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Reconstitution of the pyridoxal 5'-phosphate (PLP) dependent enzyme serine palmitoyltransferase (SPT) with pyridoxal reveals a crucial role for the phosphate during catalysis**

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Supporting information:

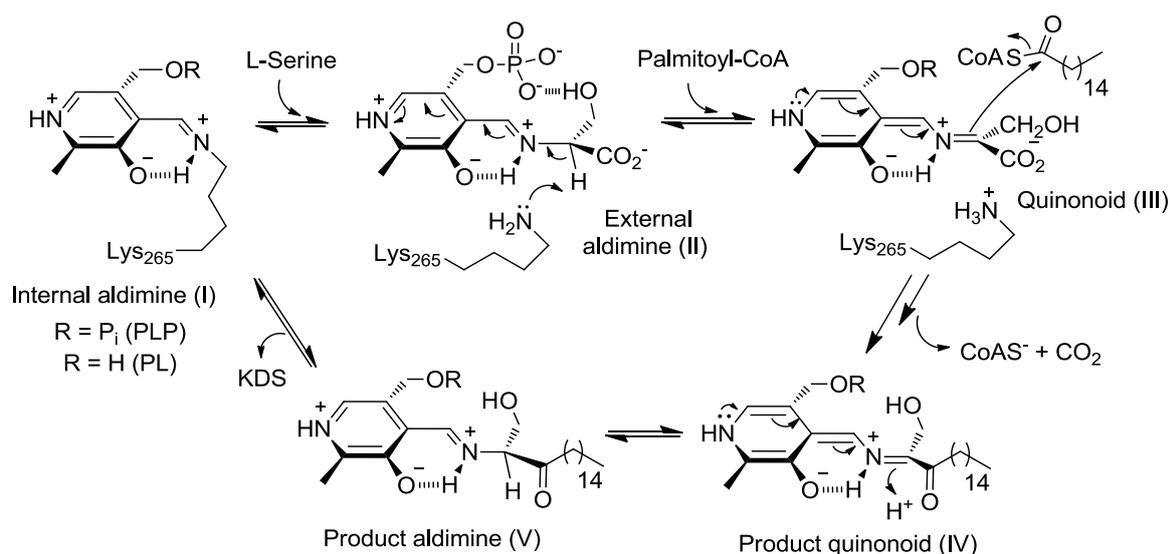
Electronic Supplementary Information (ESI) available at <http://dx.doi.org/10.1039/C3CC43001D>

Abstract

The pyridoxal 5'-phosphate (PLP)-dependent enzyme serine palmitoyltransferase (SPT) is required for *de novo* sphingolipid biosynthesis. A previous study revealed a novel and unexpected interaction between the hydroxyl group of the L-serine substrate and the 5'-phosphate group of PLP. By using pyridoxal (PL), the dephosphorylated analogue of vitamin B₆, we show here that this interaction is important for substrate specificity and optimal catalytic efficiency.

Introduction

Sphingolipid (SL) biosynthetic pathways have been extensively studied in humans, yeast and bacteria.¹ Despite their diversity, all of the pathways use the same condensation of an acyl-CoA substrate with L-serine to form 3-keto-dihydrosphingosine (KDS) to initiate *de novo* SL synthesis. In all organisms, this key step is catalysed by the pyridoxal 5'-phosphate (PLP)-dependent enzyme serine palmitoyltransferase (SPT).² PLP acts as a cofactor for a wide variety of enzyme-catalysed reactions as well as also acting as a general organic catalyst.³ For example, PLP enzymes catalyse C-C formation, amino-group transfer and racemisation reactions.⁴⁻⁶ The PLP-dependent enzyme superfamily has been classified into five different fold-types based on 3D structure.^{7,8} SPT is a member of the α -oxamine synthase family (AOS, fold type I) and a general mechanism is postulated for all members that begins with PLP bound to an active site lysine residue as an internal aldimine/Schiff's base (Scheme 1, internal aldimine I).⁹



Scheme 1. Proposed SPT mechanism showing observed intermediates and the H-bond between the 5'-phosphate and serine hydroxyl in aldimine II.

This PLP-bound lysine is documented in 229 structures in the Protein Data Bank (PDB).¹⁰ In *Sphingomonas paucimobilis* SPT (*SP* SPT) the L-serine displaces the lysine residue (Lys265) to form the PLP:L-serine external aldimine (II). Deprotonation of II gives a carbanion/quinonoid species (III) which condenses in a Claisen-like manner with the acyl-CoA thioester substrate to form a PLP:β-ketoacid, which then decarboxylates to generate the PLP:KDS product quinonoid (IV). This then reprotonates to form PLP:KDS aldimine (V) which is finally displaced by Lys265.

The PLP cofactor contains four functional groups of interest; aldehyde, hydroxyl, pyridine and phosphate. Recently, the importance of the pyridine nitrogen has been studied in three PLP enzymes from two fold types.¹¹ By replacement of the natural cofactor by the carbocyclic analogue, 1-deazapyridoxal 5'-phosphate (deazaPLP) it was demonstrated that not all PLP-dependent enzymes require the protonated pyridine nitrogen to stabilise carbanion formation of the PLP-substrate aldimine.¹²

Surprisingly, the role of the 5'-phosphate group of PLP has been less well studied. Our interest in its role arose when we observed an unexpected H-bond interaction (length 3.9 Å) between the hydroxyl group of L-serine and the 5'-phosphate in the x-ray structure of the SPT PLP:L-serine external aldimine complex II (PDB code: 2W8J).¹³ As well as this H-bond the phosphate has a number of interactions with the SPT that together make up a “phosphate binding cup” (Fig. 1).

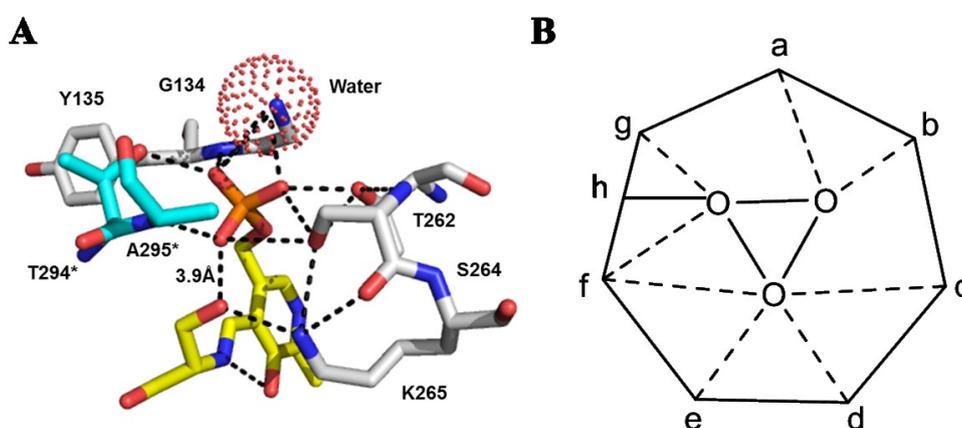


Figure 1. (A) Structure of the PLP:L-serine external aldimine intermediate (II) of *SP* SPT (PDB code: 2W8J) (B) Phosphate binding cup diagram (a) T294* (b) Y135 (c) G134 (d) water (e) T262; (f) S264 (g) A295* (h) L-serine-hydroxyl interaction (* indicates residue from opposite subunit).

The concept of this “cup” was proposed by Denesyuk *et. al* after analysis of the x-ray structures from five different fold types.^{14, 15} In *SP* SPT the cup is formed by a constellation of residues that bind the oxygen atoms but there appears to be little conservation of these across the PLP-superfamily. However, within the AOS

family (which display overall sequence homology of 30-40%) the phosphate binding ligands are well conserved (S1 and S2, ESI†). In the SPT:PLP internal aldimine structure the seven cup-forming ligands are provided by side-chains and backbones from both subunits and a water molecule. In forming the key SPT:PLP:L-serine external aldimine intermediate all of these interactions are retained and the only difference is the additional H-bond from the L-serine (Fig. 1 and S1, ESI). To probe the function of this interaction we prepared SPT with the PLP cofactor replaced by the dephosphorylated form, pyridoxal (PL). The UV-visible spectrum of the holo-form of SPT (SPT:PLP, Fig. 2A) shows peaks at 335 nm and 420 nm, characteristic of the enolimine and ketoenamine forms of PLP bound to the conserved Lys265 residue.

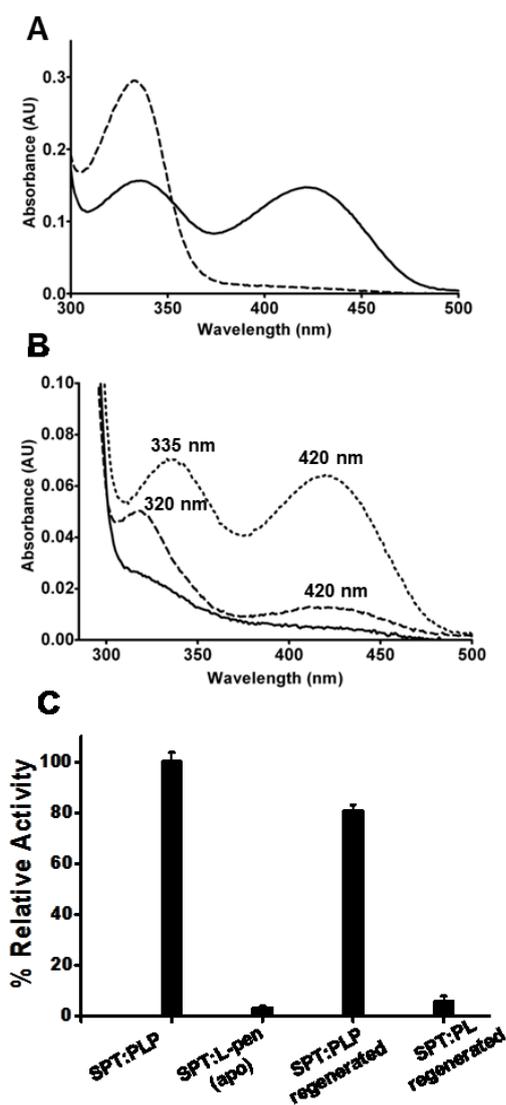


Figure 2. (A) UV-vis spectra of SPT:PLP (solid line) and after incubation with L-pen to remove the PLP (dashed line). (B) UV-vis spectra of apo-SPT (solid line), SPT:PLP formed by incubation of apo-SPT with PLP (dotted line), and SPT:PL (dashed line). Conditions: 20 mM Tris, pH 7.5 and 40 μ M enzyme. (C) Enzyme activity of SPT regenerated with PLP and PLP. Activity values are relative to the as-purified enzyme (SPT:PLP).

The PLP was removed by incubation with the aminothiols L-pencillamine (L-pen) which forms a non-covalent PLP:thiazolidine adduct which absorbs at 333 nm (Fig. 2A), that upon dialysis produces the apo-enzyme.¹⁶ In our hands the apo-SPT was prone to precipitation, and the holo-enzyme could not be recovered in high yield by simple titration of apo-SPT with PLP or dialysis against PLP-containing buffers. Therefore, both PLP and PL were added back to the enzyme by this exchange process and in doing so, we generated sufficient quantities of both regenerated SPT:PLP and SPT:PL. The SPT:PL (Fig. 2B) displays absorbance maxima at 320 and 420 nm which are shifted ~20 nm compared with SPT:PLP. It is interesting to note that the 320 nm peak was the most dominant, indicating that a different tautomer predominates in the SPT:PL complex. In order to ensure that the PL was bound to active site residue Lys265, chemical reduction of the PL-imine, followed by enzymatic digest and mass spectrometry confirmed the sequence of the peptide (S3, ESI⁺). We measured the enzyme relative activity using a continuous assay that monitors the rate of formation of the coenzyme A thiolate released during the C-C condensation reaction. The apo-SPT displayed a much reduced enzymatic activity (3%, figure 2C) and the regenerated SPT:PLP displayed 83% activity. In contrast, the regenerated SPT:PL complex displayed only 6% activity showing that catalysis in this complex was severely compromised.

We also used Michaelis–Menton enzyme kinetics to obtain kinetic constants for the regenerated SPT:PL enzyme (see Table, ESI⁺). We measured the K_m values for L-serine and palmitoyl-CoA (1.6 mM and 35.6 μ M, respectively) which are in good agreement with published data.¹³ The enzyme displayed a k_{cat} of 1.54 s⁻¹ and an efficiency (k_{cat}/K_m) of 962.5 M⁻¹ s⁻¹ for L-serine. The SPT:PLP regenerated enzyme displayed similar K_m and k_{cat} values to the as-purified SPT, with only a small increase in the K_m for L-serine (2.7 mM). In contrast, for the SPT:PL enzyme, the K_m for L-serine increased to 5 mM. The K_m for palmitoyl-CoA was unaffected (33 μ M) but we observed significantly lower k_{cat} (0.13 s⁻¹) and k_{cat}/K_m (26.3 M⁻¹ s⁻¹) values for the SPT:PL enzyme with L-serine and which are ~12 and 37-fold lower compared with the as-purified SPT. The removal of the PLP phosphate has had a combined impact by weakening L-serine binding (but not pimeloyl-CoA binding) and severely reducing the catalytic rate of the SPT:PL enzyme. This data supports our hypothesis that the L-serine:phosphate interaction plays a critical role in catalysis. In addition, we monitored KDS formation directly using a stopped assay (see methods, ESI). Using this method, no KDS was produced by the apo-SPT but the PLP regenerated SPT and the PL regenerated enzyme produced 46% and 13% KDS relative to the as-purified enzyme. This analysis provides further evidence that the removal of the phosphate impairs the catalytic activity of the SPT:PL complex.

We then sought a mechanistic basis for the requirement of the 5'-phosphate for optimal SPT activity. Addition of L-serine to SPT:PL led to the formation of a peak at 425 nm suggesting that the absence of the 5'-phosphate did not prevent the formation of the SPT:PL:L-serine external aldimine intermediate. A K_d^{L-Ser} of ~12.7 mM was obtained for the SPT:PL complex (S4, ESI) which is an 8-fold increase when compared with the L-serine K_d for SPT:PLP (1.1 mM).¹⁷ This is evidence that removing the phosphate from the cofactor impacts on serine

binding and SPT:PLP:L-serine external aldimine formation and suggests that the H-bond between the serine -OH and phosphate may play a role in substrate specificity. However, this reduced K_d for L-serine cannot, by itself, account for the dramatic reduction in activity. Therefore we also investigated if the SPT:PL:L-serine intermediate could be deprotonated by removal of the L-serine C α -H. This step generates the key carbanion/quinonoid species (III) which reacts with the incoming acyl-CoA thioester. We used the C16-CoA thioether analogue, S-(2-oxoheptadecyl)-CoA (S6, ESI $^+$) which has been shown to bind to the SPT:PLP:L-Ser complex but cannot condense with the L-serine.¹⁸ Addition of the analogue to the L-serine external aldimine form of SPT:PLP led to the appearance of a peak at 495 nm (Fig. 3A) which is thought to be the substrate quinonoid (intermediate III). In contrast, the PL-reloaded enzyme forms a broad shoulder at 495 nm under the same conditions (Fig. 3B). These results suggest that the PL cofactor may sit in a potentially ‘non-native’ environment due to the lack of the 5'-phosphate. It can still produce the external aldimine (II) with reduced affinity but crucially, it is severely compromised in its ability to form and/or stabilise the key quinonoid (III) species.

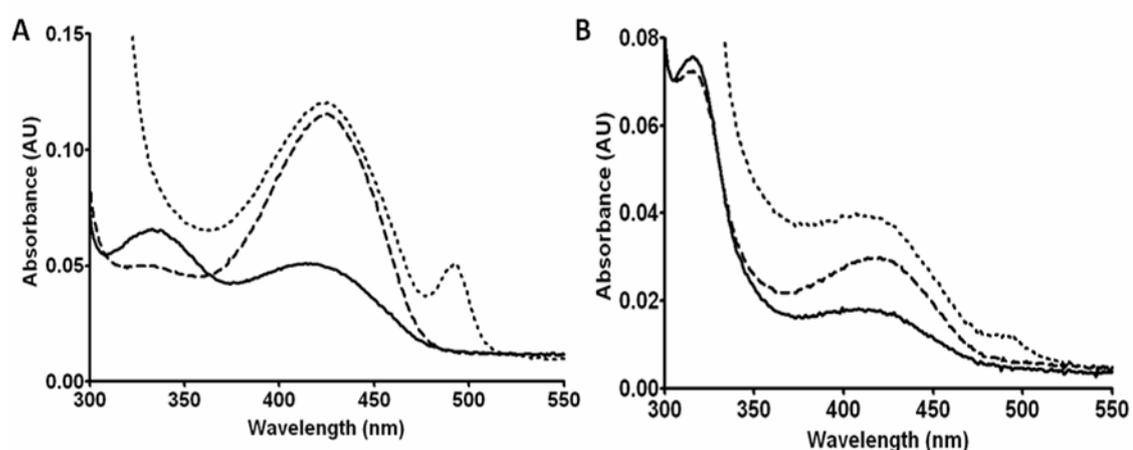


Figure 3. (A) UV-vis spectra of SPT:PLP (solid line), after addition of 40 mM L-serine (dashed line), after addition of 1.5 mM S-(2-oxoheptadecyl)-CoA (dotted line). (B) UV-vis spectra of SPT:PL (solid line), after addition of 40 mM L-serine (dashed line), after addition of 1.5 mM S-(2-oxoheptadecyl)-CoA (dotted line). Conditions: 20 mM Tris, pH 7.5 and 40 μ M SPT.

Upon addition of the product KDS to the SPT:PLP enzyme we observed changes at 335 and 420 nm, and the appearance of a peak at 505 nm (S5A, ESI) which is considered to be the SPT:PLP:KDS product quinonoid species (intermediate IV). In contrast, the SPT:PL enzyme did not form the 505 nm peak (S5B, ESI). Instead, we observed a broad signal between 330 nm and 500 nm with a shoulder at 400 nm which suggests the product binds but the PL:KDS aldimine then adopts an unusual conformation in the enzyme.

We interpret these combined results as evidence that the PL-containing enzyme cannot efficiently catalyse

the formation of the key substrate quinonoid (III). Therefore, it appears that the interaction between the 5'-phosphate of PLP and the L-serine hydroxyl group is crucial for the formation of key intermediates in the catalytic mechanism and in controlling the optimal orientation of the reactive quinonoid/carbanion that attacks the acyl-CoA thioester. This explanation lends further weight to the "Dunathan hypothesis" which was put forward over 40 years ago to explain the stereoelectronic control that the cofactor provides during PLP-dependent catalysis.¹⁹

L-alanine is a poor substrate for SPT:PLP, with a lack of detectable activity monitored at high substrate concentrations (data not shown) further emphasising the importance of the serine hydroxyl. SPT:PLP has been reported to have extremely poor affinity for L-phosphoserine with a $K_d \sim 100$ mM.²⁰ We sought to re-introduce the interaction using L-phosphoserine as a substrate for the SPT:PL enzyme. However, no substrate binding, nor detectable activity was observed, suggesting that the key phosphate-hydroxyl interaction required for optimal catalysis cannot be formed in this complex (data not shown). Older studies had looked at whether a sulphate at the 5'-position of pyridoxal could replace the phosphate.²¹ Of the three enzymes tested only arginine decarboxylase was active with this modified cofactor (23% activity compared with the PLP-containing enzyme) further emphasising the importance of the 5'-phosphate in catalysis.

The results presented herein elucidate the catalytic importance of the phosphate group of PLP in the SPT mechanism. Since SPT is the first enzyme in SL biosynthesis it should be highly specific for L-serine as a substrate and not accept L-alanine or glycine which would generate deoxySL products. Such L-alanine- and glycine-derived deoxySLs are generated by mutant forms of SPT and have recently been reported to be toxic to mammalian cells.²² Our work provides evidence that amino acid substrate specificity is not only controlled by residues from SPT but also by an interaction between the phosphate of the PLP cofactor and the L-serine side-chain. L-serine is used as a substrate by other PLP-dependent enzymes such as serine racemase, serine dehydratase and serine hydroxymethyltransferase so it will be interesting to investigate whether the 5'-phosphate also plays a catalytic role in these important metabolic proteins.

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