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A Novel Amino Acid Modification in Sulfatases That Is Defective in Multiple Sulfatase Deficiency

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Summary

Multiple sulfatase deficiency (MSD) is a lysosomal storage disorder characterized by a decreased activity of all known sulfatases. The deficiency of sulfatases was proposed to result from the lack of a co- or posttranslational modification that is common to all sulfatases and required for their catalytic activity. Structural analysis of two catalytically active sulfatases revealed that a cysteine residue that is predicted from the cDNA sequence and conserved among all known sulfatases is replaced by a 2-amino-3-oxopropionic acid residue, while in sulfatases derived from MSD cells, this cysteine residue is retained. It is proposed that the co- or posttranslational conversion of a cysteine to 2-amino-3-oxopropionic acid is required for generating catalytically active sulfatases and that deficiency of this protein modification is the cause of MSD.

Introduction

Sulfate esters occur in a wide spectrum of biological compounds ranging from glycosaminoglycans and glycolipids to hydroxysteroids. They are hydrolyzed by a group of sulfatases that are distinguished by a high substrate specificity. The sulfatases are members of an evolutionarily highly conserved gene family with substantial sequence homology among sulfatases of bacterial (Murooka et al., 1990), lower eukaryotic (Sasaki et al., 1988; Yang et al., 1989; de Hostos et al., 1989; Hallmann and Sumper, 1994), and human (Yen et al., 1987; Robertson et al., 1988; Stein et al., 1989a, 1989b; Peters et al., 1990; Schuchman et al., 1990; Wilson et al., 1990; Tomatsu et al., 1991) origins.

Of the nine human different sulfatases that have been characterized biochemically, eight are found in lysosomes, while steroid sulfatase is a membrane-bound microsomal enzyme. The biological significance of these sulfatases is emphasized by seven distinct human inherited disorders that are caused by the deficiency of specific sulfatases (for reviews see Ballabio and Shapiro, 1995; Neufeld and Muenzer, 1995). In addition, a rare autosomal recessive disorder, multiple sulfatase deficiency (MSD), is known in which the activities of all known sulfatases are severely decreased. The clinical phenotype of MSD combines features of metachromatic leukodystrophy (MLD) with that of mucopolysaccharidosis (MPS) as a result of the impaired lysosomal catabolism of sulfated glycolipids and glycosaminoglycans. In addition, an ichthyosis can develop owing to the deficiency of the microsomal steroid sulfatase (Kolodny and Fluharty, 1995).

The primary defect in MSD is unknown. Complementation studies with single sulfatase deficiency disorders have shown that the structural genes for sulfatases are not affected (Horwitz, 1979; Chang and Davidson, 1980; Fedde and Horwitz, 1984; Ballabio et al., 1985). Expression of sulfatase cDNAs in MSD fibroblasts yielded sulfatase polypeptides with a severely diminished catalytic activity (Rommerskirch and von Figura, 1992). This has led to the proposal that the basic defect in MSD affects a co- or posttranslational modification of sulfatases that is required for their catalytic activity.

We therefore searched for a protein modification that is present in catalytically active sulfatases and deficient in the inactive sulfatases from MSD. In this study we report the identification of a protein modification in the two sulfatases arylsulfatase A (ASA) and arylsulfatase B (ASB). This modification leads to the conversion of a cysteine residue into a 2-amino-3-oxopropionic acid residue and is deficient in ASA and ASB from MSD cells.

Results

Identification of an ASA Peptide with an Unknown Modification

Analysis of ASA from control and MSD fibroblasts by SDS–polyacrylamide gel electrophoresis (Rommerskirch and von Figura, 1992) or isoelectric focusing (data not shown) failed to reveal any apparent differences that would point to a modification that distinguishes ASA from control cells and MSD cells. To facilitate the detection of a modification that has only subtle effects on molecular mass, we digested ASA with trypsin prior to mass determination. For this purpose, ASA precursors were isolated from secretions of BHK cells overexpressing human ASA (Chao et al., 1990) and were subjected to reductive carboxymethylation in 6 M guanidinium hydrochloride to allow digestion with trypsin. The tryptic peptides were separated by reversed-phase high pressure liquid chromatography (RP-HPLC) (Figure 1) and characterized by mass spectrometry and N-terminal sequencing.

Tryptic digestion of ASA is predicted to yield 28 peptides. The peptides that were sequenced after separation by RP-HPLC accounted for 478 of the 489 residues of the precursor form of ASA. The missing 11 residues are predicted to reside in one dipeptide and three tripeptides. The masses of these di- and tripeptides were detected in the flowthrough fraction of the HPLC run. For all sequenced peptides, the observed and predicted masses were in perfect agreement except for three peptides. Of these three

*The first two authors contributed equally to this work.

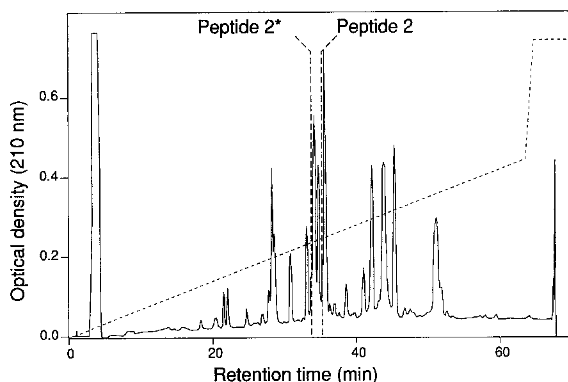

Figure 1. Separation by RP-HPLC of Tryptic Peptides from ASA

The positions of peptide 2 (carboxymethylated form with a mass of 1788 Da) and peptide 2* (modified form with a mass of 1714 Da) and the gradient from 0% to 90% acetonitrile (broken line) are indicated.

peptides, two contained N-glycosylation sites. After deglycosylation with endoglycosidase H, their masses agreed with the predicted values, indicating that N-glycosylation fully accounted for the mass deviation. The only peptide with an unexplained mass deviation was peptide 2, which comprises residues 59–73 of ASA and which had a mass of 74 Da less than predicted. Interestingly, in all preparations of ASA, a form of peptide 2 was recovered that had the predicted mass and accounted in different preparations for 10%–40% of peptide 2. The two forms of peptide 2 were separated by RP-HPLC (Figure 1). We reasoned that the major form of peptide 2 carries a modification that is missing in the minor form. This putatively modified form of peptide 2 was designated as peptide 2*.

2-Amino-3-Oxopropionic Acid Replaces the Predicted Cys-69 in Peptide 2*

Only the first ten residues of the 15-mer peptide 2* could be identified by direct sequencing. In cycles 11–15 (predicted sequence, CTPSR), no signal was obtained (Figure 2A). The presence of residues 12–15 (TPSR) could be demonstrated by amino acid analysis after strong acid hydrolysis. To verify the presence of Cys-69 (position 11 in peptide 2*), we metabolically labeled cells with [^{35}S]cysteine. [^{35}S]ASA was purified, and tryptic peptides were prepared. ASA contains 15 cysteine residues that are distributed among seven tryptic peptides. The tryptic peptides containing cysteine residues 1 and 3–15 were recovered labeled to specific activity of 270–550 dpm/pmol cysteine (Figure 3). Peptide 2 is predicted to contain the second cysteine residue of ASA. Only the minor form of peptide 2 was radiolabeled, while its major form, the modified peptide 2*, was not labeled (Figure 3). This indicated that the modification of peptide 2* was associated with a loss of the sulfur group of Cys-69.

A critical information about the nature of the modification of Cys-69 was provided by tandem mass spectrometry. When peptide 2* was embedded in a glycerol matrix for tandem mass spectrometry, it was desorbed as an adduct with glycerol. Analysis of the fragmentation products revealed that only fragments containing residue 11 (Cys-69)

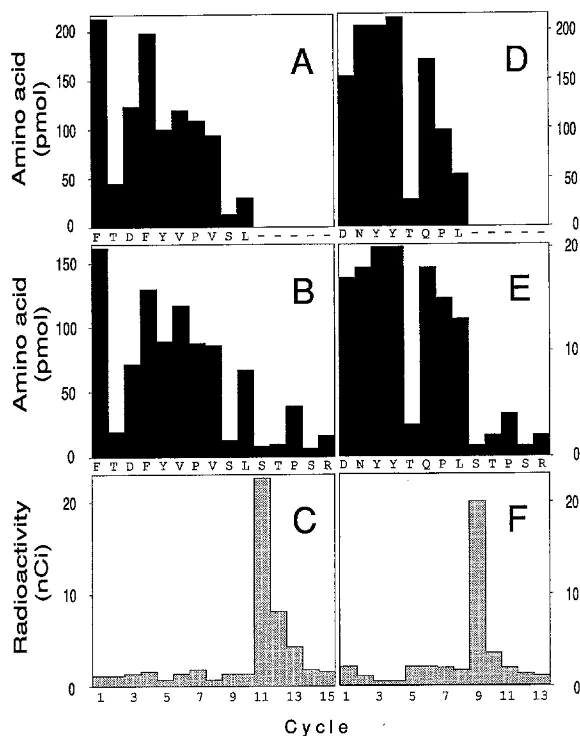

Figure 2. Sequencing of ASA Peptide 2* and ASB Peptide 3C*

Sequencing of ASA peptide 2* (A–C) and of ASB peptide 3C* (D–F) before (A and D) and after reduction with $NaBH_4$ (B and E) or [^{3}H]$NaBH_4$ (C and F). In (A), (B), (D), and (F), the amount of phenylthiohydantoine amino acid released in each cycle is given, and in (C) and (F), the radioactivity level is given.

behaved as glycerol adducts (data not shown). The formation of glycerol adducts points to the presence of an aldehyde function in position 11 of peptide 2* (Dever et al., 1989). The presence of a reducible aldehyde function in peptide 2* was confirmed in two ways. Treatment with $NaBH_4$ led to an increase of the mass by ~2 Da, from 1714.4 to 1716.2 (Figures 4A and 4B). When peptide 2* was embedded in p-nitroaniline, a product of 1835.1 Da was desorbed that represents the Schiff's base formed between peptide 2* and p-nitroaniline (Figure 4C).

The observed mass of 1714.4 Da of peptide 2* would conform to the predicted mass if the thiol group of cysteine was replaced by an aldehyde function. This would correspond to the replacement of the cysteine by a 2-amino-3-oxopropionic acid residue. If so, reduction of peptide 2* should convert residue 69 into the respective alcohol, which is serine. Indeed, when peptide 2* was reduced with $NaBH_4$ and then subjected to amino acid sequencing, a signal for serine was observed in cycle 11, and in the following cycles (12–15), signals for the predicted residues were obtained (see Figure 2B). When peptide 2* was reduced with [^{3}H]$NaBH_4$ and subjected to radiosequencing, the bulk of incorporated radioactivity was recovered in cycle 11 (see Figure 2C). The presence of a 2-amino-3-oxopropionic acid residue explains the interruption of Edman degradation in cycle 11, since the aldehyde function is predicted to interfere with the formation of the anilinothiazolinone amino acid derivative during Edman degrada-

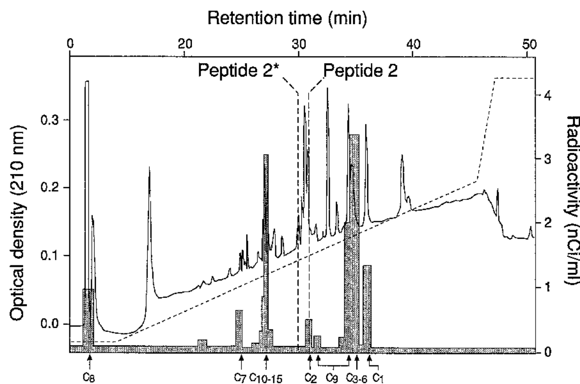

Figure 3. Separation by RP-HPLC of Tryptic Peptides from [35S]ASA

[35S]ASA from BHK cells metabolically labeled with [35S]cysteine was mixed with unlabeled ASA prior to reductive carboxymethylation and digestion with trypsin. The gradient from 0% to 90% acetonitrile (broken line) and the positions of the two forms of peptide 2 and of the tryptic peptides containing cysteine residues are indicated. The 15 cysteine residues were numbered from 1 (N-terminal) to 15 (C-terminal). The minor form of the peptide containing Cys-9 (residues 378–439) represents a truncated form of the peptide due to proteolytic processing of ASA between residues 426 and 430 (Fujii et al., 1992).

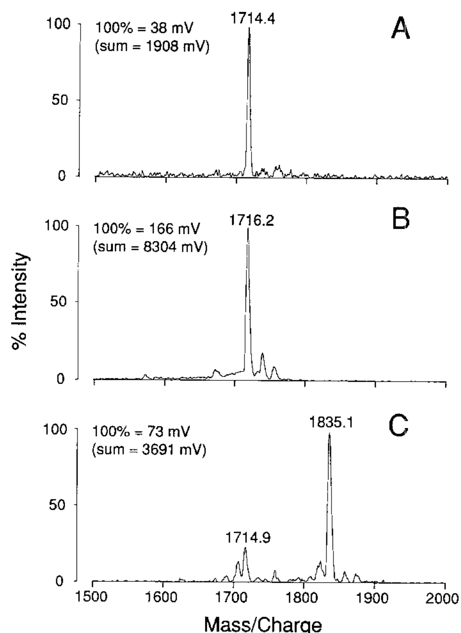

Figure 4. Mass Spectrometry of ASA Peptide 2*

ASA peptide 2* was analyzed before (A) and after reduction with $NaBH_4$ (B) or embedding in p-nitroaniline (C).

tion. We conclude from these results that peptide 2* contains a 2-amino-3-oxopropionic acid residue instead of the predicted Cys-69.

Cys-69 of ASA Is Conserved among All Known Eukaryotic Sulfatases

If the modification of Cys-69 to 2-amino-3-oxopropionic acid is considered as a candidate for the defect in MSD, Cys-69 and its modification should be conserved among all sulfatases. The available cDNA sequences of six human sulfatases indicate an identity of 20%–35% and similarity of 40%–60% among these enzymes (Wilson et al., 1990; Peters et al., 1990; Tomatsu et al., 1991). The homology extends over the entire sequence, but is highest in the N-terminal region. Among the areas of highest identity is the hexapeptide L/V-C-X-P-S-R, where X carries a hydroxyl or thiol group in five of six sulfatases (Figure 5). The cysteine in this hexapeptide corresponds to Cys-69 in ASA. Even in the four sulfatases known from lower eukaryotes (sea urchin and algae), this hexapeptide is conserved as V-C-T/C-P-S-R (Sasaki et al., 1988; Yang et al., 1989; de Hostos et al., 1989; Hallmann and Sumper, 1994).

2-Amino-3-Oxopropionic Acid in ASB

ASB was analyzed for the presence of 2-amino-3-oxopropionic acid by a slightly different approach. ASB was directly reduced with $[^3H]NaBH_4$ in the presence of 6 M guanidinium hydrochloride and then subjected to reductive carboxymethylation, tryptic digestion, and peptide separation by RP-HPLC. Incorporation of radioactivity was restricted to a single fraction (Figure 6). After rechromatography, the labeled peptide could be identified as a modified form of ASB peptide 3, designated as peptide 3*, which contains residues 69–95. The predicted cysteine residue 91 in peptide 3* is homologous to Cys-69 in ASA. If residue 91 is 2-amino-3-oxopropionic acid or (after reduction) serine, the predicted masses are 2869.2 and 2871.2 Da. The observed mass of peptide 3* was 2869 Da, indicating that the predicted Cys-91 is indeed replaced in ASB by 2-amino-3-oxopropionic acid and that only the nonreduced form of peptide 3* was detected by mass spectrometry.

For further analysis, peptide 3* was digested with endoproteinase AspN to release the 13-mer peptide 3C* containing residues 83–95. The 13-mer peptide 3C* could be separated by RP-HPLC into two forms. The radioactivity was associated with only one of the two forms (inset in Figure 7). The masses of the radioactive and the nonradioactive form were 1541 and 1539 Da, respectively. These values agree with the masses predicted for the serine (1541.6 Da)-containing and 2-amino-3-oxopropionic acid (1539.6 Da)-containing forms of peptide 3C*, suggesting that reduction of ASB had been only partial. Radiosequencing of peptide 3C* released the bulk of radioactivity in cycle 9 (see Figure 2F), demonstrating that the reducible group in ASB was associated with residue 91. Peptide 3C* was also isolated from nonreduced ASB. Sequencing of this peptide was blocked in cycle 9. After treatment of ASB peptide 3C* with $NaBH_4$, sequencing yielded a signal for serine in cycle 9 and, in the following four cycles, the signals for T, P, S, and R (see Figures 2D and 2E). It should be noted that in contrast with ASA, in which a fraction of peptide 2 was recovered in the nonmodified form containing the predicted Cys-69, only the modified form of ASB peptide 3 containing 2-amino-3-oxopropionic acid was recovered in tryptic digests of ASB.

The tryptic peptides of ASB analyzed by sequencing and mass spectrometry covered 471 of 496 residues of

Human Sulfatases:

Arylsulfatase A	S	L	C	T	P	S	R	A	A	L	L	T	G	R	L
Arylsulfatase B	P	L	C	T	P	S	R	S	Q	L	L	T	G	R	Y
Steroidsulfatase	P	L	C	T	P	S	R	A	A	F	M	T	G	R	Y
N-Acetylglucosamine-6-sulfatase	A	L	C	C	P	S	R	A	S	I	L	T	G	K	Y
Iduronatesulfatase	A	V	C	A	P	S	R	V	S	F	L	T	G	R	R
N-Acetylgalactosamine-6-sulfatase	P	L	C	S	P	S	R	A	A	L	L	T	G	R	L

Figure 5. Homology among Human Sulfatases

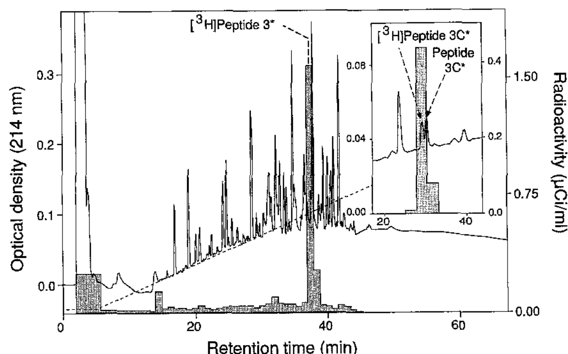

Figure 6. Separation by RP-HPLC of Tryptic Peptides from [3H]ASB
ASB was reduced with [3H]$NaBH_4$ prior to reductive carboxymethylation and digestion with trypsin. The position of the reduced [3H]peptide 3* and the gradient from 0% to 55% acetonitrile (broken line) are indicated. The inset shows the separation by RP-HPLC of ASB peptide 3C*, which was obtained by digestion of [3H]peptide 3* with endoproteinase AspN.

the ASB precursor. Except for peptide 3* and the five peptides containing the six predicted N-glycosylation sites, the observed and the predicted masses of the peptides were identical. The mass deviations of the five peptides carrying N-glycosylation sites varied between +860 and +1500 Da and are attributed to the presence of N-linked oligosaccharides. Thus, the only detectable modification in ASA and ASB apart from N-glycosylation was the modification of a conserved cysteine residue to 2-amino-3-oxopropionic acid.

2-Amino-3-Oxopropionic Acid Is Deficient in ASA and ASB from MSD Fibroblasts

To examine sulfatases synthesized by MSD cells for the modification of the conserved cysteine, ASA and ASB were purified from MSD fibroblasts that overexpressed inactive ASA or ASB polypeptides after retroviral gene transfer. As controls, ASA and ASB were expressed in fibroblasts with a deficiency of ASA (MLD) or ASB (Maroteaux–Lamy syndrome, also known as MPS type VI). After infection with recombinant sulfatase-encoding retroviruses, the MLD and MPS type VI fibroblasts synthesize catalytically active ASA or ASB (Rommerskirch and von Figura, 1992).

To demonstrate the presence or absence of Cys-69 in ASA from MSD or MLD fibroblasts, we purified ASA from these cells. ASA was reacted with [14C]iodoacetic acid to [14C]carboxymethylate the free sulfhydryl groups. The [14C]carboxymethylated ASA was then subjected to reductive carboxymethylation, tryptic digestion, and separation by RP-HPLC. In ASA from MSD fibroblasts, the three peptides containing the cysteine residues 38, 69, and 414 were labeled with ^{14}C (Figure 7A). The amount of radioactivity associated with the peptide containing Cys-69 was 139% and 116% of that associated with peptides containing Cys-38 and Cys-414, respectively. The different amounts of radioactivity associated with the three peptides is assumed to be due to different recovery rates of the peptides. We conclude from these results that in ASA from MSD fibroblasts, of the 15 cysteine residues, only three, including Cys-69, expose a thiol group and that the remaining 12 cysteine residues are disulfide bonded.

When ASA from MLD fibroblasts was analyzed, significantly less radioactivity was associated with the Cys-69-containing peptide 2 (Figure 7B). When compared with the peptides containing Cys-38 or Cys-414, the amount of radioactivity associated with the peptide 2 was 34%. This is ~3- to 4-fold lower than in ASA from MSD fibroblasts. We conclude from these results that in ASA from MSD cells, Cys-69 is accessible to iodoacetic acid, while in ASA from MLD cells, the bulk of Cys-69 (~70%) is nonaccessible. The latter is ascribed to its conversion to 2-amino-3-oxopropionic acid.

For ASB from MSD and MPS type VI fibroblasts, we could obtain direct evidence for the presence of cysteine or 2-amino-3-oxopropionic acid in position 91. ASB was subjected to reductive carboxymethylation, trypsin digestion, and RP-HPLC either prior to or after reduction with [3H]$NaBH_4$. When ASB peptide 3 was obtained from MSD cells, it had a mass of 2944.1 Da that was not affected by embedding with p-nitroaniline. This corresponds to the mass predicted (2944.2 Da) for peptide 3 with a carboxymethylated Cys-91. In contrast, ASB peptide 3 obtained from MPS type VI cells had a mass of 2868.1 Da, which corresponds to the mass predicted for the modified peptide 3* with a 2-amino-3-oxopropionic acid residue in position 91 (2869.2 Da). After embedding in p-nitroaniline, the mass increased to 2990.8 Da, which corresponds to that of the Schiff's base of peptide 3 with p-nitroaniline (2989.2 Da). The presence of reducible groups in ASB from MPS type VI fibroblasts was also apparent when ASB was reduced with [3H]$NaBH_4$ prior to tryptic digestion. The bulk of radioactivity was recovered in the fraction containing peptide 3* (Figure 7D). Interestingly, when ASB from MSD fibroblasts was treated in an identical manner, a small amount of radioactivity was recovered in the fraction containing ASB peptide 3* (Figure 7C). This indicates that in MSD fibroblasts as well, a small fraction (~20%) of ASB, which escaped detection by mass spectrometry, carries a modified residue 91. All together these results demonstrate that the modification of a conserved cysteine to 2-amino-3-oxopropionic acid in sulfatases is severely deficient in MSD.

Discussion

A Novel Co- or Posttranslational Modification in Sulfatases

We have identified in the two sulfatases ASA and ASB

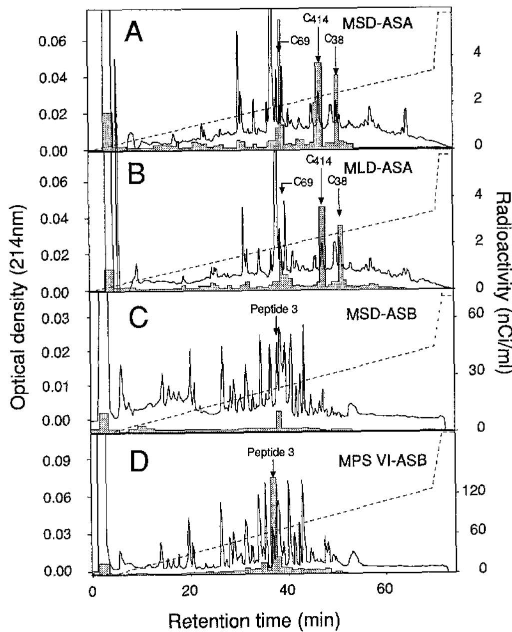

Figure 7. Separation by RP-HPLC of Tryptic Peptides from [^{14}C]Carboxymethylated ASA and [^{3}H]ASB

(A and B) ASA from MSD fibroblasts (A) or MLD fibroblasts (B) was treated with [^{14}C]iodoacetic acid prior to reductive carboxymethylation and digestion with trypsin. The gradient from 0% to 90% acetonitrile (broken line) and the position of the ASA peptides containing Cys-38, Cys-69, or Cys-414 are indicated.

(C and D) ASB from MSD fibroblasts (C) or MPS type VI fibroblasts (D) was treated with [^{3}H]$NaBH_4$ prior to reductive carboxymethylation and digestion with trypsin. The gradient from 0% to 90% acetonitrile (broken line) and the position of ASB peptide 3* (arrow) are indicated.

2-amino-3-oxopropionic acid residues at positions where the cDNA sequences predict a cysteine residue. Three observations support the notion that the 2-amino-3-oxopropionic acid residues are generated by a co- or a posttranslational modification of cysteine residues and are not directly incorporated during translation. In ASA isolated from BHK cells that overproduce ASA, a fraction of ASA contains the predicted cysteine. This fits with the view that cysteine is incorporated during translation and modified co- or posttranslationally to 2-amino-3-oxopropionic acid by a mechanism that is saturable. Catalysis of this modification by a saturable mechanism and requirement of the modification for catalytic activity (see below) can explain why overexpression of a sulfatase is associated with a severe decrease of the activities of endogenous sulfatases (Anson et al., 1993). Additional evidence for the co- or posttranslational generation of 2-amino-3-oxopropionic acid comes from the observation that ASA and ASB from MSD fibroblasts lack the modified residues and instead retain the predicted cysteines (see below).

The 2-Amino-3-Oxopropionic Residue Is Part of a Conserved Sequence

The cysteine residue that becomes modified in ASA and ASB is conserved among the known six human sulfatases and the four sulfatases from lower eukaryotes. Moreover,

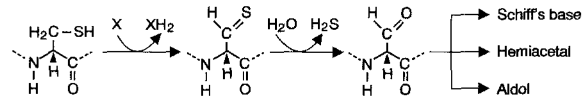

Figure 8. Conversion of Cysteine to 2-Amino-3-Oxopropionic Acid

Shown is a hypothetical mechanism for the conversion of cysteine to 2-amino-3-oxopropionic acid in sulfatases. The subsequent reaction of the aldehyde group with functional groups of amino acid side chains or of low molecular mass components is indicated.

this cysteine is embedded in a highly homologous sequence of 13 residues, nine of which are identical in at least five of the six human sulfatases (Figure 5). This consensus sequence may act as a colinear recognition motif for the machinery that is modifying the cysteine. Formally, the conversion of a protein-bound cysteine to 2-amino-3-oxopropionic acid can be conceived as the result of a two-step reaction (Figure 8), the first step being a dehydrogenase reaction yielding a thioaldehyde as intermediate, followed by a hydrolysis step yielding the aldehyde and hydrogen sulfide. Since the secreted precursor forms of lysosomal sulfatases such as ASA or ASB are catalytically active, the modifying reactions must occur within the secretory route. Steroidsulfatase has been isolated from endoplasmic reticulum in an active form (Ballabio and Shapiro, 1995), indicating that the modification occurs in the endoplasmic reticulum as an early posttranslational or even cotranslational event.

Deficiency of 2-Amino-3-Oxopropionic Acid in Sulfatases from MSD

The search for a modification in sulfatases was prompted by the hypothesis that generation of catalytically active sulfatase depends on a co- or posttranslational modification and that this modification is deficient in MSD. We therefore examined sulfatases from MSD cells for the presence of 2-amino-3-oxopropionic acid. To obtain sufficient quantities of ASA and ASB, we had to overexpress the sulfatases in MSD fibroblasts using recombinant retroviruses. For comparison, ASA and ASB were overexpressed in fibroblasts that were capable of synthesizing active sulfatases, but lacked endogenous ASA or ASB owing the mutations in the respective sulfatase genes. In ASA from MSD fibroblasts, the presence of Cys-69 rather that 2-amino-3-oxopropionic acid could be demonstrated only indirectly by the accessibility of residue 69 to carboxymethylation. Interestingly, also in ASA from controls, a fraction (~30%) of residue 69 was accessible to carboxymethylation, suggesting that under the conditions of overexpression (and possibly also at a normal expression level), part of the newly synthesized ASA escapes the co- or posttranslational modification in fibroblasts.

The presence of Cys-91 in ASB from MSD fibroblasts and its substitution by 2-amino-3-oxopropionic acid in ASB from control fibroblasts could be verified directly by mass spectrometry and reduction with [^{3}H]$NaBH_4$. By reduction with [^{3}H]$NaBH_4$, we could demonstrate that, even in ASB from MSD cells, ~20% of Cys-91 is modified. This agrees with the observation that in MSD fibroblasts the activities

of sulfatases are not completely deficient (Steckel et al., 1985). Even in fibroblasts from patients with the severe form of MSD, which were used in this study and in which the residual activity of sulfatases is usually below 5%, it may reach 15%, namely for ASB (Rommerskirch and von Figura, 1992). We conclude from the analysis of ASA and ASB from control and MSD fibroblasts that the conversion of cysteine to 2-amino-3-oxopropionic acid is catalyzed by a saturable mechanism that is severely deficient but not absent in MSD.

Role of 2-Amino-3-Oxopropionic Acid for Hydrolysis of Sulfate Esters

Comparatively little is known about the mechanism of sulfate ester bond hydrolysis by sulfatases. A SO_4–sulfatase complex has been proposed as a catalytic intermediate (Roy, 1978). This may be related to the observation that under certain conditions ASA is inactivated during hydrolysis of p-nitrocatechol sulfate and that this inactivation is associated with covalent and stoichiometric incorporation of sulfate (Waheed and van Etten, 1979, 1980).

The correlation between generation of catalytically active sulfatases and conversion of a cysteine residue to 2-amino-3-oxopropionic acid suggests that the latter may be of importance for the catalytic mechanism. It is, however, questionable whether in sulfatases the aldehyde group of the 2-amino-3-oxopropionic acid residue is free. In native sulfatases, the aldehyde function may be stabilized as an enol or linked to an amino or a hydroxyl function forming a Schiff's base or a hemiacetal. The amino or hydroxyl function may be part of the sulfatase polypeptide chain or of a low molecular weight cofactor. If the cofactor is a ketone, it could also form an aldol. In fact, preliminary experiments argue against the presence of a free aldehyde group in sulfatases. When native ASA and ASB were treated with [^{3}H]$NaBH_4$, they failed to incorporate radioactivity (T. S., unpublished data). Reduction of the 2-amino-3-oxopropionic acid residue required the denaturation of the sulfatases by guanidinium hydrochloride. This suggests that in native sulfatases the 2-amino-3-oxopropionic acid residue is stabilized as enol or is linked to one of the functional groups indicated above and that unfolding is required to shift the equilibrium to the free aldehyde.

Experimental Procedures

Purification of Sulfatases

Sulfatases were purified from secretions of BHK cells, which coexpressed human ASA (Chao et al., 1990) or human ASB (Peters et al., 1990) together with the human M_r 46,000 mannose 6-phosphate receptor, and from cell extracts (50–100 mg of cell protein) of fibroblasts from patients with MSD (cell line Mo), MLD (cell line La), or MPS type VI (cell line Ya) infected with recombinant retroviruses containing the cDNAs of human ASA or ASB (for cell lines and recombinant retroviruses, see Rommerskirch and von Figura, 1992). Where indicated, [^{35}S]ASA (0.78 mCi; 0.62 mCi/mmol) was purified from medium, and cell extracts were prepared of BHK cells metabolically labeled with [^{35}S]cysteine, as described previously (Sommerlade et al., 1994).

ASA was purified by affinity chromatography as described previously (Sommerlade et al., 1994). ASB was immunopurified using an affinity column derivatized with the monoclonal antibody ASB 4.1 (Gibson et al., 1987) and the conditions described for purification of ASA.

Reductive Carboxymethylation, Tryptic Digestion, and Separation of Tryptic Peptides

Purified ASA and ASB (10–100 μg) were freeze-dried and dissolved in 6 M guanidinium hydrochloride, 400 mM Tris–HCl (pH 8.6), and 10 mM EDTA. Dithiothreitol was added to a final concentration of 50 mM, mixed, and incubated for 1 hr at 50°C in an oxygen-free atmosphere. Following reduction, 2 M iodoacetic acid, adjusted to pH 8.6 with ammonium hydroxide, was added to a final concentration of 150 mM and incubated for 30 min at room temperature in darkness. To complete the reaction, a second cycle of reduction and carboxymethylation with 65 mM dithiothreitol and 122 mM iodoacetic acid (final concentration) followed. The sulfatases were then desalted on an HPLC system (SMART; Pharmacia) using a Sephadex G25 column (fast-desalting PC 3.2/10; Pharmacia) equilibrated with 50 mM ammonium acetate and 10% acetonitrile (pH 8.6). The desalted sulfatases were digested with 1% (w/w) (ASA) or 2% (w/w) (ASB) trypsin for 16 hr at 37°C. Tryptic peptides were separated by RP-HPLC using a 220 mm × 2.1 mm C8 column (Aquapore RP300; BAI) or, for the separation of peptides from [^{35}S]ASA, a 250 mm × 4.6 mm C18 column (Widepore RP18; Baker) equilibrated with 0.1% trifluoracetic acid in H_2O and eluted with an increasing acetonitrile concentration (0.9% acetonitrile per minute) and a flow rate of 0.3 ml per minute. Eluted peptides were collected by manual fractionation and subjected to mass spectrometry, N-terminal sequencing, or cleavage with endoglycosidase H (see below) or endoproteinase AspN as described previously (Sommerlade et al., 1994).

Digestion with Endoglucosidase H

Purified tryptic glycopeptides from ASA or ASB (~300 pmol) were dried, redissolved in 50 μl of 50 mM sodium acetate (pH 5.5), and digested with 2 mU of endoglycosidase H (Boehringer Mannheim) for 2 hr at 37°C. After a second incubation for 2 hr with 2 mU of endoglycosidase H, peptides were purified by RP-HPLC and analyzed by mass spectrometry.

Reduction with [^{3}H]$NaBH_4$

Lyophilized ASB, ASB peptide 3C*, or ASA peptide 2* (50–300 pmol) was dissolved in 20 μl of 4 M guanidinium hydrochloride, 10 mM EDTA, 25 mM Tris (pH 8.6) and incubated for 1 hr at 37°C. For reduction, $NaBH_4$ or [^{3}H]$NaBH_4$ (11.2 Ci/mmol; Amersham Corporation) from a 75 mM stock solution in 10 mM NaOH was added to a final concentration of 2.6 mM. After incubation for 1 hr at room temperature, excess of $NaBH_4$ was destroyed by addition of 1 vol of 50 mM acetic acid and incubation for 30 min at room temperature. Following reduction, ASA peptide 2* and ASB peptide 3C* were purified by RP-HPLC as described and subjected to mass spectrometry analysis and sequencing. Reduced ASB was subjected to reductive carboxymethylation, tryptic digestion, and peptide separation by RP-HPLC as described above. The HPLC fractions were analyzed by mass spectrometry, N-terminal sequencing, or liquid scintillation counting. The [^{3}H]peptide 3* from ASB from normal cells was rechromatographed by RP-HPLC using an acetonitrile gradient of 0.5% per minute and digested with endoproteinase AspN as described previously (Sommerlade et al., 1994). After purification by RP-HPLC, the [^{3}H]peptide 3C* was subjected to mass spectrometry analysis and radiosequencing.

[^{14}C]Carboxymethylation of ASA from MSD and MLD Cells

ASA purified from MSD or MLD fibroblasts was lyophilized, dissolved in 4 M guanidinium hydrochloride, 10 mM EDTA, and 25 mM Tris (pH 8.6), and incubated for 1 hr at 37°C. For carboxymethylation of free sulfhydryl groups, 10 mM [^{14}C]iodoacetic acid (53 mCi/mmol) was added to a final concentration of 0.5 mM. Following an incubation for 1 hr at room temperature in darkness, the [^{14}C]ASA was subjected to reductive carboxymethylation, tryptic digestion, and peptide separation by RP-HPLC as described above. The HPLC fractions were analyzed by mass spectrometry and scintillation counting.

Matrix-Assisted Laser Desorption Ionization Mass Spectrometry

We dried ~1 μl of indole-2-carbonic acid or 2.5-dihydroxybenzoic acid (10 mg/ml in acetone) on the sample slide to produce a thin matrix film. On this film, 0.5 μl of the HPLC fractions were dried and subjected

to mass spectrometry analysis. Alternatively, equal amounts of sample and matrix were mixed and dried on the sample slide. The samples were analyzed in a VISION 2000 (Finnigan MAT) or a MALDI III (Shimadzu) mass spectrometer. As mass standards for calibration, bovine insulin (M_r, 5733.5) and a synthetic peptide (M_r, 1979.1) were used. Mass data were evaluated using the personal computer program GPMAW (Lighthouse Data). To demonstrate the presence of an aldehyde group in ASA peptide 2* or ASB peptide 3C*, we used p-nitroaniline as a matrix, which forms a Schiff's base with aldehydes.

Amino Acid Sequencing

Sequence analysis of the tryptic peptides was performed as described previously (Sommerlade et al., 1994). For radiosequencing of [^{3}H]ASA peptide 2* or ASB peptide 3C*, the cleaved anilinothiazolinone amino acids were collected in a fraction collector and radioactivity was quantified.

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