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Reply to: LsBOS utilizes oxalyl-CoA produced by LsAAE3 to synthesize β-ODAP in grass pea

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REPLYING TO M. Goldsmith et al. *Nature Communications* https://doi.org/10.1038/s41467-024-50703-4 (2024)

Dr Goldsmith and colleagues have examined critically the findings in Edwards et al.[1] which we welcome in the spirit of constructive scientific dialogue. As the enzyme activities we described in this paper differ from the pathway described in previous literature, we took time and great care to ensure our results were robust. Several of the arguments put forward in the commentary by Dr Goldsmith and his colleagues are based on their assumption that we inferred activity of LsAAE3 using a coupled assay that measures the hydrolysis of ATP in the first part of the two-step reaction of AAE-type CoA ligases. But this was not the case, as all the results reported in our manuscript were measured using the method described in "Enzyme assays" of the Methods section.

Not only were all assays determined using this method, but we were careful to emphasise that we used this direct assay throughout the results section of our manuscript, so it is perplexing that Dr Goldsmith and colleagues state that our claims were based on measurements of ATP hydrolysis rates using an NADH-coupled enzymatic reaction. All our measurements were based on direct assays, by LC-MS of product (oxalyl-CoA or β/α-ODAP). This included the determination of the pH optimum for oxalyl-CoA synthesis which was calculated by measuring the production of the specified product directly, not by using the coupled assay. As reported earlier by Goldsmith et al.[1] we did confirm the pH-optimum for LsAAE3 using the coupled assay as pH 8.0. However, this coupled assay measures only half the reaction i.e. the hydrolysis of ATP to form oxalyl-AMP which we believe can then be converted to oxalyl-CoA or to ODAP (α- and β-ODAP) in the presence of L-DAP. However, we reported the pH optimum for LsAAE3 to make oxalyl-CoA (assessed by directly measuring oxalyl-CoA by LC-MS) as pH 6.0 in Edwards et al. with a sharp fall-off in production at pH 7.0[2].

In the remainder of this section of the commentary, Dr Goldsmith and colleagues show that LsAAE3 can catalyse an amine oxalylation of lysine similar to the mechanism we proposed for LsAAE3 plus L-DAP. They claim that this reaction is a 'chemical' rather than 'catalytic' activity, but the amine oxalylation requires LsAAE3 enzyme to produce oxalyl-AMP and therefore its formation cannot be considered as "non-catalytic". It would be interesting to know whether this 'activity' is activated by low levels of CoA, because the assay, as they conducted it, in vitro, 'in the absence of CoA' would never reflect the situation in vivo, and neither would their assay of LsAAE3 plus oxalate plus L-DAP (but without CoA) shown in Fig. 1 of their commentary.

The reason that we assayed LsAAE3 directly rather than by the coupled reaction was because following the identification of LsAAE3 and LsBOS in Emmrich[3], we undertook initial experiments that were not published but which we submit here to support the data reported by Edwards et al.[2]. These experiments cast significant doubt on oxalyl-CoA being the substrate for LsBOS, which led us to investigate possible non-canonical activities for both enzymes. These involved running the LsAAE3 reaction with CoA, oxalate, ATP and L-DAP at pH 8.0 for 30 min filtering through a size exclusion filter to remove LsAAE3 and then adding LsBOS. This effectively mimicked the coupled assay that Goldsmith and colleagues used in their purifications and their assays of the kinetic determinants of LsBOS[1]. With levels of CoA that were high enough to activate the synthesis of ODAP we saw significant activity of LsAAE3 alone resulting in the production of both α- and β-ODAP. When LsBOS was added to the filtrate without LsAAE3 there was a small increase in both α- and β-ODAP but this was variable and the difference was not statistically significant, especially when compared to the level of activity achieved by incubating LsAAE3 with LsBOS without the intervening filtration (Fig. 1a). We confirmed that LsAAE3 had been removed by our filtering (Fig. 1b). Significantly, oxalyl CoA was undetectable in the filtrate from the LsAAE3 incubation with oxalate, ATP and DAP (and CoA) (Fig. 1c). We deduced at this point that oxalyl-CoA was probably not an intermediate in the synthesis of β-ODAP under low CoA conditions. Notably, no α-ODAP was detected in the combined LsAAE3 and LsBOS incubation with oxalate, CoA (100 nM) and L-DAP (Fig. 1a).

We wished to confirm whether our interpretation of the role (or lack of a role) of oxalyl-CoA was correct and so we contacted Stefan Martens (TransMIT Gesellschaft für Technologietransfer, Gießen, Germany), to request that he synthesised oxalyl-CoA for us. We sent him the LsAAE3 expression vector, and he tried repeatedly to express the enzyme in *E. coli* and use it to produce oxalyl-CoA at pH 8.0, but in the end reported back to us that he could not synthesise oxalyl-CoA using this method—production (at pH 8.0) was too low. As an

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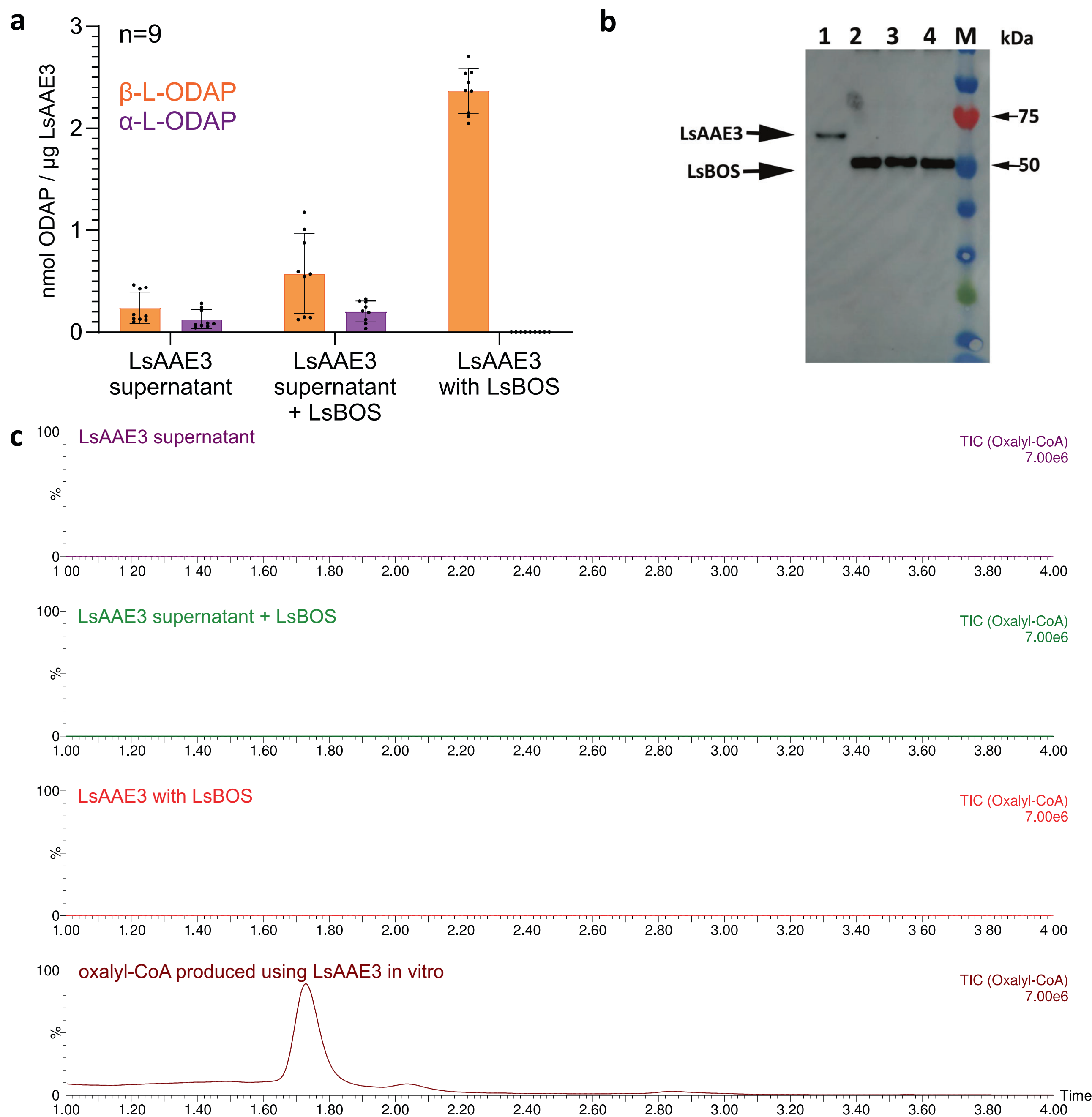

Fig. 1 | LsAAE3 enzyme assays of α/β-L-ODAP formation. Shown are measurements of the supernatant after removal of LsAAE3, removal of LsAAE3 followed by addition of LsBOS and the coupled reaction in the presence of both LsAAE3 and LsBOS. **a** concentrations of α- and β-ODAP produced. **b** Western blot showing LsAAE3 in the initial reaction (lane 1) and absent in the supernatant following filtration and addition of LsBOS (lanes 2–4), alongside Biorad Precision Plus Marker. **c** LC-MS profiles of assays showing no detectable oxalyl-CoA. The bottom trace shows oxalyl-CoA produced in vitro using purified LsAAE3 as described in Edwards et al.[2]. Source data are provided as a Source Data file.

alternative he synthesised oxalyl-CoA using chemical synthesis as first described by Quayle et al.[4] (see Methods section of Edwards et al.[2]). After we shared this compound with Dr Goldsmith and his colleagues, they tested the compound using NMR and shared results indicating that this compound was not oxalyl-CoA but another CoA-derivative with near identical mass.

In the meantime, we used experimental conditions described in Edwards et al.[2] to synthesise oxalyl-CoA using LsAAE3 in vitro. This produced a compound with the correct *m/z* for oxalyl-CoA. Using this compound as a control, we did not detect oxalyl-CoA in any of the reactions we conducted using physiologically realistic concentrations of enzymes and substrates, which nonetheless readily produce α- and β-ODAP (Fig. 1).

We considered the physiological relevance of the activity of LsAAE3 in producing oxalyl-CoA, not only by measuring the pH optimum for this reaction as pH 6.0 (substantially below the pH of plant cell cytoplasm) but also the K_m for CoA in this reaction as 5 mM. The in vitro assays of Goldsmith and colleagues were undertaken using 2 mM CoA, although it is highly unlikely that such levels of CoA are present in the cytoplasmic pool which have been broadly estimated as in the nanomolar to low micromolar range[5–8]. Indeed, Klempien et al.[9], argued and showed that the low affinity for CoA (K_m 775.2 6 ± 34.3 µM)

of the Ph CNL CoA ligase meant that it had to be localised in peroxisomes where higher levels of CoA exist, compared to the cytoplasmically-localised Ph-4CL which has a K_m for CoA of 9.96 ± 3.1 μM. These observations suggest that synthesis of oxalyl-CoA in the cytosol of grass pea by LsAAE3 is likely limited.

Dr Goldsmith and colleagues argue against our finding that CoA acts as an activator of LsAAE3 synthesis of ODAP, and yet we were able to measure the K_m for this activation as being five orders of magnitude lower than the K_m for synthesis of oxalyl-CoA (and within the estimated range for cytoplasmic CoA levels).

We were able to demonstrate activation, as opposed to competitive inhibition, of LsAAE3 synthesis of ODAP by CoA analogues which were inactive as substrates in the synthesis of oxalyl-CoA[2], supporting our conclusions. We were also able to show that the CoA analogue desulpho-CoA could activate the synthesis of α- and β-ODAP by LsAAE3 in the presence of ATP, Mg^{2+}, oxalate and DAP, conditions in which oxalyl-CoA cannot be synthesised in vitro[1] (Fig. 2).

We propose that the alternative mechanism for ODAP synthesis by LsAAE3 involves the formation of an oxalyl-AMP intermediate, the oxalyl group of which is then transferred to the amine residue of DAP. To verify this we sought to identify oxalyl-AMP in reaction of LsAAE3 with ATP, oxalate and 0.1 μM CoA in the assay shown in Fig. 2. The greatest problem with direct identification of oxalyl-AMP by LC-MS is its limited stability. However, by using a short reaction time of 10 min, loading the reaction immediately onto a Hypercarb Porous Graphitic Carbon (PGC) column combined with Q-Exactive Orbitrap LC-MS and using either full scan MS or selected ion monitoring (SIM) we were able to obtain the results shown in Fig. 3. Extracted ion chromatograms (EIC) for *m/z* 418.0406 (Fig. 3a) show no ion in the absence of LsAAE3. When LsAAE3 was added a peak (*m/z* 418.0406) was consistently observed at 17.5 min (Fig. 3a) confirming that the appearance of this peak was dependent on addition of the enzyme (ie it was catalysed by LsAAE3). The same peak was observed when LsAAE3 was incubated with desCoA replacing CoA at the same concentration. However, the peak was not detected when the oxalyl acceptor, DAP, was included in the reaction mixture, confirming that this intermediate interacted with DAP to form ODAP. We confirmed the identity of this peak as oxalyl-AMP by accurate mass and by MS2. The calculated mass for oxalyl-AMP is 418.0404 in negative ion mode and the mass observed was 418.0406 with an error of –0.36 ppm, well within the range of 5 ppm for confidence in accurate mass identification (Fig. 3b). For analysis of MS2 ions, we used electrospray ionisation (ESI) with the Q-Exactive LC-HR MS/MS analysis to detect AMP following the fragmentation of the oxalyl group from oxalyl-AMP. The calculated mass of AMP in negative ion mode is 346.05581 and the MS2 ion observed was 346.05510 with an error of – 2.05 ppm (Fig. 3c). These data confirmed that oxalyl-AMP is the intermediate formed by the LsAAE3 reaction that oxalylates the amine group of DAP to form ODAP.

Goldsmith and colleagues have argued that if the mechanism we propose were correct, L-DAP should influence the kinetics of the reaction synthesising oxalyl-CoA, but they used only the coupled reaction to measure this at pH 8.0[10] i.e. they did not measure the synthesis of oxalyl-CoA but rather the hydrolysis of ATP in the formation of oxalyl-AMP. We propose that ATP-hydrolysis is shared by both reactions, so the results of Goldsmith and colleagues are not incompatible with our findings.

Equally, we do not contest their results of weak BAHD-AT activity with acetyl-CoA and malonyl-CoA and L-DAP. We do not claim that LsBOS has no BAHD-AT activity, simply that we could detect activity in the absence of oxalyl-CoA.

Finally, Goldsmith and colleagues claim that β-ODAP is a unique metabolite found only in a small number of species and that the idea that β-ODAP synthesis can be readily replicated in a heterologous host which does not have a homologous β-ODAP synthase is 'unlikely'. However, we have tested AAE3 enzymes from a wide range of species in vitro (*Panax notoginseng, Medicago truncatula, Arabidopsis thaliana* and *Pisum sativum*) and found that all can produce small amounts of α- and β-ODAP with oxalate, CoA, ATP and L-DAP and that when combined with LsBOS all produce substantial amounts of β-ODAP. Of course, in vivo synthesis of β-ODAP in the absence of BOS requires the presence of L-DAP, which occurs in some legumes and which we reported in pea and grass pea in Edwards et al.[2]. In this respect it is very interesting that Boulfekhar et al.[11] recently detected low but reproducible levels of β-ODAP in two varieties of pea, using a new, improved-sensitivity, detection protocol. Pea lacks β-ODAP synthase[2] but does make L-DAP and encodes an AAE3 enzyme very similar to that of grass pea supporting our interpretation of the role of LsAAE3 in β-ODAP synthesis.

Collectively, our data indicate that β-ODAP synthesis in grass peas occurs through a mechanism involving a physical interaction between LsAAE3 and LsBOS (as Goldsmith et al. agreed in their arguments), and we have shown that this process can occur without oxalyl-CoA as an intermediate, instead relying on oxalyl-AMP, which is formed as part of the canonical reaction mechanisms of AAE3 enzymes. We propose that the non-canonical, oxalyl-CoA-independent mechanism is most likely to occur when CoA levels limit the production of oxalyl-CoA, which may prevail frequently, in vivo. Elucidating the exact reaction mechanism inside the micro-environment of the metabolon requires significant additional work and we have reached out to Dr Goldsmith and his colleagues to devise the most scientifically constructive way forward. We agree that a full understanding of the biochemistry of β-ODAP synthesis is important for the future improvement of this promising crop, and we hope we can come to a consensus regarding the details of the catalytic mechanisms operating under physiological conditions.

Methods

Enzyme filtration assays

Most methods used were reported in Edwards et al.[2]. For the filtration experiments shown in Fig. 1, the LsAAE3 reactions contained 100 mM Tris pH 8.0, 2 mM DTT, 5 mM ATP, 10 mM $MgCl_2$, 100 nM CoA, 3 mM Na oxalate, 0.5 mM DAP and 250 ng purified LsAAE3 in a reaction volume of 100 μL. The reaction was incubated for 30 min at room temperature before being treated in one of two ways. The reaction was filtered using an Amicon Ultra 0.5 mL filter with a 30 kDa cut off, by centrifugation at 16,000 g for 10 min at 4 °C before (1) continuing the reaction for a further 30 min (2) adding 250 ng LsBOS and incubating for 30 min. As a control reaction, LsBOS was added to the unfiltered LsAAE3 reaction and run for 30 min. Reaction products were analysed by LC-MS as described by Edwards et al.[2]. To test the efficiency of filtration, filtered and unfiltered samples were separated by SDS PAGE and His-tagged LsAAE3 detected by western blot.

Detection of oxalyl-AMP

To detect oxalyl-AMP, 50 uL reactions were set up containing: 100 mM Tris pH 8.0, 2 mM DTT, 5 mM ATP, 10 mM $MgCl_2$, 0.3 mM sodium oxalate, 0.1 μM CoA. The reaction was run without added enzyme or with purified LsAAE3 at 15 ng μL^{-1}. In one reaction the CoA was replaced by 0.1 μM desCoA and in the final reaction 1 mM DAP was added (as described for the assays shown in Fig. 2). Reactions were incubated at room temp for 10 min and samples (10 μL) were analysed immediately using a Q-Exactive LC-MS in negative ion mode (Thermo-Fischer) Res. 70,000. Collision energies were 10, 30, or 50. Inclusion was set at *m/z* 418.04055.

A Hypercarb Porous Graphite Carbon (PGC) Column; 1 × 100 mm, particle 5 um, (Thermo Scientific) was used for chromatography. The mobile phases were: A = 0.3% (79.5 mM) formic acid brought to pH 9.0 with ammonia, *B* = Acetonitrile with the following acetonitrile concentrations applied at: Time 0: 2% B, Time 20: 15% B, Time 26: 50% B, Time 27: 90% B, Time 30: 90% B, Time 31: 2% B, Time 50: 2% B, (time in

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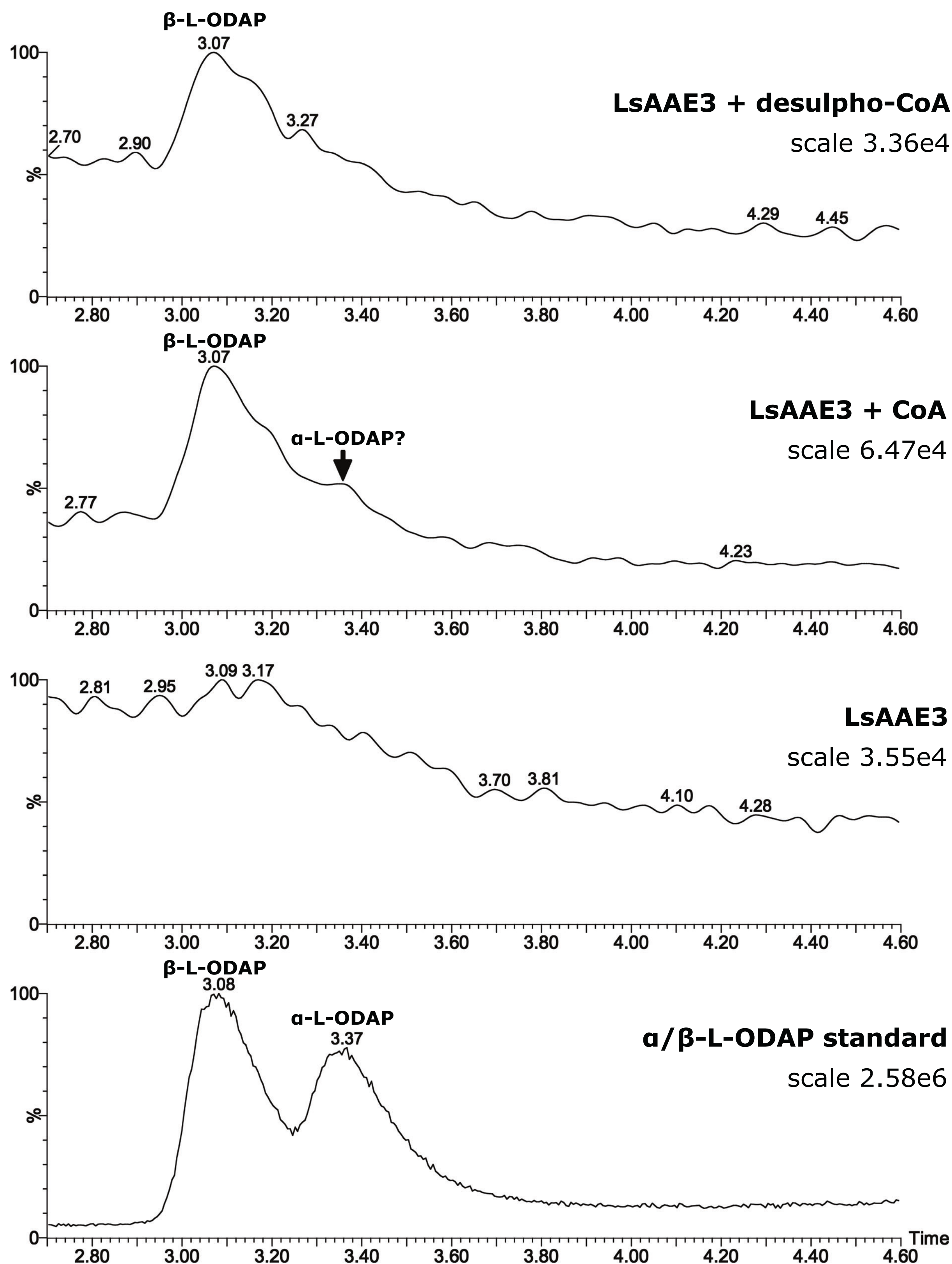

Fig. 2 | α/β-L-ODAP formation in the absence of LsBOS. LCMS spectra show *α*- and *β*-ODAP formation in vitro using oxalate and L-DAP in combination with LsAAE3 and 20 nM desulpho-CoA (track 1); LsAAE3 and 20 nM CoA (track 2); LsAAE3 only (track 3); or *α/β*-L-ODAP standard. Source data are provided as a Source Data file.

min); Column temp = 30 °C, Scan Range = 200–600 nm (UV-Vis1 265) MS -ve mode, 200–2000, 70,000 res. To facilitate peak identification standards of AMP, ADP, ATP (at 5 mM in 100 mM Tris pH8.0) were analysed for comparison.

MS2 was undertaken using LC-HR MS/MS in ESI on the Q-Exactive. MS2 = 17,500 resolution, isolation window 4 *m/z*, offset 1, negative ion mode. The mass of AMP in negative ion mode was calculated from the formula $C_{10}H_{13}N_5O_7P$ as 346.05581 (M-oxalate).

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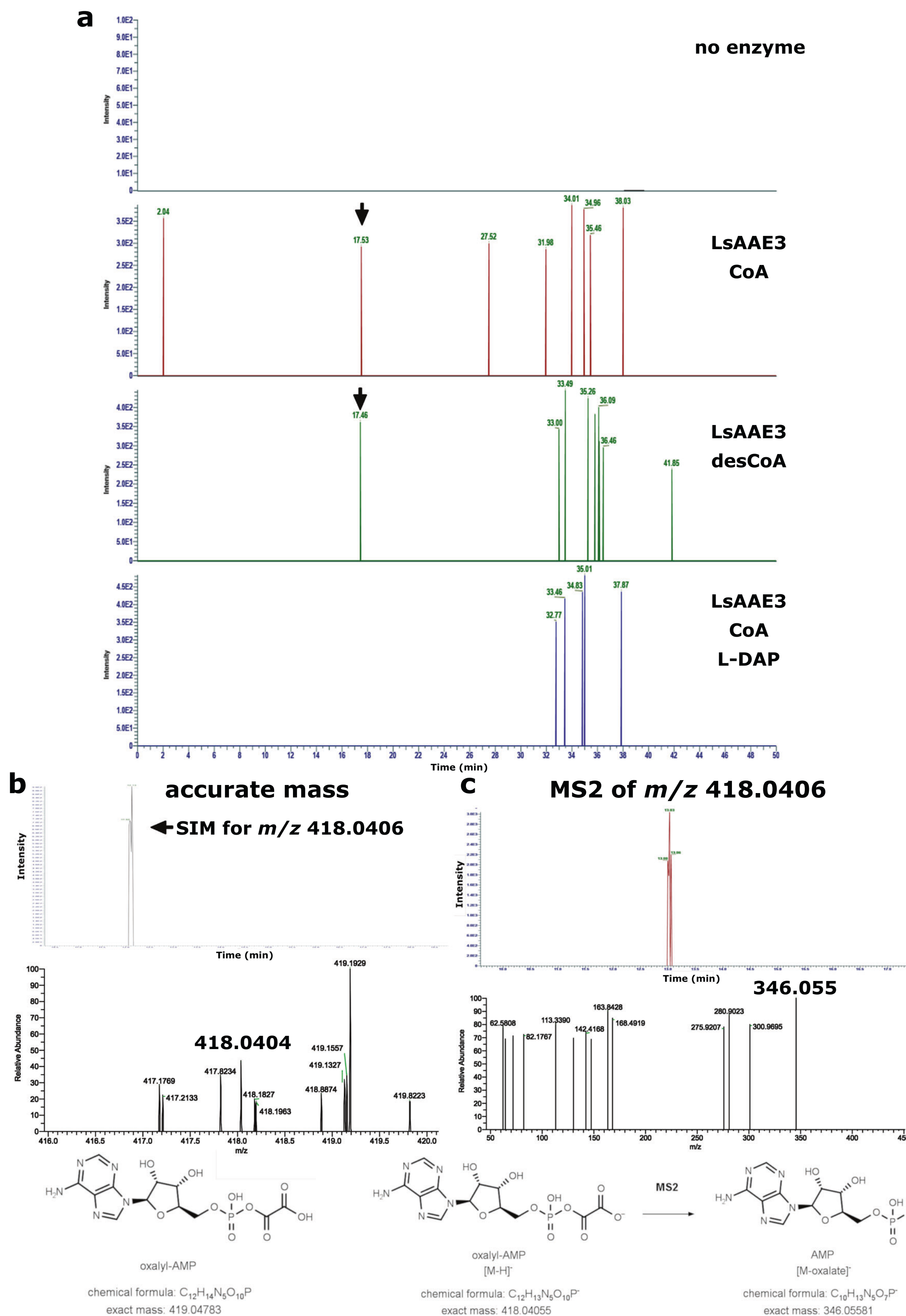

Fig. 3 | Detection of oxalyl-AMP as the intermediate in the synthesis of ODAP by LsAAE3. a Extracted ion chromatograms of incubations of 100 mM Tris pH 8.0, 2 mM DTT, 5 mM ATP, 10 mM $MgCl_2$, 0.3 mM sodium oxalate, 0.1 μM CoA with no enzyme, with LsAAE3, with LsAAE3 and desCoA replacing CoA, with LsAAE3, CoA and DAP. Black arrows indicate peak (*m/z* 418.0406) for oxalyl-AMP present in reactions with LsAAE3 and CoA or desCoA, which is absent when DAP is included as the oxalyl acceptor in the reaction. **b** Accurate mass determination with selected ion monitoring for peak *m/z* 418.0406. The calculated *m/z* was 418.04055, the observed was 418.0404 (indicated by black arrow) with an error of −0.36 ppm. **c** MS2 of selected ion *m/z* 418.0406. The calculated *m/z* for AMP in negative ion mode is 346.05581, the observed was 346.0551 with an error of −2.05 ppm. Source data are provided as a Source Data file.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Source data are provided with this paper.

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Author contributions

A.E. and Z.J. performed the enzyme assays and western blot shown in Fig. 1. S.N. performed NMR analysis of oxalyl-CoA. A.E. and M.R. performed enzyme assays and mass spectrometry to measure formation of oxalyl-AMP. C.M. and P.M.F.E. supervised the work, lead the preparation of the manuscript and prepared the final figures.

Competing interests

The authors declare no competing interests.

Additional information

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