Structural and Mechanistic Analysis of the Choline Sulfatase from *Sinorhizobium melliloti*: A Class I Sulfatase Specific for an Alkyl Sulfate Ester

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Abstract

Hydrolysis of organic sulfate esters proceeds by two distinct mechanisms, water attacking at either sulfur (S–O bond cleavage) or carbon (C–O bond cleavage). In primary and secondary alkyl sulfates, attack at carbon is favored, whereas in aromatic sulfates and sulfated sugars, attack at sulfur is preferred. This mechanistic distinction is mirrored in the classification of enzymes that catalyze sulfate ester hydrolysis: arylsulfatases (ASs) catalyze S–O cleavage in sulfate sugars and arylsulfates, and alkyl sulfatases break the C–O bond of alkyl sulfates. *Sinorhizobium melliloti* choline sulfatase (*SmCS*) efficiently catalyzes the hydrolysis of alkyl sulfite choline-O-sulfate (at = 4.8 × 10³ s⁻¹ M⁻¹) as well as arylsulfate 4-nitrophenyl sulfate (at = 12 s⁻¹ M⁻¹). Its 2.8-Å resolution X-ray structure shows a buried, largely hydrophobic active site in which a conserved glutamate (Glu386) plays a role in recognition of the quaternary ammonium group of the choline substrate. *SmCS* structurally resembles members of the alkaline phosphatase superfamily, being most closely related to dimeric ASs and tetrameric phosphonate monoester hydrolases. Although >70% of the amino acids between protomers align structurally (RMSDs 1.79–1.99 Å), the oligomeric structures show distinctly different packing and protomer–protomer interfaces. The latter also play an important role in active site formation. Mutagenesis of the conserved active site residues typical for ASs, H218O-labeling studies and the observation of catalytically promiscuous behavior toward phosphoesters confirm the close relation to alkaline phosphatase superfamily members and suggest that *SmCS* is an AS that catalyzes S–O cleavage in alkyl sulfate esters with extreme catalytic proficiency.

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Introduction

Sulfatases are ubiquitous enzymes with a variety or roles in eukaryotic and prokaryotic organisms. In humans, sulfatases are involved in lysosomal degradation of mucopolysaccharides [1,2] (leading to disease phenotypes when absent [1,3]), activation of steroid hormones [4,5] and developmental processes [6–8], which is mirrored in other vertebrates [9–11]. Elsewhere, sulfatases play a role in sulfur harvesting [12–15] and bacterial infection [16–19]. In many cases, it is not known what the primary substrate is. The only microbial sulfatases currently assumed to be specific toward one particular substrate are the choline sulfatases (CSs). CSs enable microorganisms to use choline-O-sulfate (1a in Fig. 1) as a source of sulfur, carbon and nitrogen [20–22], or contribute to osmoregulation [23]. Sulfatases have been grouped in three classes based on their catalytic mechanism [24]. Class I sulfatases (aryl)sulfatases, or ASs) hydrolyze a wide variety of sulfate esters by net attack of water on the sulfur center (Fig. 2), resulting in inorganic sulfate and the corresponding alcohol. Structurally and

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mechanistically, they belong to the alkaline phosphatase (AP) superfamily [26–34]. Class II sulfatases are specific for alkyl sulfates and convert these substrates into inorganic sulfate and the corresponding aldehyde via a reductive dioxygenase mechanism [25]. Class III sulfatases are also specific for alkyl sulfates and catalyze the same net reaction as class I sulfatases [24], but employ a different mechanism (Fig. 2). The active site nucleophile attacks at carbon rather than at the sulfur [35,36], possibly exploiting the higher reactivity at the carbon center compared to sulfur in alkyl sulfates [37]. The latter difference is reversed in aryl sulfates: resonance effects lower the reactivity of the carbon center in the aryl group. The same resonance effects facilitate leaving group departure for the reaction that involves the sulfur center. Hydrolysis of the sulfate ester bond in sulfated sugars has thus far only been shown to be catalyzed by ASs, suggesting that enzyme-catalyzed hydrolysis of sulfated sugars mainly proceeds via S–O attack (possibly due to steric hindrance preventing attack at the carbon center).

As described above, CSs enable bacteria to use choline-O-sulfate as a source of sulfur, carbon and nitrogen [22,38]. Expression of CS-encoding genes in Pseudomonads is exclusively induced by its substrate choline-O-sulfate [20–22,38], preventing the energetically costly production of an enzyme that is only useful when its eponymous substrate is available. This phenomenon can be explained by the presence of the transcriptional regulator BetR, which promotes transcription of its own gene (betR) and the CS-encoding gene betC in the presence of choline-O-sulfate [20,21]. Osteras et al. [23] showed that in addition to enabling Sinorhizobium meliloti to use choline-O-sulfate as a resource, CS is also involved in osmoregulation. In this bacterium, the product of CS-catalyzed hydrolysis of choline-O-sulfate, choline, can be readily converted into glycine betaine, which the organism could use either as an osmoprotectant or as a source of carbon and nitrogen.

Based on sequence homology analysis, S. meliloti CS (SmCS) was identified as a member of the AP superfamily and is most closely related to class I ASs [23]. However, its proposed native substrate is a primary alkyl sulfate, paradoxically a substrate expected to be converted by a class III sulfatase. This poses the three questions: (i) Can CS catalyze the hydrolysis of non-natural aryl sulfates such as 4-nitrophenyl sulfate (1b, Fig. 1) or coumarin sulfate, a phenomenon

![Fig. 1. Substrates tested with SmCS WT. 1a: choline-O-sulfate, 1b: 4-nitrophenyl sulfate, 2a: phosphoryl choline, 2b: 4-nitrophenyl phosphate, 3b: 4-nitrophenyl methylphosphate, 4b: 4-nitrophenyl methylphosphonate.](image)

**Fig. 2.** Classification of sulfatases based on their catalytic mechanism [24]. Class I and class III sulfatases catalyze the same net reaction—hydrolytic cleavage of the sulfate ester bond—via nucleophilic attack on the S–O and C–O bond, respectively (indicated with an arrow). Class II sulfatases catalyze the cleavage of the sulfate ester bond via a reductive dioxygenase mechanism [25].
observed for several of the class I sulfatases that act primarily on sulfated sugars [27,39,40]? (ii) Is CS catalytically promiscuous, a widely observed phenomenon among AP superfamily members [26,41–48]? (iii) Does CS act on its native substrate with a similar mechanism as its close relatives from the AP superfamily, that is, by nucleophilic attack on the sulfur? The latter is expected to be 14.2 kcal mol\(^{-1}\) more difficult than attack on carbon and fission of the C–O bond [37] (for details, see supporting information, SI). Nucleophilic attack on the sulfur center in alkyl sulfates has been previously shown for a class I sulfatase. *Pseudomonas aeruginosa* AS (PAS) had been shown to catalyze the hydrolysis of secondary alkyl sulfate esters with retention of configuration around the carbon center [36], suggesting that this enzyme indeed hydrolyzes alkyl sulfates via S–O cleavage. However, the majority of enzymes that are able to catalyze the hydrolysis of alkyl sulfates are expected to be class III sulfatases [24]. The enzymes in class III indeed employ nucleophilic attack on carbon, shown by both H\(_2\)\(^{18}\)O labeling studies and the inversion of configuration at the carbon atom directly connected to the leaving group for the enantioselective hydrolysis of sec-alkyl sulfates [35,49].

In order to resolve whether CS is acting as a class I or class III sulfatase and to determine its relationship to the AP superfamily, we have characterized SmCS [23] biochemically and structurally. In this study, we show that the enzyme is a tetramer and that the quaternary structure is largely mediated by a C-terminal extension. These features match those of the tetrameric phosphonate monoester hydrolases (PMHs) and the recently discovered class of dimeric ASs [26], both members of the AP superfamily. However, the oligomeric topology of SmCS presented in this study is significantly different and the oligomer interface plays a far more important role in the formation of the active site cavity. Analysis of the incorporation of oxygen isotope from H\(_2\)\(^{18}\)O showed that the enzyme attacks choline-O-sulfate at the sulfur center rather than at the chemically favored carbon atom and kinetic measurements for a series of arylsulfate and arylphosphate esters further confirmed its relation to class I ASs. Thus, the structural, phylogenetic and mechanistic analysis presented here, as well as the enzyme’s promiscuity profile confirm the close relation of CS to class I ASs and validate it as a member of the AP superfamily.

**Results and Discussion**

*S. meliloti* BetC is a choline sulfatase

SmCS, encoded by the betC gene, has been identified as a sulfatase of the AP superfamily [23,27]. We cloned betC into a protein production vector resulting in an N-terminal Strep-tag fusion and overexpressed the SmCS-encoding gene in *Escherichia coli*. SmCS contains the Cys-X-Pro-X-Arg motif that is the target of the formylglycine (fGly) generating enzyme (FGE) that catalyzes the conversion of the cysteine in the recognition motif into fGly. ASs [1,50,51] and PMHs [47,52] are well known to carry this post-translational modification that is brought about in *E. coli* by an unknown enzyme endogenous to the protein production host. Since the fGly modification is often incomplete [26,47,52], we overexpressed the SmCS-encoding gene in an *E. coli* strain that also produces the FGE from *Mycobacterium tuberculosis* H37rV (MtFGE) [53]. *E. coli*-produced SmCS was purified with a typical yield of 6 mg of pure protein per 1 g of cells. The purified enzyme showed high activity toward choline-O-sulfate (sulfate monoester 1a in Fig. 1) and \(K_{cat} / K_M\) values varied between 1.2 × 10^3 and 4.8 × 10^2 s\(^{-1}\) M\(^{-1}\) for pH 6.8–8.8 (Fig. S1, Table S1), with a \(k_{cat}\) of 2.4 s\(^{-1}\) and a \(K_M\) of 0.50 mM (\(k_{cat}/K_M = 4.8 \times 10^2\) s\(^{-1}\) M\(^{-1}\)) at the optimum pH (7.6) for SmCS (Table 1, Fig. S1). The catalytic efficiency (\(k_{cat}/K_M\)) toward choline-O-sulfate at optimum pH was ~200-fold higher than previously reported for the same enzyme fused to a C-terminal His-tag and produced without co-expression of a gene encoding a FGE [54] (Table S3), both of which are expected to affect the activity of SmCS (see SI for a more detailed explanation). However, based on the incomplete fGly modification observed in the X-ray structure (see below for details), the true catalytic efficiency of the fGly-form of SmCS WT may be higher than the activity levels observed here.

CS had been previously identified as a member of the AP superfamily closely related to the AS subgroup in this enzyme superfamily [23]. These enzymes have been assigned ASs based on their ability to catalyze the hydrolysis of non-natural aryl sulfates, such as 4-nitrophenyl sulfate (1b, Fig. 1) [26,46], and coumarin sulfate [27]. SmCS was shown to be active toward aryl sulfate monoester 1b. However, its second-order rate constant (\(k_{cat}/K_M = 12\) s\(^{-1}\) M\(^{-1}\) at pH 6.0; Fig. S1, Table 1) was considerably lower than for choline-O-sulfate (1a). Mutations in the active-site residues that are conserved between SmCS and SpAS1 [26,55] decreased catalytic efficiencies toward 4-nitrophenyl sulfate hydrolysis (Table 2), confirming that SmCS, and not a contaminating enzyme, is catalyzing the hydrolysis of aryl sulfates. The activity toward sulfate monoester 1b is modest compared to the catalytic efficiencies of many of its family members (\(k_{cat}/K_M \sim 10^2 - 10^3\) s\(^{-1}\) M\(^{-1}\)). We tested alkyl sulfates 1c–1h (Fig. S2) for activity with SmCS. No conversion of any of these sulfate esters could be detected after 24 h (suggesting a \(k_{cat}/K_M < 4 \times 10^{-5}\) s\(^{-1}\) M\(^{-1}\); see SI for details). Even 3,3-dimethyl butyl-O-sulfate 1c (Fig. S2), isosteric to choline-O-sulfate, is not converted, suggesting that SmCS hydrolyzes alkyl sulfates only when substrate carries the positively charged quaternary ammonium group.
Structure of SmCS

In order to understand the determinants of substrate specificity and catalytic mechanism, we crystallized SmCS and determined its three-dimensional structure by molecular replacement and refined the structure to 2.79 Å resolution (Table S4, Fig. 3). The enzyme crystallizes with eight molecules in the asymmetric unit, and analysis of the structure using the PISA server [56] suggests that the protein is tetrameric with each of the protomers containing two oligomerization interfaces of ~2800 and ~1600 Å² (see Figs. S3–S4 and Table S5 for details). This is consistent with analysis of SmCS by size-exclusion chromatography and multi-angle laser light scattering (Fig. S5), which shows that the protein forms tetramers in solution with ca. 240-kDa molecular mass (Mw of a protomer ~61 kDa).

The protomer structure of SmCS shows a globular protein with an α/β fold, a central β-sheet surrounded by α-helices, characteristic to proteins in the AP superfamily [30,31,42], confirming that SmCS is indeed a member of this superfamily. Similarity to the AS/PMH subgroup of the AP superfamily allows identification of the active site of SmCS by comparing the enzyme with Silicibacter pomeroyi AS1 [26] (SpAS1) and Rhizobium leguminosarum PMH [52] (RPMH) (Fig. 3c). The active site residues of SmCS match completely with the conserved active site residues of SpAS1 and are identical in 9 out of 11 residues in RPMH (Tables S6–S7). These conserved active site residues include residues Asp14, Asp296 and His297 that are expected to coordinate a divalent metal ion. MicroPIXE analysis (particle-induced X-ray emission) of the purified enzyme (Fig. S5), shows a mixture of Ca, Fe, Mn and Zn (Table S8) induced X-ray emission) of the purified enzyme (Table S8). The nucleophile (Cys54/fGly54) is showed a mixture of Ca, Fe, Mn and Zn (Table S8) with occupancies similar to the ones published [26,28,57], and the His201–Lys309 general acid pair that is likely to be responsible for the protonation of the leaving group as the transition state is approached [58]. While enzyme-catalyzed hydrolysis of the model substrate 1b with its nitrophenolate leaving group (pKₐ 7.01 [59]) may not rely very strongly on this catalytic feature, protonation of the energetically disfavored alkoxide anion expected to be formed during SmCS-catalyzed hydrolysis of choline-O-sulfate 1a (pKₐ cholate leaving group: 13.9 [60]) is essential for efficient catalysis. Lys309 interacts directly with Gln15 as described previously for the analogous active site in RPMH (residues Lys337 and Gln13) [52].

| pH   | Substrate | kcat (s⁻¹) | KM (M) | kcat/KM (s⁻¹ M⁻¹) | (kcat/KM)Kᵣ (M⁻¹) | (kcat/KM)Kᵣ a |
|------|-----------|------------|--------|-------------------|-------------------|----------------|
| 7.6 b| 1a        | 2.4 ± 0.2  | (5.0 ± 0.6) × 10⁻⁴ | (4.8 ± 0.5) × 10⁻⁷ | 1.7 × 10²⁵       | 9.6 × 10²⁶     |
| 6.0 b| 2a        | (2.67 ± 0.01) × 10⁻² | (2.1 ± 0.2) × 10⁻³ | 13 ± 1 | 2.7 × 10¹⁸ | 1.5 × 10²⁰ |
|      | 2b        | 0.22 ± 0.01 | (1.9 ± 0.2) × 10⁻² | 12 ± 2 | 6.3 × 10¹⁰ | 3.5 × 10¹² |<|
Oligomerization facilitated by the C-terminal region

The most noticeable structural differences between subgroups within the AP-type ASs and PMHs are in their C-termini, which differ in both length and sequence. In the case of SmCS, the C-terminal tail folds almost around the whole protomer and contains three α-helices that mediate oligomerization (for more details see below). Enzymes of a recently described sulfatase subgroup also contain a C-terminal α-helical tail, but in that particular case, it adopts an entirely different conformation. In PMHs, the tail is composed of the short β-hairpin, whereas monomeric PAS lacks the extended tail entirely (Fig. 3b).

We have previously shown that different subgroups of AP superfamily-type sulfatases and PMHs differ in their quaternary structures. SmCS is extending the scope of oligomeric forms further. While SmCS and PMHs are both tetrameric, they assemble into their respective oligomeric structures very differently (Fig. 4a and b). Both can be seen as a dimer of dimers, and in both cases, the C-termini interact with another protomer, yet the larger protein dimer of dimers, and in both cases, the C-termini of the short protomer with catalytic residues and in the C-terminal tail, but in that particular case, it adopts an entirely different conformation. In PMHs, the tail is composed of the short β-hairpin, whereas monomeric PAS lacks the extended tail entirely (Fig. 3b).

Table 2. Kinetic parameters* for enzyme-catalyzed hydrolysis of sulfate monoesters 1a and 1b for SmCS wild type and mutants

| SmCS mutant | $K_m$ (M) | $k_{cat}$ (s$^{-1}$) | $K_m$ (M) | $k_{cat}$ (s$^{-1}$) | ΔΔG (kcal mol$^{-1}$) |
|-------------|-----------|---------------------|-----------|---------------------|----------------------|
| WT          | (2.4 ± 0.2) × 10$^{-4}$ | (5.0 ± 0.2) × 10$^{-4}$ | (9.4 ± 0.2) × 10$^{-5}$ | (1.33 ± 0.11) × 10$^{-2}$ | <10.7 |
| C45S        | (1.24 ± 0.19) × 10$^{-3}$ | (3.47 ± 0.02) × 10$^{-3}$ | (2.02 ± 0.01) × 10$^{-2}$ | (7.8 ± 0.3) × 10$^{-4}$ | 4.7 |
| H104A       | (3.81 ± 0.26) × 10$^{-2}$ | (3.79 ± 0.08) × 10$^{-3}$ | (1.33 ± 0.11) × 10$^{-2}$ | (7.6 ± 0.3) × 10$^{-4}$ | 4.7 |
| K175A       | (1.24 ± 0.19) × 10$^{-5}$ | (3.47 ± 0.02) × 10$^{-3}$ | (2.02 ± 0.01) × 10$^{-2}$ | (7.8 ± 0.3) × 10$^{-4}$ | 4.7 |
| ΔΔG mut = RTln[$(k_{cat}/M)_{WT}/(k_{cat}/M)_{mutant}$] = 1.36Log[$(k_{cat}/M)_{WT}/(k_{cat}/M)_{mutant}$].

* Recorded at 25 °C in 100 mM Tris–HCl (pH 7.6).

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The extreme C-termini interact with the loops that cover the entrance of the active site cavity of the other protomers. We analyzed conservation of the residues in and around the active site among all CSs and found 100% conserved residues not only in the active site but also in the entrance to it, both in the subunit with catalytic residues and in the C-terminal tail (Fig. 5a). The active site is closed in this SmCS structure, and the loops that cover the entrance would need to move to allow the substrate to enter and the products to leave. Analysis of the entrance
loops shows significantly higher B-factors for the loops that limit the access to the active site, suggesting structural mobility for these structures. A hydrogen bond between Asp500 and Asn146 seems to act as a latch that stabilizes the closed conformation (Fig. 5b). The conservation extends from the mouth all the way to the active site, a narrow L-shaped cavity which is ~11.5 Å in its longest dimension, offering sufficient space to accommodate choline-O-sulfate (Fig. 5c). Three of the residues that form the tunnel come from the C-terminal region of one of the other protomers (Leu499, Leu502 and Arg507). Most of the tunnel-forming and active site residues are also part of the larger of the two oligomerization interfaces (red in Figs. S3a and S4). The other, more buried part of the active site consists mostly of hydrophobic residues, and Glu386, predicted to position the quaternary amine present in the choline ester sitting at one end (Fig. 5c).

The deeply buried binding pocket of SmCS stands in sharp contrast with the wide open, solvent exposed active site of RlPMH (Fig. S9).
tetrameric enzymes, the C-terminal tail that promotes the oligomerization extends toward the active site of the opposing protomer. However, the direct and close involvement of the oligomerization interface in the formation of the active site entrance and pocket as seen for SmCS is absent in RIPMH.

Given the apparent critical role of the C-terminus in the formation of entry to the active site of CSs, we...
explored its role in oligomerization and in enzyme catalysis in more detail. We constructed truncated mutants lacking 12 and 23 C-terminal amino acids, respectively, by replacing Thr501 and Glu490, with stop codons (SmCSΔ12 and Δ23). These two residues were chosen because they are not part of the oligomerization interface itself. The Δ12 mutant was still predominantly present in the tetrameric form (87%). However, a fraction of the enzyme was now present as a dimer (11%) and a monomer (2%) (Fig. S5). The deletion of the last 12 residues was detrimental to the enzymatic activity (Table 2), resulting in a 20- (sulfate monoester 1b) and a 2.5 × 10^3- (1a) fold decrease in $k_{\text{cat}}/K_M$, respectively. This decrease could not be explained solely by the lower proportion of the tetrameric state as compared to wild type. The effect of the truncation on catalysis of choline-O-sulfate (1a) hydrolysis ($\Delta \Delta G_{\text{mut}} = 4.6$ kcal mol$^{-1}$, Table 2) was significantly greater than for the model substrate 4-nitrophenyl sulfate (1b) hydrolysis ($\Delta \Delta G_{\text{mut}} = 1.8$ kcal mol$^{-1}$; Table 2). The 23-amino-acid C-terminal deletion caused an even more dramatic change in distribution between the various oligomeric states: now the enzyme occurs predominantly in its dimeric form (78%; Fig. S5). Catalysis was affected to a larger extent than for the Δ12 mutant and the native substrate once more suffered a larger decrease than the promiscuous substrate 1b ($\Delta \Delta G_{\text{mut}} = 7.7$ kcal mol$^{-1}$ for choline-O-sulfate (1a); $\Delta \Delta G_{\text{mut}} = 3.9$ kcal mol$^{-1}$ for 4-nitrophenyl sulfate (1b); Table 2). This observation suggests that the close interaction between oligomerization interface and active site pocket is mainly important for the ability to accept the choline leaving group, while the general ability to hydrolyze sulfate esters remains comparatively less affected.

Catalytic mechanism

In near neutral aqueous solution, the hydrolysis of alkyl sulfates, such as choline-O-sulfate, occurs predominantly via nucleophilic attack on the carbon
atom next to the bridging oxygen of the sulfate ester group (C–O attack) [37], in contrast to aryl sulfate hydrolysis, which proceeds via nucleophilic attack at the sulfur center (breaking the S–O bond). As expected, the recently discovered class III sulfatases catalyze attack at carbon (C–O cleavage) for the hydrolysis of primary and secondary alkyl sulfates [35,49] (Fig. 2) and are unable to catalyze the hydrolysis of aryl sulfates [24].

SmCS is active toward choline-O-sulfate, an alkyl sulfate, but is incapable of converting any other alkyl sulfates. However, the enzyme is able to hydrolyze aryl sulfates. This raises the question whether SmCS should be classed as a class I or III sulfatase. SmCS-catalyzed turnover of choline-O-sulfate in the presence of H$_2$^{18}$O showed no incorporation of the $^{18}$O label in the choline product (Fig. S10), indicating that SmCS hydrolyzes choline-O-sulfate via S–O cleavage, thus employing a class I mechanism (Fig. 2). The latter observation suggests that SmCS has to overcome an extra 14.2 kcal mol$^{-1}$ in activation free energy (see SI for details) as a result of using the energetically disfavored route. This means that SmCS has a catalytic proficiency ($k_{cat}/K_M$) of 1.7 x 10$^{25}$ M$^{-1}$ for choline-O-sulfate hydrolysis (Table 1); its proficiency toward the more activated 4-nitrophenyl sulfate substrate is substantially lower at 4.6 x 10$^{29}$ M$^{-1}$. Highly proficient enzymatic S–O attack on alkyl sulfate esters has been reported previously [65,66], with catalytic efficiencies reaching values as high as 10$^{29}$ M$^{-1}$ [27]. However, the amino acid sequences of these enzymes were never determined, making SmCS the most proficient fully characterized sulfatase and similarly proficient as fructose-1,6-bisphosphatase ($k_{cat}/K_M$ = 1.4 x 10$^{25}$ M$^{-1}$), one of the most proficient enzymes known to date [67].

S–O cleavage of alkyl sulfate esters has thus far only been reported for PAS [36] (although some reports make this claim without providing a gene sequence [65,66]). We tested PAS, SpAS1 [26], SpAS2 [26] and
RPMH [52], all of which are members of the AP superfamily that are expected to employ the class I mechanism (Fig. 2), for activity toward choline-O-sulfate. No choline formation could be detected with any of these enzymes ($k_{cat}/K_M$ $< 7.5 \times 10^{-5}$ s$^{-1}$ M$^{-1}$, see SI for details on the detection limit for CS activity). Class III alkylsulfatases Pisa1 [49,68] and SdsA1 [24] also showed no detectable activity toward choline-O-sulfate. The similarity of SmCS to ASs (in terms of overall structure as well as the catalytic residues) supports the case for S–O attack and is also consistent with the effect mutations in residues that are conserved between SmCS and ASs (i.e., all mutants except E386L, Δ12 and Δ23 as shown in Table 2) have on SmCS-catalyzed conversion of choline-O-sulfate. By contrast, alkylsulfatases known to utilize C–O cleavage do not catalyze the hydrolysis of 4-nitrophenyl sulfate [69], underlining the diagnostic value of this reaction.

In order to test whether the active-site residues conserved between SmCS and the previously described ASs [26–28] (i.e., all mutants in Table 2 except E386L, Δ12 and Δ23) perform the same function, we mutated several of them and determined kinetic parameters for the enzyme-catalyzed hydrolysis of sulfate monoesters $1a$ and $1b$ at pH 7.6 (Table 2). The effects of mutating these five residues were largely similar for both substrates (4 to $>10.7$ kcal mol$^{-1}$ and 4 to $>6.7$ kcal mol$^{-1}$ for sulfate monoesters $1a$ and $1b$, respectively), suggesting that they are converted via the same mechanism. The enzyme-catalyzed conversion of 4-nitrophenyl sulfate ($1b$) is inhibited in the presence of choline (Fig. S11), also indicating that both substrates use the same active site. The different pH-rate profiles (Fig. S1), however, suggest that one or more different steps are limiting for catalysis.

As mentioned above, the conserved active site residues sit at the corner of an access tunnel connecting to the solvent and a buried active pocket that is largely hydrophobic, with a glutamate (Glu386) sitting $\sim 7$ Å away from the His201–Lys309 general acid pair that is thought to protonate the alkoxy leaving group of the substrate (analogous residues have been suggested for several AP-type sulfatases [26–28]) (Fig. 6). The $\sim 10^3$-fold drop in catalytic efficiency ($k_{cat}/K_M$) in the Glu386Leu mutant (Table 2) is consistent with a role in catalysis, either via ground state binding of the substrate or via assisting with the correct positioning of the alkoxy leaving group for general acid catalysis by the His201–Lys309 pair upon departure. Furthermore, the enzyme-catalyzed hydrolysis of aryl sulfate monoester $1b$ is virtually unchanged for this mutant, confirming that Glu386 is only important for choline-O-sulfatase activity. Choline binding in other proteins has often been attributed to interactions of the quaternary ammonium with π-electrons from aromatic amino acid side chains [70–77], sometimes in combination with binding to negatively charged amino acids [78,79]. The latter is the case for P. aeruginosa phosphorylcholine phosphatase, in which two glutamates and a tyrosine are shown to contribute to binding of the quaternary ammonium group [78,80]. However, the effect of mutation of either of these residues is small ($\Delta \Delta G_{mut} \sim 0.4–2.1$ kcal mol$^{-1}$ per residue, based on data from Beassoni et al. [80]), compared to the effect of choline (Fig. S11), also indicating that both substrates use the same active site. The different pH-rate profiles (Fig. S1), however, suggest that one or more different steps are limiting for catalysis.
of the Glu386Leu mutation in SmCS ($\Delta \Delta G_{\text{mut}} = 5.1$ kcal mol$^{-1}$; Table 2). Based on the mutagenesis of the active site residues and their similarity to the active site residues in related ASs and PMHs (Fig. 3, Table S7), we propose that the catalytic cycle for SmCS-catalyzed hydrolysis of choline-$O$-sulfate is similar to that of the AS-catalyzed hydrolysis of aryl sulfates [26,55,58] and the RlPMH-catalyzed hydrolysis of phosphodiesters [52]. The specificity toward choline-$O$-sulfate is determined by the interaction of a negatively charged glutamate (Glu386) with the positively charged quaternary ammonium group of the substrate (Fig. 6).

Emergence of the CSs within the AP superfamily

As reported previously [23,54] and confirmed in our study by the similarities of the three-dimensional structure (Fig. 3), its catalytic mechanism (Fig. 6) and the conserved active site residues (Table S7), SmCS is a member of the AP superfamily and belongs to the AS/PMH subgroup within that superfamily. A structural alignment and resulting phylogenetic tree that includes SmCS and all AP-type ASs and PMHs of known structure confirmed its close relation to the recently discovered dimeric ASs and tetrameric PMHs (Fig. 7) [26].
To further explore the relationship between CSs, PMHs and dimeric ASs, we created a multiple-sequence alignment of 87 (putative) CSs with all 85 (putative) PMHs and 95 (putative) ASs contained in the previously reported phylogenetic relationship between these two subclasses of the AP superfamily [26], in effect expanding that phylogenetic tree with the CSs. The putative CS-sequences were obtained from a BLAST search with the SmCS sequence as bait on the available genome databases. Since the previously published AS/PMH tree contained only sequences from α- and β-proteobacteria [26], we limited the searchable dataset to those two bacterial classes. The final alignment included 60 sequences from α-proteobacteria (including SmCS), 27 sequences from β-proteobacteria and all the previously identified (putative) ASs [26] and PMHs [26,47,52,57] (Tables S12–S14).

The multiple-sequence alignment shows a high degree of conservation within the CS group (62%) compared to the average pairwise sequence identity of the complete alignment (36%) and the 51% and 50% identities within the AS and PMH clades, respectively. The active site residues as listed in Table S8 are 100% conserved for all CSs and align perfectly with the identical positions in ASs and PMHs. The residues that form the L-shaped active site in SmCS are largely conserved for all (putative) CSs (Fig. S8, Table S9). The C-terminal tail (Figs. 4c and S3d) of the CSs shows a similar degree of sequence conservation to the CSs for the complete alignment (57% versus 62%), whereas for the PMHs (39% versus 50%) and the dimeric ASs (27% versus 51%), there is much more variability in the C-terminal region. The latter is in agreement with the fact that the CS-specific conserved residues that form the hydrophobic part of the active site and the active site tunnel are also part of the oligomerization interface that is largely formed by the C-terminus.

The phylogenetic tree based on the multiple-sequence alignment shows CSs, ASs and PMHs as three distinct phylogenetic clades (Fig. 8). The subdivisions within the dimeric ASs and PMHs are essentially similar to the ones that were observed when only ASs and PMHs were included in the alignment [26]. The CSs are a very distinct group and appear genetically further removed from the ASs and PMHs than those two classes are from each other. The CS clade shows a clear distinction between enzymes originating from α- and β-proteobacteria (Fig. 8).

Quantification of catalytic promiscuity of SmCS

A defining feature of the phospho- and sulfohydrolases of the AP superfamily is the ability to catalyze multiple hydrolytic reactions with substantial rate accelerations [42,81]. The observation of promiscuity has been named “crosswise”; that is, the primary activities of the various family members are also promiscuous reactions in other family members [26,41–48,55]. SmCS had previously been shown to be active toward phosphoryl choline (2a, Fig. 1). These data were obtained with cell extracts [23], which means that enzymatic activity of other phosphatases present in cell extract could lead to overestimation of the actual SmCS-catalyzed phosphorylcholine hydrolysis. We confirmed the activity toward phosphoryl choline 2a for the purified enzyme and showed that SmCS converts this substrate with a $k_{\text{cat}}$ of 0.027 s$^{-1}$ and a $K_M$ of 2.1 mM ($k_{\text{cat}}/K_M = 13$ s$^{-1}$ M$^{-1}$; Table 1). The catalytic proficiency ($k_{\text{cat}}/K_M$) of 2.7 × 10$^{18}$ M$^{-1}$ for SmCS toward this promiscuous substrate rivals that of primary activities of the highly proficient carboxypeptidase b and phosphotriesterase [67]. No turnover was detected for the more reactive nitrophenyl ester 2b lacking the positive charge ($k_{\text{cat}}/K_M < 7.5 \times 10^{-4}$ s$^{-1}$ M$^{-1}$; see SI for a consideration of detection limits). When the previously observed correlation between the catalytic efficiencies ($k_{\text{cat}}/K_M$ values) of sulfatase-catalyzed hydrolysis of sulfate monoester 1b and phosphate monoester 2b [26] is extrapolated to the $k_{\text{cat}}/K_M$ value toward 1b for SmCS, the value for 2b is around 10$^{-3}$ s$^{-1}$ M$^{-1}$ (Fig. S15a), close to the detection limit of 7.5 × 10$^{-4}$ s$^{-1}$ M$^{-1}$, which is in agreement with typical class I sulfatase substrate specificity between sulfate monoester 1b and phosphate monoester 2b. Phosphate diester 3b and phosphonate monoester 4b were hydrolyzed by SmCS with $k_{\text{cat}}/K_M$ values of 0.02 and 1.5 s$^{-1}$ M$^{-1}$, respectively (measured at the optimum pH for sulfate monoester 1b, pH 6.0; Table 1), corresponding to catalytic efficiencies ($k_{\text{cat}}/K_M/k_i$) of 5.5 × 10$^7$ and 7.0 × 10$^9$ M$^{-1}$, respectively.

The observation of sulfatase-catalyzed hydrolysis of phosphate mono- (2a) and diesters (3b) and phosphonate monoesters (4b) reinforces the idea of “crosswise” catalytic promiscuity typical of the sulfatases of the AP superfamily [26,41,46]. The observed ratio of $k_{\text{cat}}/K_M$ values for the SmCS-catalyzed arylsulfate hydrolysis and the promiscuous phosphodiester and phosphonate monoester reactions matched those observed previously [26] (Fig. S15b and c). Based on the previously published correlation for AS-catalyzed sulfate and phosphate monoester hydrolysis [26], and the detection limit for enzymatic hydrolysis of phosphate monoester 2b, the lack of detectable SmCS-catalyzed hydrolysis of phosphate monoester 2b is not unexpected (Fig. S15a). The observation of conserved degrees of preference toward arylsulfate over arylphosphoesters confirms once more that SmCS is acting as a typical AS, despite its high specificity toward choline-O-sulfate (1a).

The emergence of the ability to hydrolyze choline-O-sulfate thus far appears to be unique to the CSs described here. Its evolution within the AS/PMH group (Figs. 7b and 8) of the AP superfamily is unexpected, given that these enzymes catalyze S–O cleavage of
sulfate esters, whereas for alkylsulfates such as choline-O-sulfate 1a, C–O attack would be expected. A possible factor that drives adaptation toward an alternative mechanism compared to the chemically favored C–O cleavage could be the need to accommodate both binding of the quaternary ammonium as well as efficient catalysis. It is conceivable that these molecular recognition requirements cannot easily be reconciled: the strongly electronegative character typical of a nucleophilic residue or an activated water molecule would be prone to formation of a strong interaction with the positively charged quaternary ammonium present in the substrate, resulting in a non-productive substrate binding mode. In order to prevent the latter, an additional strongly electronegative residue would have to be present in the active site. Both strongly electronegative residues or groups would benefit from some distance between them to avoid strong coulombic repulsion within the active site. In each case, efficient catalysis would be precluded. By employing S–O attack, both the issues raised here are of lesser importance: (i) the fGly nucleophile is surrounded by positive charges, which prevents non-productive interaction of the nucleophile with the quaternary ammonium group, and (ii) the nucleophile for S–O attack can simply be further removed from the negatively charged residue that interacts with the quaternary ammonium group, limiting possible coulombic repulsion as compared to a nucleophile that would be well positioned for C–O attack.

**Conclusions**

The biochemical characterization and crystal structure of the CS from *S. melliloti* (SmCS) allows rationalization of its catalytic properties. Despite its specificity for a primary alkyl sulfate, a substrate expected to be converted by a class III sulfatase via C–O attack (Fig. 2), the enzyme was shown to hydrolyze choline-O-sulfate via S–O attack. The protein structure confirms its previously postulated similarity to ASs of the AP superfamily [23]: the enzyme is active as a tetramer and its oligomerization is mediated by the C-terminal tail. The latter is essential for the catalytic activity of SmCS, in particular for its ability to catalyze the hydrolysis of choline-O-sulfate. The deeply buried, L-shaped active site is in fact largely formed at the interface between two interacting protomers. The relatedness to the AP superfamily is further confirmed by the fact that SmCS uses the same reaction pathway as the AP superfamily type sulfatases. The substrate specificity profile toward 4-nitrophenyl sulfos- and phosphoesters is also consistent with typical AS behavior. We observed that the specificity toward choline-O-sulfate can be largely attributed to the interaction of a negatively charged glutamate (Glu386) with the positively charged quaternary ammonium in the choline leaving group. A subsequent multiple-sequence alignment including >80 (putative) CSs showed us that this glutamate residue is fully conserved among CSs. The combination of its phylogenetic and mechanistic similarity to known ASs and its promiscuity profile all suggest that CS is a typical class I sulfatase that is specific toward a primary alkyl sulfate. The latter suggest that the classification of sulfatases according to the mechanism of the reaction they preferably catalyze is not necessarily an indicator of which substrate types they convert. In particular for alkyl sulfates, we have shown here and in a previous study [36] that class I sulfatases can convert alkyl sulfates via the disfavored S–O attack over C–O attack using essentially the same mechanism as for the catalysis of aryl sulfate hydrolysis.

**Materials and Methods**

**Materials**

Choline, sulfate monoester 1b, phosphate monoesters 2a and 2b were purchased from Sigma. Sulfate monoesters 1a [82], 1d–1h [49,68], phosphate diester 3b [83] and phosphonate monoester 4b [26] were synthesized as described previously. Sulfate monoester 1c was synthesized using a similar procedure to that described for alkyl sulfates 1d–1h [68]. Details on characterization are listed in the supporting information (Fig. S16). Alkylsulfatases Pisa1 [49] and SdsA1 [35], ASs PAS [46], SpAS1 [26] and SpAS2 [26], and RPMH [52] were prepared as described previously. The choline detection kit was purchased from Abcam. All restriction enzymes and T4 DNA ligase were from Fermentas. Vector pASK-IBA5plus and strep-tactin resin were purchased from Stratech Scientific. *Pfu* turbo was from Agilent.

**Cloning and mutagenesis**

The gene encoding CS from *S. melliloti* 1021 (Uniprot accession number protein sequence: O69787, gene sequence positions 1491–3029 of GenBank accession number U39940) was amplified by PCR using the appropriate forward and reverse cloning primers with commercially available genomic DNA from *S. melliloti* 1021 (ATCC 51124D-5, LGC promochem) as a template (Table S15). Primers were used at 0.4 nM in a reaction with 0.2 mM dNTPs and 0.05 U μL⁻¹ *Pfu*-Turbo® DNA polymerase. The temperature program used was 15 min at 95 °C without polymerase, followed by 30 cycles of 60 s 95 °C, 45 s 68 °C–0.5 °C per cycle (each cycle the temperature of this segment was lowered by 0.5 °C), 240 s at 72 °C, and finished with 10 min at 72 °C. The PCR product was digested with *BamH*I and *Hind*III restriction endonucleases and subsequently ligated into *BamH*I–*Hind*III digested pASK-IBA5plus
plasmid DNA using T4 DNA ligase. The ligation mixture was transformed into E. coli TOP10 by electroporation. The resulting transformants were plated on LB medium containing ampicillin (100 mg L\(^{-1}\)). Colonies were checked for insert using a PCR reaction with Taq polymerase and colony material as the template. Positive colonies were used to inoculate 5 mL of liquid LB medium containing ampicillin (100 mg L\(^{-1}\)) and grown overnight at 37 °C. Plasmid DNA was extracted and the insert was sequenced using pASK-IBA5 plus sequencing primers.

Site-directed mutants of SmCS were constructed using the QuikChange method (Agilent) using the primers listed in Table S15 with pASK-IBA5 plus SmCS WT plasmid as a template.

### Protein production and purification

Plasmids encoding Streptagged SmCS mutants C54A and C54S were produced in E. coli TOP 10. All other SmCS encoding genes were expressed in E. coli BL21(DE3) expressing MtbFGE [53] from the pRSF杜兰特MtbFGE plasmid as described previously for several other fGly-containing enzymes [26,47,52]. Expression of SmCS from the pASK-IBA5 plus vector results in a translational fusion with an N-terminal Strept-Tag. Mutants SmCS C54A and C54S were produced by growing cells to OD\(_{600}\) ~0.5 at 37 °C in 2 × YT medium containing ampicillin (100 mg L\(^{-1}\)), lowering the temperature to 28 °C, inducing expression of the pASK-IBA5 plus constructs by the addition of anhydrotetracycline (200 μg L\(^{-1}\)) followed by overnight growth at 28 °C. All other variants were expressed by growing cells to OD\(_{600}\) ~0.5 in 2 × YT medium with ampicillin (100 mg L\(^{-1}\)) and kanamycin (50 mg L\(^{-1}\)), lowering the temperature to 28 °C, inducing expression of MtbFGE by addition of IPTG (1 mM) ~30 min prior to inducing expression of the pASK-IBA5 plus construct by addition of anhydrotetracycline (200 μg L\(^{-1}\)) followed by overnight growth at 28 °C.

All overnight cultures were harvested by centrifugation and resuspended in 50 mM Tris–HCl (pH 8.0). The cells were lysed with an Emulsiflex C5 homogenizer (Avestin) and cell-free extract (CFE) was obtained by centrifugation at 30,000 g for 90 min. CFE was loaded onto a Q-Sepharose anion exchange column calibrated in 50 mM Tris–HCl (pH 8.0). The column was washed with 2 column volumes 50 mM Tris–HCl (pH 8.0) and protein was eluted with a gradient of 0–1 M NaCl in 50 mM Tris–HCl (pH 8.0) over 12 column volumes. Protein containing fractions were tested for activity toward sulfate monoester 1b. Fractions containing active protein were pooled and 1/10 of the pooled volume of 1 M Tris–HCl (pH 8.0) + 1.5 M NaCl was added. An appropriate amount of the pooled protein was subsequently loaded onto 1 mL Strept-Tactin column equilibrated with 100 mM Tris–HCl (pH 8.0) + 150 mM NaCl.

The column was washed with 100 mM Tris–HCl (pH 8.0) + 150 mM NaCl to remove unbound protein and the tagged proteins were eluted with 2.5 mM d-desthiobiotin in 100 mM Tris–HCl (pH 8.0) + 150 mM NaCl. The active protein-containing fractions were pooled and concentrated to 10–15 mg mL\(^{-1}\) protein and loaded onto a HiLoad 16/600 Superdex 200 prep grade size exclusion column (running in 100 mM Tris–HCl (pH 8.0), 150 mM NaCl). Active protein eluted at a molecular mass of ~240 kDa, corresponding to a tetramer. Protein containing fractions were concentrated to 100–200 μM, divided into the appropriate aliquots, flash frozen in liquid N\(_2\) and stored at ~20 °C.

### Enzyme assays

Initial rates (\(V_{\text{obs}}\)) for the hydrolysis of 1b–4b were determined by following the 4-nitrophenol formation at 400 nm in a SpectraMax Plus microtiter plate reader at substrate concentrations ranging 4–75 mM in 100 mM imidazole–HCl (pH 6.0–7.2), Tris–HCl (pH 7.2–8.8) or glycine–NaOH (8.8–10.0). Typical enzyme concentrations used were 0.15–0.60 μM for sulfate monoester 1b and phosphonate monoester 4b and 2–20 μM for phosphate esters 2b and 3b for SmCS WT. For the SmCS mutants, the typical enzyme concentrations were 1–20 μM. All substrate concentrations were determined more precisely by performing full turnover of the substrate stock solutions by adding excess PAS [46] (1b), AP (Sigma, cat no. P7923, 2b) or BcPMH [47] (3b and 4b) and measuring the concentration of 4-nitrophenol formed. Catalytic parameters \(k_{\text{cat}}\), \(K_{\text{M}}\) and/or \(k_{\text{cat}}/K_{\text{M}}\) were obtained from fitting the dependency of \(V_{\text{obs}}\) on substrate concentration ([S]) at constant enzyme concentration ([Enz]). In cases where \(V_{\text{obs}}\) showed saturation at higher substrate concentrations, the data were fitted to Eq. (1). In cases where the dependence of \(V_{\text{obs}}\) on substrate concentration was linear, the data were fitted to Eq. (2). In the latter, \(k_{\text{cat}}/K_{\text{M}}\) was treated as a single parameter.

\[
V_{\text{obs}} = \frac{k_{\text{cat}} \times [\text{Enz}] \times [S]}{K_{\text{M}} + [S]} \quad (1)
\]

\[
V_{\text{obs}} = \frac{k_{\text{cat}} \times [\text{Enz}] \times [S]}{K_{\text{M}}} \quad (2)
\]

Activity toward choline-O-sulfate 1a and phosphoryl choline 2a was determined by monitoring the choline formation over time using the choline/acetlycholine assay kit (Abcam, cat. no. ab65345). For SmCS WT, typically 0.9–1.5 μM enzyme was added to 1 mM choline-O-sulfate 1a in 100 mM imidazole–HCl (pH 6.8–7.2) or Tris–HCl (pH 7.2–8.8) and the reaction was left to proceed at 25 °C. Samples of 50 μL were flash frozen in liquid N\(_2\) at various time points. Prior to choline detection, the flash-frozen samples
were quickly heated to 95 °C to inactivate the enzyme. These samples were diluted 10 times and to 50 μL diluted sample 50 μL choline assay mix was added (choline assay mix was made according to the manufacturers’ instructions). The samples were incubated at room temperature in a dark place for 30 min and absorption was measured at 570 nm. If total turnover of substrate (starting at concentration \([S] = [S]_{i=0}\)) was achieved, the total progress curve of product formation ([P]) versus time could be numerically fitted to Eqs. (3) and (5) using Micromath Scientist™ to obtain \(k_{\text{cat}}\) and \(K_M\), provided that at the start of the reaction, the substrate concentration was above the \(K_M\). The reaction product choline can act as a competitive inhibitor, albeit a relatively weak one (\(K_C = 52–6.3\) mM, depending on pH; Fig. S11). When fitting Eqs. (3) and (5) to progress curves of SmCS-catalyzed conversion choline-O-sulfate 1a, the concentration of the choline product increases over time, which can cause inhibition at higher choline concentrations. The time-dependent increase in the choline concentration cannot be easily accounted for in the fitting procedure. This possible problem was circumvented by recording the progress curves starting at a low substrate concentration, in which case the maximum concentration of choline product (1 mM) would cause at most 10% reduction in observed rate, which falls within experimental and fitting error. Since 1 mM is not exceeding \(K_M > 10\)-fold, we determined an accurate value for \(k_{\text{cat}}\) by measuring initial rates of SmCS-catalyzed choline production at 10 and 20 mM choline-O-sulfate 1a. These initial rates which were used as proxy to calculate \(k_{\text{cat}}\) (provided they were identical within error). This value was subsequently fixed while fitting a product formation progress curve starting from 1 mM choline-O-sulfate 1a (i.e., \([S]_{i=0} = 1\) mM) to Eqs. (3) and (5). An example fit is shown in Fig. S16. If substrate saturation could not be achieved and \([S]_{i=0}\) was well below the \(K_M\), the data could be fitted to Eqs. (4) and (5) with \(k_{\text{cat}}/K_M\) treated as a single parameter.

\[
\frac{d[S]}{dt} = -\frac{k_{\text{cat}} \times [\text{Enz}] \times [S]}{K_M + [S]} \quad (3)
\]

\[
\frac{d[P]}{dt} = -\frac{k_{\text{cat}} \times [\text{Enz}] \times [S]}{K_M}
\]

\[ [P] = [S]_{i=0} - [S] \quad (5) \]

Kinetic measurements of active site mutants were performed with 1 mM choline-O-sulfate (1a) in 100 mM Tris–HCl (pH 7.6) and 20–30 μM SmCS. If the activity was too low to achieve total turnover within ~5 days, a lower limit for activity could be derived from the initial rate of product formation. The data for phosphoryl choline were obtained by monitoring the total turnover of 1 mM phosphate monoester 2a in 100 mM Tris–HCl (pH 7.6) catalyzed by 33 μM SmCS WT.

The errors for the individual parameters shown in Tables 1 and 2 represent those arising from the fitting procedure used. In cases where \(k_{\text{cat}}/K_M\) was calculated from values for \(k_{\text{cat}}\) and \(K_M\) (as obtained from fitting data to Eqs. (1) or (3)), the error (δ) for \(k_{\text{cat}}/K_M\) was calculated according to Eq. (6).

\[
\delta \frac{k_{\text{cat}}}{K_M} = \frac{k_{\text{cat}}}{K_M} \times \sqrt{\left(\frac{\delta k_{\text{cat}}}{k_{\text{cat}}}\right)^2 + \left(\frac{\delta K_M}{K_M}\right)^2} \quad (6)
\]

Alkylsulfatasases Pisa1 and SdsA1, ASs PAS, SpAS1 and SpAS2 and PMH RfPmH were tested for choline-O-sulfatase activity by incubating 2–4 μM enzyme with 1 mM sulfate monoester 1a in 100 mM Tris–HCl (pH 7.6) at 25 °C for 24 h, followed by choline detection as described above.

SmCS was tested for activity toward alkyl sulfates 1c–1h essentially as described previously for Pisa1 [49]. Typical incubations were done with 1.8 μM of enzyme and alkyl sulfate (4 mg mL\(^{-1}\)) in 100 mM Tris–HCl (pH 8.0). The mixtures were incubated for 24 h at 30 °C while shaking at 120 rpm. The alcohol product was extracted with ethyl acetate (1:1 with the aqueous phase) and the organic phase was derivatized to form the acetate ester by adding DMAP and acetic anhydride. The derivatized product was analyzed by chiral GC-FID [Thermo Finnigan FOCUS GC, Varian Chirasil Dex CB column (25 m × 0.32 mm × 0.25 μm film)] with the following temperature program: injector temperature, 200 °C; flow, 1.3 mL min\(^{-1}\); temperature program, 80 °C; and hold for 1.0 min, 15 °C min\(^{-1}\), to 110 °C, 4 °C min\(^{-1}\), to 130 °C, 10 °C min\(^{-1}\), to 180 °C.

Given that the Fgly modification is incomplete (see above), it is possible that the kinetic data reported underestimate the true rates by a factor of ~2. The strong effect of the Cys to Ser mutation (~1000-fold reduction in \(k_{\text{cat}}/K_M\)) on the conversion of sulfate monoesters 1a and 1b suggests that the activity of the Cys form (which is likely to be similar to the serine mutant) would contribute very little to the observed activity. This means that the comparison between the different substrates is valid.

**Crystallization and structure determination**

Following high-throughput evaluation of sparse matrix crystallization conditions in sitting drops in 96-well plates, crystals of SmCS were observed in several conditions after several days of incubation at 20 °C. After extensive screening and optimization, the best crystals (with dimensions of
600 \times 400 \times 200 \mu m) were obtained in 800 mM sodium citrate and 100 mM imidazole (pH 8.0) using hanging drop vapor diffusion with equal volumes of protein containing solution (11.2 mg mL\(^{-1}\)) and reservoir solution (3 \mu L total start volume). For cryoprotection prior to flash-freezing in liquid nitrogen, the crystals were briefly transferred to a drop containing the crystalization solution supplemented with 20% (v/v) glycerol.

Diffraction data were collected on the ID23-2 beamline at the European Synchrotron Radiation Facility, Grenoble, France. Data were processed and reduced using XDS [84] and AIMLESS [85] using [\I]/\[\O] cutoff of 2 to define the high resolution cutoff, while ensuring completeness of the data. The phases were obtained by molecular replacement with energy- and electron density-guided model building and refinement MR-ROSETTA [86] as implemented in the PHENIX suite [87], using a search model based on the coordinates of RIPMH (PDB ID 2VQR). Model improvement was monitored through \(R_l\) during rounds of density modification and reciprocal-space refinement. After extensive manual re-building in COOT [88], combined with maximum-likelihood-based restrained refinement in BUSTER-TNT [89] and phenix.refine [87], the final model was refined to 2.8-Å resolution and had \(R_{work}/R_{free}\) values of 0.205/0.253 (Table S4). The stereochemistry of the structure was assessed and validated with MOLPROBITY [90]. The metal ion in the structure was assigned as Ca\(^{2+}\), based on microPIXE experimient and the active site residue 54 modeled as a mixture of formyl glycine and cysteine, both at half occupancies. The structure and corresponding structure factors have been submitted to PDB with accession code 6FNY.

**Molecular mass determination by combined size exclusion chromatography–multi-angle laser light scattering**

The oligomeric state of wild-type SmCS and the two truncation mutants \(\Delta 12\) and \(\Delta 23\) were determined by loading 100 \mu L of 2 mg mL\(^{-1}\) protein on a Superdex 200 10/300 HR size exclusion chromatography column running in 100 mM Tris–HCl (pH 8.0) and 150 mM NaCl (0.5 mM min\(^{-1}\)) coupled to a Wyatt Dawn multi-angle light scattering detector and a Wyatt T-Rex differential refractive index detector.

**Comparison of SmCS with other AP superfamily members**

All structural homology searches and subsequent structural alignments were performed using the PDBeFold server [91] (http://www.ebi.ac.uk/msd-srv/ssm/cgi-bin/ssmserver). The conserved putative active site residues were assigned based on homology with SpAS1 [26] (PDB: 4UPI) and RPFM [52] (2VQR). The other active site residues were found using the HotSpot Wizard (loschmidt. chemi.muni.cz/hotspotwizard) [92] and manual inspection of the 3D structure.

In order to obtain an indication as to the phylogenetic relationship between SmCS and all other AP-type ASs and PMHs, we aligned SmCS with all 17 ASs/PMHs of known structure using Secondary Structure Matching [91] (see Table S11 for more details on the multiple structural alignment). The 267 positions that aligned structurally for all 18 enzymes (magenta regions in Fig. 7a) were used to build a phylogenetic tree (Fig. 7b) using RAxML HPC2 8.0.24 [93] at the XSEDE sever of the CIPRES Science Gateway [94] (http://www.phylo.org/portal2). We used the “Le and Gascuel” [95] amino acid replacement rate matrix with four category gamma rates, estimated proportion of invariable sites and empirical base frequencies. The optimal tree-building parameters and substitution matrix were calculated from the sequence alignment using ProtTest 2.4 [96].

The phylogenetic relationship between CSs, the dimeric ASs and PMHs was built from a multiple-sequence alignment of 87 (putative) CSs, 60 from \(\alpha\)-proteobacteria and 27 from \(\beta\)-proteobacteria (Table S14), with all the sequences included in the previously reported phylogenetic relationship between the PMHs and ASs [26] (Tables S12 and S13, respectively). The multiple-sequence alignment was generated using the 3D coffee mode of the T-coffee multiple-sequence alignment package [97]. The maximum likelihood phylogenetic tree was calculated using RAxML HPC2 8.0.24 [93] at the XSEDE sever of the CIPRES Science Gateway [94] (http://www.phylo.org/portal2) using the “Le and Gascuel” [95] amino acid replacement rate matrix with four category gamma rates, estimated proportion of invariable sites and empirical base frequencies. The optimal tree-building parameters and substitution matrix were calculated from the sequence alignment using ProtTest 3.2 [96].

**Accession number**

The coordinates and the structure factors have been deposited in the Protein Data Bank under accession number 6FNY.

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Appendix A. Supplementary data

Supplementary data this article contains further kinetic, phylogenetic and structural analyses as well as practical and experimental consideration. These data can be found online at https://doi.org/10.1016/j.jmb.2018.02.010.

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arylsulfatase; alkaline phosphatase superfamily; phosphatase; catalytic promiscuity; oligomerization

Abbreviations used:
AS, arylsulfatase; SmCS, Sinorhizobium melliloti choline sulfatase; CS, choline sulfatase; AP, alkaline phosphatase; PAS, Pseudomonas aeruginosa AS; PMH, phosphonate monoester hydrolase; fGly, formyglycine; FGE, fGly-generating enzyme; SpAS1, Silicibacter pomeroyi AS1; RPMH, Rhizobium leguminosarum PMH.

References

[1] G. Diez-Roux, A. Ballabio, Sulfatases and human disease, Annu. Rev. Genomics Hum. Genet. 6 (2005) 355–379.
[2] D. Ghosh, Human sulfatases: a structural perspective to catalysis, Cell. Mol. Life Sci. 64 (2007) 2013–2022.
[3] K. von Figura, B. Schmidt, T. Selmer, T. Dierks, A novel protein modification generating an aldehyde group in sulfatases: its role in catalysis and disease, BioEssays 20 (1998) 505–510.
[4] R. Hobkirik, Steroid sulfation: current concepts, Trends Endocrinol. Metab. 4 (1993) 69–74.
[5] M.J. Reed, A. Purohit, L.W. Woo, S.P. Newman, B.V. Potter, Steroid sulfatase: molecular biology, regulation, and inhibition, Endocr. Rev. 26 (2005) 171–202.
[6] J. Lai, J. Chien, J. Staub, R. Avula, E.L. Greene, T.A. Matthews, D.I. Smith, S.H. Kaufmann, L.R. Roberts, V. Shridhar, Loss of HSSL-1 up-regulates heparin-binding growth factor signaling in cancer, J. Biol. Chem. 278 (2003) 23107–23117.
[7] M. Morimoto-Tomita, K. Uchimura, Z. Werb, S. Hemmerich, S.D. Rosen, Cloning and characterization of two extracellular heparin-degrading endosulfatases in mice and humans, J. Biol. Chem. 277 (2002) 49175–49185.
[8] A.J. Obaya, Molecular cloning and initial characterization of three novel human sulfatases, Gene 372 (2006) 110–117.
[9] X. Ai, A.T. Do, O. Lozynska, M. Kusche-Gullberg, U. Lindahl, C.P. Emerson Jr., qSulf1 remodels the 6-O-sulfation states of cell surface heparan sulfate proteoglycans to promote Wnt signaling, J. Cell Biol. 162 (2003) 341–351.
[10] G.K. Dhoot, M.K. Gustafsson, X. Ai, W. Sun, D.M. Staniford, C.P. Emerson Jr., Regulation of Wnt signaling and embryo patterning by an extracellular sulfatase, Science 293 (2001) 1663–1666.
[11] T. Ohto, H. Uchida, H. Yamazaki, K. Keino-Masu, A. Matsui, M. Masu, Identification of a novel nonlysosomal sulphatase expressed in the floor plate, choroid plexus and cartilage, Genes Cells 7 (2002) 173–185.
[12] J.V. Paietta, Molecular cloning and regulatory analysis of the arylsulfatase structural gene of Neurospora crassa, Mol. Cell. Biol. 9 (1989) 3630–3637.
[13] E.L. de Hostos, J. Schilling, A.R. Grossman, Structure and expression of the gene encoding the periplasmic arylsulfatase of Chlamydomonas reinhardtii, Mol Gen Genet 218 (1989) 229–239.
[14] A. Hallmann, M. Sumper, An inducible arylsulfatase of Volvox carteri with properties suitable for a reporter–gene system. Purification, characterization and molecular cloning, Eur. J. Biochem. 221 (1994) 143–150.
[15] M.A. Kertesz, Riding the sulfur cycle—metabolism of sulfonates and sulfate esters in gram-negative bacteria, FEMS Microbiol. Rev. 24 (2000) 135–175.
[16] J.R. Govan, V. Deretic, Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia, Microbiol. Rev. 60 (1996) 539–574.
[17] H.J. Jansen, C.A. Hart, J.M. Rhodes, J.R. Saunders, J.W. Smalley, A novel mucin-sulphatase activity found in Burkholderia cepacia and Pseudomonas aeruginosa, J. Med. Microbiol. 48 (1999) 551–557.
[18] J.D. Mougous, R.E. Green, S.J. Williams, S.E. Brenner, C.R. Bertozzi, Sulfotransferases and sulfatases in mycobacteria, Chem. Biol. 9 (2002) 767–776.
[19] J. Piotrowski, A. Slomiany, V.L. Murty, Z. Fekete, B.L. Slomiany, Inhibition of Helicobacter pylori colonization by sulfated gastric mucin, Biochem. Int. 24 (1991) 749–756.
[20] T.C. Galvao, V. de Lorenzo, D. Canovas, Uncoupling of choline-5-sulphate utilization from osmoreprotection in Pseudomonas putida, Mol. Microbiol. 62 (2006) 1643–1654.
[21] B. Jovicic, V. Venturi, L. Topisirovic, M. Kojic, Inducible expression of choline sulfatase and its regulator BetR in Pseudomonas aeruginosa sp. ATCC19151, Arch. Microbiol. 193 (2011) 399–405.
[22] J.J. Lucas, S.W. Burchiel, I.H. Segel, Choline sulfatase of Chlamydomonas reinhardtii, Mol Gen Genet 218 (1989) 381–387.
[23] M. Osteras, E. Boncompagni, N. Vincent, M.C. Poggi, D. Le Rudulier, Presence of a gene encoding choline sulfatase in Sinorhizobium melliloti bet operon: choline-O-sulfate is metabolized into glycine betaine, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 11394–11399.
Choline Sulfatase from Sinorhizobium melliloti

[24] G. Hageluene, T.M. Adams, L. Wiehmann, U. Widow, H. Kolmar, B. Tummler, D.W. Heinz, W.D. Schubert, The crystal structure of SdsA1, an alkylsulfatase from *Pseudomonas aeruginosa*, defines a third class of sulfatases, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 7631–7636.

[25] I. Müller, A. Kahne, T. Pape, G.M. Sheldrick, W. Meyer-Klaucke, T. Dierks, M. Keretz, I. Uson, Crystal structure of the alkylsulfatase AtsK: insights into the catalytic mechanism of the Fe(II) α-ketoglutarate-dependent dioxygenase superfamily, Biochemistry 43 (2004) 3075–3088.

[26] B. van Loo, C.D. Bayer, S. Jonas, G. Fisher, E. Valkov, M.F. Fuchs, F. Hollfelder, Balancing Specificity and Promiscuity in Enzyme Evolution: Multidimensional Activity Transitions in the Alkaline Phosphatase Superfamily, 2016 (submitted for publication).

[27] S.R. Hanson, M.D. Best, C.H. Wong, Sulfatases: structure, mechanism, biological activity, inhibition, and synthetic utility, Angew. Chem. Int. Ed. 43 (2004) 5736–5763.

[28] I. Boltes, H. Czapinska, A. Kahne, R. von Bulow, T. Dierks, B. Schmidt, K. von Figura, M.A. Keretz, I. Uson, 1.3 A structure of arylsulfatase from *Pseudomonas aeruginosa* establishes the catalytic mechanism of sulfate ester cleavage in the sulfatase family, Structure 9 (1997) 277–289.

[29] C.S. Bond, P.R. Clements, S.J. Ashby, C.A. Collyer, S.J. Harrop, S.R. Hanson, M.D. Best, C.H. Wong, Sulfatases: structure, mechanism, biological activity, inhibition, and synthetic utility, Angew. Chem. Int. Ed. 43 (2004) 5736–5763.

[30] M.Y. Galperin, A. Bariroch, E.V. Koonin, A superfamily of metalloenzymes unifies phosphopentomutase and cofactor-independent phosphoglycerate mutase with alkaline phosphatases and sulfatases, Protein Sci. 7 (1998) 1829–1835.

[31] M.Y. Galperin, M.J. Jedrzejas, Conserved core structure and active site residues in alkaline phosphatase superfamily enzymes, Proteins 45 (2001) 318–324.

[32] F.G. Hernandez-Guzman, T. Higashiyama, W. Pangborn, Y. Osawa, D. Ghosh, Structure of human estrone sulfatase suggests functional roles of membrane association, J. Biol. Chem. 278 (2003) 22989–22997.

[33] G. Lukatela, N. Krauss, K. Theis, T. Selmer, V. Gieselmann, M. Schober, M. Kepplinger, M. Faccinelli, J. Pitzer, K. von Figura, W. Saenger, Crystal structure of human arylsulfatase A: the aldehyde function and the metal ion at the active site suggest a novel mechanism for sulfate ester hydrolysis, Biochemistry 37 (1998) 3654–3664.

[34] Y. Rivera-Colon, E.K. Schutsky, A.Z. Kita, S.C. Garman, The structure of human GALNS reveals the molecular basis for mucopolysaccharidosis IV A, J. Mol. Biol. 423 (2012) 736–751.

[35] T. Knaus, M. Schober, B. Kepplinger, M. Faccinelli, J. Pitzer, K. Faber, P. Macheroux, U. Wagner, Structure and mechanism of an inverting alkylsulfatase from *Pseudomonas* sp. DSM6611 specific for secondary alkyl sulfates, FEBS J. 279 (2012) 4374–4384.

[36] M. Schober, M. Toesch, T. Knaus, G.A. Strohmeier, B. van Loo, M. Fuchs, F. Hollfelder, P. Macheroux, K. Faber, One-pot deacetylation of sec-alcohols: enantioconvergent enzymatic hydrolysis of alkyl sulfates using stereocomplementary sulfatases, Angew. Chem. Int. Ed. 52 (2013) 3277–3279.

[37] D.R. Edwards, D.C. Lohman, R. Wolfenden, Catalytic proficiency: the extreme case of S-O cleaving sulfatases, J. Am. Chem. Soc. 114 (2012) 525–531.

[38] I. Takebe, Isolation and characterization of a new enzyme choline sulfatase, J. Biochem. 50 (1961) 245–255.

[39] J. Bielicki, M. Fuller, X.H. Guo, C.P. Morris, J.J. Hopwood, D.S. Anson, Expression, purification and characterization of recombinant human N-acetylgalactosamine-6-sulfatase, Biochem. J. 311 (Pt 1) (1995) 333–339.

[40] T. Litjens, J. Bielicki, D.S. Anson, K. Fridrici, M.Z. Jones, J.J. Hopwood, Expression, purification and characterization of recombinant caprine N-acetylgalactosamine-6-sulfatase, Biochem. J. 327 (Pt 1) (1997) 89–94.

[41] A.C. Battice, S. Bandyopadhyay, L.F. Olguin, F. Hollfelder, Efficient catalytic promiscuity for chemically distinct reactions, Angew. Chem. Int. Ed. 48 (2009) 3692–3694.

[42] S. Jonas, F. Hollfelder, Mapping catalytic promiscuity in the alkaline phosphatase superfamily, Pure Appl. Chem. 81 (2009) 731–742.

[43] J.K. Laszila, D. Herschlag, Promiscuous sulfatase activity and thio-effects in a phosphodiesterase of the alkaline phosphatase superfamily, Biochemistry 47 (2008) 12853–12859.

[44] P.J. O'Brien, D. Herschlag, Sulfatase activity of *E. coli* alkaline phosphatase demonstrates a functional link to arylsulfatases, an evolutionary related enzyme family, J. Am. Chem. Soc. 120 (1998) 12369–12370.

[45] P.J. O'Brien, D. Herschlag, Functional interrelationships in the alkaline phosphatase superfamily: phosphodiesterase activity of *Escherichia coli* alkaline phosphatase, Biochemistry 40 (2001) 5691–5699.

[46] L.F. Olguin, S.E. Askew, A.C. O'Donoghue, F. Hollfelder, Efficient catalytic promiscuity in an enzyme superfamily: an arylsulfatase shows a rate acceleration of 10^13 for phosphate monoester hydrolysis, J. Am. Chem. Soc. 130 (2008) 16547–16555.

[47] B. van Loo, S. Jonas, A.C. Battice, A. Benjdia, O. Berteau, M. Hyvonen, F. Hollfelder, An efficient, multiply promiscuous hydrolase in the alkaline phosphatase superfamily, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 2740–2745.

[48] J.G. Zalatan, T.D. Fenn, A.T. Brunger, D. Herschlag, Structural and functional comparisons of nucleotide pyrophosphatase/phosphodiesterase and alkaline phosphatase: implications for mechanism and evolution, Biochemistry 45 (2006) 9788–9803.

[49] M. Schober, P. Gadler, T. Knaus, H. Kayer, R. Birner-Grunberger, C. Guly, P. Macheroux, U. Wagner, K. Faber, A stereoselective inverting sec-alkylsulfatase for the dereac- mization of sec-alcohols, Org. Lett. 13 (2011) 4296–4299.

[50] M. Sardiello, I. Amorim, G. Roma, A. Ballabio, Sulfatases and sulfatase modifying factors: an exclusive and promiscuous relationship, Hum. Mol. Genet. 14 (2005) 3203–3217.

[51] B. Schmidt, T. Selmer, A. Ingendoh, K. von Figura, A novel amino acid modification in sulfatases that is defective in multiple sulfatase deficiency, Cell 82 (1995) 271–278.

[52] S. Jonas, B. van Loo, M. Hyvonen, F. Hollfelder, A new member of the alkaline phosphatase superfamily with a formylglycine nucleophile: structural and kinetic characterisation of a phosphonate monoester hydrolyase/phosphodiesterase from *Rhizobium leguminosarum*, J. Mol. Biol. 384 (2008) 120–136.

[53] I.S. Carrico, B.L. Carlson, C.R. Bertozzi, Introducing genetically encoded aldehydes into proteins, Nat. Chem. Biol. 3 (2007) 321–322.

[54] J.J. Sánchez-Romero, L.F. Olguin, Choline sulfatase from *Ensifer* (Sinorhizobium) *melliloti*: characterization of the unmodified enzyme, Biochem. Biophys. Rep. 3 (2015) 161–168.

[55] C.D. Bayer, B. van Loo, F. Hollfelder, Specificity effects of amino acid substitutions in promiscuous hydrolases: context-dependence of catalytic residue contributions to local fitness landscapes in nearby sequence space, Chembiochem 18 (2017) 1001–1015.
[56] E. Krissinel, K. Henrick, Inference of macromolecular assemblies from crystalline state, J. Mol. Biol. 372 (2007) 774–797.

[57] S.B. Dotson, C.E. Smith, C.S. Ling, G.F. Barry, G.M. Kishore, Identification, characterization, and cloning of a phosphonate monoester hydrolase from Burkholderia caryophylli PG2982, J. Biol. Chem. 271 (1996) 25754–25761.

[58] B. van Loo, R. Berry, U. Dokphrom, M. Golicnik, A.C. Hengge, F. Hoffelder, Pseudomonas aeruginosa arylsulfatase sulfatase and phosphatase reactions: comparative mechanistic analysis, 2018 (Manuscript in preparation).

[59] C. McWhirter, E.A. Lund, E.A. Tanfum, G. Feng, Q.I. Sheikh, A.C. Hengge, N.H. Williams, Mechanistic study of protein phosphatase-1 (PP1), a catalytically promiscuous enzyme, J. Am. Chem. Soc. 130 (2008) 13673–13682.

[60] R.M.C. Dawson, Data for Biochemical Research, Clarendon Press, Oxford, 1959.

[61] H. Ashkenazy, S. Abadi, E. Martz, O. Chay, I. Mayrose, T. A.M. Crescenzi, K.S. Dodgson, G.F. White, Purification and characterization of the D-lactate-2-sulphatase of Bacillus subtilis

[62] J.C. Wolters, R.P. Berntsson, N. Gul, A. Karasawa, A.M. Thunnissen, D.J. Slotboom, B. Poolman, Ligand binding and crystal structures of the substrate-binding domain of the ABC transporter OpuA, PLoS One 5 (2010), e10361.

[63] S.H. Smits, M. Hoing, J. Lecher, M. Jebbar, L. Schmitt, E. Bremer, The compatible-solute-binding protein OpuAC from Bacillus subtilis: ligand binding, site-directed mutagenesis, and crystallographic studies, J. Bacteriol. 190 (2008) 5663–5671.

[64] C. Horn, L. Sohn-Bosser, J. Breed, W. Welte, L. Schmitt, E. Bremer, Molecular determinants for substrate specificity of the ligand-binding protein OpuAC from Bacillus subtilis for the compatible solutes glycine betaine and proline betaine, J. Mol. Biol. 357 (2006) 592–606.

[65] A. Schiefner, G. Holtmann, K. Diederichs, W. Welte, E. Bremer, Structural basis for the binding of compatible solutes by ProX from the hyperthermophilic archaeon Archaeoglobus fulgidus, J. Biol. Chem. 279 (2004) 48270–48281.

[66] A. Schiefner, J. Breed, L. Bosser, S. Kneip, J. Gade, G. Holtmann, K. Diederichs, W. Welte, E. Bremer, Cation–π interactions as determinants for binding of the compatible solutes glycine betaine and proline betaine by the periplasmic ligand-binding protein ProX from Escherichia coli, J. Biol. Chem. 279 (2004) 5588–5596.

[67] C. Oswald, S.H. Smits, M. Hoing, L. Sohn-Bosser, L. Dupont, D. Le Rudulier, L. Schmitt, E. Bremer, Crystal structures of the choline/acyethylcholine substrate-binding protein ChoX from Sinorhizobium meliloti in the liganded and unliganded-closed states, J. Biol. Chem. 283 (2008) 32848–32859.

[68] L. Infantes, L.H. Otero, P.R. Beassoni, C. Boetsch, A.T. Lisa, C.E. Domenech, A. Albert, The structural domains of Pseudomonas aeruginosa phosphorylcholine phosphatase cooperate in substrate hydrolysis: 3D structure and enzymatic mechanisms, J. Mol. Biol. 423 (2012) 503–514.

[69] J. Kua, Y. Zhang, J.A. McCammon, Studying enzyme binding specificity in acetylcholinesterase using a combined molecular dynamics and multiple docking approach, J. Am. Chem. Soc. 124 (2002) 8260–8267.

[70] P.R. Beassoni, L.H. Otero, C. Boetsch, A.T. Lisa, Site-directed mutations and kinetic studies show key residues involved in alkylationammonium interactions and reveal two sites for phosphorylcholine in Pseudomonas aeruginosa phosphorylcholine phosphatase, Biochim. Biophys. Acta 1814 (2011) 858–863.

[71] M.F. Mohamed, F. Hoffelder, Efficient, crosswise catalytic promiscuity among enzymes that catalyze phosphoryl transfer, Biochim. Biophys. Acta 1834 (2013) 417–424.

[72] C.M. Stevens, P. Vohra, Occurrence of choline sulfate in Penicillium chrysogenum, J. Am. Chem. Soc. 77 (1955) 4935–4936.

[73] S.A. Ba-Saif, A.M. Davis, A. Williams, Effective charge distribution for attack of phenoxide ion on aryl methyl phosphate monoanion: studies related to the action of ribonuclease, J. Org. Chem. 54 (1989) 5483–5486.

[74] W. Kabsch, Integration, scaling, space-group assignment and post-refinement, Acta Crystallogr. D Biol. Crystallogr. 66 (2010) 133–144.

[75] P.R. Evans, An introduction to data reduction: space-group assignment, Acta Crystallogr. D Biol. Crystallogr. 67 (2011) 282–292.

[76] F. DiMaio, T.C. Terwilliger, R.J. Read, A. Wlodawer, G. Oberdorfer, U. Wagner, E. Vakov, A. Alon, D. Fass, H.L. Axelrod, D. Das, S.M. Vorobiev, H. Iwai, P.R. Pokkuluri, D. Baker, Improved molecular replacement by density- and energy-guided protein structure optimization, Nature 473 (2011) 540–543.

[77] P.D. Adams, P.V. Afonine, G. Bunkoczi, V.B. Chen, I.W. Davis, N. Echols, J.J. Headd, L.W. Hung, J.G. Kapral, R.W. Grosse-Kunstleve, A.J. McCoy, N.W. Moriarty, R. Oeffner, R. J. Read, D.C. Richardson, J.S. Richardson, T.C. Terwilliger, P.H. Zwart, PHENIX: a comprehensive Python-based system

Choline Sulfatase from Sinorhizobium meliloti
Choline Sulfatase from Sinorhizobium melliloti

for macromolecular structure solution, Acta Crystallogr. D Biol. Crystallogr. 66 (2010) 213–221.

[88] P. Emsley, B. Lohkamp, W.G. Scott, K. Cowtan, Features and development of Coot, Acta Crystallogr. D Biol. Crystallogr. 66 (2010) 486–501.

[89] E. Blanc, P. Roversi, C. Vonrhein, C. Flensburg, S.M. Lea, G. Bricogne, Refinement of severely incomplete structures with maximum likelihood in BUSTER-TNT, Acta Crystallogr. D Biol. Crystallogr. 60 (2004) 2210–2221.

[90] V.B. Chen, W.B. Arendall III, J.J. Headd, D.A. Keedy, R.M. Immormino, G.J. Kapral, L.W. Murray, J.S. Richardson, D.C. Richardson, MolProbity: all-atom structure validation for macromolecular crystallography, Acta Crystallogr. D Biol. Crystallogr. 66 (2010) 12–21.

[91] E. Krissinel, K. Henrick, Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions, Acta Crystallogr. D Biol. Crystallogr. 60 (2004) 2256–2268.

[92] A. Pavelka, E. Chovancova, J. Damborsky, HotSpot Wizard: a web server for identification of hot spots in protein engineering, Nucleic Acids Res. 37 (2009) W376–83.

[93] A. Stamatakis, P. Hoover, J. Rougemont, A rapid bootstrap algorithm for the RAxML Web servers, Syst. Biol. 57 (2008) 758–771.

[94] M.A. Miller, W. Pfleifer, T. Schwartz, Creating the CIPRES Science Gateway for inference of large phylogenetic trees, 2010 Gateway Computing Environments Workshop (GCE 2010) 2010, pp. 1–8.

[95] S.Q. Le, O. Gascuel, An improved general amino acid replacement matrix, Mol. Biol. Evol. 25 (2008) 1307–1320.

[96] F. Abascal, R. Zardoya, D. Posada, ProtTest: selection of best-fit models of protein evolution, Bioinformatics 21 (2005) 2104–2105.

[97] J.F. Taly, C. Magis, G. Bussotti, J.M. Chang, P. Di Tommaso, I. Erb, J. Espinosa-Carrasco, C. Kemena, C. Notredame, Using the T-Coffee package to build multiple sequence alignments of protein, RNA, DNA sequences and 3D structures, Nat. Protoc. 6 (2011) 1669–1682.