Modulation of plant Acetyl CoA Synthetase activity by acetylation

Naazneen Sofeo[1,2,3], Karina Leung[2], Basil J. Nikolau[1,2,3,*]

[1] Roy J. Carver Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA 50011, USA, [2] Engineering Research Center for Biorenewable Chemicals Iowa State University, Ames, IA 50011, USA, [3] Center for Metabolic Biology, Iowa State University, Ames, IA 50011, USA

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*To whom correspondence should be addressed: Basil J. Nikolau: 3254 Molecular Biology Building, Roy J. Carver Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA 50011-1079, USA; Email: dimmas@iastate.edu; Tel: 515-294-9423

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ABSTRACT

Acetyl-CoA synthetase (ACS) is one of several enzymes that generate the key metabolic intermediate, acetyl-CoA. In microbial cells ACS generates the precursor for fatty acid and polyketide biosynthesis, and is regulated by the post-translational acetylation of a key lysine residue that inhibits catalytic activity. In contrast, ACS in plant cells is part of a two-enzyme system that maintains acetate homeostasis. Despite these different metabolic roles, this study demonstrates that the plant ACS can also be regulated by the acetylation of a specific lysine residue. The lysine residue that is targeted for this post translational modification reaction is positioned in a homologous region of the microbial and plant ACS sequences, occurring in the middle of a conserved motif. The inhibitory effect of the acetylation of residue Lys-622 of the Arabidopsis ACS was demonstrated by site-directed mutagenesis of this residue, including its genetic substitution with the non-canonical N-ε-acetyl-lysine residue. These modifications lowered the catalytic efficiency of the enzyme by a factor of more than 500-fold, and Michaelis-Menten kinetic analysis of the mutant enzyme indicates that this acetylation affects the first half-reaction of the ACS catalyzed reaction, namely the formation of the acetyl adenylate enzyme intermediate.

INTRODUCTION

The interplay between acetylation and deacetylation of protein lysine residues is a crucial process in regulating many biological processes including chromatin structure, transcriptional regulation and the regulation of cellular metabolism (1–3). This post-translational modification mechanism is evolutionarily conserved over a range of phyla, and two enzymes play a role in this process: a) lysine acetyl transferases (KATs) that acetylates lysine residues; and b) lysine deacetylases (KDACs) that hydrolyze and remove the acetyl group from the side chain (1). In addition, non-enzymatic auto-acetylation of lysine residues occurs at alkaline pH, particularly of lysine residues that are flanked by positively charged amino acids (4).

Acetyl-CoenzymeA (CoA) synthetase (ACS) is a ~72 kDa protein that catalyzes the synthesis of acetyl-CoA from acetate and plays an important role in fatty acid and polyketide biosynthetic pathways, and this enzyme also generates the substrate used in the acetylation of protein lysine residues. In a wide variety of organisms ACS activity is known to be regulated by reversible post-translational acetylation. Specifically, the ACS from *Mycobacterium tuberculosis* can be non-enzymatically auto-acetylated (5). The enzymatic acetylation of a lysine residue as a mechanism of ACS regulation has also been studied in bacterial systems (3, 6, 7). For example, the non-acetylated ACS of

Salmonella enterica is about 480-fold more active than the acetylated enzyme (8). The deacetylation of this enzyme is catalyzed by CobB, which is a homolog of the yeast Sir2 deacetylase (9). The acetylation of this ACS occurs at the side chain of a Lys residue that is positioned in the middle of a conserved structural motif, called A10 (10). Structural studies of the *S. enterica* ACS indicate that this acetylation does not induce any conformational changes in the enzyme (11). However, acetylation significantly affects the ACS first half-reaction, presumably by aligning the acetate-substrate with the active site residue(s) (11); this protein acetylation reaction does not affect the second half-reaction catalyzed by this enzyme (8).

In plant systems, such as Arabidopsis, the metabolic function of ACS is not completely clear (12). Despite the fact that ACS is plastid localized (13), and unlike other biological systems, ACS is not the physiological source of acetyl-CoA that is the precursor of the plastid-localized *de novo* fatty acid biosynthesis process (14). In this study, we explored if the acetylation-based regulation of ACS is extrapolatable from bacteria, yeast and algae to affect the activity of this enzyme in plant systems. Specifically, this was explored by a combination of site-specific mutagenesis strategies, including a strategy for expanding the genetic code, and thereby *de novo* reconstruct the post-translational modification of this protein (15).

In this latter strategy, an already acetylated lysine residue is genetically inserted into a protein sequence at a targeted position during the *in vivo* synthesis of that protein. These methods have been used to not only incorporate N-ε-acetyl-lysine at targeted sites, but also a number of different non-canonical amino acids (16). Thus, the strategy uses an orthogonal pair of a suppressor tRNA and its respective amino acyl tRNA synthetase that recognizes a specific non-canonical amino acid, and that amino acid is genetically programmed to be incorporated during the translation process of a host, typically *Escherichia coli* (15). The position for incorporating the non-canonical amino acid is defined by the stop-codon mutagenesis of the protein-coding ORF, which is recognized by the anticodon of the suppressor tRNA that carries the non-canonical amino acid (17). The advantage offered by this strategy is the ability to generate homogeneous preparations of modified proteins, and thus is a powerful technique to study post-translational modifications. In this study, we used this strategy to explore the post-translational acetylation of an ε-amine lysine group of the Arabidopsis acetyl-CoA synthetase (atACS) as a potential regulatory mechanism.

MATERIALS & METHODS

Generation of atACS variants

The characterization of the atACS ORF was previously described (13, 14, 18, 19). Incorporating the non-canonical N-ε-acetyl-lysine (Ac-K) residue into the atACS sequence was facilitated by cloning the wild-type ORF sequence into the pCDF-1b vector, which genetically fuses a Hexa-His-tag at the C-terminus of the atACS sequence. The codon encoding for lysine-622 was mutated to the "TAG" stop codon, using Quikchange Lightning Site mutagenesis kit (Agilent Technologies, Santa Clara, CA), and this mutation was confirmed by direct sequencing of the plasmid product. The orthogonal tRNA containing *pTech* plasmid (20, 21) was obtained from Dr. Chenguang Fan (Department of Chemistry & Biochemistry, University of Arkansas) and was co-transformed with the pCDF-1b construct into *E. coli* strain BL21(DE3).

Expression and purification of wild-type and variant proteins

Wild-type atACS and the lysine-622 mutant variants were expressed and purified as earlier described (18). Namely, the *E. coli* BL21 strains carrying the atACS expression plasmid vector was grown overnight at 37 °C, with agitation at 250 rpm, in 5-10 ml LB medium containing streptomycin (50 μg/ml) and chloramphenicol (50 μg/ml). The overnight culture was used to inoculate 0.25 L LB media containing the same antibiotics. These cultures were grown at 37 °C with agitation at 250 rpm, until the OD_{600} reached ~0.6. Protein expression was then induced by the addition of IPTG to a final concentration of 0.4 mM. At this time, the culture medium was supplemented with 20 mM nicotinamide to

inhibit the endogenous cobB deacetylase (20, 22), and 2mM N-ε-acetyl-lysine (Sigma-Aldrich Co., St. Louis, MO), to ensure that this non-canonical amino acid did not limit the expression of the Ac-K atACS variant. The culture was then incubated at 22 °C for 24-48 hours with agitation at 250 rpm. The variant proteins were purified by a process similar to that of the wild-type atACS (18), except all buffers contained 20 mM nicotinamide. The purified atACS proteins were immediately dialyzed into 10 mM HEPES-KOH, pH 7.5, 10 mM KCl, 2mM TCEP, 10% glycerol, concentrated and either characterized immediately or flash frozen in liquid nitrogen and stored at -80$_o$C.

Gel Filtration Chromatography

Size exclusion gel filtration chromatography was conducted with an AKTA FPLC system (GE Healthcare Life Sciences, Pittsburg, PA) using a Superdex 200 Increase 10/300 GL gel filtration column (GE Healthcare Life Sciences, Pittsburg, PA). A 100 µl aliquot of purified atACS protein (4-10 mg/ml) was injected into the prepacked column, and chromatography was conducted with a buffer consisting of 10 mM HEPES-KOH, pH 7.5, 10 mM KCl, 2 mM TCEP, 10% glycerol, eluted at a rate of 0.4 ml/minute; the eluate was monitored using a UV absorbance detector, at 280 nm.

Autoacetylation of isolated atACS

The non-enzymatic, autoacetylation of atACS (~130 µg of purified protein) was conducted in a 1-mL volume of a buffer consisting of 10 mM potassium acetate, 10 mM $MgCl_2$, 10 mM ATP, 50 mM Tris-HCl, pH 8.0 (5). Following incubation at 37°C for 2 hours, the protein solution was dialyzed into 10 mM HEPES, pH 7.5, 10 mM KCl, 2 mM TCEP, 10% glycerol.

Protein analysis

Protein preparations were evaluated by SDS-PAGE, and the acetylation status of atACS was evaluated by Western blot analysis, using an antibody (1:1000 diluted) that reacts with *N*-ε-acetyl-L-lysine (Cell Signaling Technology, #9441).

The acetylation status of atACS was also evaluated by mass spectrometric analysis of rLysC (Promega Corporation, Madison, WI) digested protein, using Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific Inc., Waltham, MA), housed at the Iowa State University Protein Facility (http://www.protein.iastate.edu). The purified protein preparations of wild-type and Ac-K atACS variants were subjected to SDS-PAGE analysis. Following staining with Coomassie Brilliant Blue, the protein band of interest was excised and digested using an Investigator™ ProGest (Genomic Solutions, Digilab Inc, Hopkinton, MA) in 0.5 mL buffer (50 mM Tris, pH 8, 15 mM iodoacetamide and 5 mM DTT) containing 20 µg LysC per mg of purified atACS protein. The peptides were identified using Sequest-HT (23) as the search engine within Proteome Discoverer (PD) 2.2 (Version 2.2.0.388; Thermo Fisher Scientific). The peptides were analyzed against the ACS protein sequence with settings for four possible missed cleavage sites, fragment mass tolerance of 0.02 Da precursor mass tolerance of 10 ppm. The possible side-chain modifications that were analyzed include the dynamic acetylation of Lys, static carbamidomethylation of Cys, dynamic deamidation of Asn and Gln and dynamic oxidation of Met residues.

Spectrophotometric atACS Activity Assay

AtACS activity was measured by coupling the acetate- and CoA-dependent formation of AMP from ATP with the oxidation of NADH using the reactions catalyzed by myokinase, lactate dehydrogenase and pyruvate kinase (18). Progress of this reaction was monitored as a decrease in A_{340}.

Circular Dichroism Spectroscopy

CD spectra were obtained with a Jasco J-710 CD spectrometer (JASCO Analytical Instruments, Easton, MD) at the Iowa State University Chemical Instrumentation Facility (https://www.cif.iastate.edu). Spectra were taken at wavelength range of between 190 nm and 260 nm, with the following settings parameter: pitch point 1, speed 20 nm/min, response time 4 sec, bandwidth 1 nm, temperature 20 °C and data mode CD-HT. Spectra where acquired with a 1 mm dichroically neutral quartz cuvette and the average of 3 scans was used for analysis.

Acquisition of spectra were obtained with protein samples in 10 mM HEPES-KOH, pH 7.5, 10 mM KCl, 2 mM TCEP and 10% glycerol, and these solutions were diluted into double distilled H_2O to a final protein concentration of 0.1-0.2 mg/ml. A baseline-spectrum for the same dilution of the buffer was obtained and subtracted from the experimental protein spectrum. Collected spectral data were converted from millidegrees to molar ellipticity ([θ], degrees $cm^2.dmol^{-2}$). Spectra were analyzed using a suite of algorithms collectively called CDPro (24), utilizing algorithms: SELCON3, CDSSTR, and CONTIN/LL.

RESULTS

The atACS sequence shares considerable similarity with ACS sequences from a range of diverse biological phyla, and these occur in 10 conserved motifs (A1 to A10) that are distributed evenly throughout the length of these sequences (10). Three of these motifs (A3, A4 and A5) contain conserved residues that determine the carboxylate substrate specificity of this class of enzymes (11, 25, 26). Motif A10 contains a highly conserved lysine residue (at position 622 of the Arabidopsis enzyme) (Figure 1). Based on studies of homologous proteins, this lysine residue of the Arabidopsis enzyme maybe a target for reversible acetylation, a mechanism that regulates the catalytic capability of these enzymes (8).

Several different experimental strategies were used to evaluate whether acetylation of residue Lys_{622} of atACS modulates catalytic activity of the enzyme. One of these experiments focused on the characterization of mutant variants, in which the Lys_{622} residue was mutated to Ala, Gln or Arg residues. These variant proteins behaved similarly to each other and to the wild-type enzyme in such attributes as: a) stability and yield upon expression in *E. coli*, recovering 10-15 mg of purified protein/L of culture (Figure 2A); b) elution from size exclusion gel filtration chromatography occurs as a single symmetric peak, at an elution volume consistent with the molecular weight of a ~72-kDa monomer (Figure 2B); c) CD spectra show that all variant proteins are similarly folded as the wild-type enzyme, and secondary structure calculations indicate insignificant differences in the proportion of α-helices, β-sheet, turns and unordered secondary structures among the 3 variant proteins as compared to the wild-type protein (Figure 2C and 2D).

Enzymatic assays of the three Lys_{622} variants demonstrate that compared to the wild-type enzyme, catalytic competence of these variants is reduced by more than 30-fold. Specifically, the k_{cat} for the Lys_{622}Arg mutant is reduced by 30-fold (increasing K_m by 25-fold), while the catalytic activity of the Lys_{622}Ala and Lys_{622}Gln mutants is even further reduced to such low levels that catalytic constants could not be accurately determined (Figure 3A & B).

The FPLC purified wild-type and Lys_{622} mutant variants of atACS were chemically autoacetylated *in vitro*, and their acetylation status was determined by western blot analysis using the anti-acetyl-lysine antibody. While the wild-type enzyme is acetylated, as expected all the Lys_{622} variants are not acetylated (Figure 3C). Therefore, these data indicate that this residue maybe the site of acetylation of the atACS, which affects enzymatic competence of this enzyme.

Direct assessment of this hypothesis was evaluated by incorporating acetyl-lysine (AcK) specifically at position #622 of atACS. This was accomplished by first engineering a stop codon into the ORF sequence at position-622. The expression of this variant in the absence of AcK results in the expression of a truncated atACS protein that terminates at codon #622. However, when AcK is provided in the medium, because of the expression of a cognate tRNA to read the stop codon as acetyl-lysine (20, 21), AcK is incorporated at position 622, and the resultant atACS-AcK variant is translationally fused at the C-terminus to a His_6-tag that is part of the expression vector used in these studies . Thus, Ni-column affinity chromatography was used to purify this atACS-AcK-His_6-tag variant. SDS-PAGE analysis of the recovered protein preparations identified two proteins bands, close to the expected 72-kDa band of atACS (Figure S1. A). Size exclusion gel filtration chromatography of the protein preparations identified two UV-absorbing peaks (A and B)

(Figure S1. B). The fractions that encompass these the two protein peaks were pooled separately as indicated (Figure S1. B) and concentrated for further analysis.

These two protein-preparations were subjected to chymotrypsin digestion, and resulting peptides were analyzed by mass-spectrometry to determine their identity. The Peak B derived peptide sequences showed 55% coverage of the atACS sequence, whereas Peak A peptides showed only 4.5% coverage of the atACS sequence (Figure S2 & S3). Additionally, enzymatic assays of Peak A and B preparations indicate that Peak A does not support ACS catalytic activity, whereas Peak B supports the catalysis of acetyl-CoA synthesis (Figure S4). Based on these results therefore, we conclude that Peak A represents a contaminating protein, and that Peak B is the atACS-AcK variant, and all further characterizations were conducted by pooling fractions that encompassed Peak B.

Direct demonstration that this isolated atACS-AcK variant is acetylated at the targeted Lys_{622} residue was obtained by mass-spectrometric analysis of the LysC-digested protein, and these data were compared to the mass-spectrometric analysis of the LysC digested wild-type atACS protein. The sequence coverage from these analyses were 40% for the wild-type atACS protein, whereas coverage of the atACS-Ack variant was ~62% (data not shown). Although the lysine-622 containing peptide could not be identified in the LysC digest of the wild-type atACS, it was successfully detected from the atACS-Ack variant protein. The acetylated lysine-622 peptide sequence was identified as, $TRSGK_{Ac}IMRRILRK$. This was based on the monoisotopic m/z value of 415.01038 Da (+0.76 mmu/+1.84 ppm), with a charge of +4 and theoretical mass of the MH+ ion of 1657.01967 Da (Figure 4).

Comparing the Michaelis-Menten kinetic parameters (K_m and k_{cat}) of atACS to the atACS-Ack variant indicates that acetylation of the enzyme affects its ability to catalyze acetyl-CoA synthesis (Figure 5). Specifically, as compared to the wild-type atACS, the atACS-Ack variant shows a 34-fold decrease in k_{cat}, and a 15-fold increase in K_m for the acetate substrate (Figure 5).

DISCUSSION

It has become increasingly apparent that the post-translational acetylation-deacetylation cycle of the ε-amino group of lysine residues is an important mechanism for regulating many biological processes (15, 27–30). This modification reaction is one of the broader suites of mechanisms by which the chemo-physical properties of side chains of amino acid residues can be diversified from the 20 genetically encoded canonical amino acids. Moreover, because these modifications are often reversible, and enzyme catalyzed, these reactions provide a rapid mechanism for regulating biological processes. Specifically, one can rationalize that acetylation of the ε-amino group of a lysine residue changes the character of that side chain from a highly polar, positively charged residue to a non-polar, side chain that is capable of participating in weaker H-bonding interactions as both a H-bond donor or H-bond acceptor. Such drastic changes in the properties of such a side chain can generate forces that confer structural alterations in proteins, which thereby render alter functionality.

Because ACS has a role in generating the substrate for such acetylation modification reactions, the post-translational acetylation-deacetylation of this enzyme has potential to generate an autoregulatory loop that could enhance or dampen such controlling mechanisms. Indeed, in bacteria and mammals ACS catalytic activity can be regulated by the acetylation of a specific lysine residue that occurs in a conserved region of these enzymes (6, 7, 31–33). For example, acetylation of a specific lysine residue in the A10 motif of the bacterial ACS inactivates catalysis (6, 7). Similarly, with the mammalian enzyme, acetylation completely deactivates catalysis, and deacetylation by sirtuin reactivates enzymatic activity (31, 32). In contrast, acetylation of the analogous lysine residue in homologous long chain acyl-CoA synthetases increases catalytic activity (34).

Herein we have expanded upon prior studies and demonstrate by a combination of different strategies that the plastid localized plant ACS is also susceptible to post-translational acetylation mechanism, which can modulate catalytic capability. Specifically, our western blot and mass spectrometric studies demonstrate

that acetylation of Lysine-622 of atACS reduces catalytic efficiency (k_{cat}/K_m) of the enzyme by ~522-fold. The importance of Lys-622 acetylation to ACS catalysis was demonstrated by a number of mutagenesis experiments. Specifically, eliminating this side chain, as in the Lys622Ala variant eliminates catalysis. Moreover, the conservative substitution of this residue (i.e., Lys622Arg variant), which maintains the positive side chain characteristic also eliminates ACS catalysis. Furthermore, the substitution of Lys-622 with a residue that is capable of H-bond formation (i.e., Lys622Gln variant) has the same inactivation result. These mutations do not affect the overall gross folding, oligomeric state, or the *in vivo* stability of the recombinant atACS protein, even though it renders the enzyme inactive. Hence, these results indicate the importance of this lysine residue in supporting the catalytic mechanism of this enzyme.

Consistent with this role in catalysis, the experimentally determined structure of the acetylated and non-acetylated bacterial ACS (35, 36) has established that this lysine residue is near the active site pocket during the first half of the reaction, when acetate and ATP bind and react to form the acetyl-adenylate intermediate and release the pyrophosphate product (10, 35). MStructural modeling of the atACS indicates a similar configuration for Lys-622, which may indicate that acetylation inhibits the first half-reaction catalyzed by atACS. This supposition is confirmed by comparing the kinetic parameters of the wild-type and atACS-Ack variant, with acetylation of the enzyme leading to an increase in the K_m and decrease in the k_{cat} values for acetate, one of the two substrates involved in the first half-reaction catalyzed by the enzyme. Collectively therefore, these results establish that acetylation of lysine-622 of atACS results in the inactivation of its catalytic capability. Hence, reversible acetylation of this residue would be an important mechanism by which the activity of this enzyme is modulated *in vivo*, especially to accommodate changes in cellular acetate and/or acetyl-CoA levels.

The *in vivo* physiological role of ACS in plants was initially thought to be the enzymatic supplier of the acetyl-CoA substrate required for *de novo* fatty acid biosynthesis in plastids (19). However, subsequent genetic and biochemical characterizations established that ACS is not needed for this metabolic role (14), and more recent genetic characterizations establish that ACS is part of a 2-enzyme system that plants use to maintain acetate homeostasis (12). Because acetyl-CoA is a crucial intermediate of metabolism that juxtaposes anabolic and catabolic processes, and is also a critical component of many regulatory processes associated with acetylation of controlling components (e.g., histone acetylation or N-terminal and/or amino acid side-chain acetylation), one can envision that its generation is highly regulated. Hence, the post-translational acetylation mechanism identified herein for modulating atACS activity could provide plants with the ability to rapidly adapt to changing environmental and developmental conditions and maintain cellular acetyl-CoA and acetate homeostasis.

REFERENCES

1. Drazic, A., Myklebust, L. M., Ree, R., and Arnesen, T. (2016) The world of protein acetylation. *Biochim. Biophys. Acta - Proteins Proteomics*. **1864**, 1372–1401
2. Verdin, E., and Ott, M. (2015) 50 years of protein acetylation: from gene regulation to epigenetics, metabolism and beyond. *Nat. Rev. Mol. Cell Biol.* **16**, 258–264
3. Bernal, V., Castaño-Cerezo, S., Gallego-Jara, J., Écija-Conesa, A., de Diego, T., Iborra, J. L., and Cánovas, M. (2014) Regulation of bacterial physiology by lysine acetylation of proteins. *N. Biotechnol.* **31**, 586–595
4. Narita, T., Weinert, B. T., and Choudhary, C. (2018) Functions and mechanisms of non-histone protein acetylation. *Nat. Rev. Mol. Cell Biol.* 10.1038/s41580-018-0081-3
5. Li, R., Gu, J., Chen, P., Zhang, Z., Deng, J., and Zhang, X. (2011) Purification and characterization of the acetyl-CoA synthetase from Mycobacterium tuberculosis. *Acta Biochim. Biophys. Sin. (Shanghai).* **43**, 891–899
6. Noy, T., Xu, H., and Blanchard, J. S. (2014) Acetylation of acetyl-CoA synthetase from Mycobacterium tuberculosis leads to specific inactivation of the adenylation reaction. *Arch. Biochem. Biophys.* **550**–**551**, 42–49
7. Gardner, J. G., Grundy, F. J., Henkin, T. M., and Escalante-Semerena, J. C. (2006) Control of acetyl-coenzyme A synthetase (AcsA) activity by acetylation/deacetylation without NAD+involvement in Bacillus subtilis. *J. Bacteriol.* **188**, 6715
8. Starai, V. J., Celic, I., Cole, R. N., Boeke, J. D., and Escalante-Semerena, J. C. (2002) Sir2-dependent activation of acetyl-CoA synthetase by deacetylation of active lysine. *Science (80-.).* **298**, 2390–2392
9. Tsang, A. W., and Escalante-Semerenat, J. C. (1999) CobB, a new member of the SIR2 family of eucaryotic regulatory proteins, is required to compensate for the lack of nicotinate mononucleotide:5,6- dimethylbenzimidazole phosphoribosyltransferase activity in cobT mutants during cobalamin biosynthesis in Sal. *J. Biol. Chem.* **273**, 31788–31794
10. Gulick, A. M. (2009) Conformational Dynamics in the Acyl-CoA Synthetases, Adenylation Domains of Non-ribosomal Peptide Synthetases, and Firefly Luciferase. *ACS Chem. Biol.* **4**, 811–827
11. Gulick, A. M., Starai, V. J., Horswill, A. R., Homick, K. M., and Escalante-Semerena, J. C. (2003) The 1.75 Å crystal structure of acetyl-CoA synthetase bound to adenosine-5???-propylphosphate and coenzyme A. *Biochemistry*. **42**, 2866–2873
12. Fu, X., Yang, H., Pangestu, F., and Nikolau, B. J. (2019) Failure to Maintain Acetate Homeostasis by Acetate-Activating Enzymes Impacts Plant Development. *Plant Physiol.* 10.1104/pp.19.01162
13. Lin, M., and Oliver, D. J. (2008) The Role of Acetyl-Coenzyme A Synthetase in Arabidopsis. *PLANT Physiol.* **147**, 1822–1829
14. Ke, J., Behal, R. H., Back, S. L., Nikolau, B. J., Wurtele, E. S., and Oliver, D. J. (2000) The Role of Pyruvate Dehydrogenase and Acetyl-Coenzyme A Synthetase in Fatty Acid Synthesis in Developing Arabidopsis Seeds. *Plant Physiol.* **123**, 497–508
15. Chen, H., Venkat, S., McGuire, P., Gan, Q., and Fan, C. (2018) Recent Development of Genetic Code Expansion for Posttranslational Modification Studies. *Molecules*. 10.3390/molecules23071662
16. Johnson, J. A., Lu, Y. Y., Van Deventer, J. A., and Tirrell, D. A. (2010) Residue-specific

incorporation of non-canonical amino acids into proteins: recent developments and applications. *Curr. Opin. Chem. Biol.* **14**, 774–780

17. Xie, J., and Schultz, P. G. (2006) A chemical toolkit for proteins — an expanded genetic code. *Nat. Rev. Mol. Cell Biol.* **7**, 775–782

18. Sofeo, N., Hart, J. H., Butler, B., Oliver, D. J., Yandeau-Nelson, M. D., and Nikolau, B. J. (2019) Altering the Substrate Specificity of Acetyl-CoA Synthetase by Rational Mutagenesis of the Carboxylate Binding Pocket. *ACS Synth. Biol.* **8**, 1325–1336

19. Behal, R. H., Lin, M., Back, S., and Oliver, D. J. (2002) Role of acetyl-coenzyme A synthetase in leaves of Arabidopsis thaliana. *Arch. Biochem. Biophys.* **402**, 259–267

20. Venkat, S., Gregory, C., Sturges, J., Gan, Q., and Fan, C. (2017) Studying the Lysine Acetylation of Malate Dehydrogenase. *J. Mol. Biol.* **429**, 1396–1405

21. Venkat, S., Chen, H., Stahman, A., Hudson, D., McGuire, P., Gan, Q., and Fan, C. (2018) Characterizing Lysine Acetylation of Isocitrate Dehydrogenase in Escherichia coli. *J. Mol. Biol.* **430**, 1901–1911

22. Gallego-Jara, J., Écija Conesa, A., de Diego Puente, T., Lozano Terol, G., and Cánovas Díaz, M. (2017) Characterization of CobB kinetics and inhibition by nicotinamide. *PLoS One*. **12**, e0189689

23. Eng, J. K., McCormack, A. L., and Yates, J. R. (1994) An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J. Am. Soc. Mass Spectrom.* **5**, 976–989

24. Sreerama, N., and Woody, R. W. (2000) Estimation of Protein Secondary Structure from Circular Dichroism Spectra: Comparison of CONTIN, SELCON, and CDSSTR Methods with an Expanded Reference Set. *Anal. Biochem.* **287**, 252–260

25. Starai, V. J., and Escalante-Semerena, J. C. (2004) Acetyl-coenzyme A synthetase (AMP forming). *Cell. Mol. Life Sci.* **61**, 2020–30

26. Marahiel, M. A., Stachelhaus, T., and Mootz, H. D. (1997) Modular Peptide Synthetases Involved in Nonribosomal Peptide Synthesis. *Chem. Rev.* **97**, 2651–2674

27. Christensen, D. G., Xie, X., Basisty, N., Byrnes, J., McSweeney, S., Schilling, B., and Wolfe, A. J. (2019) Post-translational Protein Acetylation: An Elegant Mechanism for Bacteria to Dynamically Regulate Metabolic Functions. *Front. Microbiol.* **10**, 1604

28. Hossain, D., and Tsang, W. Y. (2019) The Role of Protein Acetylation in Centrosome Biology. in *Results and problems in cell differentiation*, pp. 17–25, **67**, 17–25

29. Hu, Y., Lu, Y., Zhao, Y., and Zhou, D.-X. (2019) Histone Acetylation Dynamics Integrates Metabolic Activity to Regulate Plant Response to Stress. *Front. Plant Sci.* **10**, 1236

30. Wang, R., and Wang, G. (2019) Protein Modification and Autophagy Activation. in *Advances in experimental medicine and biology*, pp. 237–259, **1206**, 237–259

31. Hallows, W. C., Lee, S., and Denu, J. M. (2006) Sirtuins deacetylate and activate mammalian acetyl-CoA synthetases. *Proc. Natl. Acad. Sci.* **103**, 10230–10235

32. Schwer, B., Bunkenborg, J., Verdin, R. O., Andersen, J. S., and Verdin, E. (2006) Reversible lysine acetylation controls the activity of the mitochondrial enzyme acetyl-CoA synthetase 2. *Proc. Natl. Acad. Sci.* **103**, 10224–10229

33. Starai, V. J., Takahashi, H., Boeke, J. D., and Escalante-Semerena, J. C. (2003) Short-chain fatty acid activation by acyl-coenzyme A synthetases requires SIR2 protein function in Salmonella enterica and Saccharomyces cerevisiae. *Genetics*. **163**, 545–55

34. Chen, Z., Luo, L., Chen, R., Hu, H., Pan, Y., Jiang, H., Wan, X., Jin, H., and Gong, Y. (2018)

Acetylome Profiling Reveals Extensive Lysine Acetylation of the Fatty Acid Metabolism Pathway in the Diatom Phaeodactylum tricornutum. *Mol. Cell. Proteomics*. **17**, 399–412

35. Gulick, A. M., Starai, V. J., Horswill, A. R., Homick, K. M., and Escalante-Semerena, J. C. (2003) The 1.75 Å Crystal Structure of Acetyl-CoA Synthetase Bound to Adenosine-5‘-propylphosphate and Coenzyme A†. *Biochemistry*. **42**, 2866–2873

36. (SSGCID), S. S. G. C. for I. D., Fox III, D., Davies, D. R., Calhoun, B., Edwards, T. E., Lorimer, D. D., Mutz, M. W., and Krysan, D. J. Crystal structure of Apo Cryptococcus neoformans H99 Acetyl-CoA Synthetase with an Acetylated Active Site Lysine. *TO BE Publ.* 10.2210/PDB5VPV/PDB

Author contributions

B.J.N conceived the study. N.S and B.J.N designed the experiments and wrote the manuscript. N.S and K.L conducted the experiments.

Conflict of interests

The authors declare that they have no conflicts of interest with the contents of this article.

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Figures & Tables

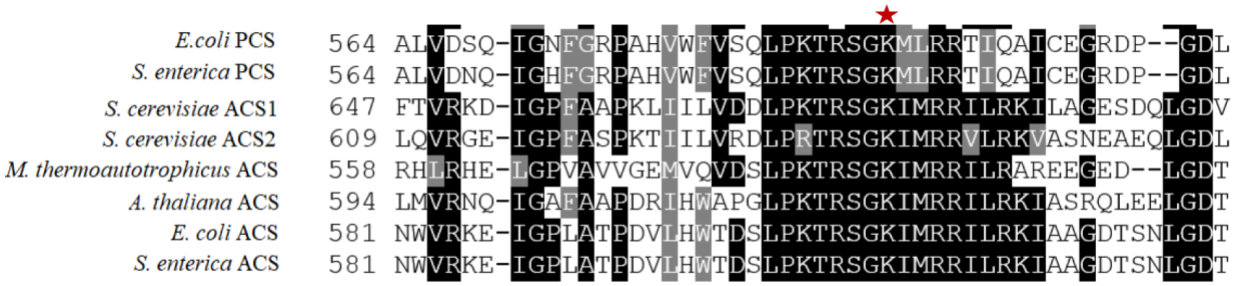


Figure 1: Comparison of the amino acid sequences of motif A10 of the AMP-forming family of acyl-CoA synthetases. Sequence alignments of enzymes that use acetate (ACS) or propionate (PCS) as substrates from different organisms. The red asterisk identifies the conserved lysine residue that is reversibly acetylated. Alignment was performed with Clustal Omega, and white letters on a black background indicate identity, white letters on a gray background indicate similarity, and black letters on a white background indicate no conservation.

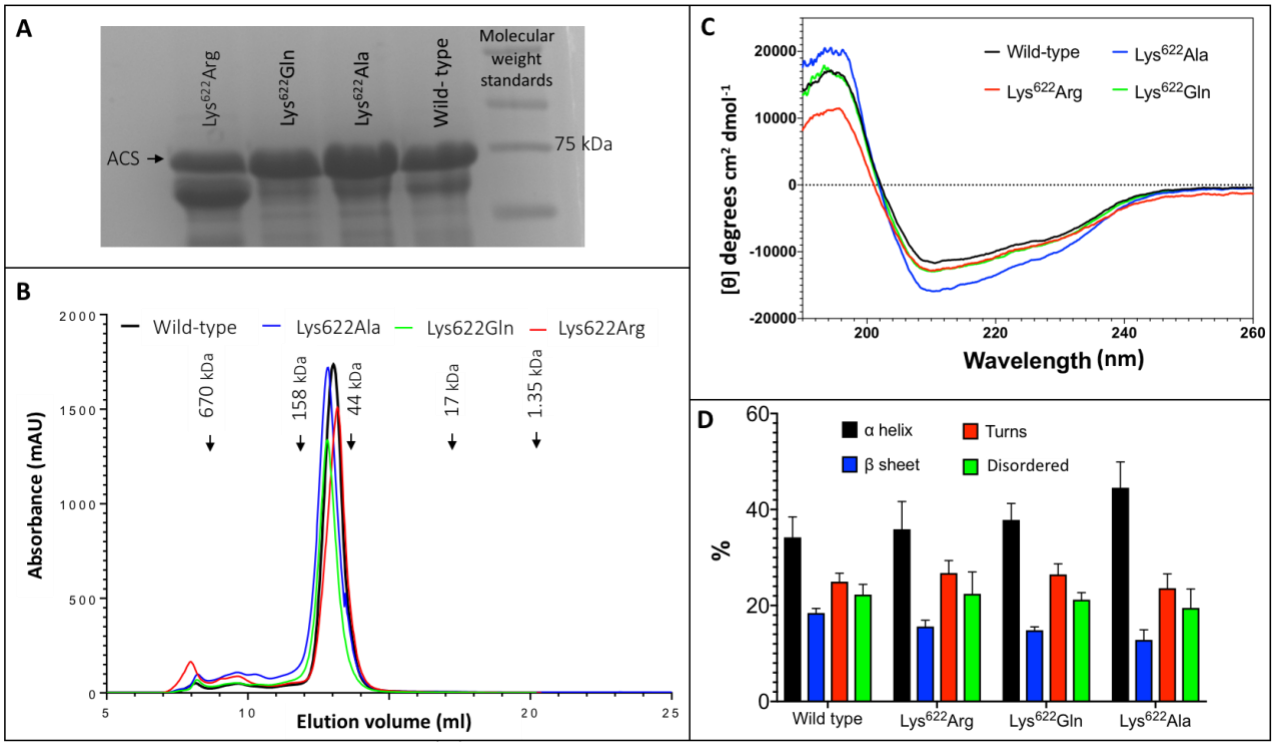


Figure 2: Characterization of ACS Lys_{622} mutants. **A**. SDS-PAGE analysis of purified wild-type and indicated Lys_{622} mutant variants of atACS. **B**. Size exclusion gel filtration chromatography of wild-type and Lys_{622} mutant variants of atACS. **C**. CD spectra of wild-type and Lys_{622} mutant variants of atACS. Measurements are an average of three replicate scans. **D**. Secondary structure composition calculated from the CD spectra of wild-type and Lys_{622} mutant variants of atACS. Errors bars indicate standard errors from 3 replicate CD spectra calculated by 3 algorithms, SELCON3, CDSSTR, and CONTIN/LL.

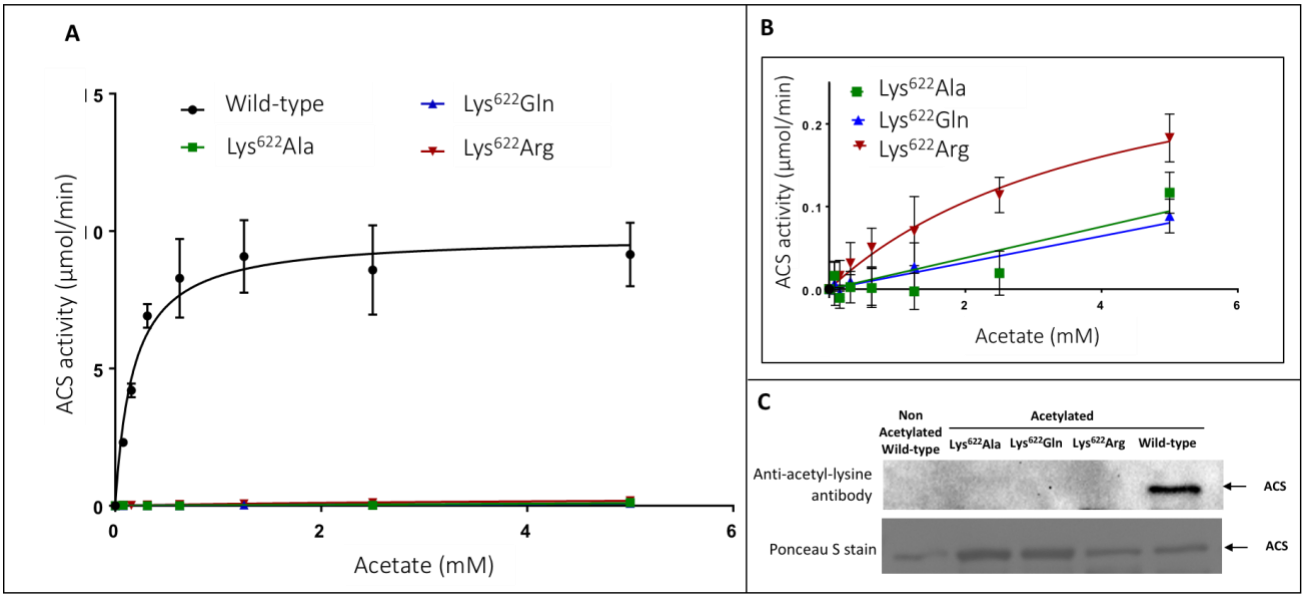


Figure 3: Effect of acetylation on catalytic properties of atACS. **A.** Michaelis-Menten kinetic analysis of atACS Lys_{622} variants. **B**. Expanded view of data presented in Panel A. **C**. Acetylation status of atACS Lys_{622} variants, analyzed by western blot analysis with anti-acetyl-lysine antibody. The top panel is the result from western blot analysis and bottom panel shows Ponceau S staining of the same membrane.

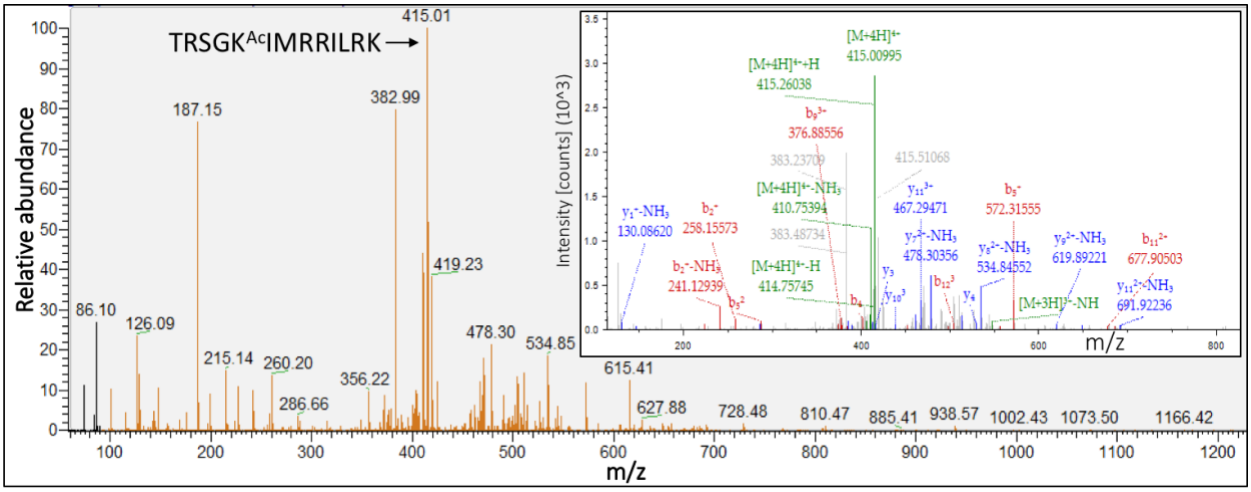
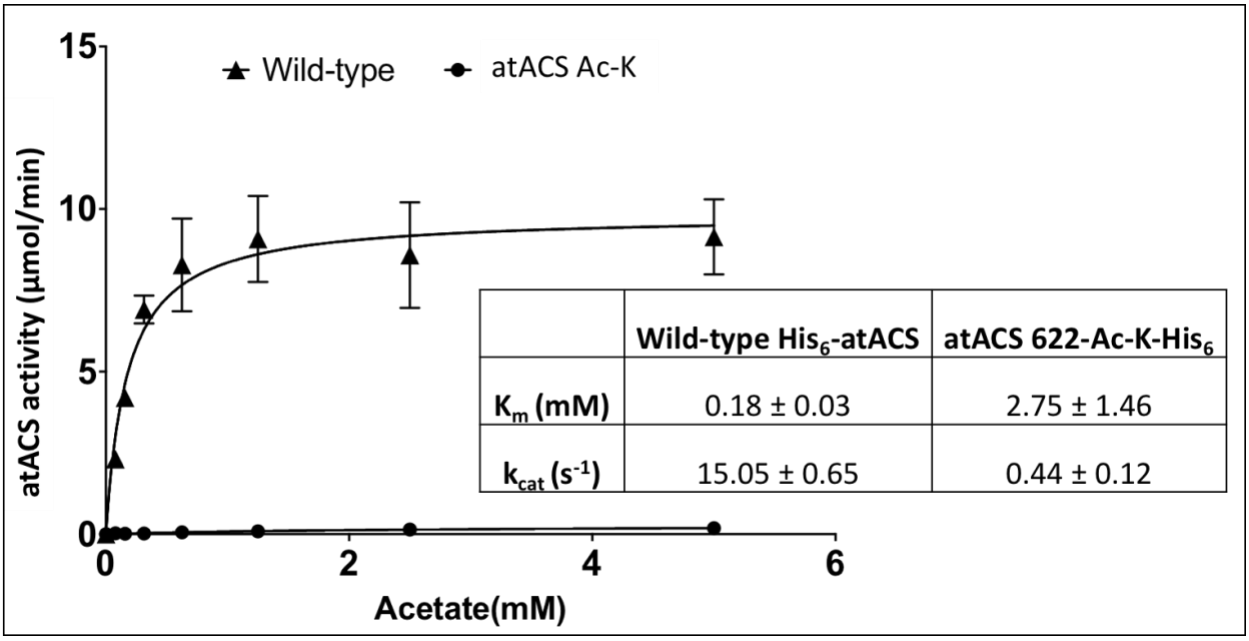


Figure 4: Mass spectrometric identification of acetylated Lys-622 containing peptide. LC-MS/MS spectra and MS2 fragmentation pattern (inset) of the LysC generated peptide containing lysine 622 from atACS Ac-K variant.



	Wild-type His_6-atACS	atACS 622-Ac-K-His_6
K_m (mM)	0.18 ± 0.03	2.75 ± 1.46
k_{cat} (s^{-1})	15.05 ± 0.65	0.44 ± 0.12

Figure 5: Catalytic capabilities of wild-type atACS (▲) and atACS Ac-K variant (●). (Inset table) Kinetic parameters of wild-type atACS and atACS Ac-K variant.