Residues Critical for Formylglycine Formation and/or Catalytic Activity of Arylsulfatase A†

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ABSTRACT: Sulfatases contain a unique posttranslational modification in their active site, a formylglycine residue generated from a cysteine or a serine residue. The formylglycine residue is part of a sequence that is highly conserved among sulfatases, suggesting that it might direct the generation of this unique amino acid derivative. In the present study residues 68−86 flanking formylglycine 69 in arylsulfatase A were subjected to an alanine/glycine scanning mutagenesis. The mutants were analyzed for the conversion of cysteine 69 to formylglycine and their kinetic properties. Only cysteine 69 turned out to be essential for formation of the formylglycine residue, while substitution of leucine 68, proline 71, and alanine 74 within the heptapeptide **L**CT**P**SR**A** reduced the formylglycine formation to about 30−50%. Several residues that are part of or directly adjacent to an α-helix presenting the formylglycine 69 at the bottom of the active site pocket were found to be critical for catalysis. A surprising outcome of this study was that a number of residues fully or highly conserved between all known eukaryotic and prokaryotic sulfatases turned out to be essential neither for generation of formylglycine nor for catalysis.

Arylsulfatase A (ASA) is a lysosomal enzyme required for desulfation of cerebroside 3-sulfate, a major constituent of myelin sheaths. Deficiency of ASA is the cause of metachromatic leukodystrophy, a fatal lysosomal storage disorder associated with severe neurological symptoms such as quadripesis, peripheral neuropathy, seizure, blindness, and dementia (*1*). ASA shares with other sulfatases a unique posttranslational modification, a formylglycine residue, which is formed in most cases by oxidation of the thiol group of a cysteine (*2*) but in one prokaryotic sulfatase by oxidation of the hydroxyl group of a serine residue (*3*). This FGly residue is located in the active site of sulfatases (*4, 5*). Its critical role for catalytic activity of sulfatases is indicated by the inactivity of sulfatases in which the cysteine is not oxidized to FGly. Multiple sulfatase deficiency is a lysosomal storage disorder in which all known human sulfatases are inactive, because this posttranslational modification is deficient (*2*).

The FGly residue is surrounded by a sequence that is highly conserved among eukaryotic and prokaryotic sulfatases (*2, 3, 6*). It is conceivable that at least some of these conserved residues are essential for the generation of the FGly residue from the parent cysteine residue and thereby indirectly also for the catalytic activity of sulfatases. Other conserved residues may be critical for the catalytic activity due to their effect on the conformation of the catalytic site or due to a direct involvement in binding or cleavage of the substrate.

In the present study we have made an alanine/glycine scanning mutagenesis of residues 68−86 in ASA. This 19mer sequence includes the active site FGly 69 residue and 13 residues that are highly conserved between sulfatases. The ASA mutants were expressed in eukaryotic cells and analyzed for their kinetic properties and the structure of residue 69. As anticipated, substitution of cysteine 69 abolished both the FGly formation and also the catalytic activity. A few substitutions impaired partially the FGly formation and the catalytic activity (L68A, P71A, and A74G). Substitution of a few residues led to a reduction of activity (T70A) or prevented (R73A, V83A) or impaired (A74G, R80A) exit from the endoplasmic reticulum and decreased protein stability. To our surprise some of the residues fully (Ser 72, Thr 78) or highly conserved between eukaryotic and prokaryotic sulfatases could be substituted by alanine without apparent loss of function.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. The codons for residues 68−88 of human ASA were replaced by codons for alanine, with the exeption of Ala 74 and Ala 75, which were replaced by glycine. Mutagenesis was performed using the pAlter cloning kit (Promega Corp.). The cDNA for human ASA in combination with the early promoter and the polyadenylation signal of the SV40 virus was inserted into the pAlter mutagenesis vector. The *Aat*II/*Dra*II-fragment from clone pBEH/HT14-CP8 (*7*) containing the early SV40 promoter, the complete cDNA for human ASA, and the SV40 polyadenylation signal was isolated. After the ends of this fragment and of the pAlter vector opened by *Hin*dIII and *Eco*RI were filled, the fragment was inserted by blund end ligation. The resulting plasmid and appropriate primers were used for mutagenesis according to manufacturer's instructions (Promega Corp.). At a ratio of repair oligonucleotide to

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mutagenesis oligonucleotide of 1 to 80 close to 100% of the clones carried the desired mutation.

Expression and Analysis of ASA Mutants in BHK21 Cells. ASA mutants were expressed in BHK21 cells as described (*7*) using pSVL2pac and pSVL2neo for selection. For transient expression cells were harvested 72 h after transfection and analyzed for ASA activity (*8*). Briefly, 40 μL of cell homogenate was incubated with 200 μL of 10 mM *p*-nitrocatechol sulfate, 0.5 M sodium acetate, pH 5.0, 1.5 M NaCl, 1 mM sodium pyrophosphate, and 1 mg/mL BSA at 37 °C for 30 min. Reaction was stopped by adding 600 μL of 1 M NaOH, and absorbance of *p*-nitrocatechol was measured at 515 nm. Activity was calculated and related to total cell protein. For calculation of specific activity (units per milligram of ASA) the concentration of ASA was determined by an enzyme-linked immunoassay using a polyclonal antibody against ASA from human liver.

For several mutants transient expression did not provide ASA expression levels sufficient for analysis. The cDNAs of these mutants (R73A, A74G, L77A, G79A, R80A, L81A, V83A, and G86A) were stably expressed in BHK21 cells. Transfection was performed as described for transient expression, but with selection of single clones in medium containing puromycin or neomycin. Activity and concentration of ASA were determined as described above. To analyze stability of the mutants, cells were metabolically labeled with [^{35}S]methionine for 2 h followed by harvesting directly after the labeling or after a chase of 24 h (*9*). For labeling phosphorylated oligosaccharide side chains of ASA, metabolic labeling was performed with [^{32}P]phosphate. After immunoprecipitation of ASA and separation by SDS−PAGE, radioactivity in ASA polypeptides was determined by autoradiography and densitometric quantification of the X-ray film.

Mutant and wild-type ASA were localized in BHK cells by immunofluorescent staining as described (*10*). Cells were fixed with 3% paraformaldehyde, 10 mM sodium phosphate, pH 7.0, and 150 mM NaCl (PBS) and permeabilized with 0.5% saponin in PBS. ASA was labeled with a monoclonal mouse antibody directed against human ASA, diluted 1:2000 (*11*), followed by incubation with fluorochrome-labeled antibody directed against IgG from mouse, diluted 1:200. Immunofluorescence was visualized by a confocal laser scanning microscope (Zeiss, Oberkochen/Germany) using 488 nm for excitation.

Expression and Analysis of ASA Mutants in mpr$^-$ MEF Cells. Thirteen ASA mutants (L68A−S72A, A74G, L77A−R80A, P82A, M85A, and H86A) and wild-type ASA were stably expressed in mouse embryonic fibroblasts deficient in mannose 6-phosphate receptors (mpr$^-$ MEF; ref *12*). ASA was purified from cell secretions by affinity chromatography (*13*). Kinetic parameters of sulfatase activity of the ASA mutants were determined using 2, 5, 10, and 20 mM *p*-nitrocatechol sulfate as substrate and the assay protocol described above. V_{max} and K_M were calculated according to the method of Lineweaver and Burk. To monitor the purity and to quantify the amount of ASA polypeptides, the preparations were subjected to reversed-phase HPLC using a Aquapore-butyl 7 μm (220 mm × 2.1 mm) column (Applied Biosystems) equilibrated with 0.1% trifluoracetic acid and eluted by an increasing gradient of acetonitrile (2%/min) at a flow rate of 0.3 mL/min. The amount of ASA protein was calculated from the integral of the ASA peak at 280 nm compared to an ASA standard of known concentration.

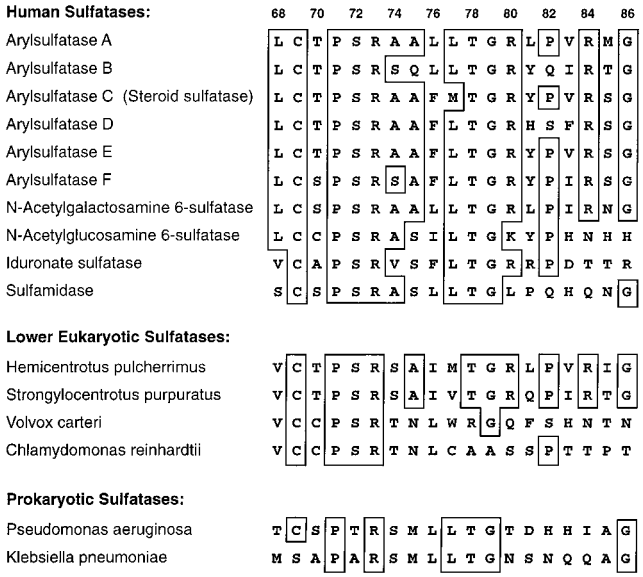

FIGURE 1: Sequence homology among human, lower eukaryotic, and prokaryotic sulfatases. Residues 68−86 are shown including cysteine 69, which is converted to formylglycine. Residues in boxes are present in at least seven of the human sulfatases. See ref *6*.

To determine pH dependence of sulfatase activity 40 μL of enzyme was added to 100 μL of sodium acetate which was adjusted to pH values ranging from 3.6 to 6.8. Then 100 μL of 20 mM *p*-nitrocatechol sulfate in H_2O was added, followed by an incubation for 30 min at 37 °C.

To determine the presence of formylglycine or unmodified cysteine at position 69, the purified ASA mutants were reduced and carboxymethylated under denaturing conditions and digested with trypsin. Peptides were purified by reversed-phase HPLC and identified by MALDI mass spectrometry (*2*, *14*). Peptide 2* containing FGly 69 and peptide 2 containing the carboxymethylated Cys 69 were quantified by amino acid sequencing (*13*).

RESULTS

Expression of ASA Mutants in BHK Cells. Residues 68−86 of human ASA comprise a sequence which is highly conserved among sulfatases. Of the 19 residues 13 are found in 7 or more of the 10 known human sulfatases. If compared with all known sulfatases from prokaryotes and eukaryotes, 6 of these residues are conserved in 14 or more of the 16 known sulfatases (Figure 1). The most highly conserved residues are found in two clusters covering the pentapeptide 69−73 (CTPSR) and the tetrapeptide 77−80 (LTGR).

Residues 68−86 were individually substituted by alanine except for Ala 74 and Ala 75, which were substituted by glycine. The cDNAs were transiently expressed in BHK cells. Three days after transfection the cells were harvested and analyzed for ASA activity and for the amount of ASA polypeptides by an ELISA. For 11 of the 19 mutants the expression levels were high enough (≥20 ng of recombinant ASA/mg of cell protein) to determine the specific activity of ASA (Table 1, transient). Substitution of four residues (L68A, C69A, T70A, and P71A) reduced the specific activity

Table 1: Transient and Stable Expression of ASA in BHK Cells

ASA−cDNA expressed	activity[a] (milliunits/mg of cell protein)	arylsulfatase A concn[a] (ng/mg of cell protein)	specific activity (units/mg of ASA)
transient			
wild type ($n = 10$)	6.0 ± 3.4	113 ± 57	61 ± 25
L68A	0.5	124	4
C69A	1.0	160	6
T70A	0.5	42	12
P71A	0.6	30	20
S72A	2.4	43	56
A75G	7.9	67	118
L76A	11.9	130	92
T78A	3.7	39	95
P82A	1.6	24	67
R84A	6.6	73	90
M85A	2.2	30	73
stable			
L77A	56.2	1107	51
L81A	15.6	311	50

[a] For transiently expressed cDNAs the activity and the concentration of ASA were determined in cell homogenates harvested 72 h after transfection. The values for ASA activity were corrected for endogenous ASA activity of BHK cells (1.7 milliunits/mg). The ELISA used for determination of ASA polypeptides detects only human ASA polypeptides.

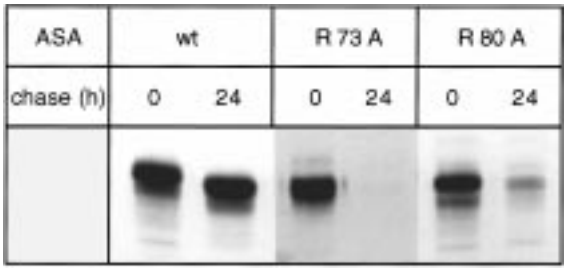

FIGURE 2: Stability of metabolically labeled ASA mutants. Cells expressing wild-type and mutant ASA were metabolically labeled for 2 h as described in Experimental Procedures. After a chase period of 0 or 24 h sulfatases were immunoprecipitated, separated by SDS−PAGE, and detected by autoradiography. In comparison to the wild-type ASA two mutants with decreased stability are shown. For quantification see Table 2.

markedly, while substitution of seven residues (S72A, A75G, L76A, T78A, P82A, R84A, and M85A) did not impair the catalytic activity.

For the remaining eight ASA mutants, the expression levels were too low for a reliable determination of the amount of ASA polypeptides. These mutants were stably expressed in BHK cells. For two of the mutants, BHK clones with high expression of ASA were obtained (Table 1, stable). The catalytic activity of these ASA mutants (L77A and L81A) was in the range of wild-type ASA.

Clones selected for expression of five of the remaining ASA mutants (R73A, A74G, R80A, V83A, and G86A) were metabolically labeled for 2 h with [^{35}S]methionine. The cells were harvested directly after the labeling or after a chase of 24 h. Radioactivity incorporated into ASA was determined after immunoprecipitation and separation by SDS−PAGE (Figure 2). In clones expressing wild-type ASA 86 ± 18% of the [^{35}S]ASA synthesized during the 2 h labeling period was recovered after the chase for 24 h. A comparable recovery was observed for the G86A mutant (Table 2). The R73A and V83A mutants were unstable as indicated by the

Table 2: Stability of ASA Mutants

ASA expressed	[^{35}S]ASA remaining after chase for 24 h (% of initial)
wild type ($n = 4$)	86 ± 18
R73A	<2
A74G ($n = 4$)	28 ± 15
R80A ($n = 2$)	32 ± 12
V83A	<2
G86A ($n = 2$)	90 ± 6

complete loss of the labeled polypeptides during the chase period (Figure 2, Table 2). The A74G and R80A mutants had a reduced stability with an intermediate recovery of labeled polypeptides after the chase for 24 h (Figure 2, Table 2).

Localization of ASA by indirect immunofluorescence revealed for the R73A and V83A mutants a fine reticular distribution concentrated around the nucleus, suggesting that these mutants are confined to the endoplasmic reticulum. This is clearly distinct from the granular distribution of the wild-type ASA which reflects its localization in lysosomes (Figure 3). The A74G and R80A mutants showed an intermediate phenotype with mostly a fine reticular distribution around the nucleus and some granular staining extending to the cell periphery (Figure 3). This suggests that a part of these mutants resides in the endoplasmic reticulum, while a fraction exits the endoplasmic reticulum and is transported to a granular lysosome-like compartment. This is supported by the incorporation of [^{32}P]phosphate into newly synthesized A74G and R80A (not shown). Phosphorylation of ASA is indicative of its modification by the *N*-acetylglucosamine 1-phosphotransferase, an enzyme localized in the cis-Golgi compartment (*15*).

Analysis of ASA Mutants. To characterize the kinetic parameters for the purified ASA mutant proteins in more detail and to determine the modification of cysteine 69 in the ASA polypeptides, a total of 13 substitution mutants were stably expressed in mpr$^-$ MEF cells. These cells are deficient in mannose 6-phosphate receptors and therefore secrete newly synthesized lysosomal enzymes such as ASA, which normally are targeted to lysosomes via mannose 6-phosphate receptors (*12*). The 13 substitution mutants included the four mutants which had a decreased specific activity, the two mutants A74G and R80A which were partly retained in the endoplasmic reticulum and partly transported to a lysosome-like compartment, and the G79A mutant for which no expressing BHK21 clone had been obtained. All mutant ASA forms were purified to apparent homogeneity by immunoaffinity chromatography and analyzed for V_{max}, K_M, the pH optimum, and the percentage of Cys 69 converted to FGly (Table 3 and Figure 4).

FGly formation was completely abolished in the C69A mutant, confirming an earlier study in which the FGly formation in a coupled in vitro translation−translocation system was determined (*14*). The C69A mutant was inactive, in agreement with the proposed function of FGly 69 in sulfate ester cleavage (*4, 5, 16*). The FGly formation was reduced to 30−50% of control in the L68A, P71A, and A74G mutants. The residual activity of these mutants was reduced to less than 15% of control, suggesting that substitution of Leu 68, Pro 71, or Ala 74 affects catalytic activity not solely by reducing the FGly formation. The L68A mutant had a

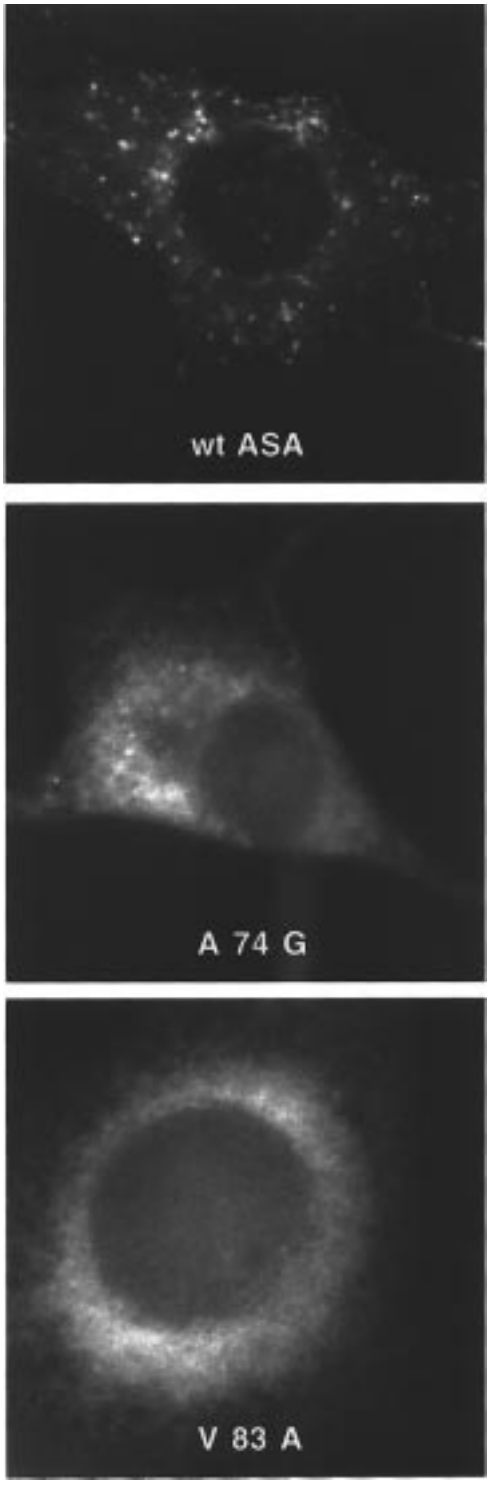

FIGURE 3: Intracellular distribution of ASA mutants**.** BHK cells expressing wild-type ASA and the ASA mutants A74G and V83A were fixed, permeabilized with saponin, and labeled with a monoclonal mouse antibody against human ASA. Bound antibody was detected by incubation with fluorochrome-labeled antibody directed against IgG from mouse.

reduced affinity for *p*-nitrocatechol sulfate (K_M 29 mM, compared to 3.5 mM in wild-type ASA), and the P71A mutant had a slightly shifted pH optimum (Figure 4).

Surprisingly, substitution of the other highly conserved residues (e.g., S72A, L77A, T78A, G79A, R80A, P82A, R84A, and G86A) did not affect the generation of FGly 69 from cysteine. The V_{max} of some of these mutants (S72A, L77A) was reduced to intermediate levels, and the pH optima

Table 3: Kinetic Parameters and FGly Content of ASA Mutants

ASA	V_{max}[a] (units/mg pf ASA)	K_M[a]	FGly 69[b] (mol/mol of ASA)
wild type ($n = 3$)	79.0 ± 7.6	3.5 ± 0.6	0.90 ± 7
L68A	7.6	29.0	0.50
C69A	< 0.2	3.5	< 0.02
T70A	11.0	14.6	0.98
P71A	5.4	1.0	0.32
S72A	70.9	1.8	0.71
A74G	11.0	4.8	0.40
L77A	22.0	2.7	0.98
T78A	79.0	4.8	0.79
G79A	92.8	1.7	0.62
R80A	54.0	2.7	0.83
P82A	73.0	1.2	0.79
M85A	28.0	2.4	0.78
G86A	43.0	5.0	0.92

[a] V_{max} and K_M were determined by measuring cleavage of *p*-nitrocatechol sulfate as described in Experimental Procedures. [b] For determination of FGly 69 see Experimental Procedures.

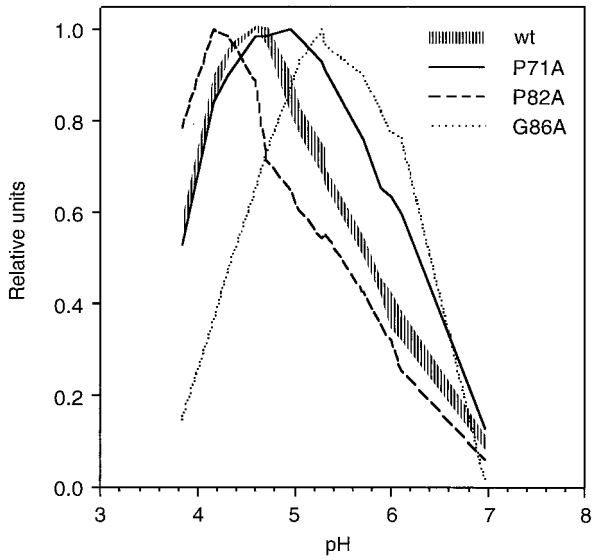

FIGURE 4: pH optimum of ASA mutants. The pH dependence of cleavage of *p*-nitrocatechol sulfate is shown for the three ASA mutants (P71A, solid line; P82A, dashed line; G86A, dotted line) which differed from wild-type ASA, for which the pH dependence observed in three experiments is indicated as shaded area.

of mutants P82A and G86A were shifted to a more acid and alkaline pH, respectively (Figure 4).

Substitution of residue 70, which in 14 out of 16 sulfatases is either a hydroxy amino acid (T or S) or a cysteine (see Figure 1), reduced V_{max} to less than 15% and increased the K_M for *p*-nitrocatechol sulfate about 4-fold. This came as a surprise as in two sulfatases (human iduronate sulfatase and the sulfatase from *Klebsiella pneumoniae*) an alanine is found in the corresponding position. Substitution of the nonconserved Met 85 had a moderate effect on V_{max} (Table 3).

DISCUSSION

Structural Requirements for the Generation of FGly 69. In eukaryotic sulfatases the FGly residue in the active site is posttranslationally generated from a cysteine residue. The structural motif which directs the conversion of the cysteine to a FGly residue is unknown. While this study was in progress, it became known that this modification is catalyzed within the endoplasmic reticulum and that a linear sequence

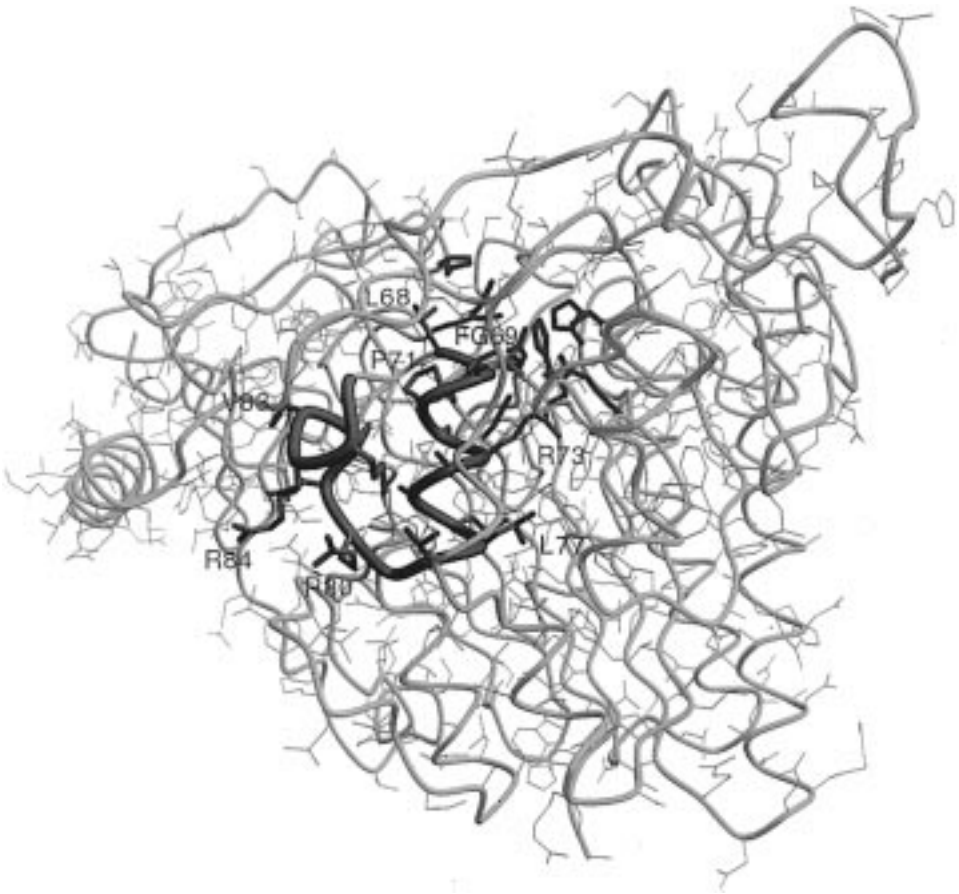

FIGURE 5: Position of amino acids 68−86 within the ASA molecule. The three-dimensional structure of the human ASA monomer is shown as determined by Lukatela et al. (*4*). The polypeptide backbone is drawn as a continuous rope from which amino acid residues protrude. The backbone and residues of amino acids 68−86 are shown in red; formylglycine residue 69 is in magenta. Other residues forming the active site are shown in green; the magnesium ion is in dark blue. Amino acids 68−86 form an α-helix (69−78) followed by a disordered structure (79−86), which turns the polypeptide chain to the surface of the molecule. The figure was drawn using computer programs MolScript 2.0.2 (*17*) and Raster3D 2.4 (*18*).

corresponding to residues 65−80 of human ASA is sufficient to direct the modification of Cys 69. It was concluded that modification of Cys 69 does not require folding of ASA to its native structure and that the modification is likely to occur either during translocation into the endoplasmic reticulum or shortly thereafter (*14*).

Due to the particularly high sequence homology among eukaryotic and prokaryotic sulfatases in the sequence following the cysteine residue to be modified, we reasoned that one of the functions of these conserved residues could be to serve as a structural motif for the generation of FGly. The specificity of this region of homology for sulfatases is underlined by the fact that the undecapeptide CXPSRXXXLTG, which is found in all eukaryotic sulfatases with the exception of steroid sulfatase, where the leucine is replaced by a methionine (Figure 1), is found only in sulfatases, when available protein sequence databases are screened.

It came, therefore, as a surprise that most of the conserved residues could be substituted by alanine without affecting the modification of Cys 69. In fact, only the substitutions of Cys 69 itself abolished the modification. This confirmed the earlier observation made in an in vitro translation−translocation system, that substitution of Cys 69 or inverting the position of Cys 69−Thr 70 to Thr 69−Cys 70 interferes with the generation of FGly (*14*). Substitution of the fully conserved Pro 71 and the less higher conserved Leu 68 and Ala 74 reduced the modification of Cys 69 to about 30−50%. None of the other residues within the sequence 68−86 turned out to be critical for modification of Cys 69. It should be noted, however, that the function of Arg 73, a residue conserved among all sulfatases, and of Val 83, a nonconserved residue, for modification of Cys 69 could not be investigated, because their substitution rendered ASA unstable.

We conclude from these results that the structural motif determining the conversion of Cys 69 to FGly is of an extended nature, tolerating the substitution of single amino acids except for Cys 69. A closer definition of this motif, to which at least four residues within the heptapeptide **LC**T**P**SR**A** contribute as shown in this study, will require the simultaneous substitution of two or more residues.

Residues Critical for Catalytic Activity of ASA. One of the possible explanations for the particularly high conservation of the sequence corresponding to residues 68−86 of ASA is that this sequence may contribute to the part of the catalytic site of sulfatases that ensures sulfate ester cleavage.

The FGly 69 is indeed part of the active site and is proposed to participate in the sulfate ester cleavage as an aldehyde hydrate (*4, 16*). The active site is distinguished by a Mg^{2+} ion which is coordinated by one of the hydroxyls of the FGly hydrate and the functional groups of Asp 29, Asp 30, Asp 281, and Asn 282. Furthermore, His 125 is

found in close vicinity to the second hydroxyl of FGly, suggesting a role in proton transfer during sulfate cleavage. The cluster of the three positively charged residues His 229, Lys 123, and Lys 302 is proposed to bind and position the sulfate group. These residues form a pocket on the surface of ASA (Figure 5).

The recent description of the crystal structure of ASA (*4*) has revealed that the residues that have been substituted in this study are not directly part of the proposed active site of ASA apart from residue 69. Residues 69−78 form an α-helix. At its top this helix is positioning the FGly 69 at the bottom of the cavity of the active site, from where the helix extends into the interior of ASA (Figure 5). Residues 78−86, which are not folded in a secondary structure, turn the polypeptide chain back to the surface of ASA, where the main chain of residues 84−86 and the residues of Arg 80, Leu 81, and Arg 84 are exposed.

In addition to Cys 69 four residues between positions 68 and 86 turned out to be critical for ASA activity (Leu 68, Thr 70, Pro 71, and Ala 74). Substitution of Pro 71 resulted in a small change of pH optimum. All are part of or directly adjacent to the α-helix extending from residues 69 to 78. This may indicate that their substitution indirectly affects the positioning of FGly 69 in the active site. Since substitution of Leu 68, Pro 71, and Ala 74 reduced also the conversion of cysteine 69 to formylglycine, part of the inactivity can also be attributed to the absence of FGly in the active site. It should be noted, however, that the reduction of V_{max} caused by substitution of Leu 68, Pro 71, and Ala 74 was much more prominent than could be explained by the impairment of FGly formation.

Substitution of Leu 68 reduced both V_{max} and affinity (K_M 29 mM as compared to 3.5 mM in wild-type ASA). Modeling of *p*-nitrocatechol sulfate into the active site of ASA suggests that the side chain of Leu 68 (R. von Bülow, unpublished) contributes to the binding of the aromatic ring of the substrate and that its loss in the L68A mutant reduces the affinity. Substitution of Thr 70, one of the few nonconserved residues in the sequence following Cys 69, decreased V_{max} and affinity (K_M 14 mM). The hydroxyl of Thr 70 may contribute to the coordination of His 125 by forming a hydrogen bond between its oxygen and the carboxyl oxygen of His 125. His 125 has been proposed to participate in the catalytic mechanism of ASA (*4*) and its substitution by alanine leads to a marked loss of activity (A. Waldow and B. Schmidt, unpublished). The substitution of Thr 70 by alanine may therefore impair ASA activity through dislocation of His 125.

The present study could not provide a clue for the functional significance of several of the most highly conserved residues in the neighborhood of FGly 69. This applies in particular to Ser 72, Thr 78, and Gly 79, which are found in all human sulfatases and also in the majority of nonhuman sulfatases, but is noteworthy also for Leu 77, Arg 80, Pro 82, and Arg 84, which are conserved in at least 9 of the 14 known eukaryotic sulfatases. Their substitution by alanine affected neither the catalytic properties nor the modification of Cys 69 to a notable extent. A functional property that is of importance for many sulfatases and therefore may impose an evolutionary constraint concerns their stability within the lysosomal environment rich in proteinases. For example, substitution of Pro 426 by leucine is one of the most common mutations found in late onset forms of metachromatic leukodystrophy, a lysosomal storage disorder caused by the deficiency of ASA (*1*). Substitution of Pro 426 by leucine affects the stability of ASA in the lysosomes, while the catalytic and transport properties are not affected (*19*). It may well be that some of the highly conserved residues which could be substituted by alanine without affecting the parameters examined may be critical for functions such as the stability in lysosomes.

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