Arylsulfatase A belongs to the sulfatase family whose members carry a Co-formylglycine that is post-translationally generated by oxidation of a conserved cysteine or serine residue. The formylglycine acts as an aldehyde hydrate with two geminal hydroxyls being involved in catalysis of sulfate ester cleavage. In arylsulfatase A and N-acetylgalactosamine 4-sulfatase this formylglycine was found to form the active site together with a divalent cation and a number of polar residues, tightly interconnected by a network of hydrogen bonds. Most of these putative active site residues are highly conserved among the eukaryotic and prokaryotic members of the sulfatase family. To analyze their function in binding and cleaving sulfated esters, we substituted a total of nine putative active site residues of human ASA by alanine (Asp29, Asp30, Asp281, Asn282, His125, His229, Lys123, Lys302, and Ser150). In addition the Mg2+-complexing residues (Asp29, Asp30, Asp281, and Asn282) were substituted conservatively by either asparagine or aspartate. In all mutants Vmax was decreased to 1–26% of wild type activity. The Km was more than 10-fold increased in K123A and K302A and up to 5-fold in the other mutants. In all mutants the pH optimum was increased from 4.5 by 0.2–0.8 units. These results indicate that each of the nine residues examined is critical for catalytic activity, Lys123 and Lys302 by binding the substrate and the others by direct (His125 and Asp281) or indirect participation in catalysis. The shift in the pH optimum is explained by two deprotonation steps that have been proposed for sulfate ester cleavage.

Mammalian sulfatases are involved in the degradation of sulfated substrates like mucopolysaccharides, cerebrosides sulfates, and sulfated steroids. The key residue for catalytic activity in sulfate ester cleavage has recently been shown to be a Co-formylglycine (FGly; Refs. 1 and 2), which is post-translationally generated from a cysteine in the endoplasmic reticulum (3). Failure of this amino acid modification leaves newly synthesized sulfatases inactive and is the cause of multiple sulfatase deficiency, a rare but fatal lysosomal storage disorder (Ref. 1; reviewed in Refs. 4 and 5). X-ray determination of the three-dimensional structures of two human sulfatases, arylsulfatase A (ASA; Ref. 6) and N-acetylgalactosamine 4-sulfatase (aryl sulfatase B, ASB; Ref. 7), revealed that the FGly residue is buried at the bottom of a cavity that is formed by positively and negatively charged amino acids. This cavity proved to be the active site by two lines of evidence. Co-cryocrystalization of ASB with vanadate, a potent inhibitor of ASB, revealed that vanadate was covalently bound to FGly (7). In addition, an ASA mutant containing serine instead of FGly was found to be covalently sulfated at the position of FGly after incubation with sulfate ester (see below and Ref. 8). The 2-fold disordered electron density of FGly was interpreted to be an aldehyde hydrate with two geminal hydroxyls protruding into the lumen of the cavity (6). In close vicinity to the aldehyde hydrate a metal ion (Mg2+ in ASA and Ca2+ in ASB) is complexed by three aspartates and one asparagine. Two lysines, two histidines, and a serine complete the arrangement of residues potentially involved in binding and cleavage of the sulfate group within the active site.

These data led to a proposal for the catalytic mechanism of sulfate ester cleavage (6) as follows (Fig. 1). In the first half-cycle, one of the two geminal oxygens of the aldehyde hydrate attacks the sulfur of the sulfate ester leading to a transesterification of the sulfate group onto the aldehyde hydrate. Simultaneously the substrate alcohol is released. In the second half-cycle, sulfate is eliminated from the enzyme-sulfate intermediate by an intramolecular rearrangement induced by the second oxygen. By this “intramolecular hydrolysis” the aldehyde group is regenerated. The essential role of FGly in forming the transient enzyme-sulfate ester has been shown indirectly (8). Mutants of ASA and ASB were expressed in which the FGly had been replaced by a serine. These mutants were able to cleave sulfate ester and to generate a sulfated enzyme intermediate by an intramolecular rearrangement induced by the second oxygen. By this “intramolecular hydrolysis” the aldehyde group is regenerated. The essential role of FGly in forming the transient enzyme-sulfate ester has been shown indirectly (8).

The function of the other residues forming the active site remained speculative. In ASA one oxygen of the aldehyde hydrate is in close vicinity to the Mg2+ ion, which is complexed by residues Asp29, Asp30, Asp281, and Asn282, and the other oxygen is close to the imidazole ring of His125. Whereas Mg2+ is thought to coordinate the oxygen that attacks the sulfur in the first step of sulfate cleavage, His125 is a candidate to deprotonate the second hydroxyl of the aldehyde hydrate, which is involved in the release of the sulfate and the regeneration of the aldehyde. By modeling a sulfate anion into the active site, the positively charged residues Lys123, Lys302, His125, and residue Ser150 were proposed to fix the sulfate group (6). To obtain experimental evidence for the function of the putative active site residues, we replaced residues Asp29, Asp30, Asp281, Asn282, His125, His229, Lys123, Lys302, and Ser150 by alanine in ASA and analyzed Vmax, Km, and the pH optimum of the

* This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Abt. Biochemie II, Gosslerstr. 12d, D-37073 Göttingen, Germany. Tel.: 49-551-395951; Fax: 49-551-395979; E-mail: bschmidt@uni-bc2.gwdg.de.

The abbreviations used are: FGly, Co-formylglycine; ASA, arylsulfatase A; ASB, N-acetylgalactosamine 4-sulfatase; HPLC, high pressure liquid chromatography.

© 1999 by The American Society for Biochemistry and Molecular Biology, Inc.

Printed in U.S.A.

From the Zentrum für Biochemie und Molekulare Zellbiologie, Abteilung Biochemie II, Universität Göttingen, Gosslerstrasse 12d, 37073 Göttingen, Germany

Amino Acid Residues Forming the Active Site of Arylsulfatase A

ROLE IN CATALYTIC ACTIVITY AND SUBSTRATE BINDING*
cleavage of p-nitrocatechol sulfate. The change of enzymatic activity observed for all mutants was interpreted on the basis of the three-dimensional structure of the active site.

**EXPERIMENTAL PROCEDURES**

**Amino Acid Sequence Alignment**—The amino acid sequences of the ten known human sulfatases, six lower eukaryotic, and three bacterial sulfatases (listed in Fig. 2), which are known to be active in cleaving sulfate esters, were retrieved from the SWISSPROT data bank (entry names: ARSA_HUMAN, ARSB_HUMAN, STS_HUMAN, ARSD_HUMAN, ARSE_HUMAN, GASS_HUMAN, GLGS_HUMAN, IDS_HUMAN, SPHM_HUMAN, ARS_HEMPS, ARS_HEMPS, ARS_VOLCA, ARS_CHLRE, ARS_PSEAE, and ARS_KLEAE) and from the GenBankTM database (accession numbers HSARSF, AFO13188, and US9492). Sequence alignments were performed using the computer programs SIMILARITY, CLUSTAL, and BLAST-2-SEQUENCES (Genetic Computer Group, Madison, WI).

**Determination of Distances and Potential Hydrogen Bonds between the Residues of the Active Site of ASA**—The atomic coordinates of human ASA (identification code 1AUK, Protein Data Bank of the Brookhaven National Laboratory) were analyzed for the distances between the functional groups of the active site residues using the computer program XtalView (9). Interatomic distances in the range of 2.5–3.2 Å between potential proton donors and acceptors were considered to be bridged by hydrogen bonds (10). All these potential hydrogen bonds are indicated in Fig. 3. The coordinates for the aldehyde hydrate residue of FGly69 were obtained from G. Lukatela (Berlin, Germany).

**Site-directed Mutagenesis**—Single amino acid substitutions were performed replacing the codons for the putative active site residues Asp29, Asp30, Asp281, Asn282, His125, His229, Lys123, Lys302, and Ser150 as possible sulfate binders with Ala (15). The putative active site residues are highly conserved among 19 eukaryotic and prokaryotic sulfatases, which are known to exhibit sulfatase activity (Fig. 2). Most of these residues are part of short sequences of conserved amino acids. Cys69 in ASA and its equivalents in the other sulfatases are converted to FGly. The sulfatase of Klebsiella pneumoniae is the only biochemically characterized sulfatase where a serine rather than a cysteine is encoded by the DNA. However, the serine is also converted to FGly (14). Asp29, Asp30, Lys123, His125, His229, Lys302, and Ser150 are conserved or conservatively replaced in 14–19 sulfatases. Ser150 is only found in ASA but replaced by threonine in 6 sulfatases. The high conservation of these residues indicates that the structure of the active site and the catalytic mechanism of sulfatel cleavage have been conserved in the sulfatase family.

**Expression of Active Site Mutants**—To obtain experimental evidence for their role in sulfate ester cleavage and sulfate binding, nine of the putative active site residues of ASA were individually substituted by alanine: Asp29, Asp30, Asp281, and Asn282 as the metal coordinating residues, His125 as one of the two FGly69 activating residues (the other one being Asp281), and Lys123, Lys302, His229, and Ser150 as possible sulfate binding residues. Cys69 has been substituted by alanine earlier (12), and the mutant C69A proved to be completely inactive. Arg73 that is interacting with Asp281 (Fig. 3) is another putative residue of FGly69 and by five δ-oxygens of Asp29, Asp30, Asp281, and Asn282. The same hydroxyl oxygen of FGly69 is in close vicinity to the second oxygen of the FGly hydrate. Therefore, His125 is in an appropriate position to de-protonate and thereby activate this second oxygen of the FGly hydrate leading to the regeneration of the oxoxygen of the enzyme and the cleavage of the enzyme-sulfate intermediate. Lys123, Lys302, His229, and Ser150 have been proposed to contribute to the binding of the sulfate group and to the activation of the sulfur atom by increasing its electrophilia (6). In addition, these residues may form hydrogen bonds with other residues of the active site: Lys123 with Asp29 and Ser150 as well as Lys302 with Asp30. All residues supposed to be involved in sulfate binding or in catalysis are able to form hydrogen bonds to neighboring residues, resulting in a net-like connection of the residues to a functional catalytic system (Fig. 3).

The putative active site residues are highly conserved among 19 eukaryotic and prokaryotic sulfatases, which are known to exhibit sulfatase activity (Fig. 2). Most of these residues are part of short sequences of conserved amino acids. Cys69 in ASA and its equivalents in the other sulfatases are converted to FGly. The sulfatase of Klebsiella pneumoniae is the only biochemically characterized sulfatase where a serine rather than a cysteine is encoded by the DNA. However, the serine is also converted to FGly (14). Asp29, Asp30, Lys123, His125, His229, Asp281, Asn282, and Lys302 are conserved or conservatively replaced in 14–19 sulfatases. Ser150 is only found in ASA but replaced by threonine in 6 sulfatases. The high conservation of these residues indicates that the structure of the active site and the catalytic mechanism of sulfate cleavage have been conserved in the sulfatase family.

**RESULTS AND DISCUSSION**

**Putative Active Site Residues of Arylsulfatase A**—The amino acid residues supposed to form the active site of ASA are shown in Fig. 3 in a three-dimensional view. FGly69 is shown as an aldehyde hydrate with two geminal hydroxyl groups. The Mg2+ ion is octahedrically coordinated by one hydroxyl oxygen of FGly69 and by five δ-oxygens of Asp29, Asp30, Asp281, and Asn282. The same hydroxyl oxygen of FGly69 is in close vicinity to one of the oxygens of Asp281. The atomic distance of 3.4 Å between FGly69 and Asp281, derived from the data of Lukatela et al. (Ref. 6 and see “Experimental Procedures”), may be smaller considering the weak resolution of the electron density in this area and the free rotatability of the Ca-Cα bond in FGly69. Thus, a hydrogen bond may be formed between the neighboring oxygens of FGly69 and Asp281 leading to an at least partial protonation of this hydroxyl of FGly69. The free oxygen would then be able to start a nucleophilic attack on the sulfur of the sulfated substrate. Coordination to Mg2+ may help to stabilize this catalytically active form of FGly69. On the other side His125 has an atomic distance of 2.7 Å to the second oxygen of the FGly hydrate. Therefore, His125 is in an appropriate position to de-protonate and thereby activate this second oxygen of the FGly hydrate leading to the regeneration of the oxoxygen of the enzyme and the cleavage of the enzyme-sulfate intermediate. Lys123, Lys302, His229, and Ser150 have been proposed to contribute to the binding of the sulfate group and to the activation of the sulfur atom by increasing its electrophilia (6). In addition, these residues may form hydrogen bonds with other residues of the active site: Lys123 with Asp29 and Ser150 as well as Lys302 with Asp30. All residues supposed to be involved in sulfate binding or in catalysis are able to form hydrogen bonds to neighboring residues, resulting in a net-like connection of the residues to a functional catalytic system (Fig. 3).

The putative active site residues are highly conserved among 19 eukaryotic and prokaryotic sulfatases, which are known to exhibit sulfatase activity (Fig. 2). Most of these residues are part of short sequences of conserved amino acids. Cys69 in ASA and its equivalents in the other sulfatases are converted to FGly. The sulfatase of Klebsiella pneumoniae is the only biochemically characterized sulfatase where a serine rather than a cysteine is encoded by the DNA. However, the serine is also converted to FGly (14). Asp29, Asp30, Lys123, His125, His229, Asp281, Asn282, and Lys302 are conserved or conservatively replaced in 14–19 sulfatases. Ser150 is only found in ASA but replaced by threonine in 6 sulfatases. The high conservation of these residues indicates that the structure of the active site and the catalytic mechanism of sulfate cleavage have been conserved in the sulfatase family.

**Expression of Active Site Mutants**—To obtain experimental evidence for their role in sulfate ester cleavage and sulfate binding, nine of the putative active site residues of ASA were individually substituted by alanine: Asp29, Asp30, Asp281, and Asn282 as the metal coordinating residues, His125 as one of the two FGly69 activating residues (the other one being Asp281), and Lys123, Lys302, His229, and Ser150 as possible sulfate binding residues. Cys69 has been substituted by alanine earlier (12), and the mutant C69A proved to be completely inactive. Arg73 that is interacting with Asp281 (Fig. 3) is another putative
active site residue. However, the R73A mutant has been expressed earlier (12) and was found to be unstable, thus pre-
venting its further characterization. The cDNAs encoding the
mutant forms of ASA were stably expressed in mpr
2
MEF cells. Due to deficiency in mannose 6-phosphate receptors, these cells
secrete the newly synthesized lysosomal enzymes such as ASA
(13). Clones stably expressing the mutant sulfatases were se-
lected and expanded. The sulfatases were purified from the
medium by affinity chromatography and analyzed for purity by
analytical reversed phase chromatography and SDS-polyacryl-
amide gel electrophoresis. In all preparations ASA polypep-
tides represented 80–90% of total protein. The major contam-
nation (5–10%) was bovine serum albumin originating from
the culture medium (data not shown).

Kinetic Properties of Active Site Mutants—
We analyzed
V
max,
Km
, and pH optimum for the cleavage of
p-
nitrocatechol sulfate
by the different substitution mutants (Table I). To control
whether a loss of activity could be accounted for by a dimin-
ished conversion of Cys69 to FGly, all mutant proteins were
analyzed for the presence of either FGly or Cys in position 69.
In all mutants FGly was found to account for 49–95% of residue
69. In wild type control ASA 94% of residue 69 were converted
to FGly (Table I). Earlier experiments have shown that this
modification efficiency depends on the expression level, and
values of 60–90% have been observed (1). Thus, the analysis of
the modification efficiency of the ASA mutants indicates that a
decrease of FGly formation can account for a not more than
50% decrease of
V
max in the mutants.

Substitution of three of the Mg 2
+ complexing residues
(Asp29, Asp30, and Asp281) by alanine led to a strong decrease of
V
max to 1–5% compared with wild type ASA, whereas substi-
tution of Asn282 by alanine decreased
V
max to 24%. This reflects
the importance of the negatively charged aspartates and, to a
lower degree, of the polar asparagine for enzyme activity by
ensuring the exact positioning of a catalytically active metal
ion or the general three-dimensional structure of the active
site. Because of their close vicinity to the first oxygen of FGly69,
a direct involvement of the Mg2
+ and the ð oxygen of Asp282 in
catalysis by stabilizing the deprotonated form of this FGly
oxygen is likely. The importance of the metal complexing res-
idues (Asp29, Asp30, Asp281, and Asn282) for the general struc-
ture of the active site is supported by the fact that in all of these
mutants the
Km was increased 3–8-fold. It should be noted,
however, that modeling of the substrate into the cavity did not predict a direct contact to one of the substituted residues. Asp$^{29}$ and Asp$^{30}$ may influence substrate binding by their coordination of Lys$^{123}$ and Lys$^{302}$, respectively, which are strong candidates for binding the sulfate group (see below).

Whereas the Mg$^{2+}$ and Asp$^{281}$ are thought to be involved in the activation of FGly$^{69}$ during the cleavage of the substrate-sulfate ester, His$^{125}$ is proposed to catalyze the desulfation of the enzyme-sulfate ester leading to the elimination of sulfate and to the regeneration of the aldehyde. Substitution of His$^{125}$ with alanine led to a decrease of $V_{\text{max}}$ to 11% and to a 7-fold increase of the $K_m$, indicating a participation in the catalytic process but also pointing to an influence on the binding of the substrate (see below).

Sulfated substrates are expected to be bound by positively charged residues, thereby orientating the sulfate group next to the catalytically active oxygen of FGly$^{69}$ and increasing the electrophilic property of the sulfur. When the potentially positively charged residues in the vicinity of FGly$^{69}$ (His$^{122}$, His$^{123}$, Lys$^{123}$, and Lys$^{302}$) were substituted by alanine, 5-fold (His$^{122}$ and His$^{123}$) and more than 10-fold (Lys$^{123}$ and Lys$^{302}$) increases of the $K_m$ were observed. Because a 3–5-fold increase of $K_m$ was also observed in other mutants, which are unlikely to interact directly with the substrate molecule, only the two lysines (Lys$^{123}$ and Lys$^{302}$) remain as strong candidates for binding the sulfate group. Additionally, the $V_{\text{max}}$ of all of these four mutants was decreased to 6–11% of wild type ASA. In the H125A mutant this is obviously due to the interference with FGly coordination (see above). For K123A and K302A it may be ascribed to their direct vicinity to Asp$^{29}$ and Asp$^{30}$, which are strongly involved in the catalytic mechanism of sulfate ester cleavage.

The pH optimum of all mutants was shifted to a more alkaline pH by 0.2–0.8 units as compared with wild type ASA (Table I). Only the D30N mutant showed a higher $V_{\text{max}}$ as compared with D30A, indicating that one oxygen of Asp$^{30}$ could partly be replaced with a nitrogen. Taken together, these results demonstrate that even relatively small changes in the metal binding complex, induced by aspartate/asparagine exchanges, affect catalytic activity and substrate binding. In line with this, the metal complexing residues are highly conserved in the sulfatase family (Fig. 2).

The pH optimum of all mutants was shifted to a more alkaline pH by 0.2–0.8 units as compared with wild type ASA (Table I and Fig. 4). This change indicates that proton abstraction is a crucial aspect in the catalytic mechanism of sulfate ester cleavage. In wild type ASA both oxygens of the aldehyde hydrate have to be activated by abstraction of a proton to facilitate the cleavage of the sulfated substrate and the release of the sulfate group from the enzyme (see above and Fig. 1). Proton abstraction may be impaired by substituting either His$^{125}$ and Asp$^{281}$, which are directly involved in these steps,
with alanine or by point mutations that alter the overall structure of the active site. An increase in pH promotes the deprotonation and could partially compensate for the loss of catalytic deprotonation. The latter appears to depend on the exact positioning of Asp$^{281}$, Mg$^{2+}$, and His$^{125}$ next to FGly$^{69}$. In line with this, the decrease in $V_{\text{max}}$ observed in all of these mutants can at least partly be ascribed to an impaired deprotonation of the aldehyde hydrate.

Conclusions—The amino acid residues neighboring FGly$^{69}$ in the active site of ASA are highly conserved among eukaryotic and prokaryotic members of the sulfatase family, indicating a similar structure of the reactive center and a common reaction mechanism. Crystal structure analysis revealed that these residues are probably interconnected by a net of hydrogen bonds, thus forming the active site. The structural data are consistent with the biochemical finding that all ASA mutants, in which putative residues of the active site were replaced by alanine, have a markedly decreased $V_{\text{max}}$. The increased pH optimum observed for all mutants is in good agreement with the decreasing $V_{\text{max}}$, because in both of the two half-reactions of sulfate ester cleavage a deprotonation step is involved. Among the residues that have been proposed to bind the sulfate group, Lys$^{123}$ and Lys$^{302}$ were shown to be most critical for substrate binding. Their substitution by alanine led to a marked increase of $K_m$. The moderate 3–5-fold increase of $K_m$ in the other mutants, except for S150A, supports the view of the active site of ASA (and other sulfatases) as a highly interconnected network of polar residues, easily disturbed by the replacement of one of its elements.

Acknowledgments—We thank Martina Balleininger and Katja Unthan-Hermeling for technical assistance, Klaus Neifer for protein sequencing, and Isabel Usoñ for critically reading the manuscript.

REFERENCES

1. Schmidt, B., Selmer, T., Ingendoh, A., and von Figura, K. (1995) Cell 82, 271–278
2. Selmer, T., Hallmann, A., Schmidt, B., Sumpfer, M., and von Figura, K. (1996) Eur. J. Biochem. 238, 341–345
3. Dierks, T., Schmidt, B., and von Figura, K. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11963–11968
4. Parenti, G., Meroni, G., and Ballabio, A., (1997) Curr. Opin. Genet. Dev. 7, 386–391
5. von Figura, K., Schmidt, B., Selmer, T., and Dierks, T. (1998) BioEssays 20, 505–510
6. Lukatela, G., Krauss, N., Theis, K., Selmer, T., Gieselmann, V., von Figura, K., and Saenger, W. (1998) Biochemistry 37, 3654–3664
7. Bond, C. S., Clements, P. R., Ashby, S. J., Collyer, C. A., Harrop, S. J., Hopwood, J. J., and Guss, J. M. (1997) Structure 5, 277–289
8. Recksiek, M., Selmer, T., Dierks, T., Schmidt, B., and von Figura, K. (1998) J. Biol. Chem. 273, 6096–6103
9. McBeau D. E. (1993) Practical Protein Crystallography, Academic Press, London
10. Kyte, J. (1995) Structure in Protein Chemistry, Garland Publishing, Inc., London
11. Stein, C., Gieselmann, V., Kreyling, J., Schmidt, B., Pohlmann, R., Waheed, A., Meyer, H. E., O'Brien, J. S., and von Figura, K. (1989) J. Biol. Chem. 264, 1252–1259
12. Knaust, A., Schmidt, B., Dierks, T., von Bulow, R., and von Figura, K. (1998) Biochemistry 37, 13941–13946
13. Kasper, D., Dittmer, F., von Figura, K., and Pohlmann, R. (1996) J. Cell Biol. 134, 615–623
14. Misch, C., Dierks, T., Selmer, T., von Figura, K., and Schmidt, B. (1998) J. Biol. Chem. 273, 4835–4837
15. Haag, E., S., and Raff, R. A. (1998) Dev. Genes Evol. 208, 188–204
16. Pajetta, J. V. (1989) Mol. Cell. Biol. 9, 3630–3637
17. Østerás, M., Boncompagni, E., Vincent, N., Poggi, M.-C., and Le Rudulier, D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11394–11399