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Review

Bacterial sulfite-oxidizing enzymes

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ABSTRACT

Enzymes belonging to the Sulfite Oxidase (SO) enzyme family are found in virtually all forms of life, and are especially abundant in prokaryotes as shown by analysis of available genome data. Despite this fact, only a limited number of bacterial SO family enzymes has been characterized in detail to date, and these appear to be involved in very different metabolic processes such as energy generation from sulfur compounds, host colonization, sulfite detoxification and organosulfonate degradation. The few characterized bacterial SO family enzymes also show an intriguing range of structural conformations, including monomeric, dimeric and heterodimeric enzymes with varying numbers and types of redox centres. Some of the bacterial enzymes even catalyze novel reactions such as dimethylsulfoxide reduction that previously had been thought not to be catalyzed by SO family enzymes. Classification of the SO family enzymes based on the structure of their Mo domain clearly shows that three distinct groups of enzymes belong to this family, and that almost all SOEs characterized to date are representatives of the same group.

The widespread occurrence and obvious structural and functional plasticity of the bacterial SO family enzymes make this an exciting field for further study, in particular the unraveling of the metabolic roles of the three enzyme groups, some of which appear to be associated almost exclusively with pathogenic microorganisms.

1. Introduction

Sulfite-oxidizing enzymes are essential for many living cells, and as a result of the severity of the sulfite oxidase deficiency syndrome in humans these enzymes have been studied in vertebrates for decades [1]. However, although the presence of similar enzymatic activities in bacteria was already reported over 40 years ago [2], the bacterial sulfite-oxidizing enzymes have remained elusive until recently, and significant progress in their study has only been made in the last 10 years [3–5].

All sulfite-oxidizing enzymes (SOE) characterized to date are molybdoenzymes that belong to the sulfite oxidase (SO) enzyme family. Contrary to earlier beliefs that this enzyme family contained exclusively eukaryotic enzymes [6–8], recent phylogenetic analyses have shown that SO-like enzymes are found in virtually all forms of life, and that protein sequences originating from bacteria make up the vast majority of SO family proteins [3,9]. Despite this fact little is currently known about the metabolic roles and the reactions catalyzed by these bacterial enzymes.

Sulfite occurs naturally in the environment, and in addition several metabolic pathways such as the degradation of sulfur-containing amino acids [10], organosulfonate metabolism [11] and sulfur oxidation pathways in chemolithoautotrophic bacteria [4] are known to lead to the formation of sulfite as an intermediate metabolite. Additional roles for sulfite as a signaling molecule in the vertebrate immune system and a potential role for sulfite in host defence against pathogens are emerging [12–15].

Due to its highly reactive nature, the sulfite anion can react with vital cell components such as DNA and proteins. Sulfite causes protein damage by reacting with disulfide bonds, and this is also the basis for some of its industrial applications [16]. In vertebrates, accumulation of sulfite caused by a sulfite oxidase deficiency causes damage to the central nervous system and increases oxidative stress [17,18]. Exposure of cells to sulfite is also known to lead to increased lipoperoxiation and to disable cellular stress defence mechanisms by depleting the glutathione pool and lowering the activity of enzymes such as catalase and glutathione peroxidase [19–21]. Therefore, both pro- and eukaryotic cells that can become exposed to externally or internally generated sulfite need to be able to detoxify it efficiently, which can occur either via reduction to the level of sulfur or sulfide, or, more commonly, by oxidation to sulfate.

In bacteria, the oxidation of sulfite to the inert sulfate anion proceeds via one of two possible routes [4]: in the indirect pathway the enzyme adenylylphosphosulfate (APS) reductase [EC 1.8.4.8] catalyzes the formation of APS, which is subsequently hydrolyzed to sulfate and either ATP or ADP by the action of sulfate adenylyltransferases [EC 2.7.7.4 or EC 2.7.7.5]. In contrast, the direct oxidation

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catalyzed by the molybdenum-containing enzymes of the SO family [4] is much more widespread and is the main topic of this review.

The SO enzyme family is one of the main families of the mononuclear molybdenum enzymes, which also comprise the Xanthine Oxidase and DMSO Reductase molybdoenzyme families [22–24]. In all of these enzymes the active site contains a single molybdenum/tungsten atom that is coordinated by one or two molecules of an organic cofactor, the so-called 'molybdopterin' or 'pyranopterin' cofactor which is also known as 'Moco' [25]. This cofactor occurs in two forms: a single molecule of the basic 'molybdopterin' (MPT or Mo-PPT) form is found in the SO and Xanthine Oxidase families (Fig. 1), while two molecules of a nucleotide modified form of MPT coordinate the Mo atom in enzymes of the DMSO Reductase family [26,27]. Both *b*- or *c*-type heme groups may be present in sulfite-oxidizing enzymes (SOEs) as additional redox centres [3], and apart from SOEs, the SO enzyme family also contains the plant, algal and yeast nitrate reductases [28] and a large number of prokaryotic enzymes of unknown function [3].

Based on their ability to transfer electrons to molecular oxygen, two types of molybdenum-containing SOEs are usually distinguished, sulfite oxidases (SO) [EC 1.8.3.1] that can use molecular oxygen as an electron acceptor, and sulfite dehydrogenases (SDH) [EC 1.8.2.1] that use other electron acceptors such as cytochromes *c* [3,29]. The general reaction catalyzed by these enzymes is $SO_3^{2-} + H_2O \rightarrow SO_4^{2-} + 2H^+ + 2e^-$, which in the case of oxygen as the electron acceptor leads to the production of hydrogen peroxide, while for sulfite dehydrogenases reduced cytochrome *c* is produced (Fig. 2). It should be noted, however, that for historical reasons, the sulfite-oxidizing enzymes found in vertebrates are usually referred to as sulfite oxidases, although they only possess a weak reactivity towards oxygen [30] and use cytochrome *c* as their preferred electron acceptor. In contrast, the recently purified sulfite oxidase from *Arabidopsis thaliana* is a true sulfite oxidase [31].

1.1. Structure of SO family enzymes: the 'SUOX-fold'

Several crystal structures are available for enzymes of the SO family, and these include representatives of three different types of SOEs [32–34], a eukaryotic nitrate reductase [28] and the bacterial YedY protein [35]. The overall structure of these enzymes varies significantly (Figs. 1 and 3) with the sulfite oxidases from plants and vertebrates being homodimers, which, in the case of the vertebrate enzymes, contain a mobile N-terminal heme *b* domain, the nitrate reductases being multidomain proteins with additional heme and FAD binding domains, while the bacterial SorAB SOE is a heterodimer comprising a molybdenum-binding subunit (SorA) and a smaller, heme *c* containing subunit (SorB) (Fig. 1). The two subunits of the SorAB SOE form a stable complex with the two redox centres being always found in close proximity (16.6 Å Mo–Fe distance, and 8.5 Å Mo to heme propionate-6 distance), and the SorAB crystal structure was the first SOE structure that allowed insights into intramolecular electron transfer between the heme and Mo redox centres in an SO family enzyme [34]. Due to the mobility of the heme domain found in vertebrate SOs, similar insights could not be derived from the crystal structure of the chicken liver SO, where the heme domain crystallized in a position far removed from the active site [32]. However, using the SorAB structure as a model, a docking site of the mobile heme domain of the chicken liver enzyme close to the Mo active site has been identified [34]. In contrast to these heme and Mo-containing SO family proteins, the *E. coli* YedY protein appears to exist in monomeric form [35] and does not contain additional redox centres (Fig. 1). Despite these differences in the overall structure and number of redox centres present in these enzymes, all available crystal structures show a conserved fold of the molybdenum domain, which has been termed the SUOX-fold [5]. It comprises a characteristic mixture of 10–12 α-helices and 2–3 β-sheets (Fig. 1). With the exception of YedY, all other SO family proteins for which crystal structures are available contain

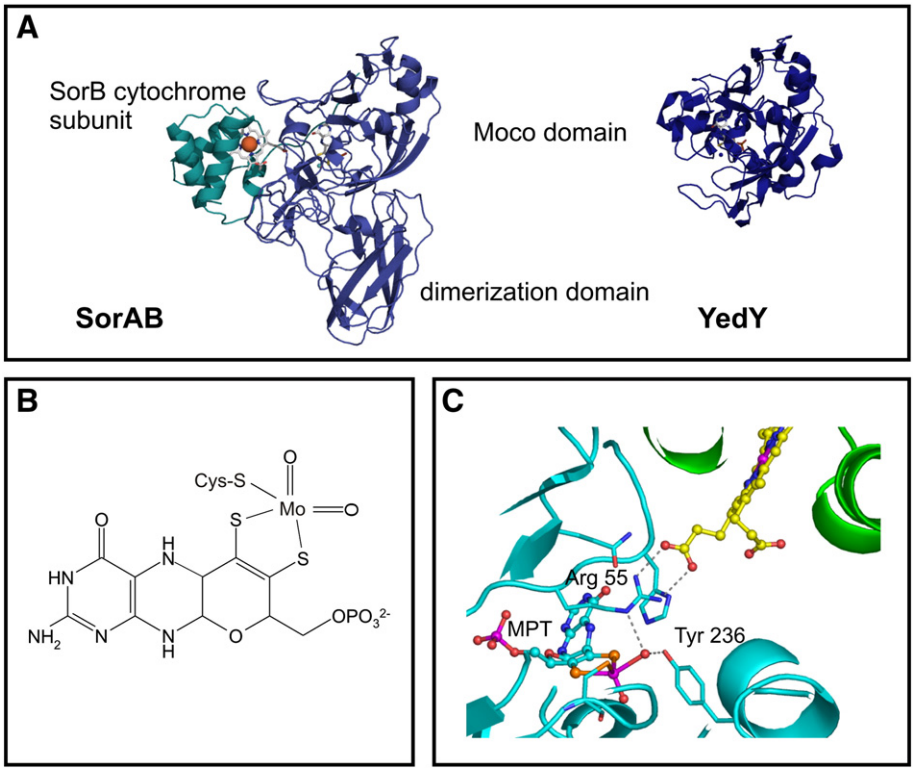

Fig. 1. Structure of bacterial enzymes of the Sulfite Oxidase family. Panel A: crystal structures of bacterial enzymes of the sulfite oxidase family. Left: *St. novella* SorAB sulfite dehydrogenase, SorA — light blue, and SorB — turquoise; Right: *E. coli* YedY protein, dark blue. Panel B: schematic representation of the molybdopterin (MPT) cofactor found in the SO family enzymes. Panel C: active site structure of the *St. novella* SorAB SDH, residues of SorA subunit — turquoise; residues or SorB subunit — green; heme group — yellow, and MPT — turquoise.

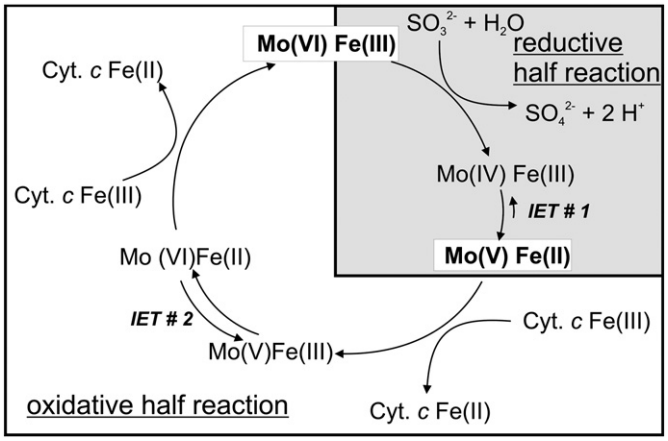

Fig. 2. General reaction mechanism for heme-containing sulfite-oxidizing enzymes.

an additional domain, the so-called dimerization domain that can mediate interactions between Mo-binding subunits e.g. of the homodimeric SOs from plants and vertebrates. However, in some bacterial enzymes (*St. novella* SorAB SDH, and *Cupriavidus necator* SOE [36,37]), the dimerization domain is present but does not appear to mediate any interactions between Mo-binding subunits. The fold of the dimerization domain is a beta-barrel structure with seven strands and is also conserved [5].

2. The molybdenum active site of SO enzymes

The central molybdenum atom at the active site of all SO family enzymes is coordinated by the dithiolene group of one MPT-type cofactor, a conserved cysteine residue and two oxo groups, resulting in a square pyramidal coordination sphere for all enzymes investigated so far (Fig. 1). Residues surrounding the Mo active site are conserved in enzymes with similar function, for example, in all confirmed SOEs a conserved tyrosine (Tyr-236, *St. novella numbering*) and up to three conserved arginine residues are found in close proximity to the molybdenum centre [32,34]. These conserved arginine residues are thought to support binding and orientation of the sulfite anion relative to the Mo-site [32,34,38]. At least two conserved Arg residues (Arg-55, Arg-109, *St. novella numbering*) are found in all so far characterized enzymes that carry out sulfite oxidation [3,5], and one of these, Arg-55, is located within hydrogen bonding distance of the Mo redox centre (Fig. 1). In contrast Arg-109 is found in the substrate access channel leading to the Mo active site. It appears as if the third conserved arginine residue which is also the one that is the furthest removed from the active site is only found in vertebrate SOs [39].

Sulfite oxidation is thought to proceed via an attack of the sulfur lone pair of electrons on the equatorial oxo group of the molybdenum centre, resulting in a reduction of the molybdenum from the Mo(VI) to the Mo(IV) state [40–44] (Fig. 2). The reaction product, sulfate, is liberated by hydrolysis in a coupled electron proton transfer reaction, which leads to a modification of the equatorial Mo oxo-ligand to a hydroxo or water ligand. This reductive half reaction is followed by a transfer of the two electrons stored in the reduced Mo centre to an external electron acceptor to regenerate the fully oxidized state of the Mo centre. If no additional redox centres are present in the enzyme (e.g. plant SO [31], *Sm. meliloti* SorT [45]), the two electrons stored in the Mo centre are directly transferred to an external electron acceptor. If additional redox centres such as heme groups are present (vertebrate SOs, and bacterial SorAB SDH), the electrons are sequentially transferred to the external acceptor via these redox centres (Fig. 2). This second mechanism requires a number of intramolecular electron transfer (IET) steps [43] and results in the intermediate formation of a stable, one electron reduced Mo(V) state of the molybdenum centre that is detectable by spectroscopy and has been widely used to characterize the molybdenum centres of sulfite-oxidizing enzymes [7,41,46,47]. In vertebrate SOs catalysis also requires a repositioning of the cytochrome *b* domain, which makes their reaction mechanism more complex than that of equivalent bacterial SOEs [43,44,48]. The heme domain of the vertebrate SOs has to move from a location distant from the Mo active site (which presumably is the position of the domain in the chicken SO crystal structure, [32]) to a position that enables electron transfer from the Mo to the heme *b* redox centre. This process requires not only the repositioning of the domain, but also efficient docking to the main body of the enzyme [49].

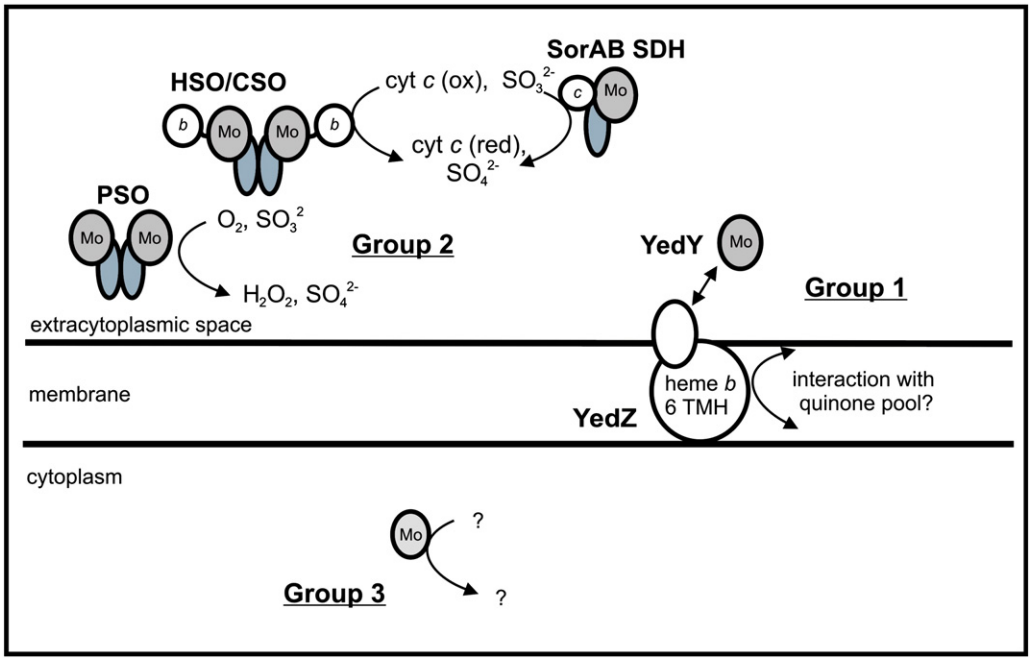

Fig. 3. Schematic representation of the structure and cellular localization of enzymes belonging to the three groups of SO family enzymes. HSO/CSO — human/chicken sulfite oxidase; PSO — plant sulfite oxidase; SorAB — *St. novella* sulfite dehydrogenase, and YedY/YedZ — YedY/YedZ proteins from *E. coli*.

The formation of a stable modified form of the enzyme such as the two electron reduced form of the heme-containing SOEs generated following sulfite oxidation is typical of a ping-pong reaction mechanism, and this type of mechanism has been shown to exist in both heme containing bacterial and vertebrate SOEs [1,36,43,50]. All characterized SOEs have $K_{Msulfite}$ values in the low micromolar range (~4–100 μM) and turnover numbers between ~26 s^{-1} (vertebrate SOEs) and ~350 s^{-1} (bacterial SOEs) [51]. Where such data has been reported (vertebrate SOs and SorAB SOE) maximal SOE activity is observed between pH 8 and 8.5, and the affinity for the substrate, sulfite, decreases almost exponentially above pH 8.5, and increases towards the low pH range. This appears to be a property of the Mo centre of these enzymes, as in non-steady state kinetics carried out on the reductive half reaction (i.e. sulfite oxidation at the Mo centre and reduction of the enzyme without any added external electron centre, Fig. 2) $K_{Dsulfite}$ showed the same pH dependence as $K_{Msulfite}$ in steady state kinetics [52,53].

The originally proposed mechanism of SOE catalysis assumes that in the reductive half reaction, i.e. the initial oxidation of sulfite, reduction of the Mo centre and hydrolysis of the bound sulfate molecule would always occur before electrons would be passed on from the Mo to additional redox centres or external electron acceptors (Fig. 2). Using a combination of kinetics, EPR and studies of intramolecular electron transfer (IET #2, Fig. 2), we have recently been able to show for the bacterial SorAB SDH that there is greater plasticity in the SOE catalytic mechanism than previously assumed, and that electrons can leave the Mo centre before the release of the sulfate molecule from the Mo centre occurs [44,54].

3. Spectroscopy of SO family enzymes

EPR spectroscopic investigations of the one electron reduced Mo (V) state of SOE redox centres were among the earliest methods used to probe the state and conformations of the Mo centre in vertebrate SOs and findings have been the subject of many excellent review papers [41,43,55–57]. Several characteristic Mo(V) EPR signals are known for SOEs, and have been named according to the conditions under which they were first identified, i.e. high and low pH signals, phosphate- and sulfite-inhibited forms and the so-called 'blocked' form of the Mo centre [54,58–62] (Table 1). All of these EPR signals were first described in the wild type vertebrate SOs, however, it appears that the Mo centres of the bacterial SOE differ from those of the vertebrate enzymes. For example, the Mo centre of the bacterial SorAB SDH exhibits only a 'high pH' EPR signal, regardless of changes in buffer pH or additions (e.g. phosphate) to the buffer system [36,56]. The sulfite reduced SorT SDH from *Sm. meliloti* also has a high pH EPR spectrum (Kappler and Enemark, unpublished). This suggests that although the crystal structures of SOEs show virtually identical Mo active sites with identical amino acids close to the Mo atom, they differ in subtle ways that influence the spectroscopic properties of the Mo centres. Both the low pH EPR signal and blocked form of the EPR spectrum, which is indicative of a sulfate bound to the Mo centre, have been observed in the bacterial SorAB SDH, but only in variant enzymes carrying mutations close to the active site (SorAB^{Y236F} — low pH signal, and SorAB^{R55Q} — blocked form) [51,54]. The SorAB^{Y236F} mutation disrupts the extensive hydrogen bonding network around the Mo active site, which appears to allow the Mo centre to adopt a conformation corresponding to the 'low pH form' of the vertebrate SOs [51]. This mutation also gave rise to a low level sulfite oxidase activity in the SorAB sulfite dehydrogenase. In contrast, the SorAB^{R55Q} substitution removes one of the conserved arginine residues from the immediate environment of the Mo centre (Fig. 1), which results in a severe slowing down of the hydrolysis of the Mo-sulfate complex and thus gives rise to the 'blocked' EPR species [54]. Interestingly, the SorAB^{R55Q} mutation corresponds to a clinically identified mutation (R160Q) in the human SO that causes sulfite oxidase deficiency syndrome [63].

An important element of SO catalysis is the transfer of electrons between the different redox centres present in both the vertebrate SOs and the bacterial SorAB SDH, and this can be probed in heme-containing SOEs by laser-flash photolysis, which measures electron transfer from the heme group to the Mo centre, i.e. electron movement in the opposite of the physiological direction [43]. Using this technique, the involvement of domain movement in the catalysis of vertebrate SOs but not in the SorAB SDH has been proven [64,65], and it was also instrumental in determining the moderating influence of the conserved arginine closest to the active site (Arg-55, *St. novella numbering*) on heme to Mo electron transfer rates [44]. Typical electron transfer rates (k_{ET}) for human SO are 411 s^{-1} at pH 6.0, while for the bacterial SorAB SDH only 120 s^{-1} was determined at the same pH [64,65]. It is intriguing to note the apparently inverse relationship of these IET rates and turnover numbers derived from steady state assays. At pH 6.0, human SO has a turnover number of 13.2 s^{-1}, for SorAB the turnover rate was 50 s^{-1} [51,53].

4. SO family enzymes from prokaryotic sources

SOEs have been reported to exist in diverse groups of Bacteria, including all groups of Proteobacteria, Firmicutes, and the Thermus lineage, as well as in Archaea [3], however, in many cases only an enzymatic activity or a basic enrichment of the proteins was reported and the earlier literature on these enzymes has been reviewed in [4]. In the last 9 years, several publications have described the purification and characterization of bacterial SOEs. A common feature of the

Table 1
Summary of EPR signals and g-values observed for different bacterial and eukaryotic SO family enzymes.

SO family group	EPR signal	SO family enzyme	g_z	g_y	g_x	References
Group 2 SOEs	High pH	CSO	1.9872	1.9641	1.9531	[90]
		HSO	1.987	1.964	1.954	[54,91]
		AT-SO	1.989	1.964	1.963	[92]
		SorAB	1.989	1.964	1.952	[36]
			1.991	1.966	1.954	[54]
	Low pH	CSO	2.0037	1.972	1.9658	[90]
		HSO	2.0037	1.972	1.9658	[91]
		AT-SOsulfite red	2.005	1.974	1.963	[60]
		AT-SOTi citr. red	2.006	1.975	1.968	
		SorAB^{Y236F}	2.004	1.973	1.965	[51]
	Phosphate inhibited	CSO	1.9917	1.9692	1.9614	[93]
	'Blocked'/sulfite-bound	CSO sulfite	1.996	1.972	1.9629	[62]
		HSO^{R160Q} *blocked*	2.006	1.971	1.951	[94]
		At-SO *blocked*	2.002	1.972	1.962	[60]
			2.005	1.974	1.963	
		SorAB^{R55Q} species 1	2.006	1.968	1.949	[54]
Group 1 SOEs	*As prepared*	YedY	2.03	1.974	1.969	[85]

characterized bacterial enzymes of the SO family is that they all appear to be located in the bacterial periplasm, i.e. in an extracellular compartment. In keeping with this, the genes encoding the molybdenum subunits of these enzymes usually encode a twin arginine-type (TAT) leader sequence for TAT-dependent export [66] of the enzymes to the periplasm [3].

At present, the bacterial SO family enzymes can be classified into two main groups, those that have been shown to catalyze sulfite oxidation [36,37,67–69] and proteins of unknown function (e.g. YedY from *E. coli* [70]). Within the group of confirmed SOEs enzymes containing only a molybdenum centre and those also containing a heme redox centre can be distinguished. Although specific chaperone proteins have been identified for prokaryotic enzymes from other molybdenum enzyme families, so far no such proteins have been identified for any enzymes of the SO family.

4.1. Bacterial heme- and molybdenum-containing SOEs

The heme-containing, heterodimeric SorAB SDH (SorA: 40.2 kDa Mo subunit, and SorB: 8.8 kDa heme *c* subunit) from the soil bacterium *Starkeya novella* is at present the best characterized bacterial SOE [36]. SorAB is expressed to high levels when *St. novella* grows as a chemolithoautotroph on thiosulfate [71], but a direct association of the enzyme with a specific dissimilatory sulfur oxidation pathway has not been established. SorAB is encoded by the *sorAB* genes and has been proposed to be under the control of an extracytoplasmic function (ECF)-type sigma factor belonging to the large subgroup ECF 26 which, at present, however, has no characterized representatives [71,72] (Figure S1).

The assembly of the bacterial heme-containing SOEs in general poses an interesting problem, as the Mo subunit contains a signal peptide targeting it for TAT-mediated export, while the heme-containing cytochrome *c* subunits are predicted to be exported via the Sec-pathway [73]. This means that the Mo subunit is exported in folded and matured form, while the heme-containing subunit matures after export to the periplasm. The assembly of the protein complex also needs to be achieved following the separate export of the two subunits to the periplasm.

The SorAB SDH resembles the vertebrate SOEs in terms of the redox centres it contains, but while catalysis in the vertebrate enzyme requires movement of the heme domain, thereby adding another level of complexity to the catalytic mechanism, the redox centres in SorAB are in a fixed position relative to one another (Fig. 1), which facilitates structure–function studies of SOE catalysis and has made SorAB a valuable model enzyme [34,44,51,52,54,56,65,74–78]. Although *St. novella* is a mesophilic organism, SorAB activity increases with temperature, and an optimum reaction temperature of 65 °C has been determined for the enzyme [36]. Some key spectroscopic and enzymological properties of SorAB have already been discussed above or are summarized in Table 2. Several variants of SorAB carrying specific mutations close to the Mo active site (Y236F, H57A, R55M, R55Q, and R55K) have been studied [44,51,52,54], and for two of them (Y236F, and R55M), crystal structures are also available [51]. All mutations caused significant changes in the SorAB catalytic parameters, the Tyr-236 mutation altered the reactivity of SorAB towards oxygen, while the series of Arg-55 mutations had profound effects on $K_{M\ sulfite}$, altering it by up to three orders of magnitude with the effect increasing with the loss of the positive charge at the active site. In contrast, the His-57 mutation had only a relatively mild effect on SorAB catalysis, but lead to a decrease in substrate affinity below pH 7, indicating that this residue is involved in mediating highly efficient catalysis at low pH.

A SorAB related SOE has been reported to exist in the human pathogen *Campylobacter jejuni*, a common causative agent of gastroenteritis [69]. The enzyme could be detected in cell extracts using anti-*St. novella* SorAB antibodies and a knockout of the gene encoding the putative cytochrome *c* subunit of this enzyme abolished all sulfite-oxidizing activity [69]. However, while these data clearly show that the cytochrome subunit is essential for 'sulfite respiration' in *Cb. jejuni*, it is unclear whether the cytochrome is indeed a subunit of the *Cb. jejuni* SOE, or whether it may be an accessory protein that is required for electron transfer. No kinetic or structural data is available for this protein at present [69].

In addition to the actual sulfite-oxidizing enzymes, there is a large group of bacterial heme-containing SO family enzymes that are core components of a multienzyme complex that enables chemolithotrophic growth of bacteria on thiosulfate [79]. These SoxCD sulfur dehydrogenases are heterotetrameric ($\alpha_2\beta_2$) enzymes with cytochrome *c* subunits that can contain one or two heme groups [80,81]. The purified SoxCD protein from *Paracoccus pantotrophus* had no sulfite-oxidizing activity, and all kinetic characterizations were done using a reconstituted multienzyme complex or SoxCD enriched protein fractions [80]. In the context of the multienzyme complex SoxCD is thought to catalyze the oxidation of the thiosulfate sulfane sulfur to a sulfone group [82], but at present SoxCD can only be assayed as part of the reconstituted multienzyme complex.

4.2. Bacterial SOEs without accessory redox centres

Although the *St. novella* SorAB SDH by virtue of being the best characterized bacterial SOE has become somewhat of a paradigm for bacterial SOEs, it would appear that SorAB-like, heme-containing enzymes might actually be less common than those lacking an associated heme-subunit. At present, five bacterial SOEs that do not contain redox centres other than the catalytic Mo centre have been purified and characterized from various sources [37,45,67,68]. Despite the fact that all of these enzymes contain a dimerization domain, some

Table 2
Comparison of the catalytic parameters of different bacterial sulfite-oxidizing enzymes.

	St. novella SorAB[a]	*Cv. necator* SorA[b]	*T. thermophilus* TTSor[c]	*Dc. radiodu rans* DraSOR[d]	*Sm. meliloti SorT*[e]
$K_{M\ sulfite}$	27 μM	50–100 μM	10.7 μM	94.5 μM	15.5 μM
$K_{M\ cyt.c}$	2 μM	n.r.	n.r.	n.r.	n.d.
$K_{M\ ferricyanide}$	n.d.	0.9 mM	6.3 μM	n.r.	3.4 μM
k_{cat}	345 s^{-1}	n.r.	53,318 s^{-1}^	n.r.	343 s^{-1}
V_{max}	422 U/mg	412.6 U/mg*	n.r.	n.r.	522 U/mg
Reaction conditions	25 °C, pH 8	n.r., pH 8	60 °C, pH 8	55 °C, pH 8	25 °C, pH 8

n.r. — not reported, and n.d. — not determined.
[a] Refs. [36,52].
[b] Performed using an enriched enzyme fraction Ref. [37].
[c] Ref. [68].
[d] Ref. [67].
[e] Ref. [45].
* Computed from the activity of 6850 mkat kg^{-1} reported in [2].
^ Value as reported in Ref. [67], a specific activity of 56.67 U/mg was reported for the purified enzyme.

of them occur as monomers, while others are dimeric in structure or can even occur in both forms. Unlike the enzymes in the previously described group, all proteins in this group have low or no activity with horse heart cytochrome *c* as the electron acceptor and are usually assayed using ferricyanide, a trait that has caused them to be described as 'atypical' sulfite dehydrogenases [83].

The first enzymes of this type were isolated from *Thermus thermophilus* and *Deinococcus radiodurans* [67,68]. Both of these species belong to the low-branching *Deinococcus–Thermus* phylum, which is unique in that it contains both Gram-negative (*Thermus* sp.) and Gram-positive bacteria (*Deinococcus* sp.) [84]. The SOE from *Thermus thermophilus* (TTSor) was purified in both native and recombinant form and is a periplasmically located, 39.1 kDa monomer containing only a Mo redox centre [68]. K_M values for sulfite and ferricyanide were in the low micromolar range (10.7 and 6.3 μM, respectively), similar to those reported for other SOEs [68] (Table 2). The activity of the enzyme did not change significantly as a function of pH, although above pH 9 a steep increase in activity was observed, which, however, as the authors point out, could be an artifact [68]. Optical spectra of the reduced enzyme indicated the presence of a molybdenum cofactor, and an optimal reaction temperature of 60 °C was determined (note that the growth temperature recommended by the German type culture collection, DSMZ, for *T. thermophilus* is 75 °C, and some other bacterial SOEs from mesophilic bacteria also showed maximal activity at 55–65 °C [36,67]). In *T. thermophilus* HB27 the TTC0961 gene encoding this enzyme occurs upstream of a gene encoding a cytochrome c_{552} that, however, does not appear to be part of the enzyme (Figure S1). Another *T. thermophilus* gene locus (TTC1044–1056) contains genes known to encode a thiosulfate oxidizing multienzyme complex, including a SoxC related protein (TTC1046).

In contrast to the situation in *T. thermophilus*, no cytochrome *c* encoding genes are found either up- or downstream of the gene encoding DraSOR (DR_A0225), the SOE from *Deinococcus radiodurans* R1 (Figure S1). DraSOR is expressed at high levels under heterotrophic conditions as well as in the presence of thiosulfate and/or molybdate [67]. The enzyme (predicted mol. mass 39.8 kDa) was overexpressed in *E. coli* and exists as a mixture of mono- and dimeric forms. Both forms are active, but the dimer may be the native enzyme form as it showed three times the enzymatic activity of the monomeric form [67]. Although DraSOR originates from a mesophilic bacterium (standard growth temperature 30 °C), all enzymatic characterizations (Table 2) were carried out at 55 °C, the reported optimum reaction temperature of the enzyme, where the enzyme has a half-life of 30 min [67]. The reported K_M value for sulfite was close to 100 μM, between 3 and 4 times higher than those reported for other bacterial SOEs (Table 2).

The remaining three characterized bacterial SOEs all originate from organosulfonate degrading Proteobacteria, namely two species belonging to the Burkholderiales within the β-Proteobacteria, *Delftia acidovorans* and *Cupriavidus necator* (formerly known as *Comamonas acidovorans* and *Ralstonia eutropha*, respectively), and the α-Proteobacterium *Sinorhizobium meliloti* that, like *Starkeya novella*, belongs to the Rhizobiales. The SOEs from these bacteria have proven to be difficult to purify from native sources [37,45,83].

The SOE from *Cv. necator* is encoded on chromosome 2 by gene H16_B0860, upstream of a cytochrome *c* encoding gene (H16_B0859), a cytochrome *c*, however, did not appear to be part of the purified enzyme preparation [37]. The enzyme has a predicted molecular mass of 40.7 kDa, and was proposed to be a monomeric enzyme based on gelfiltration experiments (est. molecular mass. 49–55 kDa) with a $K_{M\ sulfite}$ between 50 and 100 μM (Table 2). The extremely low yield of the purification precluded more detailed enzymatic characterization [37].

The SOE from *Delftia acidovorans* SPH-1 was enriched from the native organism [37] and found to be a heme-free homodimer, based on gelfiltration results (predicted molecular mass: 39.8 kDa, and 67 kDa by gelfiltration) [37]. The enzyme is encoded by an operon containing the SOE gene and a gene for a cytochrome *c* (Daci_0055 and Daci_0054), and upstream of the SOE gene genes encoding an ECF-type sigma factor and a putative anti-sigma factor are located (Figure S1), an arrangement reminiscent of the operon encoding the *St. novella* SorAB SOE, although the purified *Dt. acidovorans* enzyme is structurally distinct from the *St. novella* one. Expression of *Dt. acidovorans* SOR was reported to be inducible [37]. The two cytochromes encoded by Daci_0054 and H16_B0859 genes, respectively, encode fully soluble, periplasmic proteins. For both the *Dt. acidovorans* and *Cv. necator* enzymes, the authors predict the existence of a membrane-bound cytochrome ('SorB') that would function as the electron acceptor for the SOE.

Interestingly, while most of the bacterial SOEs described so far have about 30% identical amino acid sequences, the monomeric enzyme from *Cv. necator* is 60% identical to the heterodimeric SorAB from *St. novella*, while the *Dt. acidovorans* SOE is most closely related (61% identity) to the recently purified SorT SOE from *Sinorhizobium meliloti*. Thus in each of these cases the most closely related, characterized SOE is found in a bacterium belonging to a different group of the Proteobacteria.

SorT from *Sm. meliloti* is a homodimeric, heme-free sulfite dehydrogenase with a subunit molecular mass of 39.4 kDa. The enzyme has high affinities to both sulfite and ferricyanide (15.5 and 3.4 μM, respectively) and a turnover number of 343 s^{-1} (Table 1) [45]. Like the closely related *Dt. acidovorans* SOE, SorT is encoded in a gene region that also contains genes for an ECF sigma factor/anti-sigmafactor pair (Figure S1), and the *sorT* gene itself was shown to be co-transcribed with genes encoding other redox proteins, a cytochrome *c* and an azurin [45]. Despite this, there is no evidence for either of these two proteins being an integral part of the SorT enzyme, and SorT only shows 13% of the activity observed with ferricyanide when cytochrome *c* (horse heart) is used as the electron acceptor. SorT is located in the periplasm, and while low level SorT activity appears to be always present in *Sm. meliloti*, activity is induced 4–6 fold in the presence of taurine or thiosulfate [45]. Purified SorT shows a typical high pH EPR spectrum (Enemark and Kappler, unpublished). Unlike any other SOE that has been characterized in this respect so far, SorT has an almost pH-independent $K_{M\ sulfite}$ (pH 6–10), while in the SorAB SDH and the vertebrate SOs the $K_{M\ sulfite}$ increases exponentially above pH 8.5. This is the first time that an SOE with altered catalytic properties has been identified.

4.3. Bacterial SO family enzymes of unknown function

There is only one characterized representative of this group at present, the YedY protein from *E. coli* which does not oxidize sulfite or any other substrate tested, but has been shown to have a weak dimethylsulfoxide reductase activity (reported K_M value 12 mM, k_{cat} 4.83 s^{-1}) [35,70]. YedY is a monomeric enzyme that contains only a Mo redox centre and likely uses the membrane-bound YedZ *b*-type cytochrome as an electron donor [70]. The crystal structure of YedY clearly shows the core SUOX-fold present in all SO family enzymes (Fig. 1), however, YedY lacks the dimerization domain found in all other structurally characterized SO enzymes, as well as the conserved Arg residues (see section 2) found in SOEs, which is in keeping with a chemically different substrate molecule being used. The actual Mo redox centre of YedY, however, is virtually identical to those of other SO family proteins in terms of its geometry and coordination [35]. Redox titrations revealed that the Mo centre of YedY only shows a single redox potential (132 mV) for the Mo(IV/V) transition, contrary to what is seen in other SO family enzymes where the Mo centre cycles between a Mo(IV) and Mo(VI) state. EPR spectra of the Mo(V) state of the YedY active site differ significantly from the signals typically seen in SOEs (Table 1) and instead bear close resemblance to the EPR spectrum of the Xanthine oxidase 'very rapid' reaction intermediate [70,85]. This has been proposed to be indicative of some

of the Mo–S bonds of the YedY Mo centre assuming a more covalent character.

5. Phylogenetic analyses of the SO enzyme family

The available, biochemical data on bacterial SO family enzymes clearly highlights their great structural and possibly functional diversity as well as the fact that a meaningful classification system is necessary to distinguish between the different enzyme types.

Based on the architecture of the central catalytic molybdenum domain, the SO family enzymes can be classified as belonging to three major groups, and within each of these groups several further subdivisions can be made [3]. While groups 1 and 3 contain exclusively bacterial and archaeal proteins, group 2 contains proteins of both pro- and eukaryotic origins (Figs. 3, 4 and S2).

Sequences in group 1 originate mainly from pathogenic bacteria such as *Salmonella, Yersinia, Ralstonia* and *Burkholderia*, and the YedY protein from *E. coli* is the only characterized enzyme in this group. The molybdenum domain of group 1 proteins has a size of 30–35 kDa, and the genes encoding these proteins always occur in proximity to a gene encoding a membrane-bound cytochrome *b* subunit. Based on the nature of this cytochrome subunit, two subgroups can be distinguished — group 1A (characterized representative: YedY from *E. coli*, cytochrome *b* subunit (YedZ) with 6 transmembrane helices) and group 1B (no characterized representative to date, cytochrome *b* subunit with 4 transmembrane helices) [3].

Group 2 is comprised of all confirmed SOEs and the plant nitrate reductases. It is also the only group where the molybdenum domain always occurs in conjunction with a dimerization domain located on the same polypeptide and with the exception of YedY this group contains all the characterized bacterial SO family enzymes described above. The size of the combined Moco/dimerization domains is ~40–45 kDa in this group, but additional domains can be present on the same polypeptide, e.g. in vertebrate SOs and plant nitrate reductases. In addition to nitrate reducing and sulfite-oxidizing enzymes, there is evidence that this group also contains bacterial enzymes catalyzing largely uncharacterized reactions, such as the SoxCD sulfur dehydrogenase [3]. Three subgroups were proposed for group 2, group 2A — (eukaryotic) sulfite oxidases and plant nitrate reductases, group 2B — SoxCD-like enzymes "sulfur dehydrogenases" and group 2C — SorAB-like sulfite dehydrogenases. It was also recognized that additional enzymes are present in group 2 that could at the time not be classified due to a lack of available data. Accessory heme redox centres may or may not be present in the enzymes of this group [3,36,37,45,67,68], and one as yet unstudied subgroup of sequences appears to encode membrane-bound variants of SOEs [3,5].

SOE group 3 is composed of both bacterial and archaeal proteins, and there are at present no characterized representatives of this group of enzymes. The molybdenum domain has a size of only 20–25 kDa, the reduced size being due to the loss of an N-terminal section of the domain. The archaeal and bacterial sequences form two distinct subgroups with identity values of ~30–47% within each group and values of 25–30% for comparisons between archaeal and bacterial sequences.

As already pointed out within each of these three groups several subgroups exist, but at present there is insufficient information to refine this analysis further. The division of the SO family enzymes into three basic types based on the structure of the Mo domain corresponds well with the conserved domains defined in the CD database [86], where all the groups defined here can be easily linked to the main subgroups of the cd_00321 entry that describes 'sulfite oxidase and related enzymes' [45] (Table S1).

An alternative classification system for SO family enzymes has been proposed by Workun et al. [5]. This system distinguishes 8 clades, based on a variety of characteristics such as residues surrounding the conserved Mo-ligating cysteine, presence or absence of leader peptides and presence or absence of other conserved residues known to be important in the function of SOEs such as the arginines found close to the Mo active site (see section 2 and Table 3). The clades proposed by Workun et al. [5] can be linked to the classification based on the overall architecture of the Mo subunit described above (Table 3). At present, however, for most of the enzyme clades there is no or very little data available on the actual structure or function of the enzymes that make up the clades (e.g. clades 2, 7, and 8). In other cases the significance of the observed differences between the clades e.g. in residues surrounding the active site, is unclear, as there is no or very little functional difference observed in the characterized representatives of the respective clades,

Fig. 4. Molybdenum domain architecture of different types of Sulfite Oxidase family enzymes. Three different groups of SO family enzymes can be clearly distinguished based on the architecture of this domain: Group 1 has a Moco domain of 30–35 kDa, while in Group 2 the Moco domain always occurs in conjunction with the so-called 'Moco-dimer' domain. The third group has a reduced size Moco domain of only ~25 kDa. Within group 2 fusions of the Moco domain with additional domains have occurred in some enzymes. Only polypeptides containing the Moco domain are shown, subunits encoded on separate polypeptides are not shown. YedY — *E. coli* YedY protein, PA4882 — *P. aeruginosa* PA4882 protein, vertebrate SO — chicken/human sulfite oxidase, PSO — plant sulfite oxidase, plant NIA — plant nitrate reductase, SorA — *St. novella* SorAB sulfite dehydrogenase Mo subunit, SoxC — *St. novella* SoxCD sulfur dehydrogenase Mo subunit, SorT — *Sm. meliloti* SorT sulfite dehydrogenase, SSO3201 — *Sulfolobus solfataricus* SSO3201 protein, and Smb20584 — *Sm. meliloti* Smb20584 protein.

Table 3
Classification of Sulfite-oxidizing enzymes by Mo subunit structure and active site residues.

Mo subunit grouping [a]	Clade [b]	Mo subunit domains	Simplified classification [b]	Amino acid motif surrounding conserved Cys [a,b]	Additional domains/features	Characterized representative
Group 1						
Group 1A	Clade 1	Moco only	*tat* N C E | |	RCVExW		YedY *Escherichia coli*
Group 1B	Clade 2	Moco only	*tat* N C E | |	CVEGW		none
Group 2						
Group 2A	Clade 5	Moco-dimer	*Δtat* R C G R R	CAGNRR	Heme *b* domain fused to Moco-dimer domain	Human sulfite oxidase, and plant nitrate reductases
Group 2B	Clade 6	Moco-dimer	*tat* R C A/G | R	FxECxxN	Heme *c* subunit that is part of enzyme	SoxCD *Paracoccus pantotrophus*
Group 2C	Clade 3	Moco-dimer	*tat* R C G R |	CxGNxR		SorAB *Starkeya novella*, 'SorA' *Campylobacter jejuni*, DraSor *Deinococcus radiodurans, and* 'SorA' *Cupriavidus necator*
Group 2 others	Clade 4	Moco-dimer	*tat* R C S R |	CSGNGR		SorT, *Sinorhizobium meliloti*, TTSor *Thermus thermophilus* 'SorA', and *Delftia acidovorans*
Group 2 others	Clade 8	Moco-dimer	*Δtat* R C S | |	CVSN	Membrane bound, no heme domain	None
Group 3						
Group 3A	(Clade 7)	trunc Moco only		DFxCVTxWS	No additional domains/ subunits known	None
Group 3B	Clade 7	trunc Moco only	*Δtat* | C T | |	DFHCVTxWS	No additional domains/ subunits known	None

Vertical lines in column 4 denote the absence of residues conserved in other SOEs.
[a] Kappler, 2008.
[b] Workun et al., 2008.

while, however, clear differences in SOE subunit structure occur within some clades (clades 3, 4, and 5) (Table 3).

It would thus appear that the classification systems for SO family enzymes that are available to date (groups based on structure of Mo domain architecture, clade system) show significant congruency (Table 3). In view of the scarcity of available data, however, it would seem that the domain architecture based system for classifying SO family enzymes into different groups provides a sound basis for general classification of enzymes of unknown function based on e.g. genome data, while use of the clade system and the establishment of additional subgroups of SOEs will remain difficult until more catalytic and structural data become available for all major groupings of SOEs and e.g. the significance of the presence/absence of certain residues close to the active site for enzyme function can be fully assessed.

6. Concluding remarks

At this point in time it is becoming clear that the majority of enzymes in the SO family are of bacterial origin, and that these enzymes exhibit much greater functional and structural diversity than had previously been assumed. Moreover, the majority of the SO family enzymes encoded in the bacterial genomes sequenced so far have not been characterized, and there is evidence that in particular the enzymes from groups 1 and 3 may catalyze reactions that are distinct from those usually associated with SO family proteins, namely sulfite oxidation and nitrate reduction. Some of the recently discovered bacterial SOEs already show catalytic and spectroscopic properties that differ from those of all other well studied SOEs [45,85], and this creates exciting new possibilities for further research into Mo-mediated catalysis in these enzymes and the spectroscopic signatures that underly such changes. As an example — unlike all other SOEs for which such data has been determined, the SorT enzyme shows increasing substrate affinity above pH 8.5, where in the 'conventional' SOEs substrate affinity begins to decrease in an exponential fashion [45].

The available data on bacterial sulfite-oxidizing enzymes also clearly show that similarities in operon structure as, e.g. observed for SorAB form *Starkeya novella*, SorT from *Sinorhizobium meliloti* and the SOEs from *Campylobacter jejuni*, *Cupriavidus necator* and *Delftia acidovorans* cannot be used to deduce the structure of the SOE encoded by these genes (Figure S1). In all of the above cases the SOE Mo subunit encoding gene is followed by a gene encoding a cytochrome *c*, but the subunit structure of the actual enzymes ranges from the Mo and heme-containing SorAB heterodimer and the Mo-containing SorT homodimer to the monomeric *Cv. necator* SOE (this latter enzyme shows 61% identity to SorA from *Starkeya novella*). Clearly, much more work is needed to understand what governs the subunit association in bacterial SOEs, and whether the presence/absence of additional redox centres has any functional implications as significant differences are observed even between closely related enzymes.

These structural differences highlight another important area for future research, namely a clarification of the metabolic roles of the SO family enzymes. So far these enzymes have been implicated in the detoxification of sulfite arising from amino acid degradation in vertebrates, production of hydrogen peroxide in plant peroxisomes [87], sulfur chemolithotrophy [68,71], organosulfonate degradation [37,45], internal sulfite respiration [67] and anaerobic respiration and defence against immune system components in a human pathogen [69]. Despite these very diverse roles in which SOEs have been detected, so far no clear association of any of the known bacterial SOEs with any of the above metabolic functions has been shown. For example, although three SOEs from organosulfonate degrading organisms have been purified or highly enriched, only two of these share significant sequence similarities, while the third is 61% identical to the heterodimeric SorAB enzyme found in the sulfur chemolithotroph *St. novella*, and no evidence for the association of any particular type of SOE with organosulfonate degradation pathways was found [45]. By the same token, no association between bacterial phylogenetic groupings and the prevalence of certain types of SOEs seems to exist.

In view of the large gaps in our knowledge of the bacterial SO family enzymes the development of a consistent and informative naming system is crucial. At present a number of bacterial SOEs that clearly differ from the heterodimeric SorAB SOE in their subunit composition and possibly their catalytic and metabolic functions have all been referred to as 'SorA'. A much more systematic approach to naming these enzymes should be adopted, where only enzymes that

are clearly related in structure and function should carry the same name, while enzymes of similar function but with a differing structure should be given a distinct name to highlight these differences. Such an approach has also been adopted in the case of e.g. the trimethylamine-neoxide reductases of *E. coli*, where enzymes with distinct subunit compositions and cellular functions have been named TorABCD and TorYZ [88,89], and accordingly, the recently discovered homodimeric SOE from *Sm. meliloti* has been named SorT [45].

It is thus clear that we are only just beginning to understand the full level of functional and structural diversity within the SO enzyme family, and future work will no doubt provide important insights into the evolution of SOE structure and function in adaptation to changing metabolic requirements.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2010.09.004.

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