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L-Penicillamine is a mechanism-based inhibitor of serine palmitoyltransferase by forming a pyridoxal-5′-phosphate-thiazolidine adduct**

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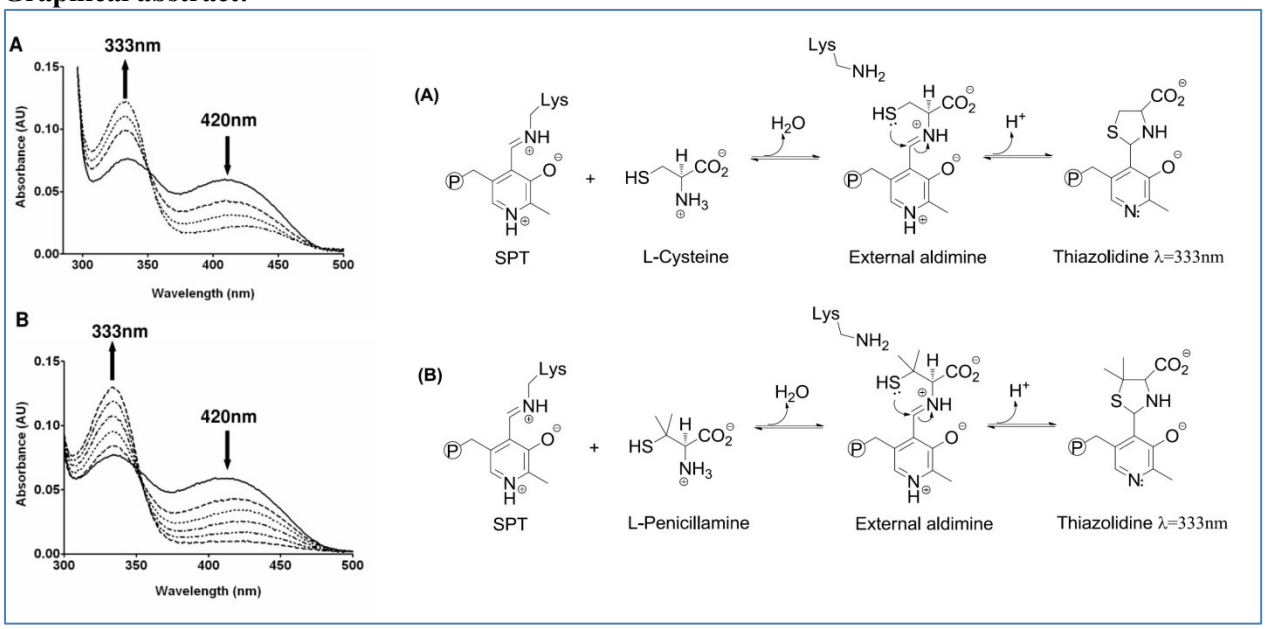
Graphical abstract:



Keywords

Serine palmitoyltransferase, pyridoxal 5'-phosphate, L-penicillamine, Lcysteine, aminothiols, thiazolidine, sphingolipids, *Sphingomonas paucimobilis*.

Abbreviations

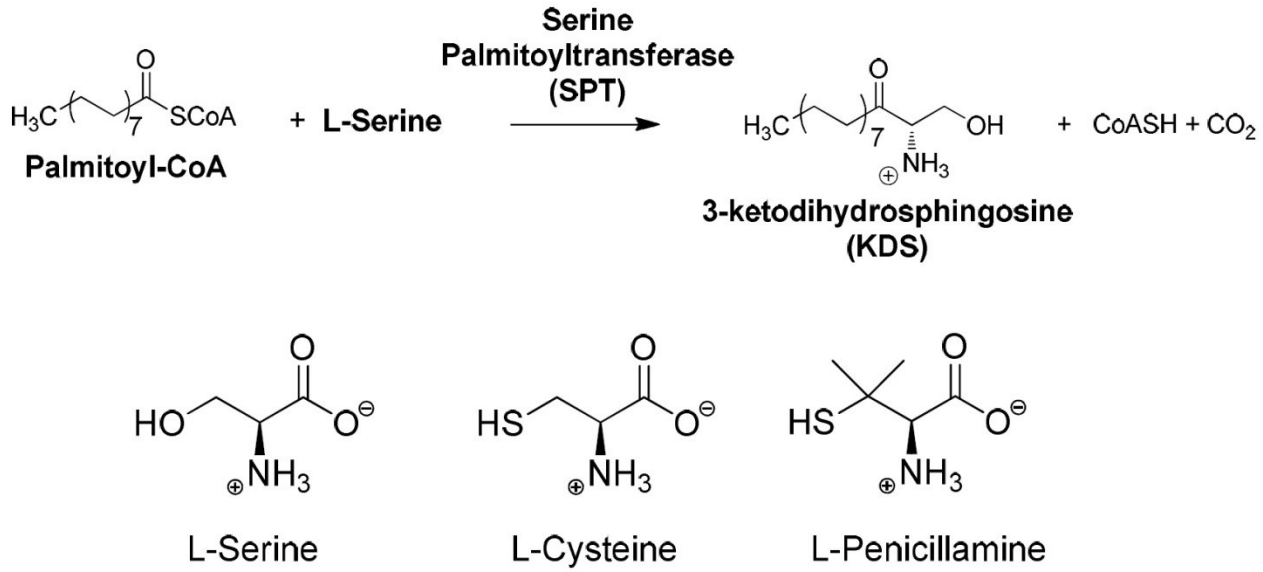
SPT, serine palmitoyltransferase; PLP, pyridoxal 5'-phosphate; KDS, 3-ketodihydrosphingosine, CoA, coenzyme A; AOS, alpha oxoamine synthase; Ser, serine; Pen, penicillamine; Cys, cysteine; pNBA, para-nitrobenzoic acid.

Abstract

Serine palmitoyltransferase (SPT) catalyses the first committed step of *de novo* sphingolipid biosynthesis. The bacterial SPT homologue from*Sphingomonas paucimobilis* is a homodimeric enzyme that contains an essential pyridoxal-5′-phosphate (PLP) cofactor bound to each subunit. Inhibitors of SPT are useful blockers of sphingolipid biosynthesis. Here we use UV-vis spectroscopy, enzyme kinetics and mass spectrometry to investigate inhibition of SPT by penicillamine (Pen), a drug with a range of useful medicinal applications.

Introduction

Penicillamine (Pen) is a sulfur-containing, non-proteinaceous amino acid (Scheme 1) and a degradation product of all penicillins following hydrolysis of the β-lactam ring.[1] Pen can be used therapeutically as an efficient copper chelator in Wilson's disease[2,3] since it is not degraded in humans. In contrast, the bacterium *Bacillus sphaericus* expresses an unusual NAD-dependent Pen-degrading enzyme that allows the organism to utilise Pen as a sole nitrogen source.[4] The naturally occurring Pen isomer is of the D-configuration but L-penicillamine is toxic.[5] Both L-Pen and D-Pen can interact with the vitamin cofactor pyridoxal-5′-phosphate (PLP)[6] but L-Pen is generally a better inhibitor of PLP-dependent enzymes because it is a better mimic of the L-amino acid substrate for these enzymes. Inhibition of several PLP-dependent enzymes by L-Pen or D-Pen or a racemic mixture have been previously studied; these include alanine aminotransferase,[7] aspartate aminotransferase,[8] glutamate decarboxylase,[9] histidine decarboxylase[10] and serine hydroxymethyl transferase.[11] Ingestion of D-Pen can also indirectly reduce activity of the PLP-dependent enzyme kynureninase by lowering pyridoxine levels.[12]



Scheme 1. Reaction catalysed by SPT and structures of L-serine (L-Ser), L-cysteine (L-Cys) and L-penicillamine (L-Pen).

Serine palmitoyltransferase (SPT) is a PLP-dependent homodimeric enzyme that catalyses the first step of *de novo* sphingolipid biosynthesis; a Claisen-like condensation of the amino acid L-serine (L-Ser) with palmitoyl-CoA to form 3-ketodihydrosphingosine. Crystal structures of the PLP-bound form (holo-SPT)[13] and PLP:L-Ser complex (external aldimine)[14] of a bacterial SPT from *Sphingomonas paucimobilis* have provided an insight into how the PLP cofactor and the L-Ser substrate are bound at an active site comprising residues from both subunits.[13,14] They have enabled studies to pinpoint active site residues essential to enzyme function.[15] The PLP cofactor is essential in a catalytic mechanism involving several crucial steps; breaking of a C–H bond (deprotonation at C_α of L-Ser), formation of a C–C bond (nucleophilic attack of the C_α carbanion at the thioester of palmitoyl-CoA), and breaking of a C–C bond (decarboxylation of the β-keto-acid intermediate). The variety of chemistry that SPT can carry out was emphasised by our laboratory in a recent study that uncovered a novel mechanism for inhibition of PLP-dependent enzymes by the antibiotic L-cycloserine.[16] Instead of forming a typical PLP–isoxazole adduct, SPT hydrolysed the cycloserine ring to form pyridoxamine 5′-phosphate (PMP) and an aldehyde product.

Interestingly SPT is not the only PLP-dependent enzyme found on the sphingolipid metabolic pathway; the homodimeric sphingosine-1-phosphate lyase is also dependent on PLP to catalyse the irreversible breakdown of the essential signalling molecule sphingosine-1-phosphate.[17,18] Therefore, studies of inhibitors that target the cofactor in PLP-dependent enzymes are of great interest therapeutically in the context of sphingolipid

biology. We extend our analysis by investigating the interaction between SPT and Pen and use a combination of enzyme assay, UV-vis spectroscopy and mass spectrometry to probe the inhibition mechanism.

Materials

Plasmids and *Escherichia coli* competent cells were purchased from Novagen. All buffers and reagents including L-Pen and D-Pen were from Sigma. Palmitoyl-CoA was from Avanti.

Methods

Cloning and expression of *S. paucimobilis* SPT wild-type enzyme: The SPT wild-type gene was cloned in pET28a expression vector (Novagen) as previously described.[14] Plasmids were transformed into *E. coli* BL21 (DE3) competent cells by heat shock at 42 °C and cells harbouring the vector were selected on LB agar plates containing 30 μg ml^{-1} kanamycin. A single colony was used to inoculate an overnight culture grown in 500 ml 2YT media (16 g l^{-1} Bacto-tryptone, 10 g l^{-1} Bacto-yeast extract, 5 g l^{-1} sodium chloride (pH 7.5)). This culture was added to 4 litres of 2YT supplemented with kanamycin and grown to OD_{600} of 0.6 before addition of isopropyl 1-thio-β-D-galactopyranoside to induce protein expression. Growth was continued for 5 hours at 30 °C before harvesting the cells by centrifugation (Sorvall RC5B centrifuge) at 4000 rpm for 15 minutes at 10 °C. Recombinant SPT was purified using IMAC on nickel resin (Qiagen). Prior to UV-vis spectroscopy and inactivation assays, the SPT was dialysed against buffers containing 25 μM PLP to ensure the enzyme was in the PLP-bound, holo-form.

UV-visible spectroscopy of SPT inhibition by aminothiols: All UV-visible spectra were recorded on a Cary 50 UV-visible spectrophotometer (Varian) and analysed using Cary WinUV software (Varian). Enzyme was dialysed against 20 mM potassium phosphate (pH 7.5) containing 150 mM NaCl and 25 μM PLP for 4 hours at 4 °C. Excess PLP was removed on a PD-10 (Sephadex G-25M) desalting column (GE Healthcare). For UV-visible assays the concentration of protein was 20 μM. The spectrophotometer was baseline-corrected with 20 mM potassium phosphate (pH 7.5) containing 150 mM NaCl and spectra were collected from 800 nm to 200 nm. Quartz cuvettes from NSG Precision Cells, Type 18-BM had a lightpath of 10 mm and a sample volume of 500 μl. Spectra were collected at intervals following the addition of inhibitor by setting the instrument to cycle mode.

SPT activity assays following inactivation by aminothiols: SPT activity was measured using a DTNB assay as previously described.[14] Assays contained enzyme, substrates and DTNB with final concentrations as follows: 0.25 μM *Sp*SPT, 25 mM L-serine, 250 μM palmitoyl-CoA, 0.2 mM DTNB in 20 mM HEPES buffer, pH 8.0. Because aminothiols react instantaneously with DTNB, SPT samples inactivated by L-Pen were

dialysed prior to activity measurements. Control *Sp*SPT samples in the absence of inhibitor were also dialysed in the same way and these rates were normalised to 100% relative activity. Activity assays were carried out in duplicate but the error bars in Fig. 1 are standard error of the mean (SEM) values for three separate inactivation experiments ($n = 3$).

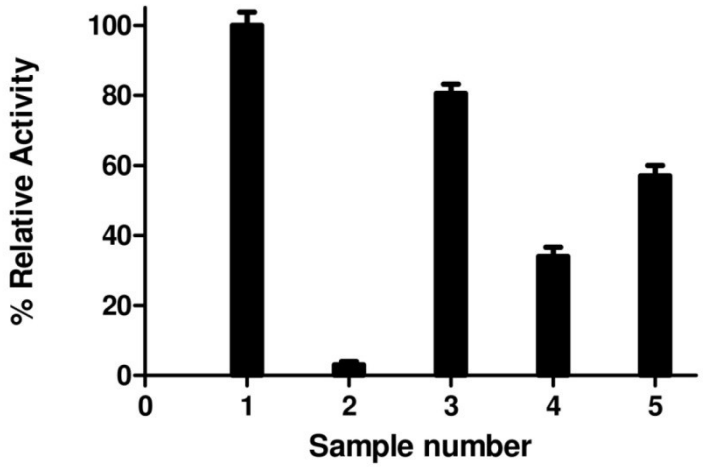


Figure 1. Enzyme activity of Pen- and Cys-inactivated *Sp*SPT is regenerated by dialysis against PLP buffer. Enzymatic rates were measured using a DTNB assay and plotted as % relative activity compared to an enzyme sample containing no inhibitor. All enzyme samples contained 20 μM SPT and were incubated for 30 minutes with the following inhibitors (1) no inhibitor (2 and 3) 5 mM L-Pen (4 and 5) 5 mM D-Pen (6 and 7) 5 mM L-Cys (8 and 9) 5 mM D-Cys. Samples 1, 2, 4, 6 and 8 were dialysed against buffer with no PLP. Samples 3, 5, 7 and 9 were dialysed against buffer containing 50 μM PLP.

Analysis of SPT by native mass spectrometry: *Sp*SPT samples for mass spectrometry analysis were buffer exchanged into 10 mM ammonium acetate buffer (pH 7.4) on a PD-10 (Sephadex G-25M) desalting column (GE Healthcare) prior to inactivation with 5 mM L-Pen. Inactivation was observed by monitoring changes in the UV-vis spectrum of the SPT-bound PLP as described above. Native mass spectrometry data was acquired on a SolariX FT-ICR mass spectrometer equipped with a 12 Tesla superconducting magnet (Bruker Daltonics). Samples, typically 2 μM, were treated with 0.5%*para*-nitrobenzoic acid prior to analysis; and ESI ionisation was employed using a syringe pump operating at a flow of 500 μl h^{-1}. RF frequencies used in all ion-transmission regions were the lowest available value: multipole 1 (2 MHz), quadrupole (1.4 MHz) and transfer line (1 MHz). Ion-funnel and skimmer voltages were 200 V (funnel 1), 130 V (skimmer 1), 8 V (funnel 2) and 3 V (skimmer 2). The collision voltage for the collision cell was varied between 10 and 20 V, to produce optimal signal. Ions were accumulated for 400 ms in the RF-hexapole ion trap before being

transmitted to the infinity ICR trap and detected between *m*/*z* 1000 and 5000 to yield a broadband 1 M time-domain transient. Typically, each spectrum was the sum of 100 mass analyses. Mass spectra were externally calibrated using ES tuning mix (Agilent). The resulting spectra were analysed using DataAnalysis software (Bruker Daltonics); spectra were smoothed using the Gaussian algorithm and a smooth width of 0.15 *m*/*z*, before maximum entropy deconvolution was performed.

Results and discussion

To investigate L-Pen and D-Pen as potential SPT inhibitors, enzyme activity was assayed following incubation of *Sp*SPT with 5 mM aminothiol (L-Pen or D-Pen) for 30 minutes at 25 °C. Activity assays used here monitor the release of enzyme-produced CoASH in the presence of the disulfide reagent DTNB.[14] Reaction of the DTNB reagent with the penicillamine and cysteine free thiol in inactivated enzyme samples resulted in high background absorbances that prevented us from a full kinetic evaluation. Because of this all samples in Fig. 1 were dialysed to remove excess inhibitor prior to activity measurements. Activities are presented as % activity relative to an enzyme sample in the absence of inhibitor. SPT activity was reduced to 3% following incubation with 5 mM L-Pen but inhibition with D-Pen reduced SPT activity to ~34% indicating specificity for the L-Pen enantiomer. Recently we observed similar enantioselectivity for inactivation of SPT by cycloserine, inhibition being ~15-fold greater with L-cycloserine compared to the D-cycloserine enantiomer.[16] In contrast inhibition by L-Cys and D-Cys reduced SPT activity to 24% and 22%, respectively. This not only shows that L-Pen is a more potent SPT inhibitor than Cys but there appears to be no stereospecificity for the Cys inhibitor. Interestingly previous work using a L-[^{3}H]-serine substrate showed that D-serine inhibited the eukaryotic SPT complex as effectively as unlabelled L-serine.[19] SPT samples inactivated with either L-Pen or D-Pen were restored to ~80% and 57% activity, respectively, following dialysis against buffer containing 50 μM PLP. Enzyme activity was regenerated to 83% and 76% for SPT samples inactivated with L-Cys and D-Cys, respectively. This suggests that the inhibition is reversible to a large extent and that the mechanism of SPT inactivation by Pen occurs by disabling the PLP cofactor. In our experience complete removal of PLP by extensive dialysis to form an apo-SPT possibly leads to collapse of the active site such that it is not possible to regenerate holo-enzyme in high yield by either titration of PLP or dialysis against a PLP-buffer. To get around this we exchanged, rather than removed, the modified cofactor following inactivation by dialysis against buffer containing fresh PLP. This explains successful regeneration of SPT activity in these samples although it appears that some active enzyme was sacrificed since only ~80% activity was recovered. An alternative explanation for inability to recover 100% activity is the possibility of additional competing reactions that irreversibly modify the protein, although we did not detect these during our analysis. To further investigate the mechanism of SPT inactivation we next monitored changes in the UV-visible spectrum of the PLP in the presence of both forms of the inhibitor. We also investigated inhibition of

SPT by L-cysteine (L-Cys) to examine whether inactivation of SPT by all aminothiols occurs *via* a common mechanism.

L-Cysteine contains a thiol sidechain where L-serine has a hydroxyl group (Scheme 1). The thiol group in L-Cys is more nucleophilic than the hydroxyl counterpart in L-Ser. ThereforeL-Cys contains, along with the nucleophilic amine, two functional groups that could potentially react with the electrophilic aldehyde carbon in free PLP. We investigated the reactivity of the thiol group by monitoring changes in the UV-visible spectrum of free PLP after addition of *N*-acetyl-L-cysteine (NAC, in this L-Cys analogue the nitrogen is blocked as an amide). Free PLP absorbs maximally at 390 nm in potassium phosphate buffer (pH 7.5) and following addition of NAC there was a small decrease in the peak at 390 nm which did not change over time (Fig. 2A). Several reports suggest PLP:thiazolidine (PLP:TA) formation occurs *via* a Schiff base intermediate rather than a thiohemiacetal.[20–22] Addition of L-Cys to free PLP resulted in the immediate loss of the 390 nm peak which slowly decreased over time with a concomitant increase in a new peak centred at 333 nm (Fig. 2B). A similar 333 nm peak was observed when L-Pen was added to PLP (Fig. 2C).

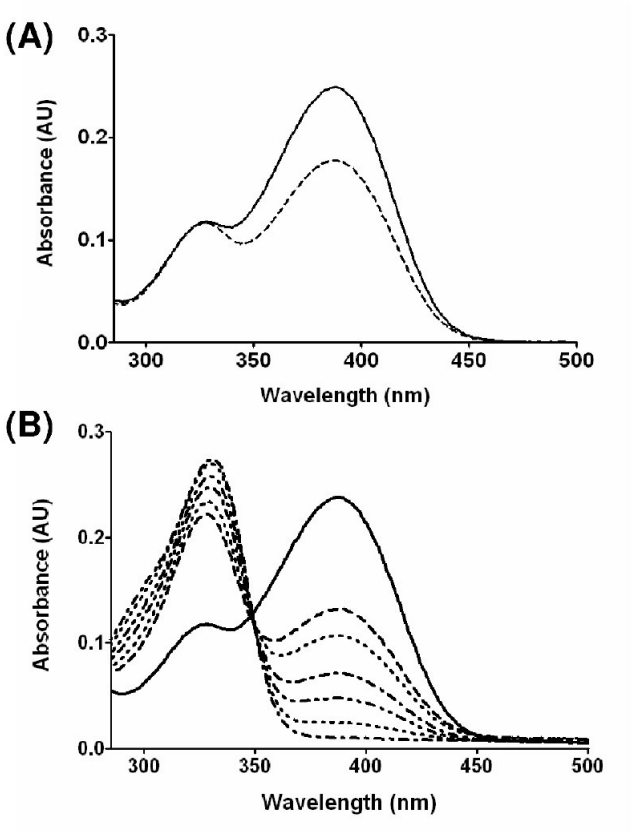


Figure 2. (A) 50 μM PLP in 20 mM potassium phosphate buffer pH 7.5 (solid line) and after the addition of 10 mM *N*-acetyl-L-cysteine (B) time-dependent formation of a thiazolidine adduct (333 nm peak) over 20 minutes upon addition of 10 mM L-Cys to 50 μM PLP in 20 mM potassium phosphate buffer pH 7.5. (C) Time-dependent formation of a thiazolidine adduct (333 nm peak) over 20 minutes upon addition of 10 mM L-Pen to 50 μM PLP in 20 mM potassium phosphate buffer pH 7.5.

In holo-SPT the PLP cofactor is bound in two forms, the enolimine (λ_{max} = 335 nm) and the ketoenamine (λ_{max} = 420 nm) (Fig. 3A, solid line). In the *Sp*SPT isozyme we find the two forms to be present in approximately equal proportions at equilibrium. Addition of 10 mM L-Cys to SPT resulted in a time-dependent decrease and broadening of the 420 nm peak with concomitant appearance of a new peak at 333 nm (Fig. 3A, broken lines). We suggest that this peak represents formation of a PLP:TA adduct at the enzyme active site, identical to the one formed in the model studies between L-Cys and free PLP. Given that transimination at Lys265 occurs when SPT binds its natural substrate L-Ser,[14] we predict that thiazolidine formation in SPT occurs *via* a PLP:L-Cys external aldimine (Scheme 2). We noted that thiazolidine formation with L-Cys was slow and decrease of the 420 nm peak did not reach baseline after 30 minutes (Fig. 2A). In contrast, thiazolidine formation in SPT appeared to reach completion in the same time period following addition of 10 mM L-Pen (Fig. 3B). This difference is likely to be due to the *gem*-dimethyl group through a Thorpe–Ingold effect favouring the cyclic form of the penicillamine.[23] In keeping with our activity data in Fig. 1, inactivation with D-Pen was slower and did not reach completion after 30 minutes suggesting increased specificity for the L-enantiomer in SPT (data not shown).

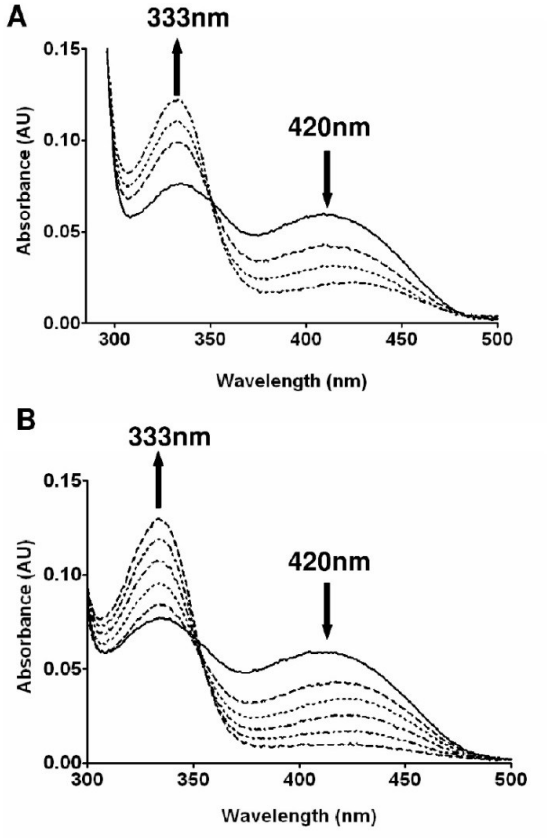
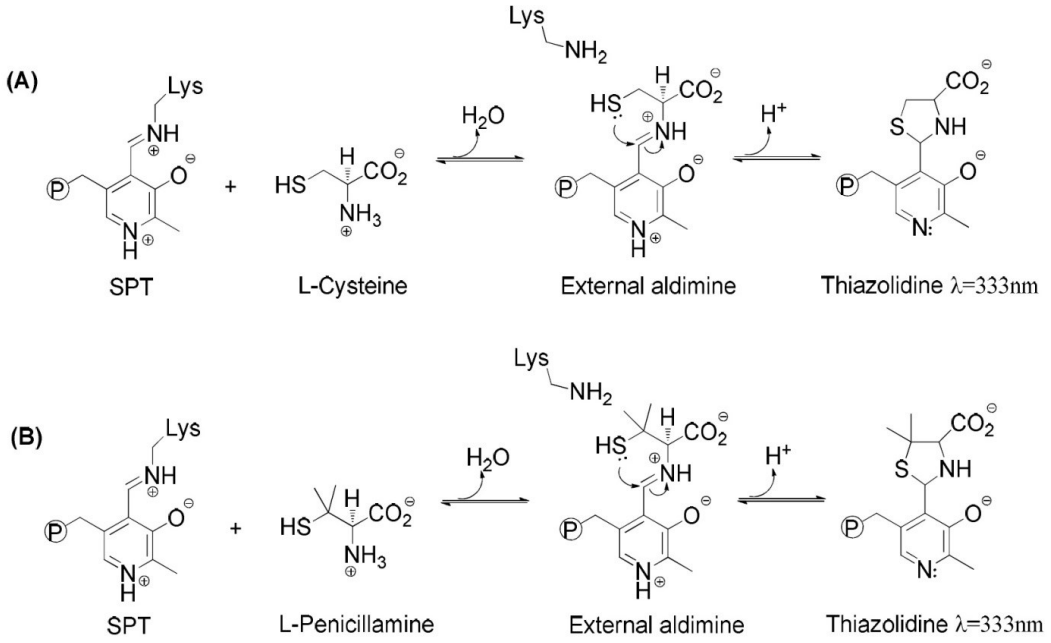


Figure 3. (A) UV-visible spectrum of 20 μM holo-SPT (solid line) shows typical peaks at 335 nm and 420 nm due to enolimine and ketoenamine forms of the PLP cofactor respectively. Addition of 10 mM L-Cys to holo-SPT (broken lines) led to formation of a thiazolidine adduct (333 nm peak) over a 30 minute period, with

concomitant loss of the 420 nm peak (B) improved thiazolidine formation in SPT over 30 minutes by addition of 10 mM L-Pen.



Scheme 2. Addition of (A) L-Cys or (B) L-Pen to SPT leads to formation of a PLP:TA adduct *via* an external aldimine intermediate (P represents phosphate, $-OPO_3^{2-}$).

The PLP:TA adduct was removed by dialysis against 20 mM potassium phosphate buffer (Fig. 4A) indicating it was bound as a non-covalent adduct in the enzyme active site. Analysis of the filtrate following immediate filtration of the L-Pen-inactivated SPT sample through a 5000 MWCO membrane confirmed that the adduct was in the low molecular weight fraction (Fig. 4B). In addition, dialysis of the L-Pen-inactivated SPT sample against 20 mM potassium phosphate buffer (pH 7.5) in the presence of PLP regenerated holo-SPT (Fig. 4C).

Formation of the L-Cys-derived PLP:TA model adduct in Tris buffer resulted in appearance of a typical 333 nm peak (Fig. S1A‡). However after prolonged incubation the 333 nm peak decreased and the 420 nm peak was regenerated, indicating that thiazolidine formation is reversible (Fig. S1‡); a similar observation was reported previously by Terzuoli *et al* [24] with free PLP and L-Cys. In contrast reversibility was not observed in an identical experiment carried out in potassium phosphate buffer (not shown). Tris buffer, which has been reported previously to form a Schiff base with PLP,[25] may be required to catalyse the reverse reaction. Reversibility of thiazolidine formation has been reported previously,[24,26] a characteristic utilised recently in producing a novel dynamic combinatorial library.[27] To confirm thiazolidine formation in SPT we next analysed the SPT complexes by mass spectrometry. Analysis of the low molecular weight material separated from the L-Pen-inactivated SPT sample (Fig. 4B) by ESI-MS in both negative and positive mode was unsuccessful. Therefore to confirm thiazolidine formation in SPT we used ESI-FT-ICR mass spectrometry to

analyse the inactivated enzyme samples under native conditions. Prior to analysis 0.5% *para*-nitrobenzoic acid (*p*NBA) was added to the sample to aid charging of the protein complex in the gas phase. The spectrum of inactivated *Sp*SPT displayed a charge state distribution from $^{[}M + 21H^{]21+}$ to $^{[}M + 27H^{]27+}$ (Fig. 5A). Maximum entropy deconvolution of the spectra revealed three signals corresponding to three individual enzyme species (Fig. 5B). The observed average masses of each of these species were (i) 92 408.5 (ii) 92 559.5 and (iii) 92 707.5 Da. These masses are in agreement with calculated average masses of (i) holo-SPT homodimer with PLP bound*via* a Schiff base to Lys265 in each active site (calculated average mass 92 409 Da), (ii) holo-PLP:TA-SPT mixed dimer with PLP bound at one active site but a PLP:TA adduct bound in the other (calculated average mass 92 558 Da) and (iii) PLP:TA-SPT homodimer with a PLP:TA adduct bound in each active site (calculated average mass 92 707 Da) (Fig. 5C).

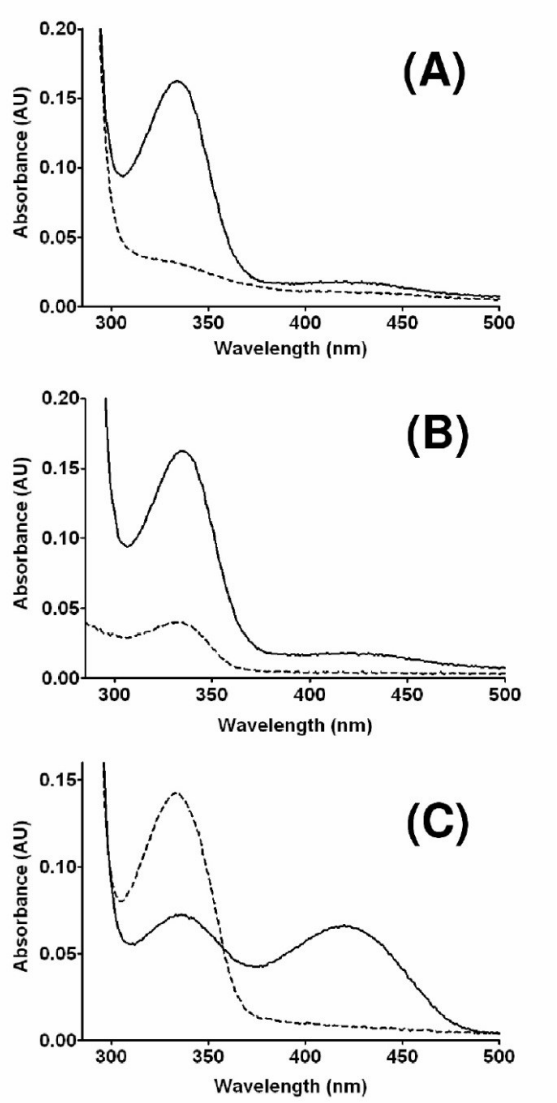


Figure 4. The PLP:TA adduct resulting from inhibition of SPT by L-Pen is non-covalently bound in the enzyme active site. (A) The L-Pen derived PLP:TA in SPT (solid line) was removed from the enzyme

following dialysis against 20 mM potassium phosphate buffer pH 7.5 (broken line). (B) A portion of the L-Pen derived PLP:TA in SPT (solid line) was removed from the enzyme following filtration through a 5000 MWCO spin filter. The low molecular weight filtrate contained the adduct (broken line). (C) The L-Pen derived PLP:TA in SPT (broken line) was replaced by PLP to regenerate holo-SPT (solid line) following dialysis against 20 mM potassium phosphate buffer (pH 7.5) containing 50 μM PLP.

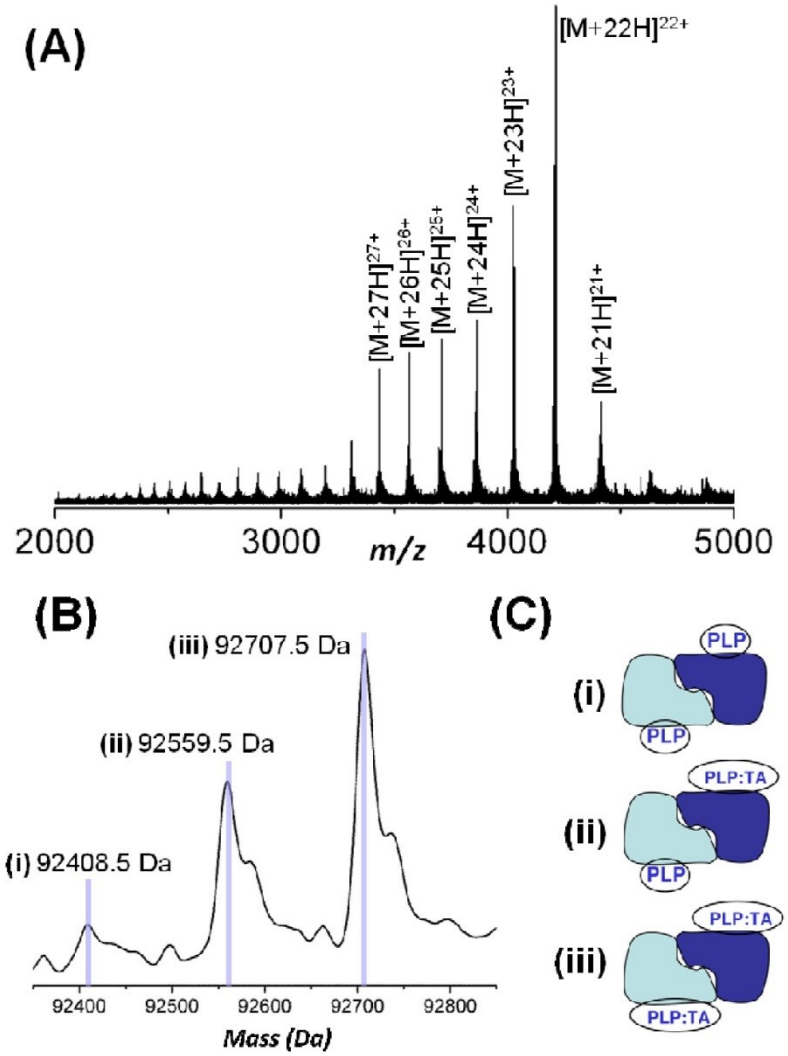


Figure 5. (A) ESI mass spectrum of holo-SPT inactivated with L-pen sprayed under native conditions. The most abundant charge states are annotated. (B) Deconvolution of the ESI spectrum revealed three species. The observed masses of each of the three signals correspond to the three species depicted in the cartoon representation (C), (i) holo-SPT mixed homodimer with PLP bound in each active site (ii) SPT homodimer with PLP bound in one subunit and the L-Pen derived PLP:TA adduct in the other (iii) SPT homodimer with a PLP:TA adduct in each subunit.

The present study shows that aminothiols that mimic L-Ser, the natural amino acid substrate of SPT, are mechanism-based inactivators of SPT with inhibition occurring by disabling of the PLP cofactor. Interestingly the PLP:TA adduct was stable in contrast to the adduct formed by the mechanism-based SPT inhibitor L-cycloserine. In that case the PLP:isoxazole adduct was metabolised to PMP and a small aldehyde product. Because L-Cys and L-Pen give rise to unsubstituted and substituted thiazolidines, respectively, that are tolerated at the SPT active site, there is now scope for structure–activity relationships investigating thiazolidines as potential inhibitors of SPT and other PLP-dependent enzymes involved in sphingolipid metabolism. Indeed modification of the sphingosine-1-phosphate lyase inhibitor THI, whereby the carbonyl group is replaced with a thiazole heterocycle, resulted in a more potent inhibitor of this key sphingolipid degrading enzyme.[28]

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