Enhancement of Residual Arylsulfatase B Activity in Feline Mucopolysaccharidosis VI by Thiol-induced Subunit Association

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ABSTRACT The molecular pathology of the deficient arylsulfatase B activity in feline mucopolysaccharidosis (MPS) VI was investigated. Compared with the highly purified normal feline hepatic enzyme, the purified MPS VI residual activity had a 100-fold higher Michaelis constant (K_m), an altered electrophoretic mobility, half the apparent native molecular weight, and markedly decreased thermo-, cryo-, and pH stabilities. Molecular weight and alkylation studies were consistent with the normal enzyme being a homodimer and the residual MPS VI enzyme a monomer. When incubated with various sulfhydryl reagents, the residual specific activity was enhanced several-fold, whereas the activity of the purified normal enzyme was unaffected or slightly inhibited. In the presence of dithiothreitol (DTT) and cysteamine, a lysosomotropic aminothiol, the residual activity had an electrophoretic mobility and native molecular weight similar to those of the normal feline enzyme. These findings suggested that the monomeric residual enzyme was dimerized in the presence of thiol-reducing agents. To determine if thiol-induced subunit association could therapeutically increase the residual activity and degrade the accumulated dermatan sulfate, in vitro and in vivo experiments were undertaken. When 2 mM DTT or cysteamine was incubated with heparinized whole blood from an MPS VI cat, the leukocyte residual arylsulfatase B activity increased 11- and 20-fold, respectively, and the accumulated dermatan sulfate was degraded in the presence of both thiol reagents. Intravenous administration of DTT (50 mg/kg) effected an immediate, but transient, increase in leukocyte residual activity; however, the substrate levels were not significantly decreased. In contrast, intravenous administration of cysteamine (15 mg/kg) increased leukocyte residual activity more than sixfold 30 min postinfusion; concomitantly, the leukocyte substrate was decreased to 35% of the initial level immediately after infusion and to about 45% of preinfusion values during the 120-min period studied. These results suggest that the defective residual activity in feline MPS VI can be therapeutically manipulated by thiol-induced subunit association. Furthermore, this animal analog provides a prototype for the investigation of human inborn errors of metabolism resulting from enzymatic defects that might be amenable to enzyme manipulation therapy.

INTRODUCTION

Dermatan sulfate mucopolysaccharidosis (MPS VI),[1] a lysosomal storage disease resulting from the deficient activity of arylsulfatase B (ASB; EC 3.1.6.1), has been described in humans (Maroteaux-Lamy disease) (1–4) and, more recently, in Siamese cats (5–7). The feline analog and the human disorder are characterized by severe dysostosis multiplex, "Hurler-like" facial dysmorphia, short stature, corneal opacities, vacuolated lymphocytes and neutrophils, normal neurologic function, and autosomal recessive inheritance (1–7). Although affected human and feline homozygotes have ~6% of normal ASB activity (2, 3, 5–9), the residual activity is insufficient and its pathologic substrate, der-

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[1] *Abbreviations used in this paper:* ASB, arylsulfatase B; DTT, dithiothreitol; MPS mucopolysaccharidosis; *p*NCS, *p*-nitrocatechol sulfate.

TABLE I

Comparative Kinetic and Physical Properties of Highly Purified Arylsulfatase B From Feline MPS VI, Normal Feline, and Normal Human Liver

Property	Feline MPS VI*	Normal feline*	Normal human‡
Specific activity, *nmol/h/mg*	2,750	106,000	800,000
Purification, *-fold*	1,880	2,840	8,400
pH Optimum	5.7	5.7	5.7
Apparent Km, *mM for pNCS*	50	0.5	3.6
Apparent V_{max}, *nmol/h/mg*	1,962	3,967	5,100
Thermostability, $t_{1/2}$ at 60°C, *min*	5	50	30
Electrophoretic mobility, R_m *on PAGE*	0.32	0.74	0.86
pI	—	7.8	8.0
Cryostability, *% initial activity after 72 h, −50°C*	42	85	85
pH Stability range	4.5–7.5	3.5–8.5	3.5–8.5
Molecular weight, Sephadex G-200	53,000	110,000	48,000
SDS PAGE	—	41,000	38,000

* Data summarized from reference 10.

‡ Data summarized from reference 11.

matan sulfate, accumulates in various tissues and fluids (1–7). Because the feline disease provides a unique model for characterization of the enzymatic defect and for the development and evaluation of experimental therapeutic strategies, an improved enzymatic method has been used to identify heterozygotes (9) for efficient breeding of affected animals.

Recently, we purified the residual ASB activity from feline MPS VI liver (10), as well as the normal feline and human hepatic isozymes for comparison.[2] As summarized in Table I, many of the physical and kinetic properties of the normal feline and human enzymes were similar. However, electrophoretic and isoelectric focusing studies revealed the homogeneous feline enzyme to be more electronegative than the homogeneous human isozyme. In addition, molecular weight and subunit alkylation studies were consistent with the normal feline enzyme being a homodimer, whereas the normal human isozyme appeared to be a monomer (10, 11). Intriguingly, the residual ASB, partially purified from feline MPS VI liver, was found to be a monomer that had an apparent K_m value for the synthetic substrate, *p*-nitrocatechol sulfate (*p*NCS), at least 100-fold greater than that for the normal feline enzyme (10).

Because the purified MPS VI residual activity was more cryolabile, thermolabile, and pH sensitive than the normal feline enzyme (Table I), an evaluation of various reagents, including sulfhydryl-reactive compounds, was undertaken in an attempt to increase the stability of the residual enzyme. In this communication, we report the enhancement of the feline MPS VI residual ASB activity by thiol-induced subunit reassociation, as well as the effect of dithiothreitol (DTT) and cysteamine on the residual activity and dermatan sulfate catabolism in the feline model.

METHODS

Specimen collection and materials. Livers from normal and affected Siamese cats were removed immediately after the animals were killed and stored at −50°C. Mixed leukocytes were isolated from heparinized whole blood by dextran sedimentation as described (12). DTT was purchased from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif. Cysteamine, cystamine, iodoacetate, iodoacetamide, *p*-chloromercuribenzoate, *p*NCS, and 1-O-methyl-α-D-glucopyranoside were obtained from Sigma Chemical Co., St. Louis, Mo. Mercaptoethanol was from Eastman Kodak Co., Rochester, N. Y. The Bio-Rad protein assay and materials for polyacrylamide gel electrophoresis were from Bio-Rad Laboratories, Richmond, Calif. Pronase from *Streptomyces griseus* was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Fluorescamine was obtained from Hoffman-LaRoche, Inc., Nutley, N. J. EDTA tetrasodium salt was from Fisher Scientific Co., Fairlawn, N. J., and LiCl was from Mallinckrodt, Inc., Paris, KT. Alcian blue 8GN was obtained from MCB Reagents, Cincinnati, Ohio. DEAE-cellulose was obtained from Whatman, Inc., Clifton, N. J. Concanavalin A-Sepharose, Sephadex G-200, and molecular weight standards (aldolase, bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome C) were obtained from Pharmacia Fine Chemicals, Piscataway, N. J. Cellulose acetate gels (14.5 × 5.5 cm; 250 μm thick) were from Kalex Scientific Co., Inc., Manhasset, N. Y. Ac-

[2] McGovern, M. M., D. T. Vine, M. E. Haskins, and R. J. Desnick. Manuscript in review.

rodisc filters were purchased from Gelman Scientific, Inc., Ann Arbor, Mich. and Amicon ultrafiltration apparatus and membranes were from Amicon Corp., Lexington, Mass. The microzone electrophoretic cell (model R-101) and Duostat power supply for cellulose acetate electrophoresis were obtained from Beckman Instruments, Inc., Fullerton, Calif. All other reagents were of highest grade available.

Enzyme assays. ASB activity was determined as described (9, 10) using the synthetic substrate, *p*NCS. Final substrate concentrations of 5 and 20 mM *p*NCS were used to assay the normal and MPS VI activities, respectively. All assays were performed in triplicate. 1 U of enzymatic activity was equal to that amount of enzyme that hydrolyzed 1 nmol of substrate per hour at 37°C. Protein was determined by the Bio-Rad assay according to the manufacturer's instructions (13).

Purification of hepatic ASB. ASB from normal feline and feline MPS VI livers was purified as described (10, 11), with final sp act of 106,000 and 2,750 U/mg protein, respectively. These highly purified preparations were used to study the effect of various sulfhydryl reagents on their physical and kinetic properties. Other studies were performed on less highly purified hepatic ASB preparations that were isolated by homogenization of normal and MPS VI liver samples (5 g) in 3 vol of 25 mM Tris-HCl buffer, pH 7.5, followed by centrifugation at 27,000 *g* for 45 min. The samples were dialyzed extensively against 25 mM Tris-HCl buffer, pH 7.5, and then chromatographed on DEAE-cellulose columns (20 × 1.5 cm), which had previously been equilibrated with the same buffer. After sample application, the columns were washed with 60 ml of buffer and then a linear NaCl gradient (0–0.3 M) was applied. Fractions (2.5 ml) were collected at a flow rate of 0.4 ml/min and assayed for ASB activity. DEAE-cellulose chromatography resulted in complete separation of ASB from arylsulfatase A. The specific activities of the normal and residual MPS VI preparations were 343.8 and 23.9 U/mg protein, respectively.

Dermatan sulfate quantitation. Dermatan sulfate levels were determined by cellulose acetate electrophoresis according to the procedure of Schuchman and Desnick (14). Briefly, the leukocyte pellets were subjected to pronase digestion (final concentration 0.5 mg/ml) at 60°C for 2 h. Protein was subsequently assayed by the fluorescamine method (15). Aliquots of each sample containing 200 μg of protein were applied to cellulose acetate gels and electrophoresis was performed for 50 min at 4°C (2.5 mA/cm gel width; 15 V/cm gel length) using an electrolyte solution containing 10 mM EDTA tetrasodium salt and 50 mM LiCl at pH 8.4. After electrophoresis, the gels were stained for 1 min in 0.1% alcian blue 8GN at 23°C. Destaining was accomplished by two serial submersions in 100 ml of 2% glacial acetic acid for 5 min each. The dermatan sulfate bands were quantitated by a modification of the procedure described by Kimura et al. (16). Each band was cut out and immersed in 2.0 ml of chloroform/methanol 9:1 (vol/vol), which dissolved the cellulose acetate. The absorbance of the solution was determined spectrophotometrically at 625 nm. For control, blank gel slices of the same size were dissolved and their absorbance determined. The standard curve for dermatan sulfate was linear over a range of 1 to 5 μg.

Effect of sulfhydryl reagents on the physiokinetic properties of purified ASB. Highly purified normal feline and feline MPS VI ASB activities were determined in the presence and absence of various concentrations of sulfhydryl-reactive agents in 25 mM Tris-HCl buffer, pH 7.5. Kinetic studies were performed at pH 7.5, the pH optimum for both enzymes, in the presence and absence of 0.25 mM DTT. Thermostability (at 60°C) and cryostability (after 72 h at −50°C) of the normal and residual enzymes were determined with and without 0.25 mM DTT. The effect of pH (3.5–8.5) on the stability of the normal and MPS VI residual activities was compared by incubating the purified enzymes for 1 h at 37°C in the presence and absence of 0.25 mM DTT in 0.14 M sodium acetate-0.14 M sodium barbital buffer, adjusted to the desired pH with HCl.

Analytical polyacrylamide gel electrophoresis was performed in 7% disc gels (0.5 × 7.0 cm) in 100 mM β-alanine-acetate buffer at pH 4.0 as described by Reisfeld et al. (17). Partially purified normal feline and feline MPS VI ASB were electrophoresed in the presence and absence of 1.0 mM DTT at a constant current of 4 mA/gel for 3 h or until the tracking dye reached the bottom of the tube. The gels were stained for ASB activity using *p*NCS as described (10). Apparent molecular weight values for each enzyme were determined in the presence and absence of 1.0 mM DTT on 5, 6, 7, and 8% native polyacrylamide gels according to the method of Hendrick and Smith (18). Aldolase (mol wt 158,000), bovine serum albumin (68,000), chymotrypsinogen (24,000), and cytochrome C (12,400) were used as standards. Apparent molecular weights were also estimated by gel filtration on Sephadex G-200 using a 1.5 × 100-cm column in the presence and absence of 1.0 mM DTT in the enzyme sample and elution buffer. Aldolase, bovine serum albumin, ovalbumin (mol wt 45,000), and chymotrypsinogen were used as standards.

In vitro studies. The effect of DTT, cysteamine, and cystamine on ASB activity and dermatan sulfate levels was determined in leukocytes isolated from 30 ml of heparinized whole blood obtained from normal and MPS VI cats. Varying concentrations of each sulfhydryl reagent, freshly prepared in 25 mM Tris-HCl buffer, pH 7.4, was added to 5-ml aliquots of whole blood in 13 × 100-mm screw-cap tubes. The tubes were sealed after gassing with N_2 and the suspensions were gently mixed on a rocking platform for 1 h at 37°C. Then the leukocytes were isolated by dextran sedimentation, washed twice with 0.15 M NaCl, suspended in 0.5 ml of distilled water, and lysed by three cycles of freeze/thaw (−70/37°C). Aliquots (50 μl) were removed from each leukocyte homogenate for determination of the dermatan sulfate concentrations and the remainder of the homogenate was centrifuged at 10,000 *g* for 10 min. The supernatant was removed, arylsulfatase A and ASB were separated by batch DEAE-cellulose chromatography as described (9), and the ASB activities and protein concentrations were determined.

In vivo studies. DTT (50 mg/kg) or cysteamine (15 mg/kg) was dissolved in 10 ml of sterile 0.15 M NaCl and passed through an Acrodisc filter (0.2-micron pore size) immediately before use. DTT or cysteamine was intravenously administered over 10 min into an 18-mo-old MPS VI female (2.27 kg) cat and her normal female sibling (3.27 kg). Heparinized blood samples (5 ml) were obtained immediately before and at 0-, 30-, 60-, and 120-min intervals after infusion. ASB activity and dermatan sulfate levels were determined in isolated leukocytes as described above. The volume of blood drawn for these studies precluded the assessment of multiple experimental infusions.

RESULTS

Effect of sulfhydryl reagents on highly purified normal and MPS VI ASB. Table II summarizes the effect of varying concentrations of selected sulfhydryl-reactive compounds on the highly purified normal fe-

line and feline MPS VI residual ASB activities. The thiol-reducing compounds, DTT and cysteamine, had a marked stimulatory effect on the residual activity. For example, DTT, at concentrations ranging from 0.025 to 0.25 mM, increased the MPS VI residual hepatic activity about fourfold; at higher DTT concentrations less stimulation was obtained. In marked contrast, DTT had no effect or slightly inhibited the purified normal enzyme. In addition, similar concentrations of alkylating or other reducing agents consistently resulted in stimulation of the residual activity, whereas the normal enzyme was not affected or was slightly inhibited. The greatest enhancement was obtained with 0.25 mM DTT and 0.25 mM cysteamine (4- and 12-fold, respectively). The inhibitory effect of EDTA on the residual activity (Table II) indicated that the depletion of divalent cations was not the mechanism responsible for residual enzyme stimulation.

The effect of DTT on the physical and kinetic properties of the highly purified normal feline and feline MPS VI hepatic ASB enzymes is summarized in Table III. DTT had essentially no effect on all the properties of the normal feline enzyme studied. However, DTT altered the specific activity, apparent V_{max}, electrophoretic mobility, and apparent native molecular weight values of the highly purified MPS VI residual activity. Although the apparent K_m value of the residual activity was unchanged in the presence of DTT, this thiol-reducing agent increased the apparent V_{max} about threefold (Fig. 1). Analytical polyacrylamide gel electrophoresis of the residual feline activity in the presence of 1.0 mM DTT (Fig. 2) or 1.0 mM cysteamine (data not shown) resulted in the migration of the residual ASB to a position similar to that of the normal enzyme, which was not changed by the presence of DTT. DTT had no effect on the altered thermo-, cryo-, or pH-stabilities of the residual MPS VI enzyme.

Most intriguingly, the apparent native molecular weight values of the residual enzyme were essentially the same as those of the normal enzyme when estimated in the presence of 1.0 mM DTT by gel filtration or by analytical polyacrylamide gel electrophoresis using increasing acrylamide concentrations according to the procedure of Hendrick and Smith (18). Analysis of the latter studies indicated that the two native enzymes had the same apparent molecular weight values (Fig. 3B) and similar, but not identical charges in the

TABLE II

Effect of Various Thiol Reagents and EDTA on Highly Purified Normal Feline and Feline MPS VI Arylsulfatase B Activities

Thiol reagent	Concentration	Normal feline	Feline MPS VI
	mM	*% Initial activity**	
None		100	100
DTT	0.025	102	411
	0.25	90	420
	2.5	77	154
Cysteamine	0.025	96	270
	0.25	95	1200
	2.5	81	800
Mercaptoethanol	0.25	86	197
p-Chloromercuribenzoate	0.25	92	152
Iodoacetate	0.25	78	152
Iodoacetamide	0.25	82	147
EDTA	5	92	74
	250	87	62
	500	88	57

* Initial activities of the normal feline and feline MPS VI residual enzymes were 313.1 and 20.3 nmol/h per ml, respectively. An aliquot of each enzyme (0.1 ml) was mixed with each sulfhydryl reagent in 25 mM Tris-HCl buffer, pH 7.5, to achieve the indicated final concentration. The solutions were gassed with nitrogen, mixed for 10 min, and then assayed by the standard procedure.

TABLE III

Effect of DTT on the Properties of Highly Purified Normal and MPS VI Residual Arylsulfatase B Activities

Property	Feline MPS VI	Feline MPS VI +DTT*	Normal feline ±DTT*
Electrophoretic mobility, R_m *on PAGE*	0.32	0.65	0.74
Cryostability, *% initial activity after 72 h, −50°C*	42	43	85
Thermostability, $t_{1/2}$ *at 60°C; min*	5	5	50
pH Stability range	4.5–7.5	4.5–7.5	3.5–8.5
K_m, *mM for pNCS*	50	50	0.5
V_{max}, *nmol/h/mg*	1,962	5,880	3,967
Molecular weight, Sephadex G-200	53,000	100,000	110,000
Native PAGE	80,000	166,000	166,000

* The physical and kinetic properties were determined in the absence and presence of 0.25 mM DTT with the exception of the molecular weight studies, which were performed with and without 1.0 mM DTT. The specific activities of the normal and residual MPS VI preparations used for the kinetic studies were 106,000 and 2,750 U/mg protein, respectively.

presence of DTT (Fig. 3A). In the absence of DTT, the molecular weight data were consistent with the residual MPS VI enzyme having approximately half the apparent molecular weight of the normal enzyme; additionally, in the absence of the thiol-reducing agent, the untreated enzymes were charge isomers. The molecular weight of the normal enzyme was estimated at 110,000 by Sephadex G-200 chromatography in the absence of DTT, while that of the mutant was 53,000. When 1.0 mM DTT was added to the highly purified enzyme sample and to the elution buffer, the molecular weight of the residual ASB was increased to 100,000, whereas that of the normal feline enzyme remained unchanged. In the presence of 1.0 mM cysteamine, the residual enzyme also had an estimated mol wt of 100,000 by gel filtration.

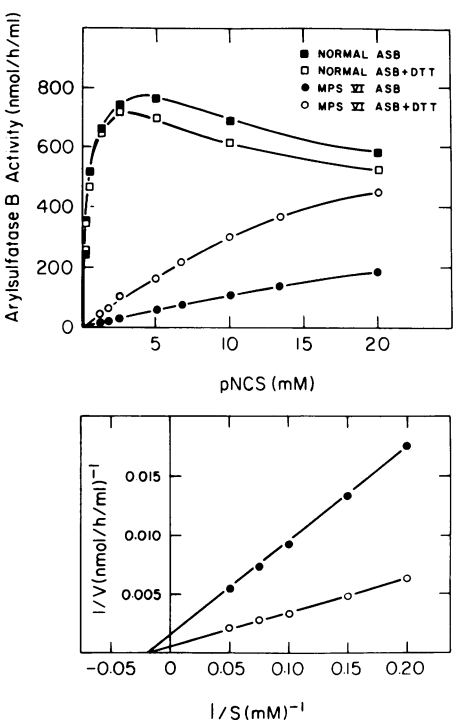

FIGURE 1 Effect of substrate concentration on normal and feline MPS VI hepatic ASB activities ±DTT. (upper panel) Velocity vs. pNCS concentration. (lower panel) Lineweaver-Burk plots. (● —— ● MPS VI ASB; ○ —— ○ MPS VI ASB + DTT)

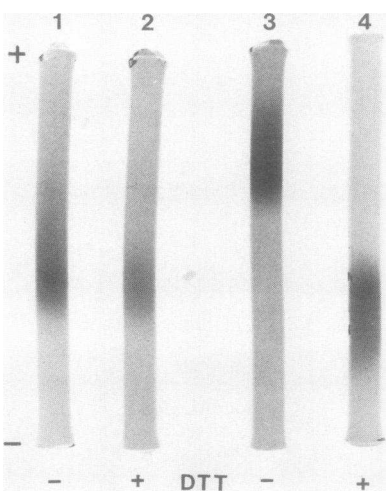

FIGURE 2 Analytical polyacrylamide gel electrophoresis of purified normal and feline MPS VI hepatic ASB±DTT. Gels 1 and 2, normal feline ASB in the absence and presence of 1.0 mM DTT, respectively. Gels 3 and 4, feline MPS VI ASB in the absence and presence of DTT, respectively. Samples were applied at the anode.

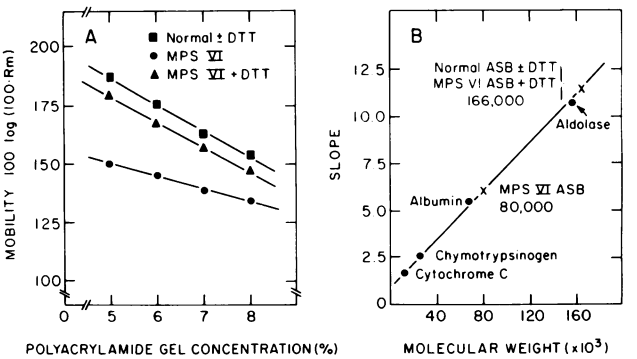

FIGURE 3 Estimation of the native molecular weights of normal and feline MPS VI hepatic ASB±DTT by polyacrylamide gel electrophoresis (18). (A) The effect of different gel concentrations on the mobility of normal feline and feline MPS VI ASB±DTT. Note that the slopes of the normal enzyme ±DTT and the MPS VI enzyme+DTT are parallel indicating that they have the same molecular weights, but are charge isomers. The slope and mobility of the MPS VI enzyme − DTT is consistent with a different molecular weight and charge. (B) The indicated molecular weight estimates were determined from the slope–molecular weight relationship for the feline enzymes ±DTT compared to protein standards (18). The feline enzymes ±DTT are indicated (X). See text for details.

In vitro effect of thiol-reducing agents on MPS VI residual ASB. Tables IV and V summarize the changes in leukocyte ASB activity and dermatan sulfate concentration following incubation of whole blood samples from an 18-mo-old affected female cat and her normal female sibling with various concentrations of DTT and cysteamine (0.1–5 mM) for 1 h at 37°C. The MPS VI leukocyte residual activity was increased up to 11- and 20-fold with DTT and cysteamine, respectively. Maximal stimulation of leukocyte residual activity occurred when either thiol reagent concentration in blood was 2 mM. The dermatan sulfate concentration in MPS VI leukocytes was markedly decreased (~5% of initial) or nondetectable at all concentrations of both sulfhydryl-reducing agents. A comparable increase in residual leukocyte activity and decrease in dermatan sulfate concentration was observed when whole blood was incubated with 5.0 mM cystamine. In contrast, none of these reagents had a significant effect on the ASB activity in normal leukocytes.

In vivo effect of thiol-reducing agents on MPS VI ASB. Intravenous infusions of DTT (50 mg/kg) or cysteamine (15 mg/kg) were well tolerated by the animals. Heart and respiratory rates, as well as glucose and hemoglobin levels, remained stable throughout the infusions. No vomiting or seizure activity was observed.

Table VI compares the ASB activities and dermatan sulfate concentrations in leukocytes from the normal and MPS VI cats before and after intravenous administration of DTT. Immediately following infusion of DTT, the MPS VI residual ASB activity in leukocytes

TABLE IV
Effect of DTT on Normal and MPS VI Feline Leukocyte Arylsulfatase B Activities and Dermatan Sulfate Concentrations In Vitro

Concentration	Normal	MPS VI	Normal	MPS VI
mM	*% Initial ASB activity**		*% Initial dermatan sulfate level‡*	
None	100	100	ND	100
0.1	100	170	ND	ND
1.0	110	510	ND	ND
2.0	110	1120	ND	ND
5.0	110	810	ND	ND

Heparinized whole blood (30 ml) from normal and MPS VI cats was incubated with various concentrations of DTT for 1 h at 37°C. The leukocytes were isolated, lysed in distilled water, and ASB activity and dermatan sulfate concentrations were determined as described in Methods.

* Initial ASB sp act were 341.9 and 49.2 nmol/h per mg protein in the normal and MPS VI leukocytes, respectively.

‡ Initial dermatan sulfate concentration was 145 μg/mg protein in MPS VI leukocytes. No dermatan sulfate was detected (ND) in normal feline leukocytes within the sensitivity of the assay (<0.01 μg/mg protein).

TABLE V
Effect of Cysteamine and Cystamine on Normal and MPS VI Feline Leukocyte Arylsulfatase B Activities and Dermatan Sulfate Concentrations In Vitro

Thiol reagent	Concentration	Normal	MPS VI	Normal	MPS VI
	mM	*% Initial ASB activity**		*% Initial dermatan sulfate level‡*	
None	—	100	100	ND	100
Cysteamine	0.1	100	600	ND	ND
	1.0	130	1,580	ND	ND
	2.0	120	2,070	ND	5
	5.0	110	1,680	ND	5
Cystamine	5.0	110	1,820	ND	ND

Heparinized whole blood (30 ml) from normal and MPS VI cats was incubated with various concentrations of cysteamine or with 5.0 mM cystamine for 1 h at 37°C. The leukocytes were isolated, lysed in distilled water, and ASB activity and dermatan sulfate concentrations were determined as described in Methods.

* Initial ASB sp act were 268 and 16 nmol/h per mg protein in the normal and MPS VI leukocytes, respectively.

‡ Initial dermatan sulfate concentration was 105 μg/mg protein in MPS VI leukocytes. No dermatan sulfate was detectable (ND) in normal feline leukocytes.

TABLE VI

Effect of Intravenous Infusion of DTT on Normal and MPS VI Feline Leukocyte Arylsulfatase B Activities and Dermatan Sulfate Concentrations

Time	Normal	MPS VI	Normal	MPS VI
min	% Preinfusion ASB activity*		% Preinfusion dermatan sulfate level‡	
Pre	100	100	ND	100
0	96	490	ND	73
30	94	140	ND	92
60	102	120	ND	100
120	98	140	ND	100

DTT (50 mg/kg) was administered intravenously over 10 min into an 18-mo-old MPS VI female cat (2.27 kg) and her normal sibling (3.27 kg). Heparinized blood samples were obtained immediately before and after infusion (0, 30, 60, and 120 min). ASB activity and dermatan sulfate levels were determined in isolated leukocytes as described in Methods.

* Preinfusion ASB sp act were 758 and 14 nmol/h per mg protein in the normal and MPS VI leukocytes, respectively.

‡ Preinfusion dermatan sulfate concentration was 64.0 μg/mg protein in MPS VI leukocytes. No dermatan sulfate was detected (ND) in the normal feline leukocytes.

TABLE VII

Effect of Intravenous Infusion of Cysteamine on Normal and MPS VI Feline Leukocyte Arylsulfatase B Activities and Dermatan Sulfate Concentrations

Time	Normal	MPS VI	Normal	MPS VI
min	% Preinfusion ASB activity*		% Preinfusion dermatan sulfate level‡	
Pre	100	100	ND	100
0	111	430	ND	34
30	98	680	ND	39
60	86	116	ND	46
120	70	98	ND	46

Cysteamine (15 mg/kg) was administered intravenously over 10 min into an 18-mo-old MPS VI female cat (2.27 kg) and her normal sibling (3.27 kg). Heparinized blood samples were obtained immediately before and after infusion (0, 30, 60, and 120 min). ASB activity and dermatan sulfate levels were determined in isolated leukocytes as described in Methods.

* Preinfusion ASB sp act were 705 and 12 nmol/h per mg protein in the normal and MPS VI leukocytes, respectively.

‡ Preinfusion dermatan sulfate concentration was 147 μg/mg protein in the MPS VI leukocytes. No dermatan sulfate was detected (ND) in normal feline leukocytes.

was increased almost fivefold. A transient decrease occurred in the dermatan sulfate level at zero time, returning to preinfusion values at 30 min.

When cysteamine was intravenously infused (Table VII), the residual activity was increased more than fourfold immediately after infusion, increasing to almost sevenfold at 30 min, and then returning to preinfusion levels by 60 min. Dermatan sulfate concentrations in peripheral leukocytes were decreased to ~35% of preinfusion levels after infusion and remained at ~45% of initial levels for at least 120 min. In contrast, cysteamine had an inhibitory, if any, effect on normal ASB activity; dermatan sulfate in normal feline leukocytes remained undetectable.

DISCUSSION

Animal models of human inborn errors of metabolism provide the opportunity to characterize the molecular nature of the enzymatic defect, and based on these studies, to rationally design and evaluate various therapeutic strategies before human trials. Using this approach, the physical and kinetic properties of highly purified hepatic residual ASB from the MPS VI cats were determined. Compared with the purified normal feline hepatic enzyme, the MPS VI residual activity had a 100-fold higher K_m, an altered electrophoretic mobility and half the native molecular weight. Notably, the residual enzyme was more thermo-, cryo-, and pH-labile, particularly when highly purified, which prompted efforts to stabilize the enzyme. When the thiol-reducing reagent, DTT, was added to the highly purified residual enzyme, its activity was increased severalfold (Table II). This observation stimulated the evaluation of other sulfhydryl-reactive agents. Cysteamine, a strong reducing agent that also is lysosomotropic (19), markedly increased the residual activity whereas the alkylating and mercurial reagents were stimulatory, but to a lesser degree. Because these reagents had no effect or slightly inhibited the normal ASB activity, an investigation was undertaken to determine the effect of DTT, the prototype reducing agent, on the physical and kinetic properties of the purified normal feline and MPS VI residual activities.

Intriguingly, in the presence of DTT, the residual activity had an increased V_{max} value and an electrophoretic mobility and native molecular weight similar to those of the normal enzyme (Figs. 1–3). In the presence of cysteamine, the native molecular weight of the residual activity also was similar to that of the normal enzyme. Previous studies of the purified native and denatured enzymes indicated that the MPS VI residual activity was a monomer, whereas the normal feline enyzme was a homodimer (10). Thus, the findings reported here suggest that DTT and cysteamine facilitated the dimerization of the residual enzyme. However, DTT did not alter the apparent K_m or stability of the dimerized residual activity.

The following model is proposed to explain the effect of the thiol-reducing agents on the MPS VI resid-

ual enzyme (Fig. 4). In the normal feline enzyme, spontaneous dimerization of the isologous subunits occurs to form the active homodimer. In feline MPS VI, we suggest that a point mutation in the structural gene results in the substitution of a cysteinyl residue at or near the substrate binding site—or a conformational change in the protein occurs due to a different amino acid substitution that alters substrate binding and, in addition, exposes a normally unavailable cysteine. This substituted or exposed cysteine forms an intramolecular disulfide bridge with another cysteinyl residue. The formation of this disulfide bridge causes a further conformational change that prevents subunit dimerization. In the presence of the thiol-reducing reagents, DTT and cysteamine, the disulfide bond is reduced, permitting subunit association as well as normalizing several other properties of the MPS VI enzyme (Table III).

Because DTT and cysteamine have been safely administered as experimental therapeutic agents in patients with cystinosis (19–25), the therapeutic use of these compounds was evaluated in the feline model. Initially, in vitro studies were undertaken to determine if thiol-induced dimerization could enhance the MPS VI residual ASB activity in leukocytes and catabolize the accumulated substrate. After incubation of fresh heparinized whole blood with DTT or cysteamine, the leukocyte residual ASB activity was increased up to 11- and 20-fold, respectively, and, most importantly, the accumulated dermatan sulfate was degraded (Tables IV and V). Based on these encouraging in vitro results, in vivo trials were conducted. Intravenously administered DTT resulted in an immediate, but transient increase in leukocyte residual ASB activity and had little, if any, effect on the leukocyte dermatan sulfate levels (Table VI). In contrast, cysteamine infusion not only enhanced the residual leukocyte activity for at least 1 h, but also resulted in the clearance of leukocyte dermatan sulfate; the accumulated substrate was reduced to 35% of the preinfusion level immediately after administration and remained at about 45% of the preinfusion level for the 120-min period studied. The differential effectiveness of these thiol-reducing reagents may have been due to the rapid inactivation (i.e., oxidation, plasma clearance, etc.) of DTT, whereas cysteamine, an aminothiol, may have been protected by its preferential uptake by lysosomes (19). The effectiveness of cysteamine and the fact that cystamine (the disulfide of cysteamine) enhanced the residual leukocyte activity in vitro (Table V) suggest that the disulfide may be of therapeutic value because it is reduced to cysteamine presumably by glutathione or other reducing agents (19). In contrast to cysteamine, cystamine is odorless and colorless, which should facilitate its palatable inclusion in the feline diet.

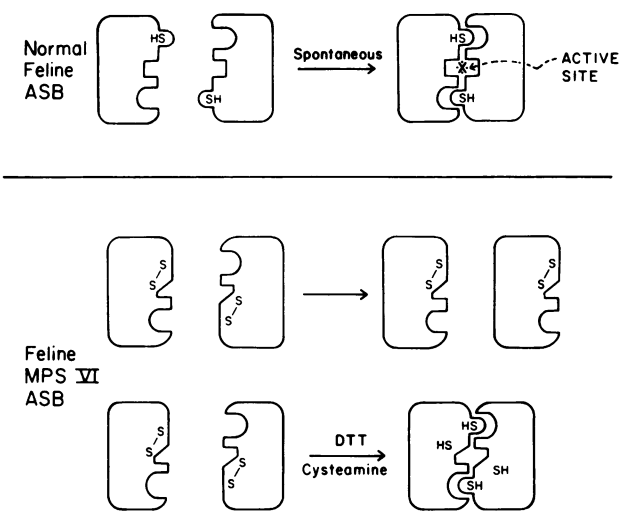

FIGURE 4 Schematic model for the thiol-induced dimerization of feline MPS VI residual ASB activity. (upper panel) The normal isologous subunits spontaneously dimerize to form the active homodimer. (center panel) Due to the structural gene mutation, a substituted cysteine or previously unavailable cysteinyl residue at or near the substrate binding site forms a disulfide bridge with another cysteine causing a further conformational change which prevents subunit dimerization. (lower panel) In the presence of DTT or cysteamine the disulfide bond is reduced allowing subunit association to occur, with a concomitant enhancement of residual ASB activity. See Discussion for details.

In summary, these studies provide the prototype for the treatment of inborn errors by enhancement of the residual activity via subunit reassociation. This approach may be useful in the design of therapeutic endeavors in human diseases in which the enzymatic defect results from mutations that alter subunit association and enzyme function. Furthermore, these findings emphasize the value of characterizing the nature of the enzymatic defect, particularly in disorders with residual enzymatic activity, for the design of novel strategies to manipulate and therapeutically enhance the function and/or stability of the defective enzyme.

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