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## Role of a conserved arginine residue during catalysis in serine palmitoyltransferase\*\*

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### Keywords:

Serine palmitoyltransferase, enzyme mechanism, quinonoid intermediate, sphingolipids, *Sphingomonas wittichii*

### Abbreviations:

SPT, serine palmitoyltransferase; PLP, pyridoxal 5'-phosphate; KDS, 3-ketodihydrosphingosine, CoA, coenzyme A; AOS, alpha oxoamine synthase

## Abstract

All sphingolipid-producing organisms require the pyridoxal 5'-phosphate (PLP)-dependent serine palmitoyltransferase (SPT) to catalyse the first reaction on the *de novo* sphingolipid biosynthetic pathway. SPT is a member of the alpha oxoamine synthase (AOS) family that catalyses a Claisen-like condensation of palmitoyl-CoA and L-serine to form 3-ketodihydrosphingosine (KDS). Protein sequence alignment across various species reveals an arginine residue, not involved in PLP binding, to be strictly conserved in all prokaryotic SPTs, the lcb2 subunits of eukaryotic SPTs and all members of the AOS family. Here we use UV-vis spectroscopy and site-directed mutagenesis, in combination with a substrate analogue, to show that the equivalent residue (R370) in the SPT from *Sphingomonas wittichii* is required to form the key PLP:L-serine quinonoid intermediate that condenses with palmitoyl-CoA and thus plays an essential role enzyme catalysis.

## 1. Introduction

Sphingolipids are a distinct class of lipid produced by all eukaryotes and some bacteria, such as the dioxin-metabolising species *Sphingomonas wittichii*<sup>[1]</sup>. We have recently begun to study bacterial sphingolipid biosynthesis using *S. wittichii* as a model organism after the completion of its genome sequence<sup>[2, 3]</sup>. Not only are they important structural components of membranes but are also necessary signalling molecules for many biological functions<sup>[4, 5]</sup>. All sphingolipids comprise a characteristic sphingoid base containing a fatty acyl chain and a polar head group derived from L-serine. These components are joined together to form 3-ketodihydrosphingosine (KDS) in the first and rate-limiting step of sphingolipid biosynthesis carried out by serine palmitoyltransferase (SPT). SPT is a member of the alpha oxo-amine synthase (AOS) subfamily of the larger group of pyridoxal 5'-phosphate (PLP)-dependent enzymes<sup>[6, 7]</sup>. Structures have been solved for several of these enzymes including 8-amino-7-oxononanoate synthase (AONS)<sup>[8]</sup>, 5-aminolevulinate synthase (ALAS)<sup>[9]</sup>, 2-amino-3-ketobutyrate CoA ligase (KBL)<sup>[10]</sup>, and cholera quorum-sensing autoinducer synthase (CqsA)<sup>[11, 12]</sup>. Bacterial SPTs are mainly cytoplasmic, homodimeric enzymes<sup>[13]</sup> encoded by single genes. In contrast, their eukaryotic counterparts are membrane-associated and encoded by two SPT structural genes (lcb1 and lcb2) with high sequence homology to each other<sup>[14]</sup>. Since lcb1 lacks the conserved PLP residues found in lcb2, it is thought that eukaryotic SPTs bind a single PLP cofactor on the lcb2 subunit. The eukaryotic SPT subunits are thought to be found in complex with the activity-enhancing small subunits (ssSPTs)<sup>[15, 16]</sup> as well as regulatory ORM proteins<sup>[17, 18]</sup>. The structures of three bacterial holo-SPTs have been solved<sup>[3, 19-21]</sup> and as well as providing molecular insight into the catalytic mechanism, we have used them to make models of the more complex eukaryotic SPTs. All AOS enzymes catalyse a Claisen-like condensation of their amino acid and acyl-CoA substrates via an ordered mechanism analogous to that proposed for SPT shown in Scheme 1. They utilise a PLP cofactor covalently-bound as an imine to the ε-amino group of a conserved lysine residue (internal aldimine, I). In SPT, the amino group of the L-serine substrate exchanges with the lysine amino group, called transimination, to form a PLP:L-serine external aldimine (II). Binding of the second substrate palmitoyl-CoA causes rotation around N-C<sub>α</sub> bond, allowing deprotonation (by the active site lysine) at C<sub>α</sub> of the putative Dunathan intermediate (III) resulting in the first quinonoid intermediate (IV). The resultant C<sub>α</sub> carbanion of IV attacks the carbonyl carbon of the palmitoyl-CoA thioester in a Claisen-type condensation to generate a β-keto acid product (V) that decarboxylates to yield the product

quinonoid intermediate (VI). Protonation at C<sub>α</sub> of VI yields the external aldimine of PLP-KDS (VII). A second transimination step releases the KDS product and regenerates the internal aldimine (I). Altering such a highly controlled mechanism by introducing substrate analogues or changing the active site environment by mutation can affect catalysis. For example, addition of the L-serine analogue cycloserine to SPT from *S. paucimobilis* resulted in the observation of novel PLP-dependent chemistry [22]. In the same SPT isoform, active site mutations resulting in subtle structural alterations at the active site had a major impact on cofactor binding and catalysis [20]. Along with stereoelectronic control by PLP itself [23], catalytic efficiency is ensured by rigorous control via interactions between active site residues and the PLP-bound intermediates. Conserved residues within SPTs include K245 (*S. wittichii* numbering) that forms the internal aldimine with PLP as well as H139 and D211 that bind the PLP cofactor non-covalently (Supplementary Scheme 1). These residues are strictly conserved in AOS enzymes and by using site-directed mutagenesis their functional roles have been investigated in this subfamily [24-26] and in the fold-type I family [27, 28]. Other active site residues that are involved in binding the amino acid moiety of the PLP:substrate complex are not strictly conserved and the broad substrate specificity in the AOS family is still unclear. One exception is R370 in the SPT from *S. wittichii*; the equivalent residues in *S. paucimobilis* SPT (R390) and *S. multivorum* SPT (R367) do not interact with the PLP cofactor or the external aldimine intermediate [20, 21]. However, sequence alignment reveals that it is strictly conserved among bacterial SPTs, across the AOS family and within the active lcb2 subunit of eukaryotic SPTs (Fig. 1). Using site-directed mutagenesis of *S. wittichii* SPT R370, combined with a non-hydrolysable thioether analog of palmitoyl-CoA, we further investigated the role of this arginine residue during catalysis. Our data suggest that this residue is involved in a step downstream of PLP:L-serine external aldimine formation and is essential for product formation.

(A)	
SwSPT	356..TARPPATPAGMFLLRCS..372
SpSPT	376..MARPPATPAGTFLLRCS..392
ZmSPT	358..LARPPATPAGMFLLRCS..374
SsSPT	353..PVVSPAVPSEESLIRFS..369
SmSPT	353..PVVSPAVPAEESLIRFS..369
BsSPT	373..PVLPPAVPKGETLIRTS..389

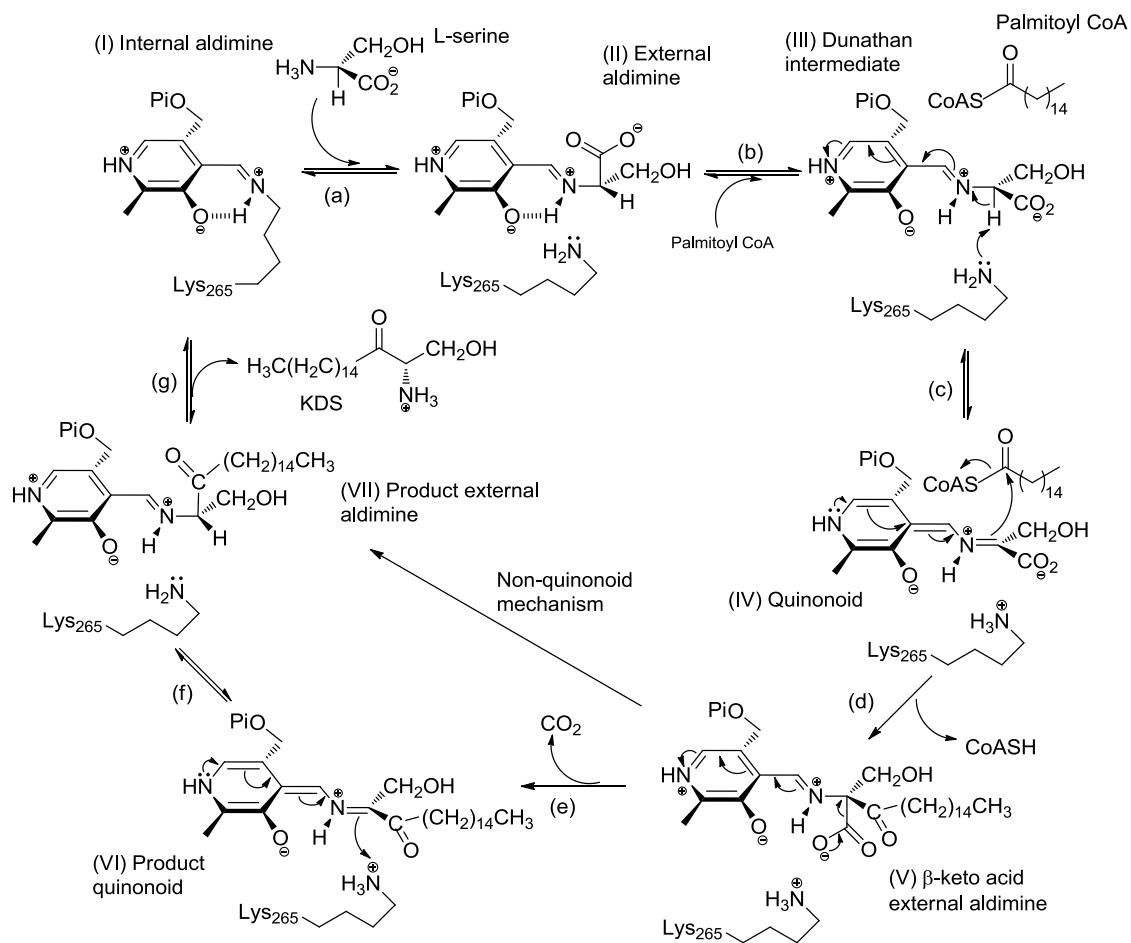
  

(B)	
SwSPT	356..TARPPATPAGMFLLRCS..372
SpSPT	376..MARPPATPAGTFLLRCS..392
AONS	347..AIRPPTVPAGTARLRLT..363
KBL	354..GFFYPVVPKGQARIRTQ..370
ALAS	360..PINFPPTVPRGTERLRF..376
CqSA	344..VFCRPATSKNKNIIRLS..360

(C)	
SwSPT	356..TARPPATPAGMFLLRCS..372
hlcb2	495..VVGFPATPIIESRARFC..511
ylcb2	482..VVAYPATPLIESRVRFC..498
ATlcb2	427..VVGFPATPLLLARARIC..443
hlcb1	419..CMN-RSIALTQARYLEK..434
ylcb1	499..ALI-NYNVLITRNTIVL..514
ATlcb1	426..ALK-EDSLLVVSSKRSF..441

**Figure 1.** Sequence alignment of (A) bacterial SPTs Sw, *Sphingomonas wittichii*; Sp, *Sphingomonas paucimobilis*; Zm, *Zymomonas mobilis*; Ss, *Sphingobacterium spiritivorum*; Sm, *Sphingobacterium multivorum*; Bs, *Bdellovibrio stolpii* (B) members of the AOS family with solved crystal structures and (C) eukaryotic SPTs (h = human, y = yeast, AT = *A. thaliana*).



**Scheme 1.** Catalytic mechanism of SPT; (a) L-serine substrate displaces K265 to form an external aldimine via a transimination step (b) binding of palmitoyl-CoA (c) deprotonation at C<sub>α</sub> by K265 (d) condensation of the substrate quinonoid and the palmitoyl-CoA thioester substrate (e) decarboxylation (f) reprotonation at C<sub>α</sub> by K265 to form the product external aldimine (g) release of the KDS product and regeneration of the internal aldimine.

## 2. Materials and Methods

### 2.1 Materials

Plasmids and *Escherichia coli* competent cells were purchased from Novagen, and all chromatography columns were from GE Healthcare. All buffers and reagents were from Sigma. Palmitoyl-CoA was from Avanti Lipids. The QuikChange site-directed mutagenesis kit was purchased from Stratagene. Oligonucleotide primers were sourced from SigmaGenosys. Quartz cuvettes were from NSG Precision Cells, Type 18BM. The synthesis of S-(2-oxoheptadecyl)-CoA was previously reported<sup>[29]</sup>.

## 2.2 Sequence alignments and cloning of SPT R370A and R370K mutants

Multiple sequence alignments were carried out using the Clustal series of programs<sup>[30]</sup>. The SPT wild-type gene was cloned in pET28a expression vector (Novagen) as previously described<sup>[20]</sup>. This template was used to prepare the R370A and R370K mutations that were introduced into the SPT gene by site-directed mutagenesis using mutagenic primers containing non-overlapping 3' ends<sup>[31]</sup>. The mutagenic bases are shown in boldface type and the sequences of the SPT mutant genes were confirmed by Big Dye sequencing:

5'-GCTGA**A**ATGCTCGCTGTGCGCCG-3' (SPT R370K forward),

5'-CGAGC**ATTC**CAGCAGGAACATGCC-3' (SPT R370K reverse),

5'- GCTGG**C**CTGCTCGCTGTGCGCCG -3' (SPT R370A forward),

5'- CGAGC**AGGCC**CAGCAGGAACATGCC -3' (SPT R370A reverse),

Protein expression of wild-type and mutant SPTs in *E. coli* has been described previously<sup>[20]</sup>. Before UV-vis spectroscopy assays, the enzyme was freshly converted to the PLP-bound form by dialysis against buffers containing 50 μM PLP.

## 2.3 Determination of Dissociation Constants (K<sub>d</sub>) for L-serine, quinonoid detection and SPT activity assays

Binding assays were carried out in 0.5-ml quartz cuvettes and typically contained 20 μM SPT in 20 mM potassium phosphate (pH 7.5). K<sub>d</sub> values were calculated from plots of Δ420 versus [L-serine] by fitting to a hyperbolic saturation curve using Sigma Plot software

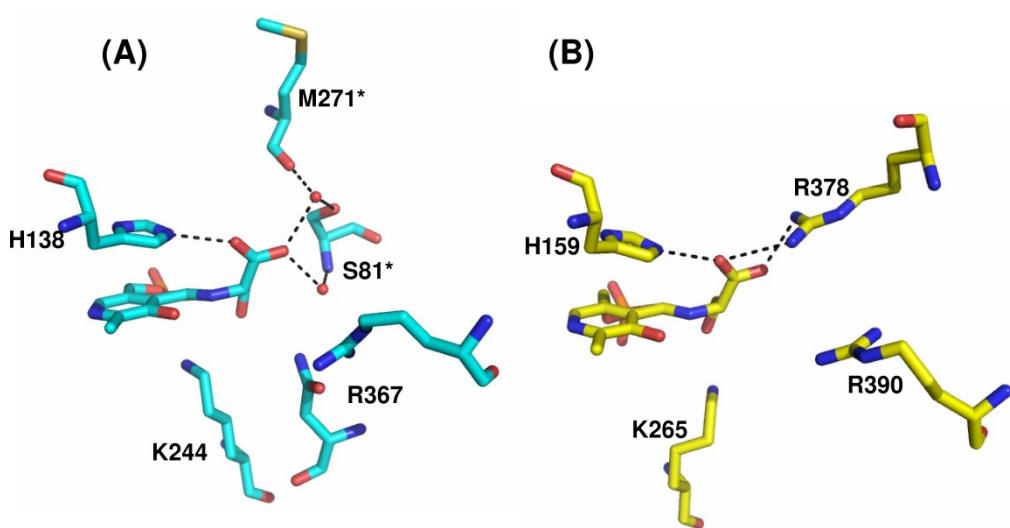
$$\Delta A_{obs} = \frac{\Delta A_{max} [serine]}{K_d + [serine]}$$

where ΔA<sub>obs</sub> represents the observed change in absorbance at 422 nm, and ΔA<sub>max</sub> is the maximal absorbance change, [serine] is the L-serine concentration, and K<sub>d</sub> is the dissociation constant<sup>[20]</sup>. To form the substrate quinonoid, 50 mM L-serine and 1.5 mM *S*-(2-oxoheptadecyl)-CoA were added to the enzyme. To form the product quinonoid, 25 μM KDS was added to the enzyme and allowed to equilibrate for 10 minutes at 25 °C. The spectroscopic data were recorded between 200 and 800 nm. SPT activity was measured using a continuous spectrophotometric assay that monitors release of CoASH from acyl-CoA substrates by its reaction with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and has been previously described<sup>[20]</sup>. The final enzyme concentrations were 0.4 μM wild-type and 20 μM for the mutant SPTs.

### 3. Results and Discussion

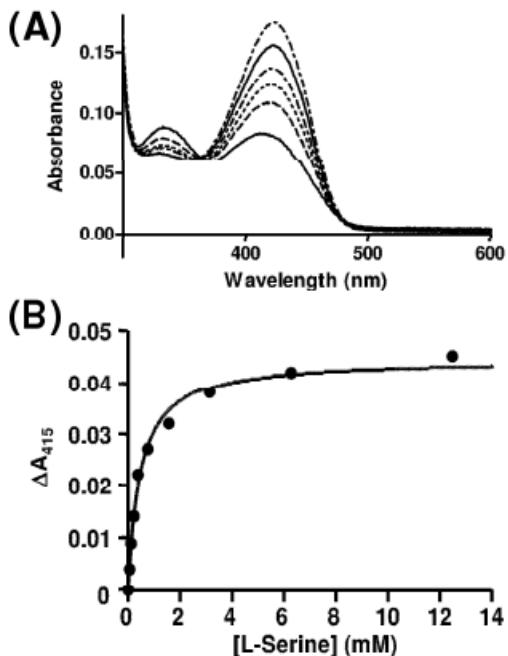
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Protein sequence alignment of the SPT from *S. wittichii* with five other bacterial SPTs and with structural homologues from the AOS family revealed that R370 is an invariant residue (Fig. 1A and 1B). The numbering for this arginine residue in each enzyme is given in Supplementary Table 1. Despite its strict conservation the exact role played by R370 in SPT from *S. wittichii* is still unknown. One function in the structural homologues ALAS<sup>[9]</sup>, KBL<sup>[10]</sup> and CqsA<sup>[11]</sup> is to bind the specific amino acid substrate; structures of the external aldimine forms showed a salt bridge between the arginine guanidinium side-chain and the carboxylate of the amino acid substrate. However, high resolution structures of two bacterial SPTs with L-serine bound revealed no such salt bridge to be present<sup>[20, 21]</sup>. In the SPT from *S. multivorum* the L-serine carboxylate group did not interact with an arginine at all but instead contacted two water molecules held in place via interactions with S81 and M271 from the opposite monomer<sup>[21]</sup> (Fig. 2A). In contrast, in *S. paucimobilis* SPT the L-serine carboxylate of the PLP:L-serine complex interacted with the non-conserved R378 from the same monomer which had swung in from its previously held position outside the active site in the holoform of the enzyme (Fig. 2B). Since the SPTs from both *Sphingomonas* strains display high sequence identity (71 %) and with the structure of the holo-form of the *S. wittichii* SPT in hand we explored the role of the conserved R370 residue in this SPT homolog. We suggest that *S. wittichii* SPT R370 plays a role after the binding of the L-serine substrate and PLP:L-serine external aldimine formation. This was confirmed by the observation that the formation of the external aldimine in the *S. wittichii* SPT R370K and R370A mutants was largely unaffected (Fig. 3);  $K_d$  values were approximately 2-fold and 3-fold lower in each mutant, respectively, compared to the wild-type enzyme (Table 1). To further investigate the role of R370 we assessed the ability of the mutant SPTs to form quinonoid intermediates (IV and VI, Scheme 1).



**Figure 2.** (A) External aldimine form of SPT from *S. multivorum* (PDB code 3A2B) shows that the L-serine carboxylate, fixed by H-bonding to H138, interacts with active site water molecules and not with the conserved R367. The (\*) denotes residues from the opposite monomer.(B) External aldimine form of SPT from

*S. paucimobilis* (PDB code 2W8J) shows that the L-serine carboxylate, fixed by a H-bond to H159, forms a salt bridge with R378 and not with the conserved R390.



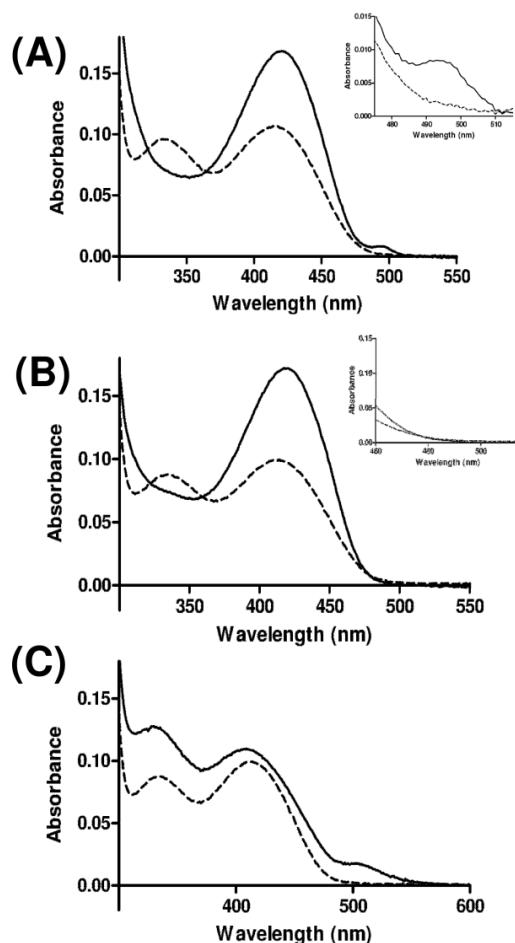
**Figure 3.** (A) Holo-form of R370K SPT containing 20  $\mu$ M enzyme (solid line). Increase of 415 nm peak due to external aldimine formation by titrating holo-SPT with increasing amounts of [L-Ser] substrate to give final concentrations of 0, 0.1, 0.4, 1.6, 12.5 mM (dotted lines). (B) Determination of  $K_d$  for binding of L-Ser to R370K.

**Table 1.** Relative activities and binding of L-serine to wild-type and mutant SPTs.

Enzyme	% Relative activity	$K_d^{L-Ser}$ (mM)
SPT <i>S. wittichii</i> wild-type	100	$0.80 \pm 0.10$
R370K	3.0	$0.46 \pm 0.04$
R370A	ND	$0.25 \pm 0.02$

Quinonoid intermediates have been observed spectroscopically under steady state conditions in SPTs and other members of the AOS family. Both quinonoid species can be distinguished by UV-vis spectroscopy; substrate quinonoids (IV, Scheme 1) absorb maximally below 500 nm while product quinonoids (VI, Scheme 1) absorb maximally above 500 nm. This clear distinction was observed with *Vibrio cholerae* CqsA which formed a substrate quinonoid at 495 nm in the presence of the amino acid substrate (*S*-2-aminobutyrate while further addition of decanoyl-CoA gave rise to the product quinonoid at 530 nm<sup>[12]</sup>. Similarly, in *E. coli* AONS, substrate and product quinonoids were observed at 486 nm and 520 nm, respectively<sup>[8]</sup>. Addition of both substrates to the SPT from

*Bdellovibrio stolpii* gave rise to a transient peak at 515 nm<sup>[32]</sup>. Similar species were not detected in the SPT from *S. paucimobilis*<sup>[20]</sup> nor for the SPT from *S. wittichii* used in this study (not shown). One way around this is to use a thioether analogue of palmitoyl-CoA which is recognised by the SPT and binds to the PLP:L-serine external form of the enzyme (Supplementary Scheme 1B). Although formation of the PLP:L-serine quinonoid intermediate can occur in the presence of the analogue, progression to the  $\beta$ -keto acid intermediate cannot be achieved because the analog lacks a thioester group. This trick allows one to observe substrate quinonoids under steady-state conditions that would not be detected in the presence of the native substrate<sup>[29]</sup>. Using this method, a substrate quinonoid was detected at 495 nm in wild-type SPT from *S. wittichii* (Fig. 4A) similar to the one previously detected in its homologue from *S. paucimobilis*<sup>[20, 29]</sup>. The absorbance value of the quinonoid peak was ~5% of the external aldimine peak suggesting that in the presence of the palmitoyl-CoA analogue, equilibrium lies largely in favour of the PLP:L-serine external aldimine in this SPT isoform. The quinonoid peak, although small, was discernable from baseline (Fig. 4A inset) and was stable for at least three hours at room temperature. No such quinonoid was detected in a similar experiment with the R370K and R370A mutants (Fig 4B) supporting the proposal that this arginine residue is necessary in orientating the carboxyl group of the PLP:L-serine:acyl-CoA ternary complex<sup>[21]</sup>. Titration of wild-type (not shown) and mutant SPTs with KDS gave rise to a new peak at 505 nm (Fig. 4C, solid line) indicative of a product quinonoid species (VI, Scheme 1). This species lacks the carboxyl group, supporting further that R370 forms a salt bridge with the L-serine carboxylate in the ternary complex prior to decarboxylation.



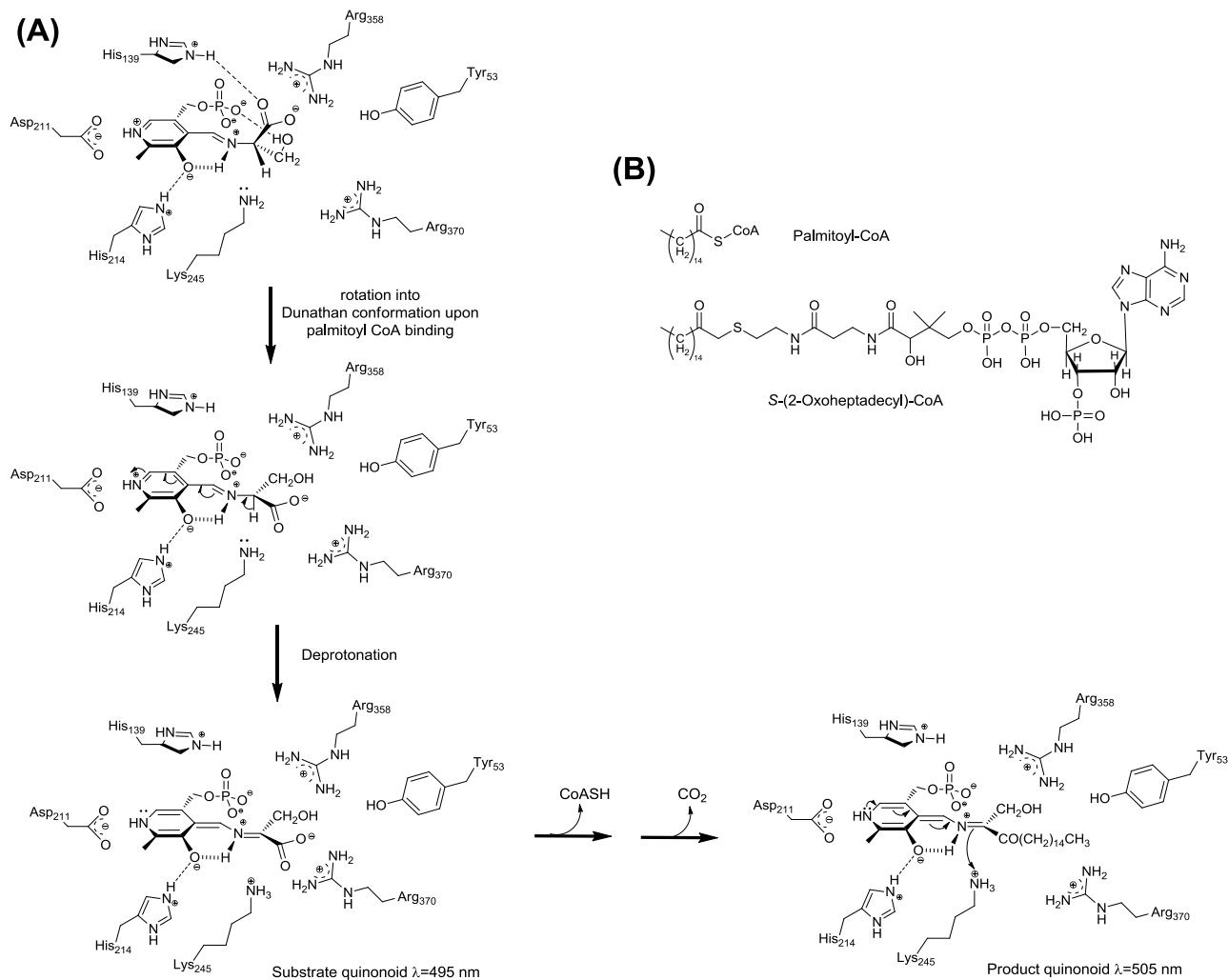
**Figure 4.** (A) Addition of 20 mM L-Ser and 500  $\mu$ M thioether analog (solid line) to 20  $\mu$ M wild-type holo-SPT (broken line). The quinonoid intermediate was visualised as a new peak at 495 nm (inset). (B) Addition of 20 mM L-

Ser and 500  $\mu$ M thioether analog (solid line) to 20  $\mu$ M R370K holo-SPT (broken line). No quinonoid intermediate was visualised (inset). (C) The holo- form of the SPT R370K (dotted line). Addition of 25  $\mu$ M KDS to 20  $\mu$ M R370K holo-SPT (broken line) resulting in formation of a product quinonoid (solid line). The product quinonoid was observed as a new peak at 505 nm (solid line).

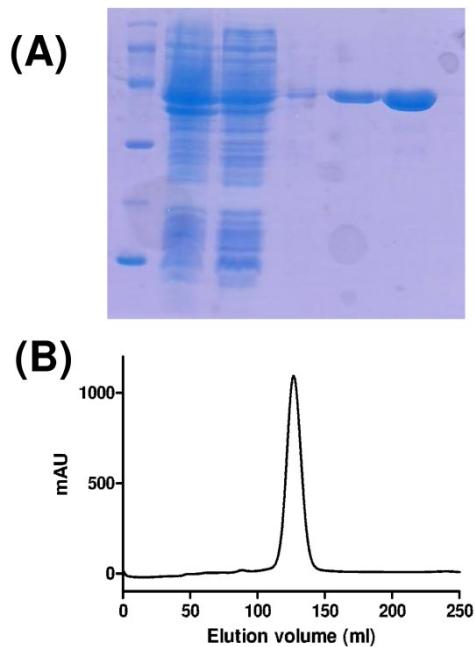
Compared to wild-type, no catalytic activity (monitoring CoASH product formation) was detected in the R370A mutant, while only 3% was recorded in the R370K mutant (Table 1). Such poor activity was not due to the instability of the mutant SPTs; the mutants behaved essentially like the wild-type enzyme with respect to expression and purification (Supplementary Fig. 1A). The mutant enzymes eluted in a single symmetrical peak from a size exclusion column indicating that dimer formation was not disrupted (Supplementary Fig. 1B). In addition, the UV-visible spectra of the mutant holo-SPTs were similar to wild-type indicating that the PLP cofactor was not perturbed (Fig. 4A, B and C; broken line) and taken together, these data suggest that R370 is crucial to enzymatic activity. It is worth noting that the equivalent arginine residue is absent from the presumably non-catalytic lcb1 subunits found in the eukaryotic SPT isoforms but conserved in the “active” lcb2 subunits (Fig. 1C).

To gain further structural insight into the role played by the R370 residue we investigated the structures of AOS members in the PDB. Structures of PLP:glycine complexes captured in CqsA (formed by a retro-aldol reaction of L-threonine) and *R. capsulatus* ALAS revealed that the glycine carboxylate forms a salt bridge with R358 and R374, respectively. In SPT the carboxylate is fixed by a hydrogen bond to H159 and so points away from R390, but no such interaction is present in these two homologues (Supplementary Figure 2). Removal of this H159 residue resulted in a severely compromised SPT that underwent abortive transamination to give pyridoxamine 5'-phosphate but did stabilise the KDS-product quinonoid<sup>[24]</sup>. The observed interaction between the conserved arginine and the amino acid substrate of the external aldimines in both CqsA and ALAS, and possibly other AOS enzymes, does not exclude a subsequent function for this residue in the ternary complex when the acyl-CoA substrate binds. Interestingly, in murine erythroid ALAS a R439L mutant had no detectable activity<sup>[33]</sup> and modification of R368 by phenylglyoxal in KBL from *E. coli* resulted in an irreversibly inactivated enzyme<sup>[34]</sup>. Also, Kelly *et al.* recently reported <0.01 % activity in a R358A *V. cholerae* CqsA mutant<sup>[12]</sup>. Likewise, in the fold-type I aspartate aminotransferase from *E. coli*,  $k_{cat}$  was reduced to 0.2% compared with wild-type in a R386K mutant<sup>[35]</sup>. Therefore, mutation or modification of the arginine residue renders the enzyme inactive and mirrors the effect of mutating R370 observed here with the *S. wittichii* SPT. These observations suggest that across the AOS family the conserved arginine plays a more crucial catalytic role rather than simply binding the amino acid substrate. It is worth noting that a recent mutagenesis study in dialkylglycine decarboxylase (which is PLP-dependent, but not an AOS member) shows that R406, which is structurally aligned with R370, not only binds substrate but also facilitates decarboxylation<sup>[36]</sup>. Our study sheds further light on how active site residues from members of the AOS family control substrate binding and catalysis in a complicated, multi-step chemical reaction.

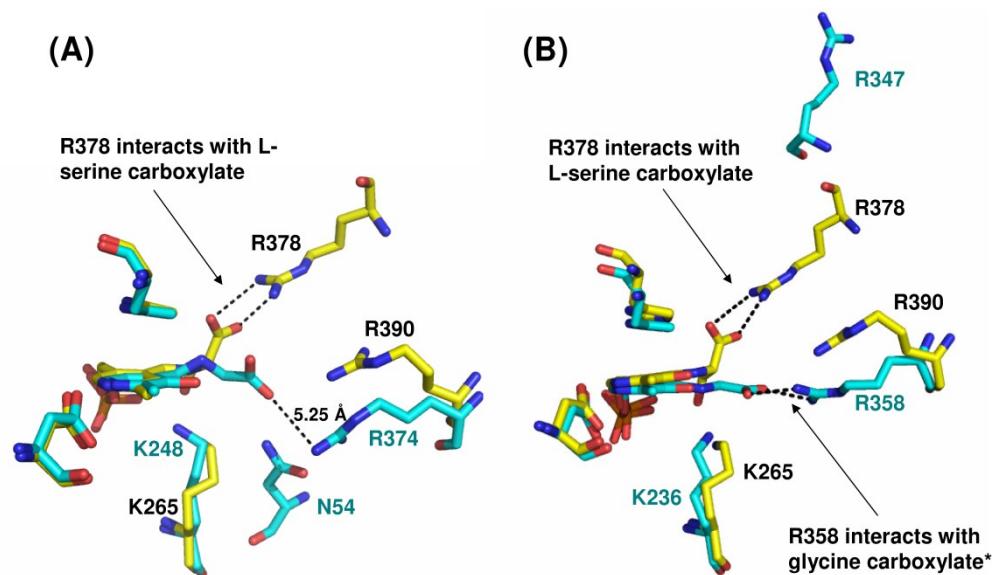
## Supplementary material



**Supplementary Scheme 1.** (A) Arg<sub>370</sub> is crucial for forming the substrate quinonoid via the Dunathan intermediate but does not interact with the product quinonoid in SPT from *S. wittichii*. (B) Structure of palmitoyl-CoA and the thioether analogue.



**Supplementary Figure 1.** (A) Expression of R370A SPT and purification by Ni-NTA affinity chromatography (B) Size exclusion chromatography of R370A on Sephadryl S-200 column.



**Supplementary Figure 2.** (A) Overlay of *S. paucimobilis* SPT (yellow) PLP:L-serine external aldimine (PDB code 2W8J) and *R. capsulatus* ALAS (cyan) PLP:glycine external aldimine (PDB code 2BWP). (B) Overlay of *S. paucimobilis* SPT (yellow) PLP:L-serine external aldimine (PDB code 2W8J) and *V. cholerae* CqsA (cyan) PLP:glycine external aldimine formed by a retro-aldol reaction from L-threonine (PDB code 2WK9).

(A)

<b>Enzyme</b>	<b>Organism</b>	<b>Numbering</b>
SPT	<i>Sphingomonas wittichii</i>	R370
SPT	<i>Sphingomonas paucimobilis</i>	R390
SPT	<i>Zymomonas mobilis</i>	R372
SPT	<i>Sphingobacterium spiritivorum</i>	R367
SPT	<i>Sphingobacterium multivorum</i>	R367
SPT	<i>Bdellovibrio stolpii</i>	R387

(B)

<b>Enzyme</b>	<b>Organism</b>	<b>Numbering</b>
SPT	<i>Sphingomonas wittichii</i>	R370
SPT	<i>Sphingomonas paucimobilis</i>	R390
AONS	<i>Escherichia coli</i>	R361
KBL	<i>Escherichia coli</i>	R368
ALAS	<i>Rhodobacter capsulatus</i>	R374
CqsA	<i>Vibrio harveyi</i>	R358

(C)

<b>Enzyme</b>	<b>Organism</b>	<b>Numbering</b>
SPT	<i>Sphingomonas wittichii</i>	R370
lcb2	<i>human</i>	R509
lcb2	<i>yeast</i>	R496
lcb2	<i>plant</i>	R441

**Supplementary Table 1.** Numbering for conserved residue *S. wittichii* R370 in (A) bacterial SPTs (B) structural homologues in the AOS family and (C) lcb2 subunits of eukaryotic SPTs.

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