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Feline Mucopolysaccharidosis VI: Purification and Characterization of the Residual Arylsulfatase B Activity

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SUMMARY

Hepatic arylsulfatase B (ASB) from normal and mucopolysaccharidosis VI (MPS VI) cats was purified over 2,800- and 1,800-fold, respectively, and their physical and kinetic properties were characterized. In contrast to the normal feline enzyme, the partially purified MPS VI residual activity had a 100-fold greater K_m value and was markedly less stable to thermal, cryo-, and pH-inactivation. In addition, the MPS VI enzyme had a more negative charge as determined by its migration on polyacrylamide gel electrophoresis and its elution profile on cation exchange chromatography. Finally, the MPS VI activity had approximately half the apparent molecular weight of the normal feline enzyme, which was a homodimer, suggesting that the genetic mutation in feline MPS VI altered the subunit association as well as the kinetic and stability properties of the mutant protein.

INTRODUCTION

Recently, an animal analog of human mucopolysaccharidosis VI (MPS VI, Maroteaux-Lamy disease) was described in Siamese cats [1]. The clinical phenotype of the feline disease was similar to that of the human disorder including a

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typical facial dysmorphia, corneal opacities, vacuolated neutrophils and lymphocytes, dysostosis multiplex, and normal neural function [1–4]. Affected cats accumulated dermatan sulfate in their tissues and urine because of the deficient activity of the lysosomal hydrolase, arylsulfatase B (ASB) (E.C.3.1.6.1) [1]. Analogous to the human disorder [5–8], 6%–10% of normal ASB activity was present in the MPS VI cats [1, 4]. Therefore, we purified and characterized the residual hepatic activity and compared its physical and kinetic properties to those of ASB purified from normal feline liver.

MATERIALS AND METHODS

Specimen Collection

Livers from normal Siamese cats and from a cat with MPS VI were removed, frozen, and stored at −50°C immediately after the animals were sacrificed.

Enzyme Assays

Arylsulfatase A (ASA) and ASB activities in crude liver homogenates were determined by the method of Baum et al. [9] using p-nitrocatechol sulfate (pNCS) (Sigma, St. Louis, Mo.) as substrate. Following separation of the ASB from ASA by DEAE-cellulose batch chromatography, the Roy procedure [10] was used with the following modifications. Enzyme source (0.2 ml) was incubated with 0.2 ml of pNCS (10 mM for the normal enzyme; 40 mM for the mutant residual activity) in 0.5 M sodium acetate, pH 5.7, for 1 hr at 37°C. The reaction was terminated by the addition of 0.1 ml of 2 N NaOH, and the adsorbance was determined at 515 nm using a Gilford Model 2400 spectrophotometer. All assays were performed in duplicate. One unit (U) of enzyme hydrolyzed 1 nmol of substrate per hr at 37°C. Protein was determined using the Bio-Rad assay according to the manufacturer's instructions [11].

Purification of ASB

Hepatic tissue from a MPS VI (30 g) and a normal Siamese cat (80 g) was homogenized in 3 vol of 0.01 M Tris-HCl buffer, pH 7.5, in a Virtis homogenizer (Gardiner, N.Y.). Following three cycles of freeze-thaw, each suspension was further homogenized with a Brinkman Polytron (Brinkman, Westbury, N.Y.) for 20 seconds and then centrifuged at 27,000 *g* for 45 min. The ASB and ASA in the supernatants were separated by batch anion exchange chromatography. The supernatants were added to 2 vol of 0.1 M Tris-HCl buffer, pH 7.5, containing DEAE-cellulose (6 ml DEAE/g protein; DE 52, Whatman, Clifton, N.J.), which was previously equilibrated with the same buffer. The suspension was stirred overnight at 4°C. Under these conditions, the ASA was bound to the resin, but the ASB was not. The DEAE-cellulose suspension was suction-filtrated over a Buchner funnel, and the filtrate containing the ASB activity was diluted sixfold with 25 mM Tris-HCl buffer, pH 7.5, containing 0.5 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$. The diluted filtrate was mixed with Concanavalin A (Con A)-Sepharose (Pharmacia, Piscataway, N.J.) (3 ml Con A-Sepharose/g protein), and stirred for 3 hrs at room temperature. The Con A-Sepharose was removed by suction-filtration over a coarse-porosity fritted glass funnel, then poured into a 10-ml disposable syringe fitted with a glass wool plug. The column was washed extensively with the above buffer until the absorbance (OD_{280}) of the eluted fractions was less than 0.03 OD. Then the bound glycoprotein was eluted with 1 M 1-O-methyl-α-D-glucopyranoside (Sigma) at a flow rate of 60 ml/hr. The fractions containing the ASB activity were pooled, concentrated to 14 ml by ultrafiltration (Amicon, Lexington, Mass.) using a PM-10 membrane, and dialyzed against 4 liters of 25 mM Tris-HCl buffer, pH 7.5, with three changes of buffer over 12 hrs. The dialyzed concentrate was chromatographed over

DEAE-cellulose (6 × 1.5-cm and 16 × 1.5-cm columns for the MPS VI and normal preparations, respectively), which had been equilibrated previously with 25 mM Tris-HCl buffer, pH 7.5. The column was eluted with 60 ml of the same buffer, before a linear NaCl gradient (0–0.3 M) was applied. Fractions (2.5 ml) were collected at a flow rate of 0.3 ml/min. Fractions containing the ASB activity were pooled, concentrated to about 3 ml by ultrafiltration and then chromatographed over Sephadex G-100 (0.5 × 100 cm column; Pharmacia). The column was eluted with 0.05 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl, at a flow rate of 5 ml/hr. The fractions (2.5 ml each) containing the ASB activity were pooled and concentrated as above. These partially purified preparations were used to characterize their physical and kinetic properties as described below.

Cation Exchange Chromatography

Aliquots of the normal and MPS VI enzyme were dialyzed overnight against 25 mM sodium acetate-acetic acid buffer, pH 5.0, and individually chromatographed over CM-cellulose (CM 52, Whatman; 10 × 0.8 cm column) that was previously equilibrated in the same buffer. The column was eluted with 30 ml of buffer, and a linear NaCl gradient (0–0.5 M) was then applied. Fractions (1.5 ml) were collected at a flow rate of 0.3 ml/min, and assayed for ASB.

Electrophoretic Studies

Polyacrylamide disc gel electrophoresis was performed in 7% gels (7.0 cm) using β-alanine-acetate buffer, pH 4.0, as described by Reisfeld et al. [12]. Enzyme (80 U) was applied to each gel, and the gels stained for ASB activity by incubation with pNCS (10 mM for the normal enzyme; 40 mM for the residual MPS VI activity) at 37°C for 1 hr; for visualization of enzymatic activity, the gels were then transferred into 2 N NaOH for 2–3 min. Enzyme protein (40 μg) was applied to the gels stained for protein using Coomassie Brilliant Blue G-250 [13].

Cellulose acetate electrophoresis (Cellogel, Kalex, Manhasset, N.Y.) was performed by the method of Rattazzi et al. [14]. ASA and ASB activities were visualized with 4-methylumbelliferyl sulfate as substrate (RPI, Elk Grove Village, Ill.).

Kinetic and Stability Studies

Kinetic studies were performed at pH 5.7, the pH optimum for both enzymes, using pNCS as substrate. The thermostability of each of the purified enzymes was determined at 60°C in the presence of 1 mg/ml of bovine serum albumin. Cryostability studies were performed by freezing aliquots of the two enzymes at −5°C for 72 hrs. The effect of pH on the stability of the normal and MPS VI residual activity was compared by storing each enzyme at 4°C for 72 hrs over a pH range of 3.5 to 8.5 in 0.14 M sodium acetate-0.14 M sodium barbital buffer, adjusted with 0.1 M HCl.

Molecular Weight Studies

The molecular weights of the partially purified normal and MPS VI enzymes were determined by polyacrylamide gel electrophoresis according to the method of Hendrick and Smith [15]. The apparent molecular weights of the two glycoprotein enzymes were also determined by gel filtration on Sephadex G-200.

Subunit Structure Studies

The subunit structure of the normal feline enzyme was determined by treatment with thiol reagents, as described by Fisher et al. [16]. Two electrophoretically different forms of ASB were prepared by treating 3,000 U of purified normal feline enzyme with 14 mM iodoacetate and 3,000 U with 14 mM iodoacetamide. The alkylated enzymes were dissociat-

ed into subunits by dialysis against 0.1 M Tris-HCl buffer, pH 7.0, containing 0.4 M sucrose, 1 M NaCl, and 2 M urea. An equal amount of the iodoacetate-treated enzyme was mixed with the iodoacetamide-treated preparation. Aliquots of the iodoacetate-treated ASB, the iodoacetamide-treated ASB, and the mixture were frozen at −55°C for 2 hrs, thawed, and then dialyzed against 10 mM Tris-HCl buffer, pH 7.4, to recombine the subunits. In addition, a control mixture was made that contained equal amounts of the iodoacetamide- and iodoacetate-treated enzymes, after each was dissociated and reassociated. Each preparation was subjected to analytical polyacrylamide gel electrophoresis as described above. As a further control, aliquots of normal feline ASB were also treated with varying concentrations of iodoacetate (0.14–14 mM), dialyzed against 10 mM Tris-HCl buffer, pH 7.4, and analyzed on polyacrylamide gel electrophoresis without subjecting the preparations to the dissociation-reassociation procedure.

RESULTS

Demonstration of Residual ASB Activity in MPS VI Liver

Figure 1 shows the cellulose acetate electrophoretic profiles of ASB and ASA in the crude homogenate and after separation of the enzymes by batch DEAE-cellulose chromatography, the first purification step. The ASB activity was cleanly separated from ASA; the recovery of both enzymes was essentially 100%. Following the ion exchange step, the mean normal ASB activity was 32.5 U/mg protein (no. = 5), whereas the mean MPS VI residual activity was 2.1 U/mg protein (no. = 3) or 7% of mean normal hepatic activity.

Purification of Feline ASB from Normal and MPS VI Livers

Table 1 summarizes the typical results obtained for the purification of ASB activity from normal and MPS VI livers. As noted above, batch DEAE-cellulose chromatography with 0.1 M Tris-HCl buffer, pH 7.5, readily separated the ASB from ASA activity. Both the normal and residual MPS VI ASB activities were bound to Con A-Sepharose (~ 80% of applied activity), and elution resulted in 50% recovery with 140- and 60-fold purification, respectively. Subsequent column chromatography on DEAE-cellulose equilibrated with 25 mM Tris-HCl, pH 7.5,

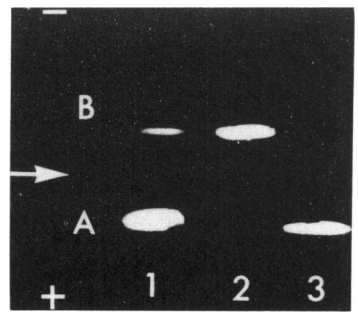

FIG. 1.—Cellulose acetate gel electrophoresis of ASA and ASB activities from normal feline hepatic tissue. The ASA and ASB activities were separated by batch DEAE-cellulose chromatography. *Lane 1*, feline liver total homogenate; *lane 2*, unbound activity; *lane 3*, bound activity eluted from DEAE-cellulose. *Arrow*, point of application; *A*, ASA; *B*, ASB. See text for details.

TABLE 1

PURIFICATION OF ASB FROM NORMAL AND MPS VI FELINE LIVERS

STEP	NORMAL FELINE*			FELINE MPS VI*		
	Specific activity U/mg	Purification fold	Yield %	Specific activity U/mg	Purification fold	Yield %
Crude homogenate	39.2	1	100	1.5	1	100
Preparative DEAE-cellulose	46.0	1.2	97	3.1	2	97
Con A-Sepharose	6,380	172	46	185	123	52
DEAE-cellulose	21,800	589	35	820	547	31
Sephadex G-100	106,000	2,840	20	2,750	1,880	16

* Based on 80 and 30 g of normal and MPS VI liver, respectively.

resulted in elution of the normal feline enzyme at 50 mM NaCl, whereas the MPS VI residual ASB activity did not bind to the resin and was recovered in the buffer wash. Following the gel filtration step, the final specific activities were 106,000 U/mg protein for the normal feline ASB and 2,750 U/mg protein for the feline MPS VI enzyme, which represented purifications of 2,840- and 1,880-fold, respectively. The final yields were 20% and 16% for the normal and MPS VI hepatic enzymes. On native polyacrylamide gel electrophoresis, these preparations each contained two major protein bands, only one of which stained for ASB activity. These partially purified preparations were used for characterization of the kinetic and physical properties as described below.

Electrophoretic Properties

Charge differences between the normal and MPS VI ASB enzymes were revealed by both cation exchange chromatography and polyacrylamide gel electrophoresis. On CM-cellulose chromatography, both the partially purified normal and MPS VI ASB activities bound to the resin; however, the normal feline activity eluted at 25 mM NaCl, whereas the MPS VI residual activity eluted a NaCl concentration of 14 mM (fig. 2). The normal enzyme bound to DEAE-cellulose, but the residual enzyme did not bind, contrary to expectation. The anomalous behavior of MPS VI ASB activity on anion exchange chromatography may be due to the fact that the residual enzyme's charge and/or binding properties may have been altered at pH 7.5. On polyacrylamide gel electrophoresis, the normal feline activity migrated more cathodally than the feline MPS VI enzyme (fig. 3), consistent with the more negative charge of the MPS VI activity on cation exchange chromatography. In addition, the normal and residual enzymes were shown to be charge isomers by the method of Hendrick and Smith [15] as described below.

pH Optima and Kinetic Studies

pH activity profiles were determined using pNCS as substrate. The pH optimum of both the freshly purified normal and MPS VI ASB activities was 5.7. However, when the profiles were repeated following several days of storage at 4° C, the MPS

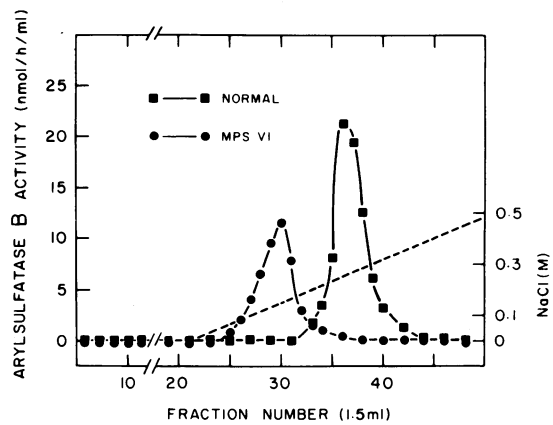

FIG. 2. —CM-cellulose chromatography of normal feline (■——■) and feline MPS VI (●——●) hepatic arylsulfatase B. Column was washed with 25 mM sodium acetate-acetic acid buffer, pH 5.0, and activities eluted with NaCl gradient as described in MATERIALS AND METHODS.

VI activity had a triphasic curve with optima at pH 5.2, 5.7, and 6.2; after further storage at 4°C, the pH profile was biphasic, with optima at pH 5.2 and 6.2. In contrast, the pH optimum of the normal hepatic enzyme was not affected by storage at 4°C.

Kinetic studies at pH 5.7 demonstrated that the purified MPS VI activity could not be saturated at final substrate concentrations up to 20 mM (the maximum solubility of the substrate), while the normal feline activity was maximal at 2.5 to 5.0 mM pNCS and was inhibited at higher substrate concentrations (fig. 4*A*). K_m values calculated for pNCS from Lineweaver-Burk plots were 0.5 mM for the normal feline enzyme (fig. 4*B*) and 50 mM for the mutant enzyme (fig. 4*C*). Based on these studies, the final substrate concentrations used in the routine assay for the normal and MPS VI activities were 5 and 20 mM pNCS, respectively.

Stability Studies

Figure 5 shows the effect of thermal inactivation at 60°C on the partially purified normal and MPS VI ASB activities. The normal enzyme had a half-life of

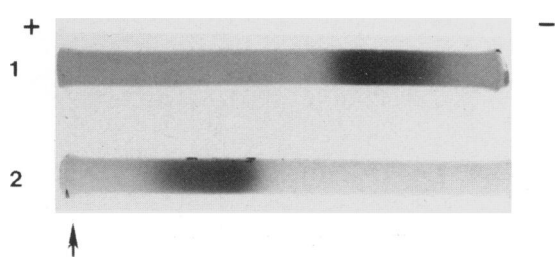

FIG. 3. —Analytical polyacrylamide gel electrophoresis of partially purified (post Con A-Sepharose) normal feline and feline MPS VI hepatic ASB. *Gel 1*, normal feline ASB; *gel 2*, MPS VI ASB. *Arrow* indicates point of application. See MATERIALS AND METHODS for details.

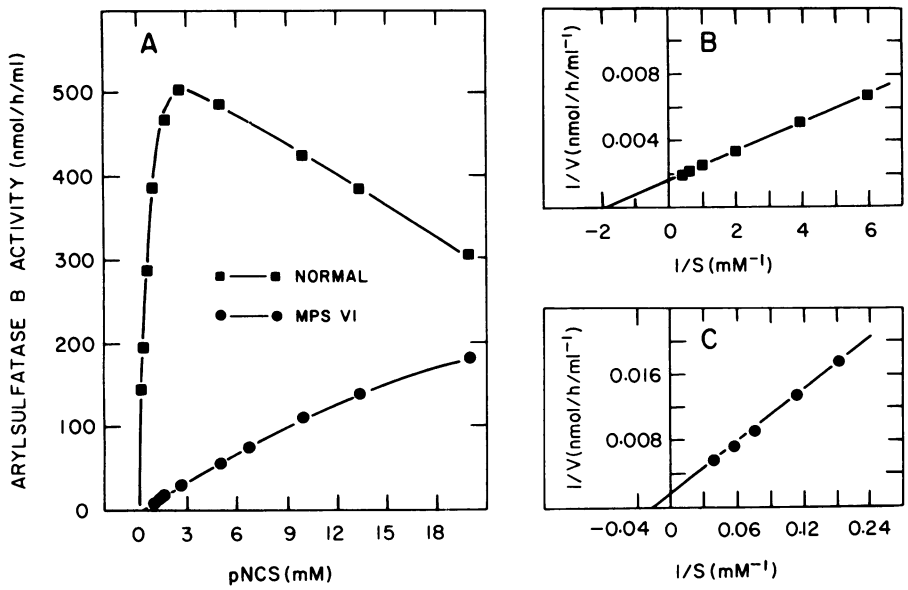

FIG. 4.—*A*, Effect of substrate concentration on partially purified (post Sephadex G-100) normal and MPS VI feline hepatic ASB activities; (■——■) normal, (●——●) MPS VI. *B* and *C*, Lineweaver-Burk plots for hepatic ASB from normal feline (K_m = 0.5 mM) and feline MPS VI (K_m = 50 mM), respectively.

about 50 min, while the mutant enzyme had a markedly shorter half-life of less than 5 min. The MPS VI activity also was markedly more cryolabile than the normal feline enzyme. Following 72 hrs of freezing, 85% of the initial normal activity was retained, while only 42% of the MPS VI activity was recovered. The normal activity was stable when stored at pH values ranging from 3.5 to 8.5. In contrast, the mutant activity was rapidly lost when stored below pH 4.5 or above pH 7.5 (fig. 6). Therefore, the partially purified preparations were routinely stored at pH 6.5 at 4°C.

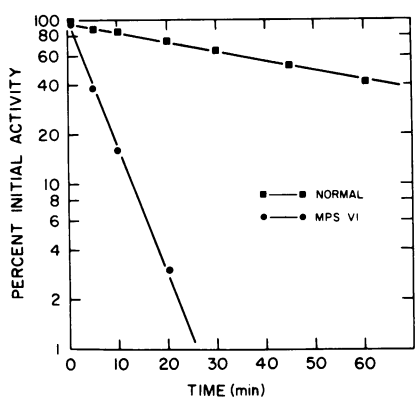

FIG. 5. —Thermal inactivation of partially purified (post Sephadex G-100) normal feline (■——■) and feline MPS VI (●——●) hepatic ASB at 60°C, pH 7.5.

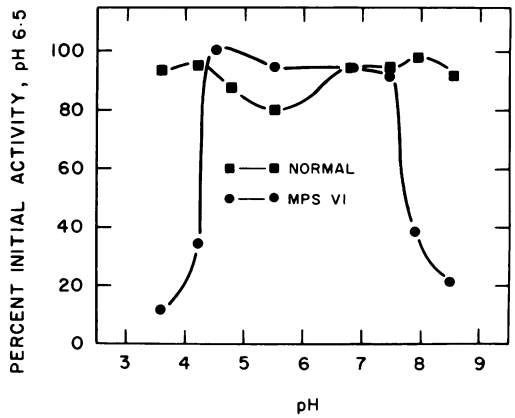

FIG. 6.—Effect of pH on stability of normal feline (■——■) and feline MPS VI (●——●) hepatic ASB activities. See MATERIALS AND METHODS for details.

Molecular Weight Studies

The apparent molecular weights of the normal and mutant ASB activities determined by gel filtration on Sephadex G-200 were 110,000 and 53,000, respectively (fig. 7). The native molecular weights of the two enzymes also were determined by the analytic polyacrylamide gel electrophoretic method of Hendrick and Smith [15] using 5%, 6%, 7%, and 8% gels (fig. 8). The plots of gel concentration vs. log Rm for the two enzyme preparations were not parallel and did not intersect at the ordinate, indicating that the normal and residual activities differed in molecular

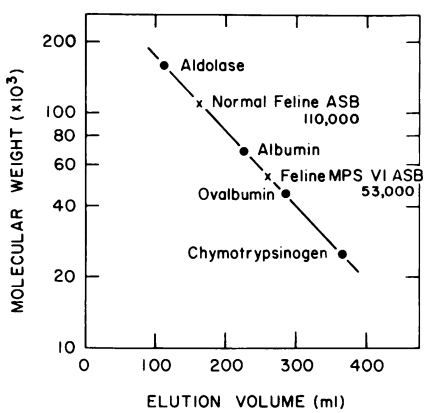

FIG. 7.—Determination of molecular weight of normal feline and feline MPS VI hepatic ASB. Normal and MPS VI enzymes (post Sephadex G-100) were analyzed in separate chromatographic runs. Enzyme (2,000 U of normal feline ASB; 200 U of mutant residual activity) and 2 mg of each protein standard were applied to a column of Sephadex G-200. Protein standards used were aldolase (mol. wt. 158,000), bovine serum albumin (68,000), ovalbumin (45,000), and chymotrypsinogen (24,000). Elution points of the normal feline and feline MPS VI ASB are indicated *X*.

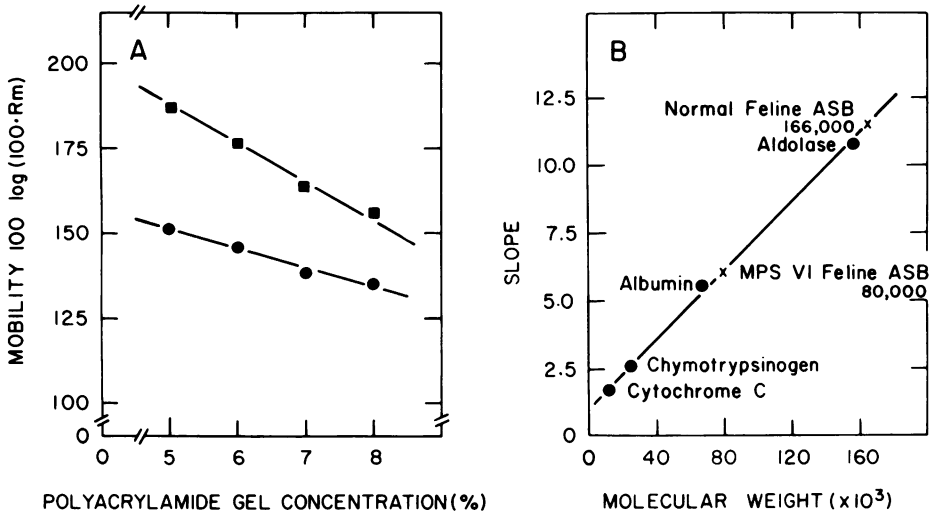

FIG. 8.—Native molecular weights of partially purified (post Sephadex G-100) normal feline and feline MPS VI hepatic ASB determined by polyacrylamide gel electrophoresis by method of Hendrick and Smith [15]. *A*, Gel concentration vs. log Rm for normal feline (■——■) and feline MPS VI (●——●) ASB. Lines do not intersect at the ordinate, indicating that the enzymes are charge isomers. *B*, Estimation of the molecular weights from the slopes of the normal feline (slope = 6) and feline MPS VI (slope = 11.5) ASB. Protein standards used were aldolase (mol. wt. 158,000), bovine serum albumin (68,000), chymotrypsinogen (24,000), and cytochrome C (12,400). Normal feline and feline MPS VI ASB are indicated *X*.

weight as well as charge (fig. 8*A*). Molecular weights estimated from the slopes of these plots were 80,000 for the residual ASB activity and 166,000 for the normal feline enzyme (fig. 8*B*).

Subunit Structure of Normal Feline ASB

Figure 9 compares the polyacrylamide disc gel electrophoretic profiles of the reassociated thiol reagent-treated subunits. The iodoacetate-treated enzyme (gel 1) migrated considerably less cathodally than the iodoacetamide-treated preparation (gel 2), which was not altered in electrophoretic mobility. Electrophoresis of the reassociated mixture of iodoacetate- and iodoacetamide-treated ASB preparations demonstrated a third activity band that migrated to a position intermediate between those of each alkylated enzyme preparation (gel 3). The intermediate activity band presumably represented a hybrid molecule containing one iodoacetate- and one iodoacetamide-treated ASB subunit. These findings suggest that normal feline ASB is a homodimeric enzyme. In support of this, a control mixture, prepared by mixing the already reassociated thiol reagent-treated enzymes, contained only two activity bands, which corresponded to the positions of the respective alkylated enzymes when individually electrophoresed (not shown). The iodoacetate-treated enzyme (gel 1) had a second, very faint activity band that migrated more cathodally than the major band. This band also was observed when the purified enzyme was treated with iodoacetate concentrations between 3.5 and 14

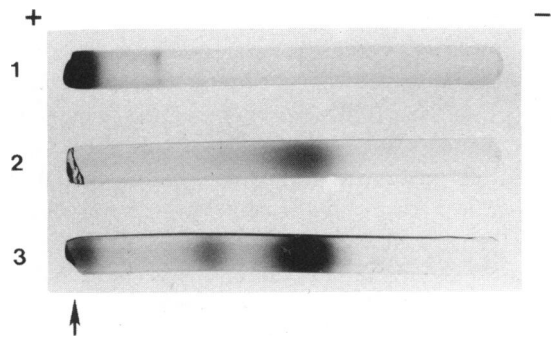

FIG. 9.—Analytical polyacrylamide gel electrophoresis of partially purified normal feline hepatic ASB (post Sephadex G-100) treated with iodoacetate (*gel 1*) and iodoacetamide (*gel 2*) to generate two electrophoretically different forms of the enzyme. "Hybrid" sample is a 50:50 mixture of two preparations (*gel 3*). Each preparation was first dialyzed against 0.1 M Tris-HCl buffer, pH 7.0, containing 0.4 M sucrose, 1.0 M NaCl, and 2.0 M urea, then frozen at −55°C for 2 hrs to further dissociate subunits. Each preparation was then thawed and dialyzed against 10 mM Tris-HCl, pH 7.4, to reassociate the subunits. The iodoacetate-treated enzyme migrated less cathodally, whereas the iodoacetamide-treated enzyme was not altered in electrophoretic mobility. In the mixture, only one "hybrid" enzyme was generated, which migrated to an intermediate position. Minor band in *gel 1* represents a partially alkylated form of the enzyme. *Arrow* indicates point of application.

mM, and directly subjected to polyacrylamide gel electrophoresis (no dissociation-reassociation procedure); these findings indicated that the faint minor band represented an incompletely alkylated form of the enzyme.

DISCUSSION

Attempts to investigate the molecular pathology of inborn errors of metabolism have focused on the characterization of the primary enzymatic defect. The presence of residual enzymatic activity permits the purification of the defective enzyme and comparison of its physical and kinetic properties with those of the normal enzyme. Among the human lysosomal storage diseases, several have defective enzymes with residual activity including Gaucher type 1 disease [17], mannosidosis [18], and MPS VI [5–8]. However, purification and characterization of the residual activity usually has been precluded by the unavailability of adequate fresh, human material. Thus, animal analogs of human enzyme deficiency disorders provide the opportunity to characterize the defective enzyme and gain insight into the molecular pathology of the enzymatic lesion.

This study represents the first purification of the residual ASB activity in feline MPS VI and comparison of its properties to those of the normal feline enzyme. The MPS VI enzyme differed from its normal counterpart in electrophoretic mobility, kinetic properties, stability, and molecular weight. Compared with the partially purified normal hepatic enzyme, the MPS VI ASB activity had at least a 100-fold greater K_m value and was markedly more thermo-, cryo-, and pH-labile. In addition, the molecular weight of the native MPS VI residual activity was about half that of the native normal feline enzyme as determined by both gel filtration and polyacrylamide gel electrophoresis. The difference in the molecular

weight values for each enzyme obtained by the two procedures was presumably due to the known anomalous behavior of glycoproteins on gel filtration [19]. These results and the demonstration that the normal feline enzyme was a homodimer (fig. 9) suggest that the mutation in the structural gene for feline ASB altered the gene product such that it was unable to maintain its normal dimeric subunit conformation. Although the defective enzyme retained partial activity, the inability for subunit association (e.g., dimerization) presumably rendered the MPS VI protein more defective catalytically and markedly unstable.

It is of interest that the mammalian ASB enzymes characterized to date [20–26], including the normal human, bovine, and ovine forms, were presumed to be monomers having molecular weights of about 40,000 to 54,000, as determined by gel filtration. The normal feline enzyme was shown here to be a dimer, having a molecular weight about twice that of the other normal mammalian enzymes. Perhaps during evolution, a monomeric ASB enzyme evolved following a structural gene mutation in the gene encoding a more primitive homodimeric form. The resulting monomeric enzyme retained catalytic activity and stability, but had an altered site for subunit association. Further characterization of the ASB proteins in phylogeny, including studies of immunologic similarity, is needed to support this speculation.

It is notable that human MPS VI also has been shown to have about 4%–15% of normal ASB activity in liver [5], cultured fibroblasts [6, 7], and leukocytes [8]. In contrast to the defective ASB activity in the feline disease, the human MPS VI residual activity was similar to the normal human enzyme in pH optimum, apparent K_m, electrophoretic mobility, and thermostability at 60° C [6, 7]. Immunologic studies of the human MPS VI enzyme demonstrated that the ratio of immunoreactive protein to residual activity was about 6.7 [7]. Thus, we conclude that the structural gene mutations causing the defective ASB activities in human and feline MPS VI differ in their molecular nature.

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