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# Anaerobic Sulfatase-Maturating Enzymes: Radical SAM Enzymes Able To Catalyze in Vitro Sulfatase Post-translational Modification

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Sulfatases are widespread enzymes, found from prokaryotes to eukaryotes, and are involved in many biochemical processes.[1] They can be divided into two classes, "Ser-type" or "Cys-type", according to the critical residue encoded in their active site, respectively a serine or a cysteine.[1] To be active, all known sulfatases undergo a unique post-translational modification leading to the conversion of the critical residue into a $C_\alpha$-formylglycine (FGly).[2,3] This conversion is catalyzed by one of two nonhomologous systems. The formylglycine-generating enzyme system (FGE), an oxygen-dependent oxidoreductase, which has been extensively studied, catalyzes the maturation of "Cys-type" sulfatases.[4] The other system, which is responsible for "Ser-type" sulfatase maturation, has undergone only preliminary characterization, and only the enzyme of *Klebsiella pneumoniae*, AtsB, has been the focus of biochemical studies.[5]

Until recently, it was thought that each class of sulfatase had a corresponding maturation system. We recently reported the discovery of an enzyme in *Clostridium perfringens* that shares 48% similarity with AtsB and is physiologically responsible for the maturation of the unique "Cys-type" sulfatase encoded by this bacterial species.[6] AtsB and the *C. perfringens* enzyme not only share a high sequence similarity but also possess the $Cx_3Cx_2C$ amino acid consensus motif of the "radical SAM" super-family[7] as well as two additional strictly conserved cysteine clusters (Figure 1). As these enzymes account for the maturation of both classes of sulfatases under anaerobic conditions, we named this group of enzymes anSME (**an**aerobic **S**ulfatase **M**aturating **E**nzymes).[6]

On the basis of the presence of a "radical SAM" motif, a speculative mechanism was proposed involving the reductive cleavage of *S*-adenosyl-L-methionine (SAM), followed by a proton abstraction from the critical amino-acid residue, leading to the formation of FGly.[5] However, experimental data to support this hypothesis have been lacking. Indeed, in contrast to FGE, in vitro maturation has never been obtained with this type of enzyme.[5] This is probably due to the inability to obtain sufficient amounts of soluble enzyme, as members of the anSME group are unstable proteins and occur as inclusion bodies when overexpressed.[5] Furthermore, most radical SAM enzymes have oxygen-sensitive clusters, which require strict anaerobic conditions for catalysis.

Herein, we report the first purification and anaerobic reconstitution of an anSME. This allowed us to give the first experimental evidence of the presence of iron−sulfur centers in anSME and to investigate their activity toward SAM and a peptide, containing the cysteine target of the post-translational modification.

The gene encoding the anSME enzyme in *C. perfringens*[6] was cloned and overexpressed as a His-tagged protein in *Escherichia coli* (see Supporting Information). To prevent fast protein precipitation, the enzyme was purified in the presence of DTT. After purification with Ni-NTA Sepharose and Superdex S200HR, the protein was essentially pure (Figure 2A). The enzyme preparation exhibited the typical brownish color of iron−sulfur proteins, and UV−visible spectroscopy analysis revealed a characteristic absorption band around 420 nm (Figure 2B). The enzyme purified under aerobic conditions contained only 3.0 ± 0.2 mol of iron per mole of monomer. It was thus reconstituted under strict anaerobic conditions by incubation with a 10-fold excess of iron and sulfur, followed by size exclusion chromatography purification.

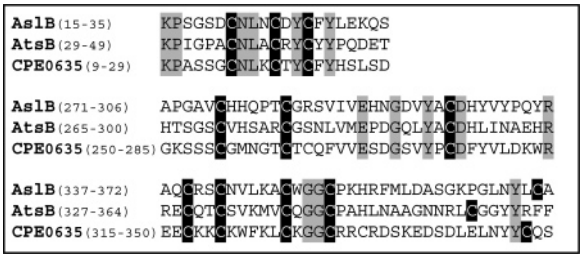


***Figure 1.*** Sequence alignment of the three anSME putative clusters. AslB (*E. coli*), AtsB (*K. pneumoniae*) and CPE0635 (*C. perfringens*). Sequence positions in the proteins are in brackets. In black are the conserved cysteines, and in gray are the other conserved residues.

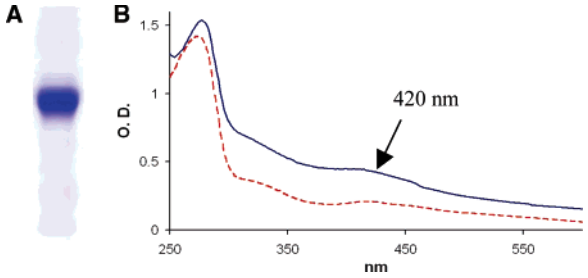


***Figure 2.*** (A**)** Gel electrophoresis analysis (SDS-PAGE) of *Clostridium perfringens* anSME. (B**)** UV−visible spectra of the "as isolated" (dashed line) and the reconstituted (solid line) enzyme.

The reconstituted enzyme had an increased iron content, leading to a protein with 6.0 ± 0.3 mol of iron per mole of monomer. As a result, the absorption band at 420 nm increased and the $A_{420}/A_{280}$ ratio shifted from 0.15 to 0.3 (Figure 2B).

Radical SAM enzymes are characterized by the presence of a [4Fe-4S] center, which is involved in the formation of a 5′-deoxyadenosyl radical from SAM. In general, the reductive cleavage of SAM is coupled to subsequent reactions that are thermodynamically favored. However, in the presence of highly reducing electrons several members of the radical SAM superfamily are able to produce AdoH and methionine in the absence of substrate.[7]

Thus, the reconstituted anSME enzyme (40 $\mu$M) was incubated with SAM in an anaerobic glove box in the presence of DTT and

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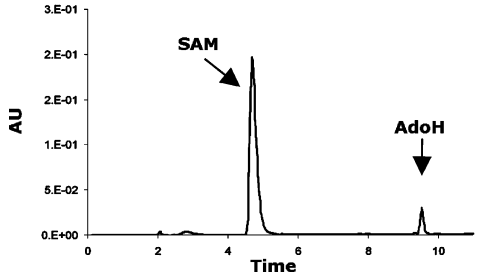



***Figure 3.*** Reversed-phase HPLC analysis of SAM incubation with *C. perfringens* anSME after 6 h under anaerobic conditions. Detection was made at 260 nm.

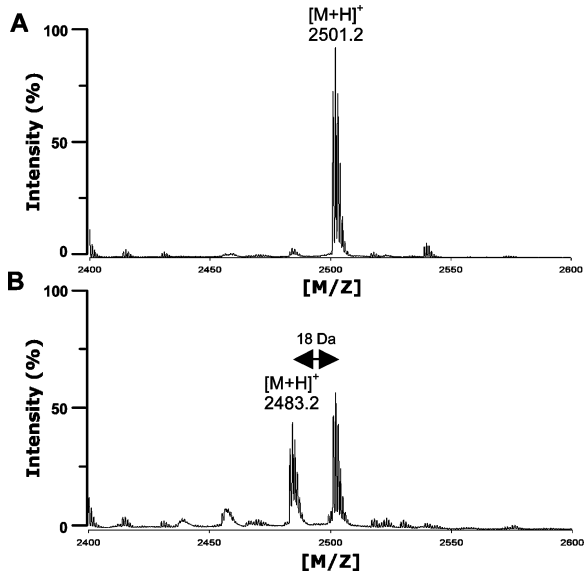


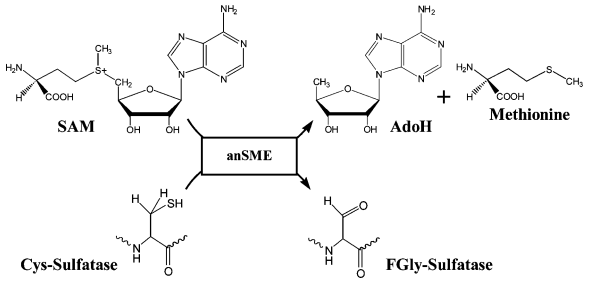

***Figure 4.*** Mass spectrometry analysis of a 23-mer peptide (350 $\mu$M) incubated with *C. perfringens* anSME (40$\mu$M), SAM, DTT, and dithionite under anaerobic conditions at $T = 0$ (A) and $T = 6$ h (B) at 25 °C (see Supporting Information).

dithionite. Analysis of the reaction by reversed-phase HPLC showed that *C. perfringens* anSME is able to produce AdoH from SAM, as assessed by its elution time (9.5 min), its UV absorption maximum, and by mass spectrometry analysis (Figure 3 and Supporting Information). anSME are thus authentic radical SAM enzymes able to perform in vitro the reductive cleavage of SAM.

Sulfatase maturation is thought to be a co- or post-translational modification. Therefore, we used, as substrate, a 23-amino acid peptide derived from the *C. perfringens* sulfatase sequence and containing the critical cysteine (see Supporting Information). This peptide was incubated under strict anaerobic conditions with SAM and the reconstituted anSME. Mass spectrometry analysis clearly showed its conversion into a new peptide with a mass loss of 18 Da (Figure 4). This shift was consistent with the conversion of cysteine into FGly. To further ascertain the nature of the modification we used dinitrophenyl hydrazine (DNPH) as a matrix for MALDI-TOF MS analysis.[8]

As expected, the DNPH efficiently and specifically reacted with the newly formed aldehyde group, leading to a hydrazone derivative

***Scheme 1.*** Reaction Catalyzed by anSME, Leading to the Conversion from Unmature (Cys-sulfatase) to Mature (FGly sulfatase) Sulfatase




with a mass increment of 180 Da (see Supporting Information). To definitively ascertain the localization of the modification on the peptide, we also incubated anSME under the same conditions with a peptide containing an alanine (instead of a cysteine) (see Supporting Information). This peptide was not an enzyme subtrate, thus confirming that the modification occurred on the cysteine residue. Thus, anSME catalyzes the reaction described in Scheme 1. This reaction leads to the cysteine oxidation and the formation of AdoH in strict anaerobic conditions with an oxygen-independent radical-based mechanism.

This study is the first report of in vitro FGly formation catalyzed by anSME. Our experiments demonstrate that anSME belong to the radical SAM superfamily and, in contrast to what was previously thought,[5] are able to catalyze the conversion of cysteine into FGly (Scheme 1). The iron content assay and the UV spectrum, of the reconstituted protein, also suggest that anSME are likely to contain, in addition of the typical [4Fe-4S] center of radical SAM enzymes, at least one other iron−sulfur center. Further characterization of anSME is ongoing to ascertain this hypothesis.

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**Supporting Information Available:** Experimental procedures, HPLC and MALDI-TOF analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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