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Choline sulfatase from *Ensifer* (*Sinorhizobium*) *meliloti*: Characterization of the unmodified enzyme

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ABSTRACT

Ensifer (*Sinorhizobium*) *meliloti* is a nitrogen-fixing α-proteobacterium able to biosynthesize the osmoprotectant glycine betaine from choline sulfate through a metabolic pathway that starts with the enzyme choline-*O*-sulfatase. This protein seems to be widely distributed in microorganisms and thought to play an important role in their sulfur metabolism. However, only crude extracts with choline sulfatase activity have been studied. In this work, *Ensifer* (*Sinorhizobium*) *meliloti* choline-*O*-sulfatase was obtained in a high degree of purity after expression in *Escherichia coli*. Gel filtration and dynamic light scattering experiments showed that the recombinant enzyme exists as a dimer in solution. Using calorimetry, its catalytic activity against its natural substrate, choline-*O*-sulfate, gave a $k_{cat}=2.7\times10^{-1}\,s^{-1}$ and a $K_M=11.1$ mM. For the synthetic substrates *p*-nitrophenyl sulfate and methylumbelliferyl sulfate, the k_{cat} values were $3.5\times10^{-2}\,s^{-1}$ and $4.3\times10^{-2}\,s^{-1}$, with K_M values of 75.8 and 11.8 mM respectively. The low catalytic activity of the recombinant sulfatase was due to the absence of the formylglycine post-translational modification in its active-site cysteine 54. Nevertheless, unmodified *Ensifer* (*Sinorhizobium*) *meliloti* choline-*O*-sulfatase is a multiple-turnover enzyme with remarkable catalytic efficiency.

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1. Introduction

Ensifer meliloti (formerly *Sinorhizobium meliloti*) is a nitrogen-fixing α-proteobacterium that establishes root nodule symbiosis with legume plants, providing ammonia to their hosts and receiving nutrients from them [1]. In free life or in symbiosis, these bacteria have to deal with adverse environmental conditions such as droughts, rain or floods, which cause severe changes in their extracellular osmolality. An immediate response to cope with these situations is to accumulate or release ions and selected low-molecular-weight organic molecules called osmolytes that counteract the osmotic gradient [2–5]. Glycine betaine is a potent and well-characterized osmoprotectant widespread in nature [5,6], and *E. meliloti* can efficiently transport it to its interior through high-affinity uptake protein systems [4,7]. Alternatively, it can synthesize glycine betaine from choline-*O*-sulfate by a three-step pathway with choline and betaine aldehyde as intermediates (Fig. 1) [8]. The genes involved in this metabolic pathway constitute the operon *betICBA*, which is composed of a regulatory gene (*betI*) and three structural genes: *betC* (choline-*O*-sulfatase, or COS), *betB* (betaine aldehyde dehydrogenase) and *betA* (choline dehydrogenase) [8].

The metabolic pathway is controlled by BetI, a repressor that regulates the expression of *bet* genes in response to the inducer choline [9]. Transcription of the operon can also be initiated to a lesser extent by the presence of choline-*O*-sulfate or acetylcholine, but not by the presence of high salt concentration alone [9]. However, as the product of the route, glycine betaine, is a potent osmolyte that accumulates in *E. meliloti* under salt stress [10,11], choline-*O*-sulfate or choline can allow the proliferation of *E. meliloti* under high salt concentration through their transformation to glycine betaine [8,9]. Under no salt stress, *E. meliloti* can import choline or choline-*O*-sulfate from its surroundings and transform them to glycine betaine, which can be further metabolized to cope entirely with the carbon and nitrogen cell demands in the absence of other nutrients [8,11]. The inorganic sulfate produced by the hydrolysis of choline-*O*-sulfate can also be used as the only source of sulfur by *E. meliloti* [8]. Choline-*O*-sulfate, has been shown to be biosynthesized and accumulated by a variety of plants, marine and soil fungi and red algae where it serves as an osmoprotector and sulfur reservoir [12–18]. In contrast, certain bacteria, as *Bacillus subtilis, Escherichia coli and Salmonella typhimurium,* accumulate

Abbreviations: COS, *E. meliloti* choline-*O*-sulfatase; FGly, α-formylglycine; FGE, α-formylglycine-generating enzyme; anSME, anaerobic sulfatase maturing enzyme; pNPS, *p*-nitrophenyl sulfate; MUS, 4-methylumbelliferyl sulfate; ITC, isothermal titration calorimetry; DLS, dynamic light scattering; MALDI-TOF, matrix assisted laser desorption ionization time-of-flight; DTNB, 5,5′-Dithiobis(2-nitrobenzoic acid); DTT, DL-Dithiothreitol; TCEP, Tris(2-carboxyethyl)phosphine hydrochloride; UPLC-ESI-Q-TOF-MS, Ultra-performance liquid chromatography-electrospray ionization-quadrupole time-of-flight-mass spectrometry

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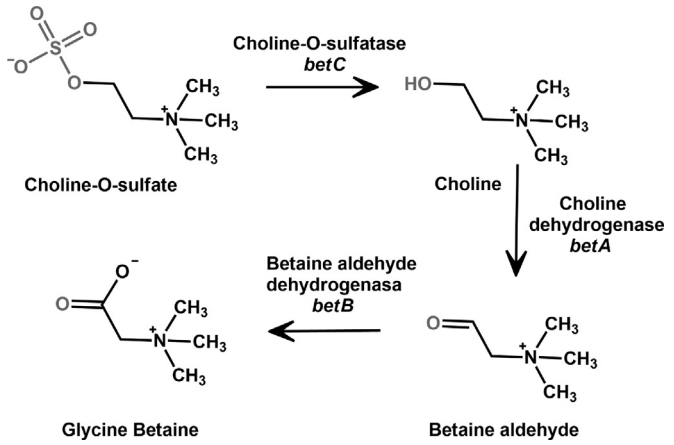

Fig. 1. Synthesis of glycine betaine from choline sulfate in *E. meliloti*.

choline-*O*-sulfate as osmoprotector without further metabolization [19,20]. Choline-*O*-sulfate can be released to the environment through root exudation or microbial cell decay [4,21] and once in the soil, it can be taken by other microorganisms as an important source of sulfur, carbon and nitrogen [22,23]. The extent of choline-*O*-sulfate in soil or other environments is not well characterized, however, its direct or indirect role as osmolyte and the widespread presence of choline sulfatase genes in microbes [24] suggest an important role of this compound and these enzymes in the biological sulfur cycle.

Amino acid sequence analysis of COS revealed that this enzyme belongs to the type I sulfatases family (previously named arylsulfatases) [8,24]. These enzymes share high degree of conservation in sequence, structure and enzymatic mechanism among all life kingdoms and hydrolyze many diverse sulfate esters present in mono- oligo- and polysaccharides, proteoglycans, amino acids, steroids and glycolipids [25]. Recent genetic analyzes identified a few peptide signatures that seem to be specific for choline sulfatases [24]. In *E. meliloti* and other members of the *Rhizobiaceae* family, the gene *betC*, is in the operon *betICBA* mentioned above, but in the rest of microbes *betC* has a different genetic environment and is mainly found associated to an ABC-type betaine periplasmic binding protein and to an ATP-binding protein with a putative sulfate permease activity [22,24]. All type I sulfatases, including COS, have a highly conserved amino acid sequence in their active site: (C/S)-X-(P/A)-X-R [25–27]. This sequence is critical, since it is the recognition site for a post-translational modification of an active site cysteine or serine residue, to the catalytically functional residue α-formylglycine (FGly). This modification is catalyzed by a formylglycine-generating enzyme (FGE), or by an anaerobic sulfatase maturing enzyme (anSME) depending on the organism [27]. *E. coli* is only able to modify cysteine residues and the identification of the enzymatic machinery responsible for this modification in this bacterium has been elusive [28].

Given the apparent ubiquity of choline-*O*-sulfatases in microorganisms [24], their study is important in understanding sulfur metabolism in microorganisms and soil, and in some contexts the osmoprotection capacity of their substrate. To our knowledge, there are only three reports of biochemical characterization of choline-*O*-sulfatases, but in all of them only crude extract or partially purified proteins were studied [14,29,30]. In this work the heterologous expression of COS was performed in *E. coli* BL21 (DE3), followed by complete purification and biochemical characterization.

2. Materials and methods

2.1. Molecular cloning and recombinant expression

COS gene (*betC*) sequence [AAC13371.1] was codon optimized for *E. coli* expression (Supplementary material Fig. S1), synthesized, and sequenced by GenScript USA Inc. Then, it was subcloned in pET26b+ with a C-terminus His_6-tag sequence. Overexpression of COS was performed in *E. coli* BL21 (DE3) using IPTG (1.0 mM) at 30 °C for 7 h.

2.2. Enzyme purification

Cells were centrifuged and resuspended in 1/25 of their original volume in Tris–HCl buffer (200 mM) with imidazole (20 mM) pH 7.5. They were treated with lysozyme (1 mg/ml final concentration) for 30 min at 4 °C and lysed by sonication. The crude extract obtained after centrifugation was loaded into a His-Trap FF column (GE-Healthcare) and eluted with a linear gradient of 20–250 mM imidazole in 10-column volumes. Fractions were analyzed by 10% SDS-PAGE and those containing the enzyme were pooled and further purified by size exclusion chromatography (HiLoadSuperdex 200 16/600 GL; GE-Healthcare) using Tris–HCl (20 mM) buffer pH 7.5. Fractions containing the pure enzyme were mixed and its concentration calculated spectrophotometrically at 280 nm using a theoretical molar extinction coefficient of 97,750 $M^{-1}\,cm^{-1}$ [31]. The enzyme was aliquoted and stored at −20 °C. Typical yields of purified protein were in the range 30–35 mg of protein/L of culture.

2.3. Biochemical characterization and enzymatic activity

The molecular weight of the native enzyme was determined with a gel filtration analytical column (Superdex 200 10/300 GL; GE-Healthcare) using as standards: thyroglobulin (670 kDa), gamma globulin (158 kDa), ovoalbumin (44 kDa), myoglobin (17 kDa) and vitamin B12 (1.35 kDa) (Gel Filtration Standard; Bio-Rad).

Masses were determined with a Bruker Microflex matrix assisted laser desorption ionization time-of-flight (MALDI TOF) instrument (Bruker Daltonics GmbH) equipped with a 20-Hz nitrogen laser at $\lambda = 337$ nm. Spectra were recorded in reflector and/or linear positive mode for the mass range of 25,000–250,000 Da. 1.0 μL of sample solution was mixed with 5 μL of 30% acetonitrile, 70% water, 0.1% trifluoroacetic acid, and saturated with sinapinic acid. Then, 1.0 μL of this solution was deposited onto the MALDI target and allowed to dry at room temperature.

Dynamic light scattering (DLS) measurements were performed at 25.0 °C using a Malvern Nano S (Malvern, Ltd.) instrument equipped with laser NIBS (Non Invasive Back Scattering) technology and a Peltier temperature controller. The hydrodynamic radius was calculated using the Zeta Sizer software provided with the equipment.

Enzyme-catalyzed hydrolysis of choline-*O*-sulfate (Cambridge Isotope Laboratories) was measured by Isothermal Titration Calorimetry (ITC) using a VP-ITC microcalorimeter (Microcal Inc.) at 25 °C in the Reaction Buffer (200 mM Tris–HCl pH 7.5 and 500 mM NaCl). The kinetic parameters were obtained following the procedure of multiple injections [32] in which a COS solution (2.46 μM) was incubated in the calorimetric cell and a solution of choline-*O*-sulfate (300 mM) was injected multiple times (2×5 μL and then 18×10 μL) to acquire a calorimetric thermogram. The calorimetric data were transformed to initial rate vs substrate concentration plots using the ITC Data Analysis in Origin 7.0 (Microcal Inc.) [32]. The enthalpy of the reaction was determined by triplicate using the single injection method in which a COS

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