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Research Article

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Mucopolysaccharidosis Type I Subtypes

Presence of Immunologically Cross-reactive Material and In Vitro Enhancement of the Residual α-L-Iduronidase Activities

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Abstract

The enzymatic and immunologic properties of the defective residual α-L-iduronidase activities were investigated in fibroblast extracts from the three subtypes of mucopolysaccharidosis type I, Hurler (MPS IH), Scheie (MPS IS), and Hurler-Scheie (MPS IH-S) diseases. Using 4-methylumbelliferyl-α-L-iduronide (4MU-α-Id), the activities in fibroblast extracts from all three subtypes were less than 0.1% of normal. Rocket immunoelectrophoresis with monospecific rabbit anti-human α-L-iduronidase polyclonal antibodies, as well as immunoblots using a monoclonal antibody, revealed the presence of cross-reactive immunologic material (CRIM) in extracts prepared from each subtype. When the samples were equalized for β-hexosaminidase A activity, 38–105% of normal enzyme protein was detected. The sequential addition of cystamine, $MgCl_2$ and pyridoxal phosphate increased the residual 4MU-α-Id activities in subtype extracts up to about 35% of normal mean fibroblast activity. Cystamine, $MgCl_2$ or pyridoxal phosphate alone enhanced the residual activities two- to fourfold, whereas the sequential addition of all three compounds was required for maximal effect. Of the six B_6 vitamers evaluated, only the negatively charged forms, pyridoxamine (PLN), pyridoxamine phosphate (PNP), and pyridoxal phosphate (PLP), stimulated the residual activities. The addition of dermatan sulfate or heparan sulfate to the subtype extracts, followed by treatment with the effector compounds, similarly inhibited both the normal and enhanced MPS I activities. Heat inactivation experiments confirmed the fact that the mutant iduronidase activity was reconstituted and that the observed increase in enzymatic activity was not an artifact of the fluorogenic assay. These results suggest that the presence of certain thiol reducing agents, divalent cations and negatively charged B_6 vitamers can alter the conformation of the mutant α-L-iduronidase in vitro such that the hydrolysis of 4MU-α-Id is enhanced into the heterozygote range.

Introduction

The type I mucopolysaccharidoses represent three clinically delineated, autosomal recessive disorders (Hurler disease, MPS IH; Scheie disease, MPS IS; and Hurler-Scheie disease, MPS IH-S)[1] that result from the deficient activity of the lysosomal hydrolase, α-L-iduronidase (α-L-iduronide iduronohydrolase, E.C. 3.2.1.76) (1, 2) and the subsequent lysosomal accumulation of the glycosaminoglycans (GAGs), dermatan sulfate (DS), and heparan sulfate (HS) (3). MPS IH is characterized by severe dysostosis multiplex, a characteristic facial dysmorphia, corneal clouding, hepatosplenomegaly, and severe mental retardation with demise usually before the second decade of life. In contrast, affected individuals with MPS IS have mild dysostosis multiplex, mild corneal clouding, aortic valvular involvement, normal mentation, and a normal lifespan. Patients with MPS IH-S present with an intermediate phenotype (4). Although it had been proposed that MPS IH-S was the genetic compound of MPS IH and MPS IS alleles (5), reports of patients with consanguineous parents indicated that different mutant alleles at the α-L-iduronidase locus were responsible for the intermediate phenotypic expression of the disease (6).

Only limited information is available on the nature of the enzymatic defects in the different MPS I subtypes. Characterization of the physicokinetic properties of the defective enzymes in fibroblast and leukocyte extracts has been difficult due to the low levels of residual activity; estimates have ranged from nondetectable to ~ 1% of normal levels depending on the substrate and assay conditions used (7–10). However, Hopwood and Muller (7) reported that the apparent K_m values of the residual activity in MPS IH fibroblast extracts were elevated 4- to 10-fold toward a radiolabeled disaccharide substrate derived from heparan sulfate, whereas the apparent K_m value for the MPS IS and MPS IH-S residual activities were in the normal range. These investigators also found the pH optima, inhibition by sulfate and chloride ions and thermostability properties of the MPS IH and MPS IS fibroblast enzymes to be essentially normal. Using a radiolabeled disaccharide derived from dermatan sulfate, no activity was detected in a MPS IH fibroblast extract, whereas a MPS IS extract had residual activity with a normal apparent K_m (10). To date, the only reported immunologic study of α-L-iduronidase enzyme protein was in a MPS IH fibroblast extract and no enzyme protein was detected (11).

Recently, we purified to homogeneity the high and low uptake forms of human α-L-iduronidase (12), determined the physicokinetic and immunologic properties of each enzyme form (13) and localized the gene to the chromosomal region, 22pter → q11 (14). Since the purified enzyme was relatively

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1. *Abbreviations used in this paper:* CRIM, cross-reactive immunologic material; DS, dermatan sulfate; GAGs, glycosaminoglycans; HS, heparan sulfate; MPS, mucopolysaccharidosis; MPS IH, Hurler disease; MPS IH-S, Hurler-Scheie disease; MPS IS, Scheie disease; 4MU-α-Id, 4-methylumbelliferyl α-L-iduronide; PLN, pyridoxamine; PLP, pyridoxal phosphate; PNP, pyridoxamine phosphate.

unstable, the effects of various sulfhydryl reactive reagents and divalent metal cations on enzyme stability and activity were evaluated. Sulfhydryl reducing reagents and the metals, Mg^{2+} and Ca^{2+}, were found to stabilize the purified activity (13). In addition, the effects of various vitamin cofactors were assessed and it was observed that PLP reactivated partially inactivated normal enzyme. Therefore, an investigation was undertaken to evaluate the effects of these "effector" compounds on the residual activities in fibroblast extracts from the MPS I subtypes. The availability of monospecific polyclonal and monoclonal antibodies raised against the purified enzyme (12) permitted the analysis of the enzyme protein present in subtype extracts using sensitive rocket immunoelectrophoretic and immunoblotting tehniques. This manuscript reports the presence of cross-reactive immunologic material (CRIM) in individuals from each of the three MPS I subtypes and the identification of effector compounds that enhance the residual activities in subtype extracts into the heterozygote range.

Methods

Materials. 4MU-α-Id was purchased from Calbiochem-Behring Co., San Diego, CA. Cystamine-di-HCl, dithiothreitol (DTT), iodoacetamide, methyl-α-D-mannopyranoside and all B_6 vitamers were from Sigma Chemical Co., St. Louis, MO. $MgCl_2$ and $CaCl_2$ were from Aldrich Chemical Co., Inc., Milwaukee, WI. Concanavalin A Sepharose was from Pharmacia Fine Chemicals, Piscataway, NJ. Seakem agarose was obtained from the FMC Corporation, Rockland, ME. RPMI 1640 tissue culture media and fetal calf serum were from Gibco, Grand Island, NY. Fluorescamine was obtained from Pierce Chemical Co., Rockford, IL. High purity acetonitrile was purchased from Burdick and Jackson Laboratories, Muskegon, MI. A model 250/2.5 power supply was obtained from Bio-Rad Laboratories, Richmond, CA. MPS I mutant cell lines were obtained from the Mutant Cell Repository, Camden, NJ. Purified DS and HS were supplied by Dr. M. B. Matthews and J. A. Cifonelli, who prepared these standards under a contract (No. 1 AM-52205) from the National Institutes of Health.

Enzyme and protein assays. α-L-Iduronidase activity was determined using 4MU-α-Id as substrate (1.0 mM final concentration) (15). Protein concentrations were measured by a modified fluorescamine assay (16). β-Hexosaminidase A enzyme assays were performed as previously described (17).

Preparation of polyclonal and monoclonal antibodies and immunologic studies. Monospecific polyclonal antibodies were produced in New Zealand white rabbits against purified α-L-iduronidase as previously described (12). For quantitative rocket immunoelectrophoresis, the α-L-iduronidase activity in cell extracts was concentrated about 10-fold by chromatography on Con A-Sepharose and immunoelectrophoresis was performed in 6.5 × 8.0-cm agarose gels as described (14). Each gel contained 10.0 μl of the antibody preparation dissolved in 4.5 ml of 1% agarose. After electrophoresis, the rockets were incubated overnight in a moist chamber, and precipitin bands were either observed directly or visualized by a peroxidase-conjugated second antibody staining system (18). Equal amounts of extract protein from the MPS I subtype and control fibroblasts were electrophoresed.

In order to further characterize the enzyme protein present in each of the MPS I subtypes, a monoclonal anti-α-Id antibody was raised in mice by previously described methods (19). For immunoblotting experiments, fibroblast extracts were prepared from normal and MPS I individuals and electrophoresed through a native polyacrylamide gel in a β-alanine/acetate buffer, pH 4.0, as described (20). For quantitative experiments, equal amounts of β-hexosaminidase A activity were applied for each sample. Immunoblotting was performed according to the manufacturer's instructions using a Bio-Rad trans-blot apparatus.

In vitro enhancement assay. Cultured fibroblasts from MPS I subtype and normal fibroblasts were harvested from 75 mm^2 culture flasks with a rubber policeman, centrifuged at 5,000 *g* for 10 min and then washed twice with a total of 10 ml of Dulbecco's phosphate-buffered saline. The cells were resuspended in 100 μl of 0.4 M sodium formate buffer, pH 3.5, containing 0.2% Triton X-100, and then were sonicated for 90 s (three bursts, 30 s each) at 4°C with a cup sonicator (model 200; Branson Sonic Power Co., Danbury, CT). The sonicates were centrifuged at 20,000 *g* for 30 min and the supernatants (fibroblast extracts) were either used immediately or frozen at −20°C for subsequent use.

For the standard enhancement assay, 25 μl of fibroblast extract was preincubated sequentially with 25 μl of 200 mM cystamine, 100 mM $MgCl_2$, and 1.0 mM PLP. The effector compounds were dissolved in distilled water and each was preincubated for 5 min at 37°C with the fibroblast extract before addition of the next effector. After the 15-min preincubation period, 25 μl of 4MU-α-Id (1.0 mM final concentration) was added and the reaction mixture was incubated for 1 h at 37°C. The fluorescence was quantitated as previously described (15). For each fibroblast line, the stimulation assay was performed in duplicate on cells harvested at midconfluency from three different passages. Substrate blanks were prepared by sequentially preincubating each effector compound with the fibroblast extracts, incubating the mixture for 1 h at 37°C, and then adding substrate after the reaction was stopped. Enzyme blanks were prepared similarly with the fibroblast extract added after the reaction was stopped.

Kinetic studies. K_m determinations were made for the 4MU-α-Id activity present in fibroblast extracts from normal and MPS I individuals after treatment with the effector compounds. Enzyme assays were performed at six different substrate concentrations and K_m values were calculated from Lineweaver-Burk plots. Inhibition experiments were performed at two different 4MU-α-Id concentrations using purified DS and HS; K_i values were determined from Dixon plots.

Heat inactivation studies. For the heat inactivation experiments, fibroblast extracts from normal and MPS I subtypes were incubated at 68°C for 120 min. Aliquots were removed every 15 min and were either assayed directly or were incubated with the effector compounds for 15 min before assay.

Results

Demonstration of CRIM in fibroblast extracts from the MPS I subtypes. Table I shows that the residual 4MU-α-Id activities in the MPS I subtype extracts were less than 0.1% of normal mean fibroblast values using the assay conditions described in the methods section. The levels of residual activity varied within and among the subtypes. When equal amounts of β-hexosaminidase A activity from normal (MS 1163), MPS IH (GM 0034), MPS IH-S (GM 0963), and MPS IS (GM 1256) fibroblast extracts were subjected to quantitative rocket immunoelectrophoresis, ~ 96, 102, and 98% CRIM was detected using a monospecific polyclonal rabbit anti-α-Id antibody (Fig. 1). There was ~ 10% variability observed when equal amounts of β-hexosaminidase activity from the same normal sample was electrophoresed. Quantitative rocket immunoelectrophoresis of seven additional MPS IH, MPS IS, and MPS IH-S fibroblast extracts demonstrated that the amount of CRIM ranged from ~ 38 to 105% of that found in normal individuals. To further characterize the enzyme protein present in each of the MPS I subtypes, a monoclonal anti-α-Id antibody was used for immunoblotting studies. Fig. 2 shows a native polyacrylamide gel stained for activity using 4MU-α-Id (*A*) and an immunoblot of an identical gel incubated with the

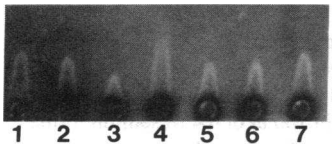


Figure 1. Rocket immunoelectrophoresis of fibroblast extracts from MPS I subtypes. Rocket immunoelectrophoresis was performed as described in the text. Lanes *1* and *5:* normal fibroblast extracts (MS 1163) containing 5U of β-hexosaminidase A activity. Lanes *2, 6,* and *7:* 5U of β-hexosaminidase A activity from cell lines GM 0034 (MPS IH), GM 0963 (MPS IH-S) and GM 1256 (MPS IS), respectively. Lane *3:* 2U of β-hexosaminidase A activity from cell line GM 0034. Lane *4:* normal fibroblast extract (MS 1163) containing 15U of β-hexosaminidase A activity.

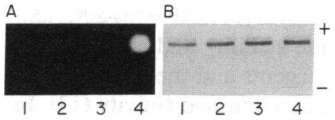


Figure 2. Immunoblotting of fibroblast extracts from MPS I subtypes. Native polyacrylamide gel electrophoresis and immunoblotting was performed as described in the text. *A* shows a native polyacrylamide gel stained for 4MU-α-Id activity; lanes *1–4:* extracts from fibroblast cell lines GM 0034 (MPS IH), GM 0963 (MPS IH-S), GM 1256 (MPS IS), and MS 1163 (normal), respectively. *B* shows an immunoblot of an identical native polyacrylamide gel reacted with a monoclonal anti-α-Id antibody. Each lane contained 20 U of β-hexosaminidase A activity.

monoclonal anti-α-Id antibody (*B*). Although no enzymatic activity was detected in each of the MPS I subtypes (*A*), about normal amounts of enzyme protein were identified (*B*).

In vitro enhancement of the residual activity in the MPS I subtype fibroblast extracts. Preincubation of the sulfhydryl reducing compounds, DTT, and cystamine for 5 min at 37°C before the addition of 4MU-α-Id, increased the mean residual activities in fibroblast extracts from all three subtypes 2.6- to 14.7-fold (Table I). The mean residual activities in MPS IH, MPS IH-S, and one MPS IS extract, were increased about threefold by either DTT or cystamine. The residual activity in extracts from the other MPS IS individual was increased ~ 9- and 15-fold by DTT and cystamine, respectively. For comparison, the normal mean fibroblast activity was stimulated 1.3- and 1.6-fold by DTT and cystamine, respectively. In contrast, iodoacetamide, which alkylates reduced sulfhydryl groups, had little, if any, effect on the mean residual activities in the subtype extracts and inhibited the mean activity in normal fibroblasts by ~ 50%. The divalent metal cations, Mg^{2+} and Ca^{2+}, also enhanced the residual activities in the subtype extracts. Mg^{2+} and Ca^{2+} increased the mean residual activities about two- to eightfold and about two- to threefold, respectively, whereas the normal enzyme was essentially unaffected by either cation (Table I).

Investigation of the effects of various vitamin cofactors on the residual activities in subtype extracts revealed that only certain B_6 vitamers enhanced the residual activities, as shown in Table II. PLN, PNP, and PLP each stimulated the mean residual activities from 2.5- to 16-fold, while pyridoxal and pyridoxine essentially had no effect. None of the B_6 vitamers enhanced the normal fibroblast enzymatic activity. Fig. 3 shows that the enhancement effect of cystamine, $MgCl_2$, and PLP on the residual activities in MPS I extracts was concentration dependent, with the optimal concentrations about 5.0 mM cystamine, 25 mM $MgCl_2$, and 0.25 mM PLP.

Next, the effect of the sequential addition of the effector compounds was evaluated. The sequence in which the effector compounds were added in the stimulation assay proved critical, with optimal results obtained when the extracts were sequentially preincubated with cystamine, $MgCl_2$, and PLP. When the subtype extracts were preincubated in the optimal effector concentrations and sequence, the residual activities

Table I. Effect of Sulfhydryl Reactive Reagents and Divalent Cations on the Residual α-L-Iduronidase Activities in MPS I Subtype Fibroblast Extracts

Addition		Enzymatic activity*			
		Normal controls (n = 5)	MPS IH (n = 5)	MPS IH-S (n = 3)	MPS IS (n = 2)
		nmol/h/mg	*nmol/h/mg*	*nmol/h/mg*	*nmol/h/mg*
None	Mean	2,840	1.76	1.44	
	Range	885–4,440	0.83–3.97	0.26–2.65	0.33; 3.62
DTT	Mean	3,610	4.65	4.02	
	Range	1,970–5,430	1.65–23.0	0.85–11.5	0.92; 14.5
Cystamine	Mean	4,550	4.83	5.58	
	Range	3,010–6,720	1.14–13.5	3.96–18.0	2.95; 12.5
Iodoacetamide	Mean	1,240	1.72	1.37	
	Range	723–2,000	0.67–3.65	0.32–2.97	0.44; 3.08
$MgCl_2$	Mean	2,670	3.35	4.46	
	Range	1,010–5,060	1.98–16.5	3.01–14.0	2.66; 17.2
$CaCl_2$	Mean	3,150	3.08	3.92	
	Range	1,160–5,720	1.99–4.66	1.05–5.52	1.06; 6.67

Fibroblast extracts were preincubated with the effector compound for 5 min at 37°C before the addition of 4MU-α-Id. * α-L-Iduronidase assays were performed as described in Methods.

Table II. Effect of Various B_6 Vitamers on the Residual α-L-Iduronidase Activities in MPS I Subtype Fibroblast Extracts

Addition		Enzymatic activity			
		Normal controls (n = 5)*	MPS IH (n = 5)	MPS IH-S (n = 3)	MPS IS (n = 2)
		nmol/h/mg	*nmol/h/mg*	*nmol/h/mg*	*nmol/h/mg*
None	Mean	1,887	1.47	1.37	
	Range	914–3,335	0.66–2.65	0.28–2.44	0.25; 3.09
Pyridoxamine	Mean	2,006	3.65	4.08	
	Range	992–3,725	1.98–14.6	3.04–19.2	3.66; 21.7
Pyridoxamine phosphate	Mean	1,882	4.08	4.45	
	Range	1,008–3,666	2.65–20.8	3.66–16.5	3.85; 19.5
Pyridoxal phosphate	Mean	1,803	4.66	4.72	
	Range	906–3,448	2.07–18.5	3.07–20.7	4.04; 19.3
Pyridoxal	Mean	1,765	1.36	1.65	
	Range	877–3,642	0.20–1.85	0.30–2.09	0.39; 2.87
Pyridoxine	Mean	1,795	1.41	1.47	
	Range	896–3,447	0.45–2.93	0.36–2.28	0.33; 2.97

Fibroblast extracts were preincubated with the B_6 vitamers for 5 min at 37°C before the addition of 4MU-α-Id. * α-L-Iduronidase assays were performed as described in Methods.

were increased to levels from less than 0.1% to almost 37% of the mean normal fibroblast activity (Table III). The percents of normal mean activity (and ranges) after in vitro enhancement for 10 MPS IH extracts was 22.8% (9.2 to 37.3%), for seven MPS IH-S extracts was 20.6% (3.5 to 31%), and for two MPS IS extracts was 26.0% (22.8 and 29.2%). Note that there was no direct relationship observed between the degree of enhancement and the percentage of CRIM detected in the various MPS I extracts (Table III).

Kinetic studies. Fig. 4 shows a Lineweaver-Burk plot of normal and MPS I fibroblast α-L-iduronidase after in vitro enhancement with the various effector compounds. The K_m values for patients with each of the MPS I subtypes was about the same as that of normal individuals (70 μM). Fig. 5 shows representative Dixon plots for the effects of DS (*A*) and HS (*B*) on normal and MPS I fibroblast α-L-iduronidase with and without stimulation. The K_i values of DS determined for the normal and MPS I enhanced activities were 0.05 and 0.03 mg/ml, respectively. The stimulation cocktail did not alter the K_i of the normal fibroblast enzyme. Similar results were obtained using HS, where the K_i values were 0.06 and 0.08 mg/ml for the normal and MPS I activities, respectively. For both physiologic substrates, the inhibition was apparently noncompetitive, as was reported in previous work using purified normal α-L-iduronidase (13). No significant inhibition was observed of either the normal or "enhanced" MPS I activities below 10 mg/ml DS or HS. To further characterize the antagonistic effects of the GAG substrates on activity enhancement, fibroblast extracts from each of the subtypes were assayed before and after removal of endogenous GAG by cetylpyridinium chloride. The residual activity did not change after the precipitation of endogenous GAG.

Heat inactivation studies. Fig. 6 shows heat inactivation profiles of normal and MPS I fibroblast extracts with and without enhancement. Note that the slope for the normal enzyme was similar to those of the MPS I subtypes after enhancement. The $t_{1/2}$ values for the normal enzyme with and without enhancement were 11.4 and 10.8 min, respectively. Interestingly, the $t_{1/2}$ value for the enhanced MPS IH, MPS IH-S, and MPS IS were 3.7, 3.6, and 6.1 min, respectively. In contrast,

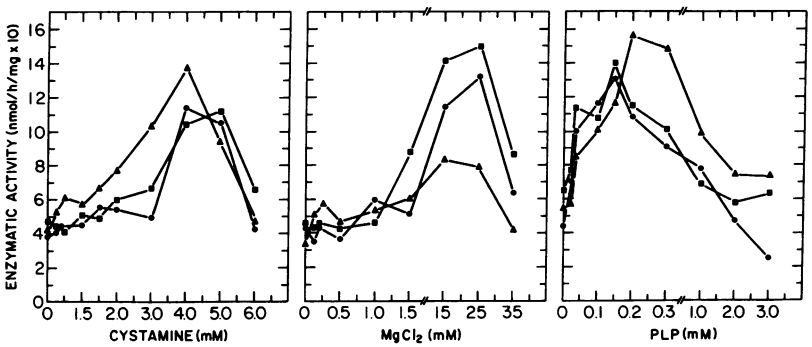


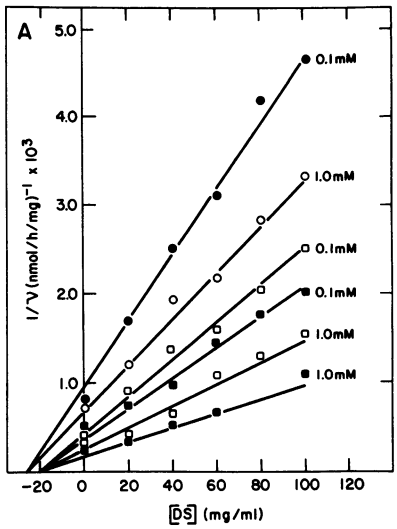
Figure 3. Effects of various cofactors on the residual α-L-iduronidase activities in fibroblast extracts from MPS I subtypes. α-L-Iduronidase assays were performed as described in the text. Each sample was preincubated with the indicated amounts of the cofactor for 5 min at 37°C before the addition of 4MU-α-Id. ●, GM 0034 (MPS IH); ■, GM 0963 (MPS IH-S); ▲, GM 1256 (MPS IS).

Table III. Effect of Cystamine, $MgCl_2$, and Pyridoxal Phosphate on the Residual α-L-Iduronidase Activities in MPS I Subtype Fibroblast Extracts

Fibroblast line	% CRIM	Enzymatic activity*	
		Pretreatment	Posttreatment
	% normal	*nmol/h/mg*	*nmol/h/mg*
MPS IH			
GM 0034	96	0.95	1,000
GM 0798	78	0.88	828
GM 0887	71	0.46	268
GM 1053	65	1.03	674
GM 1257	45	1.12	208
GM 1391	38	0.22	1,090
MPS IH-S			
GM 0512	62	1.03	400
GM 0963	102	1.92	866
GM 1254	58	1.38	103
GM 1898	44	1.08	928
GM 2845	105	0.66	624
GM 2846	68	0.88	801
GM 2847	80	0.32	496
MPS IS			
GM 1256	98	0.85	1,024
GM 1323	84	0.97	667
Normal (n = 8)	100 (88–119)	2,670±182	2,920±103

* In vitro enhancement assays and CRIM determinations were performed as described in Methods and the values represent the means of duplicate experiments.

the activity of the heat inactivated extracts from the MPS I subtypes without enhancement was not detectable at the 15 min timepoint.



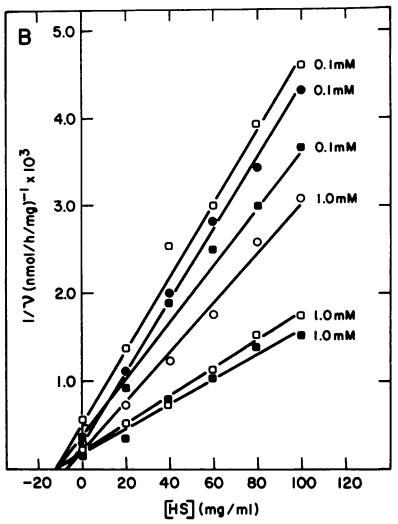


Figure 5. Inhibition of normal and enhanced MPS I-H α-L-iduronidase activities by DS and HS. In vitro enhancement and α-L-iduronidase assays were performed at two concentrations (0.1 and 1.0 mM) as described in the text. *A* and *B* show representative Dixon plots using DS and HS, respectively. ○, GM 0034 (MPS IH, 1.0 mM, enhanced); ● GM 0034 (MPS IH, 0.1 mM, enhanced); □, MS 1163 (normal); ■ MS 1163 (normal, enhanced).

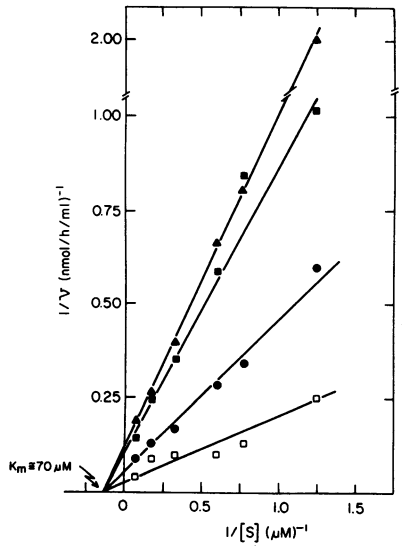


Figure 4. Lineweaver-Burk plots of α-L-iduronidase activity in fibroblast extracts from MPS I subtypes after in vitro enhancement. α-L-Iduronidase assays and in vitro enhancement were performed as described in the text. ●, GM 0034 (MPS IH); ■, GM 0963 (MPS IH-S); ▲, GM 1256 (MPS IS); □, MS 1163 (normal).

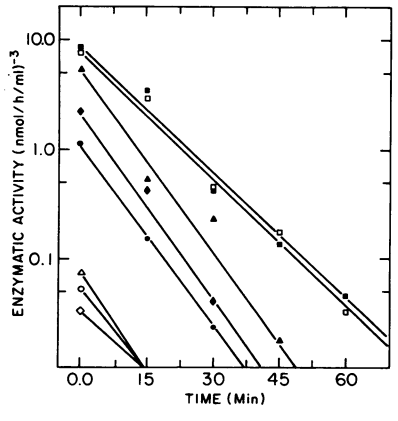


Figure 6. Heat inactivation profiles (68°C) of α-L-iduronidase activities in normal and enhanced MPS I fibroblast extracts. In vitro enhancement and α-L-iduronidase assays were performed as described in the text. ○, GM 0034 (MSP IH); ●, GM 0034 (MPS IH, enhanced); ◇, GM 0963 (MPS IH-S); ◆, GM 0963 (MPS IH-S, enhanced); △, GM 1256 (MPS IS); ▲, GM 1256 (MPS IS, enhanced); □, MS 1163 (normal); ■, MS 1163 (normal, enhanced).

Discussion

Although the immunologic and physicokinetic properties of normal human purified α-L-iduronidase from several sources have been well documented (11–13, 21–30), limited information is available on the nature of the defective enzyme in the MPS I subtypes. In this communication, fibroblast extracts from each subtype were evaluated for the presence of CRIM using monospecific polyclonal and monoclonal anti-human α-L-iduronidase antibodies. When equal amounts of β-hexosaminidase A activity were electrophoresed in a quantitative rocket immunoelectrophoretic system, the subtype extracts were found to have from 38 to 105% of normal α-L-iduronidase enzyme protein using the anti-α-Id polyclonal antibodies. It should be noted that these extracts had < 0.1% of normal enzymatic activity. The demonstration of CRIM by immunoblotting (Fig. 2) using an anti-α-Id monoclonal antibody corroborated the results obtained with rocket immunoelectrophoresis. These findings indicated that the different mutations in the MPS I subtypes examined did not dramatically alter the transcriptional activity of the α-L-iduronidase gene or the stability of the mutant enzyme proteins, despite the fact that the catalytic properties of the mutant proteins were severely impaired. It was notable that CRIM-negative mutations were not detected in any of the MPS I lines examined. However, the one MPS IH line previously analyzed for an α-L-iduronidase maturation study appeared to be CRIM negative (11). This may be explained by differences in antibody preparations used in the two studies, as well as differences in the immunologic techniques. Based on the results presented here, it is likely that the majority of MPS I mutations are CRIM positive.

Since sulfhydryl reducing reagents and divalent cations were required for optimal activity of the normal purified enzyme (12, 13, 30), and since it had been determined that enzyme proteins were present in fibroblast extracts from the MPS I subtypes, studies were conducted to determine the effects of these effector molecules on the residual activities in the MPS I subtypes. The finding that the sequential addition of cystamine, $MgCl_2$, and PLP enhanced the residual activities in fibroblast extracts up to ~ 35% of normal was unexpected as similar observations had not been reported previously for other lysosomal enzymes, with one notable exception. The residual arylsulfatase B activity in leukocyte extracts from feline mucopolysaccharidosis VI was enhanced over 10-fold by the addition of DTT or cysteamine (31). Studies indicated that the purified mutant feline enzyme was a monomer that dimerized in the presence of thiol-reducing agents, thereby regaining partial enzymatic activity toward both artificial and natural substrates (31, 32). It is unlikely that thiol-induced subunit association was responsible for the activity enhancement in the MPS IH subtypes since normal human α-L-iduronidase is a monomer (12–14, 30).

Although the specific mechanism responsible for enhancement of the residual activities is not known, several possibilities can be considered. The fact that removal of the GAG substrates by cetylpyridinium chloride precipitation from subtype extracts did not enhance the residual activity toward 4MU-α-Id suggests that the mutant enzymes were not inhibited by bound excess natural substrate. Therefore, the effector molecules may have a direct effect on the mutant proteins as opposed to interacting with the natural substrates present in the extracts. Inhibition studies of α-L-iduronidase from normal and "enhanced" MPS I fibroblast extracts using the natural substrates, DS and HS, have revealed that: (*a*) these physiologic substrates inhibit the 4MU-α-Id activity in fibroblast extracts in a manner similar to that previously observed with purified human enzyme (13). Dixon plots at two different substrate concentrations indicated that the inhibition was apparently noncompetitive, as was observed in the previous work mentioned above, and (*b*) these substrates inhibit the activity of the "enhanced" enzymatic activity in the MPS I extracts in a manner similar to that observed in the normal samples. Thus, the use of 4MU-α-Id as a substrate accurately reflected the enhanced activity of the mutant enzymes toward their natural substrates and was a reliable measurement of reactivated enzyme. Finally, heat inactivation studies confirmed the fact that the enhancement effect was due to the presence of active mutant enzyme and was not an artifact of the fluorogenic assay. Although the $t_{1/2}$ of the enhanced activities from the MPS I subtypes was less (3.5–6.1 min) than that observed for the normal activities (10.8–11.4 min), the linear inhibition curves for normal and mutant enzymes were similar, indicating that the enhancement effect was dependent on the amount of non-heat denatured mutant enzyme protein.

It is likely that the mutant enzyme proteins in each of the subtypes have altered conformations and are modified by the effector molecules such that their normal integrity is partially restored and residual activity toward 4MU-α-Id is markedly increased. This hypothesis assumes that different mutations in the structural gene for α-L-iduronidase each result in enzyme proteins which are relatively stable, but have markedly reduced activities toward the natural and artificial substrates, i.e., presumably, each mutant allele encodes a monomeric enzyme in which the protein conformation is altered at or near the active site of the enzyme. This concept is supported by the following two findings: immunologic demonstration of CRIM in each of the MPS I subtypes, and enhancement of the activities in normal and subtype fibroblast extracts by thiol reducing reagents alone (Table I), indicating that reduction of crucial sulfhydryl groups at or near the active site may be important for obtaining optimal activity and involved in maintaining the active conformation. We previously reported that thiol reducing reagents stimulated the 4MU-α-Id activities of purified α-L-iduronidase, whereas *p*-chloromercurobenzoate and maleate were potent inhibitors (13). Similar findings for the low uptake kidney enzyme also have been reported using a different artificial substrate (23). Furthermore, Clements et al. have found that DTT helped prevent a gradual loss of enzyme activity during the purification of α-L-iduronidase from human liver and kidney (30).

The fact that Mg^{2+} and Ca^{2+} cations stimulated the residual enzymatic activity in the MPS I subtypes may relate directly to this concept, as well as to their mechanistic role as effectors of enzyme enhancement. The metal cations may bind the reduced sulfhydryl groups in the enzyme and protect these critical residues at or near the active site from oxidation. Such a mechanism, involving reduced sulfhydryl groups that are maintained by zinc cations and PLP, has been suggested to prevent inactivation of δ-aminolevulinate dehydratase (33–36). Although a B_6 vitamer is not known to be a requirement for normal α-L-iduronidase activity, clearly, PLP, PLN, or PNP enhanced the residual activities in the MPS I extracts

(Table II). The pyridoxal moiety may covalently bind the mutant protein through a Schiff base mechanism and alter the active site conformation, making it more accessible to the 4MU-α-Id substrate. Alternatively, and more likely, PLP may bind to the metal cations, further preventing the oxidation of crucial sulfhydryl groups and maintaining the active conformation. It should be noted that B_6 vitamers are strong chelaters of divalent metal cations (35) and that only negatively charged vitamers stimulated the residual activities.

In summary, the data presented here clearly demonstrate the presence of mutant enzyme protein in each of the MPS I subtypes and, although these studies were performed using an artificial substrate, 4MU-α-Id, the results suggest that it may be possible to enhance the residual enzymatic activity in patients with MPS I by further investigation of methods to manipulate the conformation and substrate binding of the mutant enzyme. However, caution should be exercised in the extrapolation of in vitro findings to in vivo expectations. For example, several investigators have shown that normal α-mannosidase A and B were stimulated by zinc ions in vitro (37, 38). Zinc also was found to enhance the residual acidic α-mannosidase activity in various sources from patients with mannosidosis (37–40). However, a clinical trial of oral zinc supplementation did not alter the level of residual activity in plasma, leukocytes, or tears from the treated patients (41). Thus, in vivo trials of α-L-iduronidase enhancement should be performed in animal analogues (42, 43) before human experimentation, particularly since the residual activity in feline MPS I can be significantly enhanced (Schuchman, E. H., M. E. Haskins, and R. J. Desnick, unpublished results).

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