The Iron Sulfur Protein AtsB Is Required for Posttranslational Formation of Formylglycine in the Klebsiella Sulfatase*

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The catalytic residue of eukaryotic and prokaryotic sulfatases is a α-formylglycine. In the sulfatase of Klebsiella pneumoniae the formylglycine is generated by posttranslational oxidation of serine 72. We cloned the atsBA operon of K. pneumoniae and found that the sulfatase was expressed in inactive form in Escherichia coli transformed with the structural gene (atsA). Coexpression of the atsB gene, however, led to production of high sulfatase activity, indicating that the atsB gene product plays a posttranslational role that is essential for the sulfatase to gain its catalytic activity. This was verified after purification of the sulfatase from the periplasm of the cells. Peptide analysis of the protein expressed in the presence of AtsB revealed that half of the polypeptides carried the formylglycine at position 72, while the remaining polypeptides carried the encoded serine. The inactive sulfatase expressed in the absence of AtsB carried exclusively serine 72, demonstrating that the atsB gene is required for formylglycine modification. This gene encodes a 393-amino acid residue iron sulfur protein that has a cytosolic localization and is supposed to directly or indirectly catalyze the oxidation of the serine to formylglycine.

Mammalian sulfatases (see Ref. 1) are involved in the turnover of endogenous sulfated substrates. Sulfatases of lower eukaryotes and bacteria, on the other hand, are expressed under conditions of sulfur starvation and function in sulfate scavenging from exogenous substrates (2). Despite their different functions all these sulfatases form a highly conserved protein family showing strong homology on the level of both primary (3, 4) and three-dimensional structure (5, 6). Furthermore, sulfatases of prokaryotic, lower eukaryotic, and human origin share a unique amino acid residue, a α-formylglycine (FGly), 1 that is essential for catalytic activity (7–10). Like the FGly all other putative active site residues are conserved (11). This reflects the importance of the catalytic mechanism underlying sulfate ester cleavage, during which the FGly acts as the catalytic residue (6, 12). Failure to generate the FGly residue is the cause of multiple sulfatase deficiency, a rare but fatal human lysosomal storage disorder (10, 13).

In eukaryotic sulfatases the FGly is generated in the endoplasmic reticulum by oxidation of a conserved cysteine residue (14, 15). This oxidation occurs during or shortly after translocation of the nascent polypeptide into this compartment and is directed by a linear sequence motif starting with the residue to be modified. As shown in vitro for human arylsulfatase A (16), this motif consists of the dodecamer sequence CTTPRSAALLTGR comprising an essential core element (CTXPR) and a stimulating auxiliary element (AALLTGR). The core element is fully conserved, and the auxiliary element is partially conserved, among all eukaryotic members of the sulfatase family and also in the well characterized sulfatase of Pseudomonas aeruginosa (17).

Unlike this prokaryotic cysteine-type sulfatase, which is located in the cytosol, the other well characterized prokaryotic sulfatase, the arylsulfatase of Klebsiella pneumoniae (18), is a serine-type sulfatase, which is located in the periplasm and which carries a FGly residue that is generated by oxidation of a serine rather than a cysteine (7). Nevertheless, the two sequence motifs (SXPXR and SMLLTGN) are also conserved in the Klebsiella sulfatase. After expression of this protein under strongly inducing conditions, 60% of the polypeptides carried the FGly residue, and the remaining 40% carried the serine predicted from the DNA sequence (7).

Conversion of serine to FGly obviously is catalyzed also by Escherichia coli, since the Klebsiella sulfatase can be expressed in E. coli as an active enzyme (18). This organism furthermore is able to quantitatively oxidize cysteine 51 to FGly after overexpression of the cysteine-type sulfatase of P. aeruginosa (8). Surprisingly, no FGly modification was observed when a mutant of the Pseudomonas sulfatase was expressed, in which cysteine 51 was substituted by a serine. This suggests that E. coli harbors two FGly generating systems or that a common modification system is modulated by a cofactor (8).

Transformation of E. coli with the structural gene encoding the Pseudomonas sulfatase (atsA) is sufficient to obtain catalytically active and FGly-containing sulfatase protein. Active expression of the Klebsiella sulfatase, however, was reported to require not only the sulfatase gene (atsA) but in addition an adjacent non-sulfatase gene termed atsB (18). AtsB therefore was considered to function as a positive regulator of sulfatase expression in Klebsiella. In the present study we characterized this regulation in more detail. Data presented here show that regulation by AtsB is not due to a function as a transcriptional activator, as had been suggested originally (19, 20). AtsB rather plays a crucial role in a posttranslational event, namely the conversion of serine 72 to FGly.
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**EXPERIMENTAL PROCEDURES**

**Cloning of the atsBA Operon—Genomic DNA of K. pneumoniae DSM 681 (Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany) was prepared according to Ref. 21. For generation of a sub-genomic bank, 110 μg of genomic DNA were digested with *Bam*HI. After Southern blotting of 10% of the digest, a positive signal was obtained in the 6.8-kbp region using a 585-bp probe generated by PCR amplification of *atsA* nucleotides 2495–3079 (see Ref. 18) and labeled with [-³²P]dCTP (Rediprime DNA Labeling, Amersham Pharmacia Biotech). After electrophoretic separation of the remaining digest, 5–8-kbp fragments were recovered from the gel, cloned into pBluescript II KS (Stratagene) and transformed into *E. coli* DH5α by electroporation. Positive clones were identified first by colony hybridization using the probe described above and, second, by sulfatase assays using 5-bromo-6-chloro-3-indoxyl sulfate (Bioynth) or p-nitroacetohex sulfite (Sigma) as a substrate (7, 17). The *atsA* and *atsB* ORFs were localized by restriction mapping (cf. Ref. 18) and DNA sequencing. Thereby it turned out that the *atsB* ORF was 30 bp shorter and the *atsA* ORF 339 bp longer than published previously (18). The entire *atsBA* operon was subcloned as a 3832-bp *Bam*HI/XcmI fragment (clone pATSBA#R5), and both DNA strands were sequenced using Big-Dye Terminator Cycle Sequencing (Perkin-Elmer Biosystems). The sequence obtained was deposited in the EBI data base (accession number AJ131525).

**Protein Expression and Purification—**For protein expression the single *atsA* and *atsB* and also the bicistronic *atsBA* ORFs were placed under control of the *lac* promoter of pBluescript II KS or pBluescript II KS (22) (see Fig. 1). To facilitate insertion into the multi cloning sites of these vectors we introduced a *Bam*HI site 3’ of the *atsA* stop codon (noncoding primer: CCGGATCCTAGGATACGAGCCTGCTCGAG) and a *Hind*III or *Kpn*I site directly 5’ of the ribosome binding sites of *atsA* (coding primer: CCAAGCTTGAAACGGTAGCTGAGCGATCTG) or *atsB* (coding primer: GGGGTACCAACAGTACCGGTCATTAACCG), respectively, using PCR methods. Disruption of the *atsB* ORF was achieved after deletion of a 882-bp *Nhe*I/StuI fragment and in-frame religation of the blunt-ended ends (see Fig. 1).

To facilitate purification of the expressed sulfatase protein, a C-terminal Arg-Gly-Ser-(His)_₆ tag was added to the *AtsA* protein. This was achieved after adding a corresponding oligonucleotide (noncoding sequence: CCGGATCCTAGGATACGAGCCTGCTCGAG) and a *Hind*III or *Kpn*I site directly 5’ of the ribosome binding sites of *atsA* (coding primer: CCAAGCTTGAAACGGTAGCTGAGCGATCTG) or *atsB* (coding primer: GGGGTACCAACAGTACCGGTCATTAACCG), respectively, using PCR methods. Disruption of the *atsB* ORF was achieved after deletion of a 882-bp *Nhe*I/StuI fragment and in-frame religation of the blunt-ended ends (see Fig. 1).

Protein expression was achieved after transforming *E. coli* DH5α with pBluescript II KS containing the described *ats* constructs. For coexpression of *atsA* and *atsB* from two different plasmids (Fig. 2A) a double transformation was performed using *atsA* cloned into pBluescript II KS and *atsB* cloned into pBluescript II KS. Double transformants were selected using their ampicillin and chloramphenicol resistance. The presence of the two genes was verified by PCR analysis. The transformed cells were grown aerobically in Luria-Bertani medium with constant shaking at 37 °C. After 2–3 h of incubation isopropyl thiogalactopyranoside was added and growth continued for another 5–6 h. Preparation of periplasm from these cells and purification of the His₆-tagged proteins on nickel-nitrilotriacetic acid-agarose (Qiagen) under native conditions was carried out according to the protocols (The QiAexpressionist) given by the manufacturer.

**Protein Analysis—**Expression of the recombinant sulfatase protein...
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The material eluting from the RP column (Fig. 4A and B), is indicated. The UV absorbance and the resulting tryptic peptides were separated by RP-HPLC. The UV absorbance and the radioactivity (not shown) and amino acid sequencing (Fig. 4A and B), is indicated.

The purified AtsA-His6 protein was almost devoid of any contaminating proteins (>95% purity), as checked by SDS-PAGE (Fig. 3, A and C) and RP-HPLC on a C4 column (not shown). The presence of FGly at the protein level was determined after subjecting the purified AtsA-His6 protein to treatment with NaB[3H]H4 under denaturing conditions. After desalting, aliquots were subjected to SDS-PAGE followed by Coomassie Blue staining (Co) and fluorography (Fl). The amount of protein loaded on the gel was 2 μg (A, C) corresponding to 145 milliunits (A) or 0 milliunits (C) in the original protein preparation. B, D, the major part of the desalted sulfatase proteins was digested with trypsin, and the resulting tryptic peptides were separated by RP-HPLC. The UV absorbance and the radioactivity (shaded area) associated with the peptides are shown. The position of the peptide 2 (P2), as identified by mass spectrometry (not shown) and amino acid sequencing (Fig. 4A and B), is indicated. The material eluting from the RP column 1 min earlier than P2 and containing radioactivity two times above background (B, D) is not a derivative of P2, and its radioactivity was not released during 15 cycles of radiosequencing (not shown, cf. Fig. 4C).

RESULTS

Cloning of the atsBA Operon—In order to study the role of the atsB gene on expression of active Klebsiella arylsulfatase, encoded by the atsA gene, we needed the cloned atsBA operon. Since the atsBA plasmid described previously (18) was not available, we generated a subgenomic bank of Klebsiella DNA and identified the entire atsBA operon on a 6.8-kbp BamHI fragment (see “Experimental Procedures”). The DNA sequence of a subcloned 3832-bp BamHI/XcmI fragment (accession number AJ131525) revealed that the atsB ORF codes for an iron sulfur protein consisting of 395 amino acid residues, i.e. 10 residues less than published previously (Ref. 18, GenBank™ accession number M31938), and lacking a signal peptide (Fig. 1). The atsA ORF, on the other hand, encodes the arylsulfatase protein consisting of 577 amino acid residues, i.e. 113 residues more than published by Murooka et al. (18), and including a 20 residues signal peptide directing translocation of this protein into the periplasm (Fig. 1). The correctness of the revised atsA sequence was verified on the protein level by mass spectrometry and amino acid sequencing of several tryptic peptides of purified Klebsiella arylsulfatase protein (7), including peptide 44 (GLTAGDAPWQ, residues 487–496), which is located within the extra 113 residues predicted from the revised DNA sequence. This C-terminal part of the protein was found to be essential for enzyme activity (data not shown). The protein sequence directly following the serine 72 to be converted to FGly (SAPARSMLTGQ, residues 72–83) is homologous to the sequence motif directing FGly modification in human arylsulfatase A (Ref. 16, see Introduction).

Expression of Active AtsA in E. coli Depends on Coexpression of AtsB—Although E. coli carries three sulfatase-related genes (25), active endogenous sulfatases have not yet been found in this species. Transformation of E. coli with the atsA gene of P. aeruginosa (8) or with the Klebsiella atsBA operon (18), however, leads to the expression of active arylsulfatases. Since in
prokaryotes arylsulfatase expression is repressed during growth in the presence of sulfate, we cloned the atsBA operon without its endogenous promoter into an expression vector downstream of the lac promoter (Fig. 1), allowing controlled expression at logarithmic growth in Luria-Bertani medium. E. coli DH5α transformed with the atsBA plasmid was found to express high arylsulfatase activities reaching a maximum of 2–3 units/mg of cell protein after aerobic growth for 5–6 h in the presence of isopropyl thiogalactopyranoside (Fig. 2, lane 1). The recombinant AtsA protein was detected by Western blotting using antibodies against the purified Klebsiella arylsulfatase (Fig. 2) and was found to be located in the periplasm (not shown). Its electrophoretic mobility was in agreement with the predicted mass of 62,230 Da (Figs. 2 and 3A) and was identical to the mobility of the arylsulfatase purified from Klebsiella (not shown).

AtsB and AtsA are coexpressed from a bicistronic transcript. Since we wanted to study AtsA and AtsB independently, we cloned each of the two ORFs separately downstream of the lac promoter into two vectors carrying different selection markers, thus allowing selection of double transformants. The AtsA protein expressed by these double transformants showed a similar specific activity when compared with the bicistronic expression, as concluded from the activities and Western blot signals determined (Fig. 2A, lanes 1 and 2).

Most interestingly, expression of atsA alone did not lead to any detectable sulfatase activity (<1 milliunits/mg of cell protein), although the AtsA protein was produced at normal levels (Fig. 2A, lane 3). Since expression of atsB alone also did not lead to any sulfatase activity (Fig. 2A, lane 4), it has to be concluded that the atsB gene product has a posttranslational function that is essential for the AtsA arylsulfatase to gain its enzymatic activity. The dependence of active AtsA expression on a functional atsB gene was confirmed by the bicistronic expression of atsA together with an atsB fragment that carried a 882-bp in-frame deletion corresponding to amino acid residues 89–382 of AtsB (see Fig. 1). As a consequence of this deletion, no arylsulfatase activity was measurable, although the AtsA protein was present (Fig. 2A, lane 5). This rules out that the dissection of the bicistronic gene organization abolished active AtsA expression. The atsB gene product rather acts in trans on the AtsA protein, as shown by the coexpression of AtsB and AtsA from two different plasmids.

**Generation of FGly Depends on AtsB**—In order to analyze the expressed AtsA protein for the presence of FGly in position 72, the recombinant arylsulfatase had to be purified. To facilitate purification we expressed the AtsA protein in a His-tagged form (AtsA-His6). This protein showed a similar catalytic activity as wild-type AtsA (Fig. 2B, compare lanes 1 and 2). The AtsA-His6 protein was purified from the periplasm of the cells by chromatography on nickel-agarose, yielding a homogenous protein preparation (>95% purity), as checked by SDS-PAGE (Fig. 3A) and RP-HPLC (not shown). This preparation showed a specific enzymatic activity of 73 units/mg of purified protein (Fig. 2B, lane 4). As expected, the AtsA-His6 protein purified from E. coli expressing only the structural gene but not the atsB gene showed no activity (Fig. 2B, lane 3, and Fig. 3C).

To examine whether serine 72 was converted to FGly, the purified AtsA-His6 proteins, expressed in the absence (AtsA-His6) or presence of AtsB (AtsA-His6/AtsB), were denatured and incubated with NaB[3H]H4. This treatment reduces the formyl group of FGly leading to formation of a [3H]serine residue (7–10). After gel filtration aliquots of the protein samples were analyzed by SDS-PAGE, followed by Coomassie Blue staining and fluorography. Thereby it turned out that AtsA-His6/AtsB carried a [3H] label, whereas AtsA-His6 did not (Fig. 3, A and C). The proteins were subjected to digestion with trypsin, and the tryptic peptides were separated by RP-HPLC. The radioactivity recovered during chromatography of the...
AtsA-His$_6$(-B) protein was denatured and digested with trypsin. During RP-HPLC of the resulting tryptic peptides two adjacent peaks were recovered (A) containing peptides with masses of 1588 Da (B) and 1590 Da (C), which agree with the masses of the FGly 72 and serine 72 containing forms of peptide 2 (P2* and P2), respectively. Mass determination was carried out by matrix-assisted laser desorption ionization mass spectrometry using indole-2-carboxylic acid (ICA) as a matrix (B, C); the masses given are corrected for protonation. The presence or absence of FGly 72 in P2* and P2, respectively, was demonstrated when using p-nitroaniline (pNA) as a matrix, which led to the formation of a 1708-Da Schiff base of p-nitroaniline with P2* (D) but not P2 (E). Furthermore, amino acid sequencing was blocked in P2* at the position of the FGly (X in F) but not in P2 lacking FGly (G), as had been described for the FGly-modified and nonmodified peptides of other sulfatases (see “Results”). From the amount of amino acids recovered in cycles 1, 3, 4, and 5 (F and G), a modification efficiency (P2*/(P2* + P2)) of 48 ± 2% is calculated. It should be noted that P2* and P2 were not quantitatively separated during HPLC (A), as indicated by the observation that low amounts of P2* contaminating the P2 fraction gave rise to Schiff base formation with p-nitroaniline (E).

**FIG. 5.** Half of the recombinant *Klebsiella* sulfatase polypeptides undergo FGly modification after coexpression with AtsB in E. coli. AtsA-His$_6$(-B) protein was denatured and digested with trypsin. During RP-HPLC of the resulting tryptic peptides two adjacent peaks were recovered (A) containing peptides with masses of 1588 Da (B) and 1590 Da (C), which agree with the masses of the FGly 72 and serine 72 containing forms of peptide 2 (P2* and P2), respectively. Mass determination was carried out by matrix-assisted laser desorption ionization mass spectrometry using indole-2-carboxylic acid (ICA) as a matrix (B, C); the masses given are corrected for protonation. The presence or absence of FGly 72 in P2* and P2, respectively, was demonstrated when using p-nitroaniline (pNA) as a matrix, which led to the formation of a 1708-Da Schiff base of p-nitroaniline with P2* (D) but not P2 (E). Furthermore, amino acid sequencing was blocked in P2* at the position of the FGly (X in F) but not in P2 lacking FGly (G), as had been described for the FGly-modified and nonmodified peptides of other sulfatases (see “Results”). From the amount of amino acids recovered in cycles 1, 3, 4, and 5 (F and G), a modification efficiency (P2*/(P2* + P2)) of 48 ± 2% is calculated. It should be noted that P2* and P2 were not quantitatively separated during HPLC (A), as indicated by the observation that low amounts of P2* contaminating the P2 fraction gave rise to Schiff base formation with p-nitroaniline (E).
To determine the FGly content in the recombinant AtsA-His6(+B) protein, we analyzed its tryptic peptides without prior treatment of the protein with NaB[3H]H4. In the HPLC chromatogram a peptide with a mass of 1588 Da was identified eluting at a 0.4 min earlier retention time than the 1590-Da P2 (Fig. 5, A–C). A mass of 1587.8 Da is predicted for the FGly 72-containing form of peptide 2 (P2*). The presence of the FGly was verified when using p-nitroaniline as a matrix for matrix-assisted laser desorption ionization mass spectrometry, which led to a mass of 1708 Da for P2* (Fig. 5D). The increase in mass by 120 Da, which was not observed for P2 (Fig. 5E), is due to a Schiff base formation of p-nitroaniline and the formyl group of P2* (7–10). The presence of the FGly in P2* could also be demonstrated by amino acid sequencing. Whereas the entire sequence of P2 could be determined (Fig. 5G), almost no recovery of the residues C-terminal of position 72 (X in Fig. 5F) was observed in the case of P2*. In addition, the signal for methionine 71 was reduced in P2*. The presence of a FGly residue is known to block Edman degradation at the position of the FGly and to reduce its efficiency in the preceding cycle (7–10). From the sequencing data (Fig. 5, F and G) the FGly content of AtsA-His6(+B) was calculated to be 48 ± 2%. Thus, the modification degree observed for the recombinant sulfatase in E. coli is similar to the degree of 60% determined previously for the protein purified from Klebsiella (7).

### DISCUSSION

The present study demonstrates that the atsB gene product is required for FGly modification in the aroylsulfatase of K. pneumoniae. This modification is a prerequisite for sulfatase activity, as had been shown previously for other pro- and eukaryotic sulfatases (8–10). In the absence of a functional atsB gene inactive sulfatase polypeptides were synthesized lacking the FGly. In the presence of atsB, however, 48% of the recombinant sulfatase molecules, expressed in His-tagged form in E. coli, carried the FGly leading to an overall specific activity of 73 units/mg of purified protein. This approximately agrees with the modification efficiency of 60% and a specific activity of 123 units/mg determined for the wild-type protein purified from K. pneumoniae. Extrapolated to 100% FGly content, activities of 152 or 205 units/mg, respectively, are calculated for the two protein preparations. These results rule out that AtsB acts as a transcriptional activator, as had been suggested originally (19, 20). AtsB rather plays an essential role in the posttranslational oxidation of a conserved serine to FGly.

The data, furthermore, show that FGly formation involves an enzyme-mediated process. This agrees with the finding that in man a genetic defect is the cause for the lack of FGly in sulfatases from multiple sulfatase deficiency patients (10, 13). In E. coli, absence of AtsB does not lead to a general deficiency of FGly formation. While oxidation of serine to FGly is abolished under these conditions, oxidation of cysteine in the sulfatase of P. aeruginosa occurs with maximum efficiency (8). Thus, E. coli obviously harbors a second FGly-generating system that is independent of atsB and may specifically oxidize cysteine but not serine. The latter is concluded from the observation that substitution of the critical cysteine by serine abolished FGly formation in the Pseudomonas sulfatase (8). Whether or not the Klebsiella atsA gene would promote FGly formation in this substitution mutant remains to be investigated. Modification of both serine-type and cysteine-type sulfatases most likely occurs in the cytosol, since in the former case the AtsB protein, and in the latter case the sulfatase itself, lack a signal peptide.

Although no endogenous sulfatase activity has ever been measured in E. coli, this species carries two genes encoding putative serine-type sulfatases termed aslA and f571 (GenBank™ accession numbers M87049 and U00096). Like the Klebsiella atsA gene, also aslA and f571 have an adjacent gene in the same operon (aslB and f590, respectively) encoding an AtsB homolog of about 400 amino acids (25). These homologs did not take over function in the absence of AtsB. All three AtsB homologs are 34–41% identical and represent iron sulfur proteins that comprise three conserved cysteine clusters, each consisting of 3–5 cysteines with short and conserved distances between these cysteines (25). Most iron sulfur proteins are involved in redox reactions and function as electron transfer proteins (26). Therefore we speculate that also AtsB functions as an oxidoreductase oxidizing the critical serine of the unfolded sulfatase polypeptide during or shortly after synthesis and, at the same time, transferring electrons to an acceptor molecule. AtsB may act directly on the sulfatase polypeptide or may oxidize and thereby regenerate the electron acceptor.

The role of AtsB homologs in sulfatase activation is highlighted by a paper reporting that Bacteroides thetaiotaomcron mutated in the atsB-related chuR gene is defective in the utilization of two sulfated substrates, namely chondroitin sulfate and heparin (27). No chondroitin sulfatase activity was detectable in this mutant, which, however, was ascribed to transcriptional regulation of chondroitin sulfate and heparin utilizing genes by chuR.

Further AtsB homologs that are similar in size, but carry only 1 or 2 cysteine clusters, can also be found among a group of proteins involved in the synthesis of cofactors such as PQQ, molybdopterin, Fe-Mo cofactor, tungsten cofactor, or heme d1 (28–32). Interestingly, one of the homologs without known function is YidF of E. coli and is encoded in the yid operon also coding for the cysteine-type sulfatase YidJ (33). YidF is a 165-amino acids protein showing 19% identity to the C-terminal half of AtsB. It may therefore be involved in FGly modification of cysteine-type sulfatases (25).

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