

Activating Power of the Phosphopantetheine Arm in α -Oxoamine Synthase Catalysis

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Abstract

PLP-dependent enzymes are attractive biocatalysts due to their ability to perform complexity building transformations on amine-containing, readily available substrates from the chiral pool such as amino acids. Although many classes of PLP-dependent enzymes are routinely employed in synthesis, select families remain underutilized due to limitations in practical scalability. For example, some PLP-dependent enzymes act on prohibitively expensive substrates such as coenzyme A-bound substrates. To address this challenge, we have investigated the mechanism of action of coenzyme A in gating catalysis of one α -oxoamine synthase, SxtA AOS. Through investigation of the reactivity of SxtA AOS with panels of substrates and in the presence of various coenzyme A mimics as well as monitoring PLP absorbances and the behavior of SxtA AOS variants, we have determined that activity is gated through the binding of the eastern phosphodiester group by the residue Arg142 that is located in a tunnel that leads to the active site. To circumvent this gating mechanism, a synthetic CoA mimic additive was designed that allows for enhanced reactivity with non-CoA substrates. These findings outline a strategy for employing AOS enzymes without the need for cost-prohibitive coenzyme A substrates.

Introduction

As highly functionalized molecules from the chiral pool, α -amino acids are valuable building blocks in

synthesis. Building forward from α -amino acids allows for inclusion of several motifs commonly embedded in pharmaceutical agents, such as amine substituents and carboxylic acid groups, as well as diverse side chains that can provide multiple functional handles for diversification and an established stereocenter.¹ Specifically, α -amino acids are a tantalizing starting point for the synthesis of α -amino ketones, such as those shown in Figure 1A. Despite formally requiring breaking one bond and forming just one bond, synthetic approaches towards such motifs often require alternative starting materials as amino acids often present a chemoselectivity challenge, requiring protection and deprotection steps to furnish the desired target (Figure 1B).²⁻⁶

By contrast, the liability created by the need to protect the amino group for traditional methods is an asset in the biocatalytic elaboration of α -amino acids. In particular, pyridoxal phosphate (PLP)-dependent enzymes leverage the amino group to condense onto the PLP cofactor and initiate catalysis (7, Figure 1B).⁷⁻¹⁰ PLP-dependent transaminases have become ubiquitous in biocatalytic syntheses, appearing in the synthesis of sitagliptin, among other compounds.^{8,11} Transaminases are popular due to their simplicity; L-amino acids and small molecule biologically relevant compounds are their substrates, which are often inexpensive starting materials whose simplified scaffolds are conducive to directed evolution.¹²⁻¹⁴ Racemases have also become valuable biocatalysts, for similar reasons.^{8,15} Perhaps an under-exploited

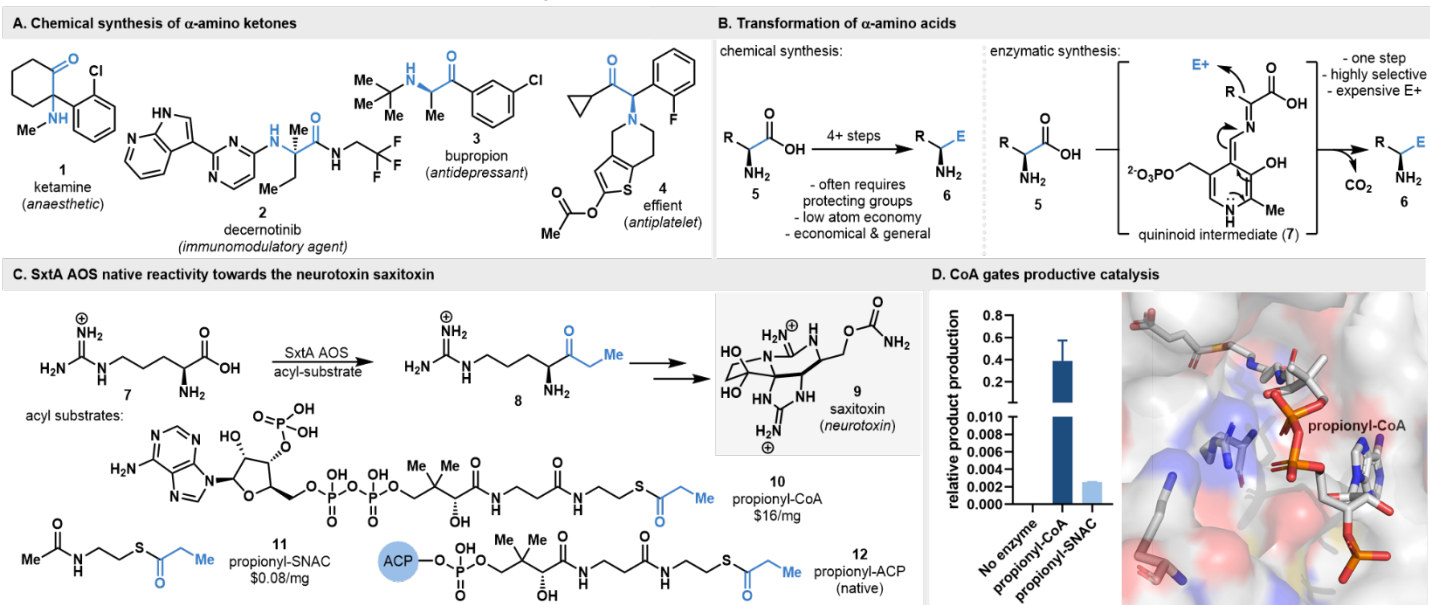


Figure 1 | A. Four pharmaceutical and natural product targets containing α -modified amino acids B. Comparison of chemical and enzymatic synthesis of α -modified amino acids. C. SxtA AOS's native reaction, the natural product saxitoxin, along with three potential acyl-substrates: propionyl-CoA, a commercial substrate that stands in for the native substrate; propionyl-ACP, the native substrate; and propionyl-SNAC, the smallest activated substrate accepted by the enzyme. D. Graph of the difference in activity between propionyl-CoA and propionyl-SNAC, and a model of the acyl-substrate docked into SxtA AOS. AOS: α -oxoamine synthase, CoA: coenzyme-A, ACP: acyl-carrier protein, SNAC: N-acetylcysteamine

enzymatic transformation is carbon-carbon bond formation, and one class of PLP-dependent enzyme that can mediate C–C bond formation is the α -oxoamine synthase (AOS).¹⁶ These catalysts rely on initial condensation of an amine substrate onto the PLP cofactor to generate an external aldimine intermediate.¹⁶ The pK_a of the α -proton is significantly lower in the aldimine intermediate, allowing for deprotonation by a proximal basic residue to form a nucleophilic quinonoid intermediate (see 7, Figure 1B) which can subsequently attack an electrophile. AOS reactions on α -amino acids are terminated by decarboxylation and stereoselective protonation to form products such as 6.

The potential of this C–C bond-forming enzyme class is attenuated as AOSs require the electrophile to either be carrier protein-bound or a CoA-activated thioester (see Figure 1C). Carrier protein-bound acyl substrates require stoichiometric amounts of protein and pre-generation of the substrate loaded, holo protein. Alternatively, the use of free thioester substrates currently requires coenzyme A-activated acyl substrates for productive turnover, a reagent with high market value. An example of these limitations is highlighted in the transformation of L-arginine to ketone 8 with propionyl-CoA where a five-gram reaction would cost over \$50,000 for the propionyl-CoA alone.

The cost of coenzyme A is therefore prohibitive for the use of AOSs as biocatalysts. To avoid the requirement for a stoichiometric CoA substrate or carrier protein-bound substrate, the role of the acyl substrate in gating catalysis must be more clearly understood. Previous studies have suggested two possible interactions between the enzyme and the thioester substrate: (1) with the carbonyl oxygen and (2) the sulfur atom. Ikushiro and coworkers previously explored the interaction of the carbonyl oxygen with an active site histidine of a serine palmitoyltransferase, another member of the AOS family.^{17,18} Rapid acceleration of deprotonation of L-serine was observed upon addition of palmitoyl-CoA or a thioether analog to the reaction. Based on kinetic

studies and analysis of the crystal structure, Ikushiro asserts that a major factor in driving catalysis was the shift of this active site His (Supplementary Figure 4). This residue serves as a hydrogen bond partner for the carboxylate group of the amino acid substrate. Upon entering the active site, the acyl substrate is proposed to induce a shift in the nature of the histidine interactions, to interact with carbonyl oxygen of the acyl substrate. This change is proposed to position the amino acid for deprotonation and activate the carbonyl group of the acyl-CoA substrate for electrophilic attack. A second proposal surrounding the interaction of the acyl substrate and the enzyme focuses on a moiety outside the active site.^{19–23} Specifically, it is proposed that the aliphatic arm and the phosphodiester groups of CoA/ACP are involved in binding;^{19–23} however, no additional studies have defined the specifics of this binding event. Much of the research into this interaction occurs with overall binding studies of the acyl-CoA substrate, or observations of AOS crystal structures.^{19–23} Multiple research groups have identified the coenzyme A substrate as essential for catalysis through kinetics studies with related enzymes, including 5-aminolevulinate synthase,¹⁹ BioF,²⁴ and a serine palmitoyltransferase.²⁵ Experiments led by Alexeev identified a possible interaction with the expensive electrophile by observing a concentration of positively charged residues proximal to the phosphate groups at the tail end of the acyl-coenzyme A substrate in a crystallographic study of BioF (PDB 1BSO).²⁶

Understanding the role that the natural acyl substrate plays mechanistically is essential for engineering AOSs into effective catalysts. We anticipated that a detailed understanding of the acyl substrate-protein interaction could inform the engineering of a system that is CoA- or ACP-free and thus suitable for preparative-scale biocatalytic reactions.

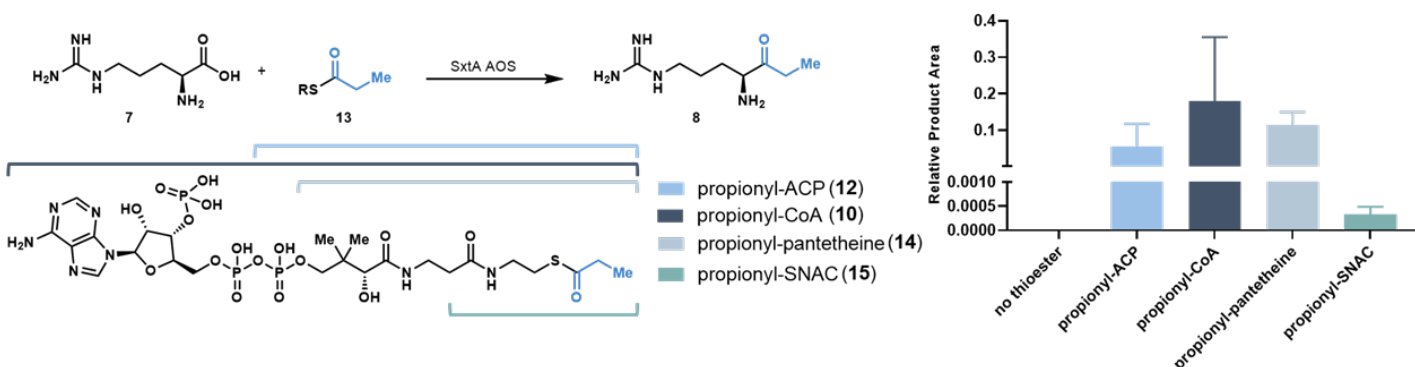


Figure 2 | Native acyl-substrate reactivity with wild-type enzyme. As the acyl substrate is truncated, activity decreases, as seen by HPLC-MS areas of the extracted ion chromatogram compared to tryptophan internal standard.

Results and discussion

Toward understanding the role of the acyl substrate in gating AOS chemistry, we directed our focus to an AOS with demonstrated acyl substrate promiscuity. SxtA AOS is the last domain of a larger multidomain protein, SxtA, the first enzyme in the biosynthesis of saxitoxin (**9**, Figure 1C).²⁷ This PLP-dependent enzyme transforms L-Arg into the corresponding ethyl ketone **8** with a propionyl group provided by the upstream ACP domain. In addition to acting on ACP-bound acyl substrates, SxtA AOS readily accepts propionyl-CoA as a substrate (Figure 2). To assess the reactivity with various thioester substrates, initial experiments were performed with 20 μ M SxtA AOS, 2 mM of the native amino acid substrate L-Arg and 0.5 mM of four propionyl-thioester substrates: propionyl-ACP (**12**, the native thioester of SxtA AOS), propionyl-CoA (the native thiol of most characterized AOSs), and truncated substrates propionyl-pantetheine (**14**) and propionyl-SNAC (**15**). As shown in Figure 2, the simplification of this substrate readily decreased the yield of ketone product **8** in the AOS reaction. In reactions with propionyl-SNAC (**15**), the most truncated electrophile accepted as a substrate, less than one percent of the product was generated in compared to reactions with propionyl-CoA (**10**).

To further interrogate which portion of coenzyme A was playing a role in modulating this reaction, we sought to investigate the impact of compounds on the PLP absorbance spectra. In these experiments, the UV-vis absorbance from 300 to 550 nm of 20 μ M enzyme was measured in the presence of 1 mM thioester substrate (Figure 3). In samples containing SxtA AOS, two peaks associated with the PLP absorbance spectra are prominent, one at 330 nm and the other at 420 nm. Analysis of SxtA AOS purified with no added thioester, the 330 nm peak was most prominent. When propionyl-CoA (**10**) was added and the mixture incubated for five minutes, the ratio between the 330 nm and 420 nm peaks was perturbed, with the 420 nm peak becoming the highest intensity peak. Because addition of this close analog of the native ACP substrate results in an increase of the 420 nm peak, we propose that the 420 nm peak is the active conformation of the enzyme that will proceed with productive catalysis. An alternative explanation is that this peak shift was due to consumption of L-Arg that had co-purified with the enzyme. To discriminate between these two scenarios, an experiment in which CoA salt was added to SxtA AOS was performed (Figure 3). This experiment reveals a similarly altered peak ratio, favoring the the proposed active form of the enzyme, despite this mixture lacking the acyl group required for product formation. This experiment provided evidence that some portion of the CoA substrate was causing a change of the electronics around PLP, rather than being a function of a chemical reaction. To further probe which portion of the thiol induced this change, 1 mM propionyl-pantetheine was added to purified enzyme. This thioester did not induce the same change, as 330 nm remained the prominent peak. Comparing the results obtained from these three substrates, we hypothesize that the striking differences in the PLP-spectra in the presence of CoA-containing substrates indicate that some portion of the diphosphate group, sugar moiety, or nucleoside base is essential to accessing the catalytically active conformation of the enzyme. With the western-most phosphate group, sugar and base not included in the native propionyl-ACP substrate (**12**), we hypothesized that the eastern phosphodiester group was the moiety inducing the increased product formation. Furthermore, we hypothesized that specific interactions between the eastern phosphodiester group (shown in green, Figure 3) of CoA and SxtA AOS induce a conformational change that is critical for productive catalysis. To probe this idea, a

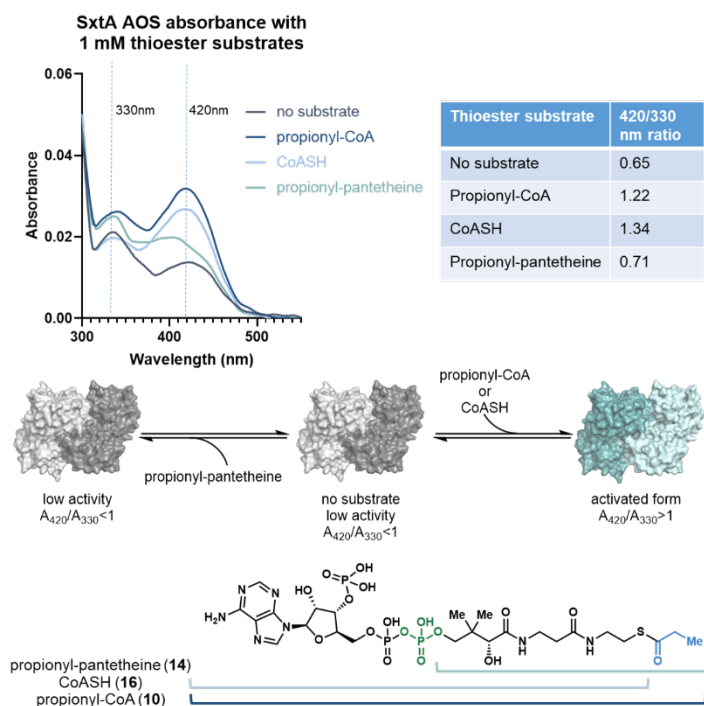


Figure 3 | PLP-absorbance changes observed in SxtA AOS with three thioester substrates in two PLP-based peaks—proposed inactive form at 330 nm and activated form at 420 nm. The equilibrium is shown in the middle scheme, with observed absorbance spectra with each thioester substrate in the top left and the individual ratios of the two peaks shown in the top right. At the bottom, brackets show the structure of each acyl-substrate used in this experiment.

homology model of the SxtA AOS domain was generated by the Phyre2 server based on the crystal structure of the related AOS 2-amino-3-ketobutyrate CoA ligase (PDB:1FC4, Figure 4).²⁸ The position of the acyl substrate was modeled based on the structure of succinyl-CoA bound in 5-aminolevulinic synthase (PDB:2BWO).²⁹ This was the only available crystal structure with an acyl-CoA substrate docked, and no structures have been published showing interactions between an ACP and AOS. Examination of the region proximal to phosphodiester group in question revealed two positive residues, an Arg and a Lys, anticipated to engage in stabilizing interactions with the substrate. To test this hypothesis, site-directed mutagenesis was performed to access variants in which either residue was substituted with alanine. These variants along with wild type enzyme were evaluated in reactions with L-Arg and either propionyl-CoA or propionyl-pantetheine. These two substrates were used as propionyl-CoA contains the eastern phosphodiester group, while propionyl-pantetheine does not. As such, we anticipated that if the Lys and Arg residues were interacting with this phosphodiester group, then the increase in activity typically observed with propionyl-CoA would not be as significant. Reactions containing 20 μ M SxtA AOS or variant, 2 mM L-Arg, and 0.5 mM propionyl-CoA or propionyl-pantetheine in buffer were incubated overnight and product formation was measured by LC-MS. The extent to which the activity is attenuated between propionyl-CoA and propionyl-pantetheine in the R142A variant is less than in the wild type enzyme, suggesting that this residue is indeed involved in the phosphodiester interaction (Figure 4, chart).

With support for the importance of the eastern phosphodiester in gating catalysis, we envisioned a synthetic mimic of Nature's substrates could enable a productive reaction without the need for stoichiometric CoA or carrier protein-bound substrate. First, a series of auxiliary substrates were synthesized, including those found in Table 1. Next, the efficacy of 0.5 mM of each auxiliary was evaluated in reactions of 2 mM L-arginine and 0.5 mM propionyl-SNAC. The amount of product formed in reactions with synthetic mimic added was compared to the amount of product formed in reactions with propionyl-SNAC alone. Both the mimic without the phosphate and with an unprotected phosphate group showed no increase in reactivity. This was unsurprising as the mimic without the phosphate was not able to form the hydrogen bonds proposed to increase reactivity, and the

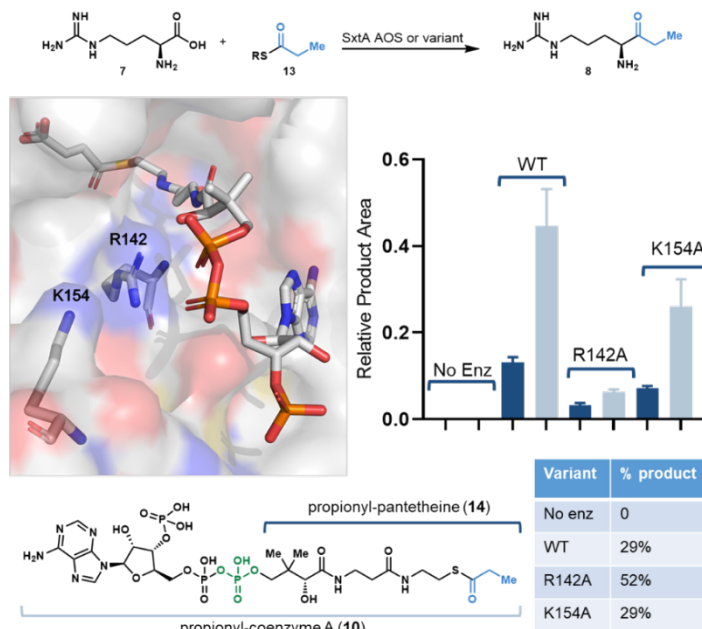


Figure 4 | Native reactivity of propionyl-pantetheine and propionyl-CoA with Ala variants of residues though to be involved in CoA binding. At top, the reaction scheme. At left, a SxtA AOS model showing the propionyl-CoA and the two residues substituted: Lys154 and Arg142. At right, a graph demonstrating the change in activity when each residue is varied to Ala. At bottom, brackets showing the substrates involved.

unprotected phosphate mimic added negative charge density not found in the native substrate. Only the mimic containing the protected phosphotriester moiety (entry 5, Table 1) showed increased product formation. Not only does this provide further evidence that the phosphate is essential for activity, as the same mimic without the phosphate does not induce the increase in activity, but it also provides support for the idea that a mimic can be used to improve activity, negating the need for expensive coenzyme A substrates.

Table 1 | Reactions with wild-type SxtA AOS enzyme, demonstrating propionyl-SNAC's activity improves with a methylated phosphodiester mimic, demonstrating the possibility of improving reactivity without utilizing an expensive CoA substrate.

Rxn #	Acyl-substrate and mimic	Relative product
1	propionyl-CoA	153 \pm 72.9
2	propionyl-SNAC	1 \pm 0.0191
propionyl-SNAC with:		
3	ACP-OH	N.D.
4		4.89 \pm 1.40
5		0.909 \pm 0.0959
6		0.959 \pm 0.0176

Conclusions

AOS enzymes are attractive candidates for biocatalysis, however, these enzymes naturally require acyl-CoA substrates or carrier protein-bound substrates for productive catalysis, making these catalysts unsuitable for preparative-scale reactions. To overcome this challenge, we sought to identify the key protein-acyl substrate interactions and devise an approach to circumventing this gatekeeping mechanism. We have demonstrated that coenzyme A has critical interactions with the AOS, specifically with the eastern phosphodiester group, and that synthetic mimics containing this group can serve as additives that avoid the need for expensive coenzyme A substrates. This work provides evidence that a mimic can be used to turn the switch of activity, and provides an avenue for the application of AOSs as useful biocatalysts for the transformation of α -amino acids.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: **xx**
Supplemental figures and methods (PDF)

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Notes

The authors declare no competing financial interests.

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