C5-formylglycine (FGly) is the catalytic residue of sulfatases. FGly is generated by post-translational modification of a cysteine (prokaryotes and eukaryotes) or serine (prokaryotes) located in a conserved C(S)/SiXPXR motif. AtsB of Klebsiella pneumoniae is directly involved in FGly generation from serine. AtsB is predicted to belong to the newly discovered radical S-adenosylmethionine (SAM) superfamily. By in vivo and in vitro studies we show that SAM is the critical co-factor for formation of a functional AtsBSAM-sulfatase complex and for FGly formation by AtsB. The SAM-binding site of AtsB involves S4GGE and possibly also a juxtaposed FeS center coordinated by Cys29 and Cys35, as indicated by alanine scanning mutagenesis. Mutation of these and other conserved cysteines as well as treatment with metal chelators fully impaired FGly formation, indicating that all three predicted FeS centers are crucial for AtsB function. It is concluded that AtsB oxidizes serine to FGly by a radical mechanism that is initiated through reductive cleavage of SAM, thereby generating the highly oxidizing deoxyadenosyl radical, which abstracts a hydrogen from the serine-C5H2OH side chain.

In most eukaryotes FGly residues are generated by the recently discovered FGly-generating enzyme (FGE), and it is the genetic deficiency of FGE that causes multiple sulfatase deficiency (9, 10). FGE acts in the lumen of the endoplasmic reticulum shortly after import of the nascent sulfatase polypeptide and modifies a cysteine residue located in a conserved CXPXR motif (11–14). This cysteine undergoes Cβ-oxidation and S-elimination by an as yet unknown reaction mechanism. FGE is encoded in the SUMF1 gene and belongs to a protein family consisting of a domain of unknown function that does not allow prediction of any aspect of its mechanism of action (15). It was proposed that a cysteine cluster, located at the highly conserved C terminus of FGE, may be involved in catalysis, because this region is a hot spot for mutations in multiple sulfatase deficiency patients (9).

SUMF1 genes are lacking in Caenorhabditis elegans, Schizosaccharomyces pombe, and Neurospora crassa that are known to express active sulfatases, which indicates the existence of a second FGly-generating system in eukaryotes. In a variety of prokaryotes SUMF1 orthologs can be found in sulfatase operons (15). As in eukaryotes, some bacteria known to generate FGly residues by cysteine modification, e.g. Escherichia coli and Pseudomonas aeruginosa (16), lack SUMF1 orthologs. Thus, in bacteria a second cysteine-modifying system also exists, orthologs of which may be operative in those eukaryotes lacking FGE, thereby functionally closing the phylogenetic gap mentioned above. It should be noted that FGE as well as the E. coli and P. aeruginosa cysteine-modifying systems are strictly cysteine-specific (6, 16).

The remarkable versatility of bacteria to generate the critical FGly residue is highlighted by the fact that a number of species express sulfatases in which a serine residue is oxidized to FGly, a modification directed by a homologous SXPXR motif (17). These serine-type sulfatases, in contrast to the cystosolic cysteine-type sulfatases, show periplasmic localization (18, 19) and are specifically modified by a cytosolic protein termed AtsB in Klebsiella pneumoniae (19, 20). AtsB is co-expressed with the sulfatase AtsA (arylsulfatase of K. pneumoniae) being encoded on the same ats operon. Co-expression of AtsB was shown to be strictly required for AtsA modification in E. coli where it acts in trans on AtsA (20). AtsB interacts with the FGly modification motif of AtsA in a serine-specific manner, as was shown in yeast two-hybrid and GST pull-down experiments (19). FGly generation from cysteine and serine share the C(Si)XPXR modification motif. Furthermore, in vivo FGE and AtsB both rely on transmembrane translocation of the sulfatase or at least on the presence of a signal peptide in the sulfatase substrate (11, 19). Thus, AtsB-mediated FGly generation may occur when the unfolded sulfatase protein is being translocated, acting on the cis-side of the membrane, in contrast to the microsomal FGE, which acts on the trans-side.

When studying AtsA modification in E. coli, its two chromo-
somal *atsB* orthologs did not substitute for *Klebsiella* *atsB*, most likely because bacterial sulfatase operons generally are repressed under conditions of sulfur supply. On the contrary, the cysteine-modifying system of *E. coli* fulfills also further, possibly essential functions and that FGly is present also in some non-sulfatase proteins.

In contrast to FGly generation from cysteine, where no mechanistic information for the modification reaction is available, some aspects of AtsA-mediated serine oxidation may be inferred by bioinformatics. AtsB was proposed to belong to the so-called radical SAM protein superfamily that generates a deoxyadenosyl radical via reductive cleavage of *S*-adenosylmethionine (SAM) (21). This radical in turn could generate a substrate radical that undergoes further reaction (22–24). Accordingly, the oxidation of serine to FGly, i.e. serine semialde- hyde, would involve two single electron transfer steps. These electrons are likely to be donated/accepted by FeS centers, three of which have been predicted for AtsB on the basis of its conserved cysteine clusters (25). Here we experimentally test this hypothetical mechanism, investigating the involvement of SAM as a co-factor and of the predicted FeS centers as redox catalysts for FGly generation in *vivo* and *in vitro*.

**EXPERIMENTAL PROCEDURES**

**Materials**—The pT-groE plasmid was a gift of Dr. S. Ishii (Ibaraki, Japan). For generation of anti-AtsA and anti-AtsB antibodies see Ref. 20 and 19, respectively. SAM-chloride and 2,4-dinitrophenyl hydrazine were obtained from Fluka; SAHC and GSH-agarose were from Sigma; [methyl-3H]SAM (TRK865, 80 Ci/mmol) and anti-GST antibodies (goat) were obtained from Fluka; SAHC and GSH-agarose were from Sigma; and 2,4-dinitrophenyl hydrazine was obtained from Aldrich/Sigma, TRK865 (80 Ci/mmol) and anti-GST antibodies (goat) were from Amersham Biosciences, and trypsin was from Promega.

**Site-directed Mutagenesis**—All of the mutations were generated by the QuiKChange method (Stratagene) using *atsB* (cloned into pBlue-script II Ks, 20) as template and complementary mutagenesis primers. The coding sequences of these primers were: CACCCCTCGCAATGGCGG-CCGGGGCCGCAACCTAATGCTGCTCA (atsA-st7/2A/Ph74/AtB/76A), CTTAGGGCGGCAGCGCCGAGGGC (atsB-G38A), CTGGCGGAGGCGCGAGGCTGTAGAC (atsB-P284A), CCAGGGGCCGGCGGCTGCAGTACC (atsB-E58A), CGAGGGGGGGCGGGCCTGATCGCC (atsB-P860), CGGCGGCGGCGGGCTGACC (atsB-L387A), GGCAGAGCGCCTGAGACCGCCGGCCTGAG (atsB-L589A), GACACTCGGCGCCGATCCCTATTGCTTA (atsB-C398A), GCCTCGCGGCTGAGCTTACATCAGAG (atsB-C342A), CGACGGCGGAGCCGCTGCAGAACGGC (atsB-C270A), CTGCCGGCGGAAGGCGACTGTGAGCA (atsB-C331A), and GCGAATGGCGA-GACTGTGCGTAAAAATGTG (atsB-C343A). For construction of GST-AtsA, AtsA-residues 21–112 were fused with GST using the pGEX-KG vector, as described earlier (19). All of the mutations and constructs were analyzed by full-length sequencing of coding regions to predict any PCR-derived errors.

**Protein Expression**—*E. coli* DH5α was transformed with the following plasmids (for construction see above): pGEX-KG containing either gst-atsA or gat-atsA-ST72/AtB/77A or pBlueScript II containing either atsA or atsB (atsA with or without mutations, as indicated for each experiment). SAM-binding site mutants of AtsB were co-expressed with Gto/LES in double transformants containing also the pT-groE plasmid (26). The presence of the two plasmids was maintained in selective medium containing ampicillin and chloramphenicol and was routinely checked by PCR analysis. Overnight cultures were used to inoculate 100 ml of fresh liquid medium (1% inoculum). For induction of protein expression, isopropyl-β-D-thiogalactopyranoside was added to 0.2% for pGEX or 0.25 mg/ml for pBlueScript, respectively, at an A600 value of 0.3–0.5, and culture was maintained for another 3 h at 37 °C. For SAM-binding site mutants of AtsB only, expression was performed in overnight (14 h) cultures and inoculated from glycerol stocks without the addition of isopropyl-β-D-thiogalactopyranoside.

**In Vivo Interaction and FGly Modification of AtsB**—Bacteria expressing GST-AtsA were transformed, washed once in PBS (pH 7.4), resuspended in PBS (pH 7.4) containing 8 μl urea, and disrupted in a French press cell. The soluble material (75,000 × g supernatant) was subjected to dialysis against PBS to remove the urea. The dialyzed material was cleared by centrifugation (as above). For *in vitro* interaction, 0.75 ml of the supernatant was mixed with 0.75 ml of an *E. coli* French press lysate (in PBS), containing AtsB protein, and incubated at 37 °C for 15–60 min in the absence or presence of different concentrations of SAM. Where indicated, [3H]SAM (1 μCi) was added as tracer. After incubation, the interaction mixture, corresponding to ~5 mg of total *E. coli* protein, was loaded on a GSH-agarose column (0.2 ml of bed volume), and the flow-through was immediately collected without further incubation. The column was washed six times with 4 column volumes of PBS and then eluted three times with 1 column volume of 10 mM glutathione in PBS (pH 8.0). The eluate fractions were analyzed for GST-AtsA and AtsB by Western blotting and for [3H]SAM by liquid scintillation counting. To test for *in vitro* FGly generation, the incubation of the interaction mixture described above was carried out at 37 °C for 5 min in the presence of ~5 μM SAM. Where indicated (see Refs. 3, 4C, and 5C), another 500 μM SAM was added to the interaction mixture half of the incubation time had passed. After incubation, the GST-AtsA fusion protein was purified by GSH-agarose, separated by SDS-PAGE, and subjected to tryptic digestion and mass spectrometry (see below). It should be noted that all experiments could also be performed using purified GST-AtsA for interaction and modification reactions. However, using purified AtsB led to poor interaction with GST-AtsA. Rather, the *E. coli* lysate containing AtsB had to be freshly prepared avoiding exposure to room temperature.

**Protein and Peptide Analysis**—AtsA, AtsB, or GST fusion proteins were detected by Western blotting using anti-AtsA, anti-AtsB, or anti-GST as primary antibodies. ECL signals of corresponding secondary antibodies were detected by Typhoon1000 imaging system (Raytest) and quantitated by densitometry of digital images using Aida 3.10 software (Raytest). For SDS-PAGE see Ref. 27. The sulfatase activity of expressed AtsA was determined in duplicate assays at saturating substrate concentration, as described earlier (17).

The presence of FGly in AtsA and GST-AtsA was analyzed by MALDI-TOF mass spectrometry at the level of their tryptic peptides. These proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. From the gels, the stained AtsA or GST-AtsA bands were excised and subjected to in-gel digestion with trypsin and extraction of tryptic peptides according to standard protocols (28). The presence of FGly in the tryptic AtsA peptide 2 (P2) was verified by mass spectrometry on a matrix-assisted laser desorption ionization-time of flight Reflex III instrument (Bruker Daltonics), using as matrix 1.3 mg/ml recrystallized 2,4-dinitrophenyl hydrazine in 50% aqueous acetoni-trile solution containing 0.5% trifluoroacetic acid. The samples were prepared by the drying droplet method, drying 0.5 μl of each sample and matrix solution on a stainless steel target. For further details and for MALDI post-source decay MS analysis, see the work of Peng et al. (29).

**RESULTS**

**Formation of a Functional Complex of AtsB with AtsA Requires S-Adenosylmethionine**—AtsA has been predicted to belong to the long radical SAM superfamily generating substrate- or enzyme-located radicals with the help of SAM (21). To test for a possible involvement of SAM in AtsB-mediated FGly modification of AtsA, we performed *in vitro* AtsB-AtsA interaction experiments in the absence or presence of SAM or its demethylated analog SAHC. Using an AtsA fragment (amino acid residues 21–112) fused to the C terminus of glutathione S-transferase (GST-AtsA) and an *E. coli* cell lysate containing overexpressed AtsB, we observed an association of AtsB with GST-AtsA that both bound to and co-eluted from GSH-agarose, as was described earlier (see Ref. 19). It turned out that addition of 500 μM SAM significantly improved AtsB binding, whereas 500 μM SAHC completely inhibited AtsB binding (Fig. 1A). The association of GST-AtsA and AtsB and its stimulation by SAM required incubation of all interaction partners at 25–37 °C for ≥5 min; at 0 °C interaction was rather inefficient (Fig. 1B). This suggests that formation of a stable complex, which can be pulled down by GSH-agarose, involves a rate-limiting step that is temperature-dependent. In the presence of SAM, formation of this complex increased with time over a 60-min period (Fig. 1C). Without the addition of SAM, the complex formed initially but was lost upon incubation for longer than 30 min. This transient complex is attributed to the
presence of some endogenous SAM in the *E. coli* lysate, which initially promotes complex formation and decomposes during the 37 °C incubation, thus destabilizing the complex. Control experiments showed that GST-AtsA-AtsB complex formation in the presence of 50 μM SAM reached a maximum after ~15 min at 37 °C. At ≥300 μM SAM the complex was stable for more than 60 min (Fig. 1C and data not shown).

To prove that SAM indeed is part of a ternary GST-AtsA-AtsB-SAM complex, we added 3H-labeled SAM as a tracer to the incubation mixture containing 50 μM SAM. After loading this mixture to GSH-agarose and extensive washing of the column, the GSH eluate was collected and analyzed for AtsB by Western blotting and for SAM by liquid scintillation counting. It turned out that significant amounts of radioactivity bind to the column and co-eluted with AtsB. No radioactivity was recovered in the elute of GSH-agarose loaded with an incubation mixture lacking AtsB (Fig. 2A, compare first and third lanes). In a further control GST-AtsA-S72A/P74A/R76A was used as bait. This mutant bound neither AtsB nor SAM (Fig. 2A, second lane).

SAM stimulated GST-AtsA-AtsB interaction in a concentration-dependent manner. Under standard conditions (incubation for 15 min at 37 °C), half-maximum stimulation was observed at ~20–40 μM SAM, and saturation was observed at ~150–300 μM SAM (Fig. 2B). Also the amount of [3H]SAM co-eluting from GSH-agarose together with GST-AtsA and AtsB showed a clear dependence on the applied SAM concentration. At ≥300 μM SAM nearly equimolar amounts of AtsB and SAM were recovered in the elute (Fig. 2B); in three experiments SAM and AtsB bound in a ratio of 0.69 ± 0.06. At nonsaturating SAM concentrations, the amount of bound SAM was clearly lower than the amount of bound AtsB (Fig. 2B). This difference may reflect a cooperative stimulation of AtsB binding by SAM to avoid deoxyadenosyl radical formation in the absence of substrate (see “Discussion”). However, we cannot exclude that some SAM is lost from the AtsB-GST-AtsA complex because of catalytic turnover (see below) and/or because of the non-steady-state conditions on the affinity column during washing and elution. Previous results have shown that AtsB and GST-AtsA associate with each other in a ratio of 0.54 (19). Taken together these data suggest that binding of SAM to AtsB efficiently promotes formation of a ternary AtsB-AtsB-SAM complex and that the three interaction partners are likely to be present in this complex in a 1:1:1 ratio.

**SAM-dependent in Vitro FGly Formation by AtsB**—The data shown above suggest that FGly modification of serine depends on SAM. To directly measure the effect of SAM on the FGly-generating activity of AtsB, an *in vitro* system with limiting SAM levels had to be established. In a previous study, we failed to observe FGly formation in an *in vitro* system, when GST-AtsA was incubated with a soluble extract of *E. coli* overexpressing AtsB (19). Here we used essentially the same components, this time supplementing the modification reaction with 500 μM SAM. After incubation at 37 °C, GST-AtsA was isolated from the mixture by GSH-agarose and subjected to digestion with trypsin and MALDI-TOF MS analysis using DNP-hydrazone as a matrix. This method efficiently converts the FGly-containing tryptic peptide of AtsA (1588 Da) into the corresponding hydrazone (1768 Da) upon laser-assisted desorption from a DNP-hydrazone matrix, and the resulting product can be detected with high sensitivity (29). Formation of the FGly-
modified peptide of AtsA, detected as the DNP-hydrazone (1768 Da), was indeed observed when the incubation was performed in the presence of SAM (Fig. 3A) but not in its absence (not shown). The molecular identity of the DNP-hydrazone peptide was confirmed by the indicative 2-oxodihydroimidazole-derivatized fragment ion (524 Da; Fig. 3C) desorbing with high efficiency upon MALDI post-source decay analysis of the 1768-Da parent ion (for generation of this fragment from FGly-containing peptides, see Ref. 29). The formation of FGly was dependent on AtsB and on the FGly modification motif of AtsA. Substitution of Ser72 by alanine in AtsA prevented FGly formation by AtsB (data not shown). The \textit{in vitro} modification reaction, however, was found to be rather inefficient (Fig. 3B). It should be noted that the peak heights of the FGly-DNP-hydrazone peptide (1767.9 Da) and of the unmodified peptide (1590 Da) in Fig. 3A cannot be used to quantitate FGly modification, because during MALDI-TOF MS the former peptide desorbs much better from the used DNP-hydrazone matrix than the latter peptide (29). The efficiency of FGly formation could be increased up to 5-fold by repeated addition of SAM during the incubation (Fig. 3B). This effect is attributed to the limited thermostability of SAM. Nevertheless, from a comparison with AtsA of known FGly content (see Ref. 29) we estimate that at best \( \frac{1}{10} \) of the GST-AtsA substrate was modified under conditions where GST-AtsA was in a roughly 10-fold excess over AtsB. Apart from the poor efficiency of the \textit{in vitro} system, the data obtained show unequivocally that AtsB-mediated FGly formation relies on SAM as an essential co-factor.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{SAM-dependent \textit{in vitro} FGly formation by AtsB. A and B, \textit{in vitro} reactions were set up, as described under “Experimental Procedures,” incubating GST-AtsA and AtsB in the presence of E. coli protein and 500 \( \mu \text{M} \) SAM for up to 3 h at 37 °C, as indicated in B (squares). Two samples (B, triangle and circle) were supplemented a second time with SAM, corresponding to 500 \( \mu \text{M} \), after 30 or 60 min of incubation, as indicated by the arrows. GST-AtsA was purified on GSH-agarose and subjected to SDSPAGE, Coomassie staining, and in-gel digestion with trypsin. The extracted tryptic peptides were analyzed by MALDI-TOF MS using DNP-hydrazone as a matrix. A shows the result of the sample obtained after 2 h at 37 °C with the readdition of SAM (B, circle). The FGly-containing form of tryptic peptide P2 is detected as the corresponding DNP-hydrazone (1767.9 Da), whereas the serine-containing P2 gives a signal at 1589.8 Da. The relative abundance of the m/z 1767.9 signal is given in B for all \textit{in vitro} reactions as a function of their incubation time. C, MALDI post-source decay analysis of the 1787.9-Da ion led to formation of the predicted 2-oxodihydroimidazole-derivatized fragment ion (524.4 Da, see Ref. 29), thereby verifying the molecular identity of the DNP-hydrazone. The fragmentation giving rise to the 524.4-Da product is indicated. Control reactions in which AtsB or SAM were omitted did not lead to FGly modification, because only the serine-containing form of P2 was detected (not shown).}
\end{figure}
the radical SAM superfamily (21). Yet it should be noted that the individual sequence of each family member may vary considerably from the semi-consensus sequence.

To study the importance of the putative SAM-binding site for AtsB-mediated FGly formation in vivo, we performed an alanine scanning mutagenesis for residues 83GGEPLL88 (Pro86 additionally was substituted by glycine to allow for enhanced structural flexibility at this position). After co-expression of these mutants with AtsA in E. coli, the specific sulfatase activity of AtsA was determined as an indirect indicator of the FGly modification activity of AtsB. It turned out that the specific sulfatase activity of AtsA, i.e. the ratio of activity over Western blot signal, was drastically reduced when Gly83, Gly84, or Glu85 of AtsB was replaced by alanine (313-, 31-, and 50-fold lower specific activities, respectively, as compared with AtsA co-expressed with wild type AtsB; Fig. 4A). For the other mutants, at positions 86–88, wild type sulfatase activities were observed. The expression level of AtsB and its solubility were comparable with those of wild type AtsB. These results indicate that the FGly modification requires the presence of the SAM-binding site.

**Fig. 4. Alanine scanning mutagenesis of the putative SAM-binding site of AtsB.** A, AtsA was co-expressed in E. coli together with wild type (WT) AtsB or with a G83A, G84A, E85A, P86A, P86G, L87A, or L88A mutant form of AtsB. The Western blot shows the AtsB and AtsA polypeptides, as recovered from the soluble fraction of total cell lysates. The sulfatase activities present in the samples loaded for SDS-PAGE are given below each lane. The AtsA protein, after SDS-PAGE, Coomassie staining, and in-gel digestion with trypsin was analyzed for the presence of FGly by MALDI-TOF MS using DNP-hydrazine as matrix (see “Experimental Procedures”). The relative abundance of the detected P2-FGly-DNP-hydrazone signal (cf. Fig. 3A and B) is given as follows: ++, high abundance (signal/noise ratio >20); +, low abundance (signal/noise ratio, 2–5); –, no signal. B, in vitro interaction reactions of GST-AtsA and AtsB, expressed in wild type, G83A, G84A, E85A, or P86G mutant form, were incubated for 15 min at 37 °C in the presence of 50 μM [3H]SAM (cf. Fig. 2A). The components of these reactions binding to and eluting from GSH-agarose were analyzed by Western blotting and liquid scintillation counting. The positions of the detected proteins, the relative amounts of AtsB, detected by quantification of the Western blot signals, and the amount of SAM-associated radioactivity recovered in a 33% aliquot of the GSH eluate are given. C, for in vitro FGly modification, GST-AtsA and AtsB expressed in wild type, G84A, or P86G mutant form were incubated in the presence of E. coli protein and 500 μM SAM or SAHC, as indicated, for 2 h at 37 °C, with the readdition of 500 μM SAM or SAHC after 1 h (cf. Fig. 3B). The presence (+) or absence (–) of FGly in GST-AtsA is given, which was detected as DNP-hydrazone derivative by MALDI-TOF MS analysis of its tryptic peptides.
not affected by the mutations (Fig. 4A).

To directly determine the AtsB-mediated FGly formation, the co-expressed AtsA protein was subjected to FGly analysis using the same semi-quantitative MALDI-TOF MS method described above, which detects the FGly-modified peptide as a DNP-hydrazide derivative of 1768 Da. The intensity of this 1768-Da signal was strongly reduced in the case of AtsA co-expressed with AtsB mutants G84A and E85A and was not at all detectable in case of co-expression with AtsB-G83A (Fig. 4A). Thus, the drastically reduced AtsA activities, caused by these three AtsB mutants, fully agree with the impaired FGly modification of AtsA.

In vitro AtsA-AtsB interaction experiments in the presence of SAM demonstrated that binding of AtsB mutants G83A, G84A, and E85A to GST-AtsA was totally abolished and that binding of the AtsB-P86G mutant was reduced to 60% (Fig. 4B). In the corresponding eluates from the GSH-agarose columns, measurable amounts of [3H]SAM only were detected for the wild type AtsB control, indicating that none of the AtsB mutants tested was able to bind SAM (Fig. 4B).

The AtsB-G84A and P86G mutants, being either fully (G84A) or only partially (P86G) impaired in binding of AtsA and SAM, were selected to directly test their FGly-generating activity using the in vitro assay described above. It turned out that the latter but not the former mutant was able to catalyze FGly generation in an SAM-dependent and SAHC-sensitive manner (Fig. 4C). Thus, although apparently having a clearly reduced affinity for SAM, the AtsB-P86G mutant obviously is catalytically active.

Taken together these findings strongly suggest that 83GGE86 indeed constitutes the SAM-binding site of AtsB, or a part thereof, and that its occupation by SAM is required for the formation of a ternary AtsB-SAM-AtsA complex that catalyzes the FGly modification of AtsA. Out of this tetrapeptide sequence, 83GGE86 is crucial for AtsB function. At position 86, substitution of proline by glycine was tolerated in vivo, although in vitro it partially impaired the formation of the ternary complex.

Requirement of Conserved Cysteine Residues in AtsB for FGly Generation in Vivo and in Vitro—Radical SAM proteins generate deoxyadenosyl radicals through reductive cleavage of SAM (see the Introduction). Reduction requires an FeS center that is coordinated by a specific cluster of three conserved cysteines (CXXCXXC), with two aromatic residues often flanking the last cysteine. This cysteine cluster usually is located near the N terminus and some 30–50 residues upstream of the putative SAM-binding site (21). In the corresponding cluster of AtsB we mutated two cysteines, Cys39 and Cys42, to alanines. We also mutated three further conserved cysteines that have been predicted to coordinate two additional FeS centers, Cys270 on the one hand and Cys351 and Cys354 on the other (25). All five mutants could be expressed in E. coli at levels comparable with that of wild type AtsB, and the proteins were largely soluble (Fig. 5A). When measuring the activity of co-expressed AtsA, it was found that all five mutants were fully impaired in activating AtsA. After MALDI-TOF MS analysis of AtsA for FGly modification it turned out that in all five cases not even trace amounts of FGly were detectable (Fig. 5A).

In vitro interaction experiments showed that only AtsB mutants C39A and C42A were able to associate with GST-AtsA, albeit with reduced efficiency (55 and 16% of wild type AtsB control, respectively; Fig. 5B). The other mutants showed no interaction. Binding of [3H]SAM also was markedly reduced. Only AtsB-C39A still bound residual amounts of [3H]SAM (~10% of control; Fig. 5B). For all other mutants radioactivity in the GSH eluate was in the background range. Three of the five mutants also were analyzed for their capability of in vitro FGly formation. In line with the in vivo data, none of the cysteine mutants, including AtsB-C39A and AtsB-C42A, was able to generate FGly in the AtsA substrate (Fig. 5C). In conclusion, all three predicted FeS centers seem to be required for FGly modification. Mutation of the second and third cysteine cluster abolishes in vitro binding of SAM and the AtsA substrate, whereas mutation of the first cluster allows for some residual binding (Fig. 5B). This residual binding is blocked in the presence of SAHC (Fig. 5C), suggesting that binding to GST-AtsA is SAM-dependent also for AtsB-C39A. The ternary AtsB-SAM-AtsA complex of this mutant, however, shows no turnover, indicating that AtsB-C39A is catalytically inactive.

Differential effects on AtsA-AtsB interaction and FGly-generating activity were also observed when adding iron chelators such as EDTA or o-phenanthroline to the in vitro reaction. Whereas EDTA but not o-phenanthroline blocked AtsA-AtsB association, both compounds fully impaired FGly modification (Fig. 5D). These results corroborate the conclusion drawn from the cysteine mutants of AtsB, namely that AtsB carries FeS centers that are essential for its enzymatic function as a radical SAM protein.

DISCUSSION

AtsB Acts as a Radical SAM Protein—Here we show that AtsB acts as a radical SAM protein involving SAM and at least one FeS center as critical co-factors. The essential requirement for SAM was shown in vivo, after mutagenesis of the putative SAM-binding site (see below) and also in in vitro experiments. Here binding of AtsB to AtsA was stimulated by SAM in a concentration-dependent manner, was impaired by its demethylated analog SAHC, and was also dependent on an intact SAM-binding site. In fact, [3H]-labeled SAM was recruited into a ternary AtsB-SAM-AtsA complex in a roughly 1:1:1 ratio (Fig. 2). Most significantly, we could demonstrate in vitro FGly generation in the GST-AtsA substrate mediated by freshly prepared AtsB in dependence of SAM. This in vitro modification was furthermore dependent on temperature and incubation time and required an intact serine-type modification motif in GST-AtsA (Fig. 3 and data not shown). It should be noted that already the formation of a stable AtsB-SAM-GST-AtsA complex was a slow and strictly temperature-dependent process (Fig. 1, B and C). This suggests that recognition of the linear modification motif by AtsB is rate-limiting under the applied in vitro conditions and may require unfolding of the AtsA (residues 21–112) appendix of the GST domain. In fact, elongation of the AtsA appendix at its N or C terminus interfered with in vitro interaction (not shown). In vivo, chaperones or components of the early secretory pathway may maintain the modification competence of newly synthesized AtsA polypeptides. Also our earlier observation that the signal peptide of AtsA, targeting the protein for export to the periplasm, stimulates FGly modification 50–100-fold, supports the idea that an export-competent, largely unfolded conformation is required for efficient FGly modification (19).

SAM-binding Site of AtsB—By site-specific mutagenesis we found that the sequence 83GGE86 is crucial for AtsB function. Substitution of each of these three residues by alanine drastically impaired FGly modification activity in vivo (Fig. 4A). In vitro, none of the mutants was able to bind SAM, to interact with AtsA, or to generate FGly (Fig. 4, B and C). Mutation of the juxtaposed Pro86 impaired formation of the ternary AtsB-SAM-AtsA complex partially. GGE perfectly matches a semi-consensus sequence in an alignment block of the radical SAM superfamily (21). Another member of this huge family is the HemN protein of E. coli, which catalyzes oxygen-independent oxidative decarboxylation of coproporphyrinogen-III.

...
HemN carries the corresponding GGTP sequence at positions 112–115. A double mutant in which Gly^{113} and, in addition, Gly^{111} that directly precedes GGTP, both were substituted by valine no longer showed enzymatic activity (31). The binding of SAM by wild type HemN and its inactivation in the mutant, however, was not assayed. The crystal structure of HemN, which is the first three-dimensional structure of a radical SAM protein, was solved recently (32). It verified that Gly^{112} and Gly^{113} make contact to two SAM molecules bound in the active site tunnel close to the FeS center (see below). The requirement of two SAM molecules may be specific for HemN, which catalyzes two consecutive oxidative decarboxylation reactions on FIG. 5.

**A**. Mutagenesis of conserved cysteines coordinating putative FeS centers in AtsB. A, AtsA was co-expressed in *E. coli* together with wild type (WT) AtsB or with a C39A, C42A, C270A, C331A, or C334A mutant form of AtsB. The Western blot shows the AtsB and AtsA polypeptides, as recovered from the soluble fraction of total cell lysates. The sulfatase activities present in the samples loaded for SDS-PAGE are given below each lane. The presence (+) or absence (−) of FGly in AtsA is given, which was detected as DNP-hydrazone derivative by MALDI-TOF MS analysis of its tryptic peptides. B, *in vitro* interaction reactions of GST-AtsA and AtsB, expressed in wild type, C39A, C42A, C270A, C331A, or C334A mutant form, were incubated and analyzed as described for Fig. 4B. The positions of the detected proteins, the relative amounts of AtsB, and the amount of SAM-associated radioactivity recovered in a 33% aliquot of the GSH eluate are given. C and D, *in vitro* FGly modification, GST-AtsA and AtsB expressed in wild type, C39A, C42A, or C270A mutant form were incubated and analyzed as described for Fig. 4C. The presence (+) or absence (−) of FGly in GST-AtsA is given, which was detected as DNP-hydrazone derivative by MALDI-TOF MS analysis of its tryptic peptides. In D, 500 μM SAM and, where indicated, 2 mM EDTA or 2 mM o-phenanthroline were present during the *in vitro* reaction.

**Fig. 5**. Mutagenesis of conserved cysteines coordinating putative FeS centers in AtsB. A, AtsA was co-expressed in *E. coli* together with wild type (WT) AtsB or with a C39A, C42A, C270A, C331A, or C334A mutant form of AtsB. The Western blot shows the AtsB and AtsA polypeptides, as recovered from the soluble fraction of total cell lysates. The sulfatase activities present in the samples loaded for SDS-PAGE are given below each lane. The presence (+) or absence (−) of FGly in AtsA is given, which was detected as DNP-hydrazone derivative by MALDI-TOF MS analysis of its tryptic peptides. B, *in vitro* interaction reactions of GST-AtsA and AtsB, expressed in wild type, C39A, C42A, C270A, C331A, or C334A mutant form, were incubated and analyzed as described for Fig. 4B. The positions of the detected proteins, the relative amounts of AtsB, and the amount of SAM-associated radioactivity recovered in a 33% aliquot of the GSH eluate are given. C and D, *in vitro* FGly modification, GST-AtsA and AtsB expressed in wild type, C39A, C42A, or C270A mutant form were incubated and analyzed as described for Fig. 4C. The presence (+) or absence (−) of FGly in GST-AtsA is given, which was detected as DNP-hydrazone derivative by MALDI-TOF MS analysis of its tryptic peptides. In D, 500 μM SAM and, where indicated, 2 mM EDTA or 2 mM o-phenanthroline were present during the *in vitro* reaction.
the same substrate (see Ref. 32).

Interestingly, Shields et al. (33) located $^{85}$GXG$^{100}$ and $^{18}$EE$^{148}$ motifs in phosphatidylethanolamine N-methyltransferase that seem to cooperate in SAM binding. These separate sequence elements were found in subgroups of SAM-utilizing enzymes. From the crystal structure of several SAM-dependent methyltransferases, it is known that a glycine-rich motif (motif I) and a separate motif (post I) containing a negatively charged amino acid make direct contact to SAM (30). Also the HemN structure revealed that the negative charges of Asp$^{209}$ and Glu$^{145}$, the latter in direct proximity to Gly$^{143}$, make contact to ribose hydroxyls of the two bound SAM molecules (32). The linear GGE motif of AtsB includes both the glycines and a negatively charged residue that, as shown here, are critical for binding of SAM.

**Mechanism of AtsB Function**—Like other radical SAM proteins, AtsB needs a specific FeS center to facilitate reductive cleavage of SAM. Apart from the correct position, 30–50 residues upstream of the SAM-binding site, and perfect match of the $^{18}$XGX$^{35}$$^{18}$cysteine cluster of AtsB with the corresponding semiconsensus sequence of radical SAM proteins (21), we could provide experimental evidence for the critical role of this cluster. Mutagenesis of the corresponding cysteines fully inactivated the FGly-generating activity of AtsB in *in vivo* and *in vitro* (Fig. 5, A–C). Interestingly, the same result was obtained after mutagenesis of two additional cysteine clusters, suggesting the existence of two further important FeS centers in the C-terminal half of AtsB, which are not shared with radical SAM proteins in general. Direct evidence for the participation of metal ions came from the observation that chelating agents such as EDTA or o-phenanthroline completely blocked the FGly-modification activity of AtsB (Fig. 5D).

The specific FeS center of radical SAM proteins in its [4Fe-4S]$^{+}$ form is active for reductive cleavage of SAM (23–24). Because it is coordinated by only three cysteines, one iron coordination position is vacant, thereby explaining its oxygen sensitivity (22, 32). On the other hand, the unique iron, which is not coordinated by a cysteine, allows for SAM binding. Because it is coordinated by only three cysteines, one iron coordination position is vacant, thereby explaining its oxygen sensitivity (22, 32). On the other hand, the unique iron, which is not coordinated by a cysteine, allows for SAM binding. The latter can directly abstract a hydrogen from the two bound SAM molecules (32). The linear GGE motif of AtsB includes both the glycines and a negatively charged residue that, as shown here, are critical for binding of SAM.

**In view of the model outlined above, the critical [4Fe-4S]$^{+}$ center with its unique iron contributes to SAM binding. We observed that mutating the corresponding cysteine cluster (Cys$^{28}$ and Cys$^{42}$) led to catalytically inactive AtsB protein in which SAM binding was much stronger affected than association with AtsA (Fig. 5B). This may reflect the contribution of the [4Fe-4S] center to SAM binding.**

Further insight into molecular aspects of AtsB function most likely will require analysis under anaerobic conditions and to work with AtsB protein (wild type and mutant) that is characterized with respect to its iron content. Studies of its FeS center(s) by EPR spectroscopy in the absence and presence of SAM and a substrate peptide should clarify basic properties of those centers and their interconversions. For understanding the mechanism of the radical SAM family in general, consisting of an enormous variety of enzymes, the first three-dimensional structure that is now available (32) will be extremely valuable. The structures of other family members will reveal common principles and also the variations underlying radical SAM-mediated catalysis.

**Acknowledgments**—We thank Klaus Neifer for peptide and DNA sequencing and Dr. S. Iishi (RIKEN, Tsukuba, Japan) for providing the p7-groK plasmid.

**REFERENCES**

1. Schmidt, B., Selmer, T., Ingendoh, A., and von Figura, K. (1995) *Cell* **82**, 271–278.
2. Lukatela, G., Krause, N., Theis, K., Selmer, T., Gieselmann, V., von Figura, K., and Saenger, W. (1998) *Biochemistry* **37**, 3854–3864.
3. von Figura, K., Schmidt, B., Selmer, T., and Dierks, T. (1998) *BioEssays* **20**, 505–510.
4. Dierks, T. (2001) in *Encyclopedia of Molecular Medicine* (Creighton, T. T., ed) pp. 974–976, John Wiley & Sons, New York.
5. Boltes, I., Czapinski, H., Kanhert, A., von Bulow, R., Dierks, T., Schmidt, B., von Figura, K., Kertesz, M. A., and Usoin, I. (2001) *Structure* **9**, 483–491.
6. Beckers, M., Selmer, T., Dierks, T., Schmidt, B., and von Figura, K. (1998) *J. Biol. Chem.* **273**, 6966–6971.
7. von Bulow, R., Schmidt, B., Dierks, T., von Figura, K., and Usoin, I. (2001) *J. Mol. Biol.* **305**, 269–277.
8. Hopwood, J. J., and Bullabio, A. (2001) in *The Metabolic and Molecular Bases of Inherited Disease* (Scrivener, C. R., Beaudet, A. L., Valle, D., and Sly, W. S., eds) pp. 3725–3732, McGraw-Hill, New York.
9. Dierks, T., Schmidt, B., Borissenko, L. V., Peng, J., Presurer, A., Mariappan, M., and von Figura, K. (2003) *Cell* **113**, 435–444.
10. Cosma, M. P., Pepe, S., Annunziata, I., Trott, D. A., Parenti, G., and Bullabio, A. (2003) *Cell* **113**, 445–456.
11. Dierks, T., Schmidt, B., and von Figura, K. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 11963–11968.
12. Dierks, T., Lecca, M. R., Schmidt, B., and von Figura, K. (1998) *FEBS Lett.* **423**, 61–65.
13. Dierks, T., Lecca, M. R., Schlotterhose, P., Schmidt, B., and von Figura, K. (1999) *EMBO J.* **18**, 209–219.
14. Fey, J., Balleiniger, M., Borissenko, L. V., Schmidt, B., and von Figura, K. (2001) *J. Biol. Chem.* **276**, 47024–47028.
15. Landgrebe, J., Dierks, T., Schmidt, B., and von Figura, K. (2003) *Gene (Amst.* **316**, 47–56.
16. Dierks, T., Miech, C., Hummerjohn, J., Schmidt, B., Kertesz, M. A., and von Figura, K. (1998) *J. Biol. Chem.* **273**, 25560–25564.
17. Miech, C., Dierks, T., Selmer, T., von Figura, K., and Schmidt, B. (1998) *J. Biol. Chem.* **273**, 4835–4837.
18. Murooka, Y., Ishibashi, K., Yasumoto, M., Sasaki, M., Sugino, H., Azakami, H., and Yamashita, M. (1998) *J. Bacteriol.* **172**, 2131–2140.
19. Marguerat, C., Fang, Q., Will, E., Peng, J., von Figura, K., and Dierks, T. (2003) *J. Biol. Chem.* **278**, 22122–22128.
20. Szamet, C., Miech, C., Balleiniger, M., Schmidt, B., and von Figura, K. (1999) *J. Biol. Chem.* **274**, 15375–15381.
21. Sophia, H. J., Chen, G., Hetzler, B. G., Reyes-Sindolo, J. F., and Miller, N. E. (2001) *Nucleic Acids Res.* **29**, 1097–1106.
22. Frey, P. A. (2001) in *Annu. Rev. Biochem.* **70**, 121–148.
23. Cheek, J., and Broderick, J. B. (2001) *J. Biol. Inorg. Chem.* **6**, 209–226.
24. Jarrett, J. T. (2003) *Curr. Opin. Chem. Biol.* **7**, 174–182.
25. Schriner, A., and Kober, R. (1996) *Chem. Biol.* **3**, R181–R186.
26. Yatsuka, T., Kanie-Ishii, C., Markiewa, T., Fujimoto, J., Yamamoto, T., and Iishi, S. (1995) *J. Biol. Chem.* **270**, 25328–25331.
27. Dierks, T., Volkmer, J., Schlenstedt, G., Jung, C., Sandholzer, U., Zachmann, K., Schlotterhose, P., Neifer, K., Schmidt, B., and Zimmermann, R. (1996) *EMBO J.* **15**, 6931–6942.
28. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) *Anal. Chem.* **68**, 6550–6555.
29. Peng, J., Schmidt, B., von Figura, K., and Dierks, T. (2003) *J. Mass Spectrom.* **38**, 80–86.
30. Newmeyerzycka, A., and Clarke, S. (1999) *J. Biol. Chem.* **274**, 814–824.
Formylglycine Modification by Radical SAM Protein AtsB

31. Layer, G., Verfurth, K., Mahlitz, E., and Jahn, D. (2002) J. Biol. Chem. 277, 34136–34142
32. Layer, G., Moser, J., Heinz, D. W., Jahn, D., and Schubert, W. D. (2003) EMBO J. 22, 6214–6224
33. Shields, D. J., Altarejos, J. Y., Wang, X., Agellon, L. B., and Vance, D. E. (2003) J. Biol. Chem. 278, 35826–35836
34. Walsby, C. J., Hong, W., Broderick, W. E., Cheek, J., Ortíllo, D., Broderick, J. B., and Hoffman, B. M. (2002) J. Am. Chem. Soc. 124, 3143–3151
35. Cosper, N. J., Booker, S. J., Ruzicka, F., Frey, P. A., and Scott, R. A. (2000) Biochemistry 39, 15668–15673
36. Ugalava, N. R., Frederic, K. K., and Jarrett, J. T. (2003) Biochemistry 42, 2708–2719
Post-translational Formylglycine Modification of Bacterial Sulfatases by the Radical S-Adenosylmethionine Protein AtsB
Qinghua Fang, Jianhe Peng and Thomas Dierks

J. Biol. Chem. 2004, 279:14570-14578.
doi: 10.1074/jbc.M313855200 originally published online January 28, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M313855200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 34 references, 14 of which can be accessed free at
http://www.jbc.org/content/279/15/14570.full.html#ref-list-1