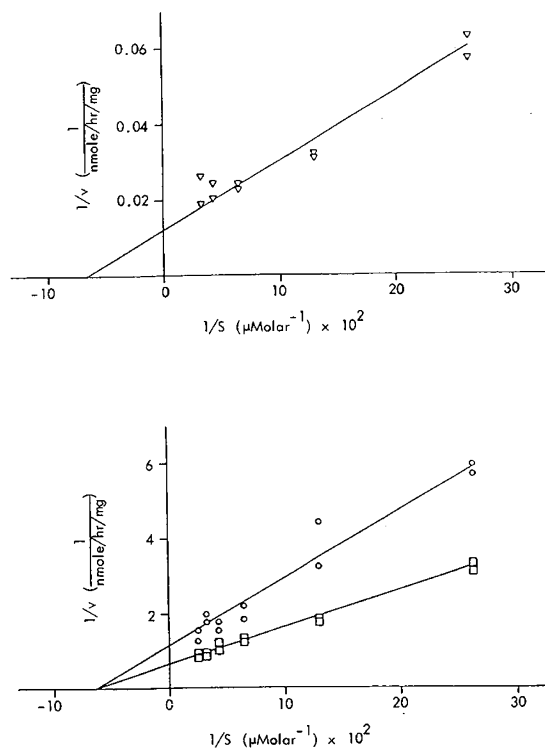


Figure 2:

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., \square) and severe form (\circ) of Morquio disease type A was measured in 20 mM sodium acetate buffer, pH 4.0.

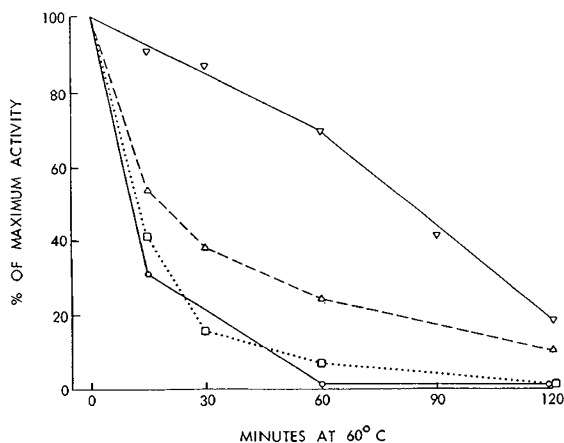


Figure 3:

Heat stability of GalNAc-6-S sulfatase of normal fibroblasts (∇) and of fibroblasts from patients de S. (\square), B.B. (Δ) and from a patient with severe phenotypic expression of Morquio disease type A (\circ). 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.