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Application of Acetyl-CoA synthetase from *Methanothermobacter thermautotrophicus* to non-native substrates

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ABSTRACT

The substrate selectivity of the Trp^{416}Gly mutant of *Methanothermobacter thermautotrophicus* acetyl-CoA synthetase (Trp^{416}Gly MT-ACS1) was explored. The goal was to identify its substrate range, particularly for functionalized carboxylic acid substrates that would allow post-synthesis functionalization of CoA thioesters or downstream products using metathesis or Click chemistry. Relative activities were determined by *in situ* formation of acyl-hydroxamate iron (III) complexes. Trp^{416}Gly MT-ACS1 showed good activities for saturated straight chain carboxylic acids from C_2 to C_8, for ω-alkenyl straight chain carboxylic acids from C_4 to C_7 and for ω-alkynyl straight chain carboxylic acids from C_5 to C_7. Carboxylic acids showing ≥ 20% conversion in screening reactions were used in preparative conversions that completely consumed the added CoASH.

1. Introduction

Acyl-CoA synthetases (ACSs) play fundamental roles in the metabolism of both prokaryotic and eukaryotic cells and are essential to many biochemical transformations. ACSs belong to the adenylate-forming enzyme superfamily, which encompasses acyl- and aryl-CoA synthetases, the adenylation domains of nonribosomal peptide synthetases, and firefly luciferase. [1] All of these enzymes catalyze a two-step reaction in which an enzyme-bound acyl-adenylate is formed in the first half-reaction, then the acyl-CoA thioester is formed from this intermediate in the second half-reaction (Fig. 1) [2–4].

ACSs can be divided in four different sub families. Both very long-chain and long-chain acyl-CoA synthetases (EC 6.2.1.3) activate ≥ C_{14} fatty acids, allowing them to enter mitochondria, the nucleus or the Golgi apparatus where subsequent metabolism occurs. Their fates can include β-oxidation, inhibition of tri-iodothyronine (T3) binding and protein transport respectively. [5–7] By contrast, medium-chain acyl-CoA synthetases conjugate C_4–C_{10} fatty acids as well as xenobiotics such as benzoate to CoASH, which prepares them for the glycine conjugation pathway of detoxification. [7] Finally, short-chain acyl-CoA synthetases (EC 6.2.1.1) yield acetyl- or propionyl-CoA, a key link between different anabolic and catabolic pathways.

Individual ACSs that can form a wide diversity of CoA thioesters with additional functional groups would be highly useful in several bioorganic applications. For example, acyl groups with appropriate functionality could be used for post-synthesis functionalization of acyl-CoAs. The resulting conjugates might be highly useful for *in vivo* imaging or for other studies. By using biotinylated Hoveyda-Grubbs second generation catalyst, it is possible to do metathesis reactions in the periplasm of *E. coli* [8,9]. Click reactions could also be used in living cells and in animals because of the biological inactivity of triple bonds. It is not an uncommon strategy which has been used by Kuerschner et al. to follow and understand fatty acid metabolism or Bertozzi et al. to visualize fucosylated glycans in developing zebrafish [10,11]. We therefore decided to address the growing need for CoA thioesters with alkenyl or alkynyl functional groups by identifying an ACS with wide substrate range.

Our interest in α-oxoamine synthases [12,13] provided a second motivation for seeking ACSs with broad substrate tolerance. These enzymes catalyze a thio-Claisen condensation between a small amino acid and an acyl-CoA, resulting in *C-C* bond formation. Serine palmitoyltransferase (SPTase) has attracted our particular interest since this enzyme catalyzes the first committed step in sphingolipid biosynthesis using serine and palmitoyl-CoA (Fig. 2). By employing palmitoyl-CoA analogs that included alkene or alkyne functionality, SPTase could also yield sphingolipid analogs suitable for post-synthesis functionalization, analogous to those described above for the acyl-CoAs. All of these applications depend on ACSs that accept more than simple *n*-alkyl acids.

Methanothermobacter thermautotrophicus ACS (MT-ACS1), a member of the short-chain ACS family, has the expected high activity for acetate

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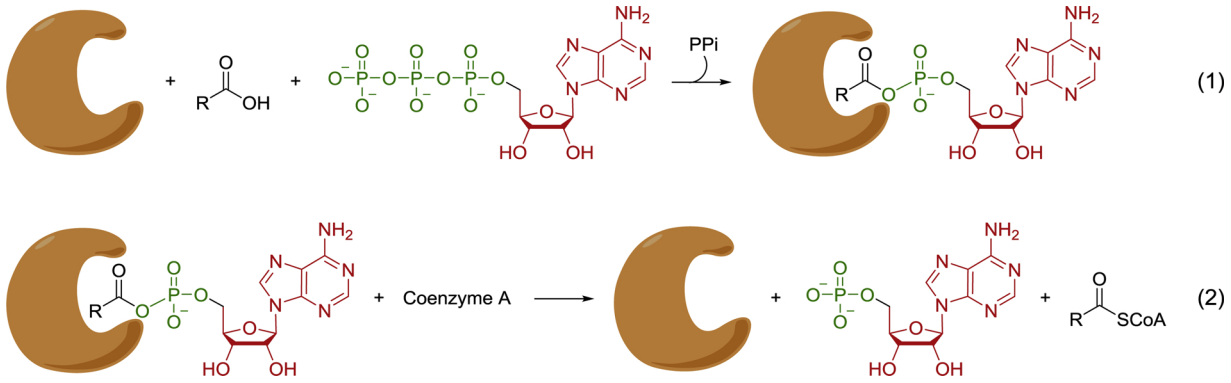

Fig. 1. Reactions catalyzed by acyl-CoA synthases (ACSs). Formation of an acyladenylate (Reaction 1) activates the carboxyl and allows formation of the corresponding thioester and AMP (Reaction 2).

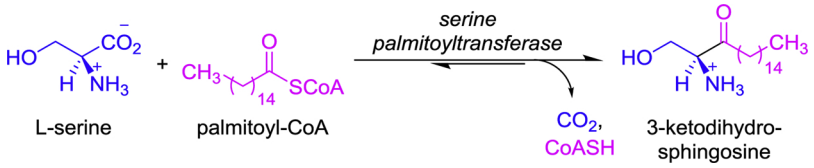

Fig. 2. α-Oxoamine synthases.

and propionate. Surprisingly, the Trp^{416}Gly variant showed significantly broader substrate selectivity, accepting straight chain acids ranging from C_2 to C_8 as well as some branched chains such as 3-methylvalerate and 4-methylvalerate. [14,15] Based on these promising results, we decided to explore the substrate scope of this enzyme further, concentrating particularly on functionalized acids containing halogen, hydroxyl, alkene and alkyne moieties.

2. Results and discussion

In order to test a variety potential substrates forTrp^{416}Gly MT-ACS1 efficiently, a fast and reliable assay to measure enzyme activity was needed. A colorimetric assay originally described by Lipmann and Tuttleand and by Rose for quantitating acyl-phosphates – a motif with similar reactivity to acyl-CoAs – was adapted. [16] In the presence of excess hydroxylamine and $FeCl_3$, acyl-phosphates form hydroxamic acids that in turn yield mono-acyl-hydroxamate / Fe^{III} complexes with λ_{max} = 510 nm [16–18]. We experimentally determined the ε^{510} value by mixing various concentrations of authentic acetohydroxamic acid with an excess of $FeCl_3$ [19].

Prior to screening, it was critical to ensure that no acyl-adenylate intermediates were released into solution by the enzyme (since this would give a false positive signal in the hydroxylamine / Fe^{III} assay). Indeed, it has been reported that Thr^{313}Lys MT-ACS1 showed activity even in the absence of CoASH, whereas no activity was detected for the wild-type. [15] This observation suggested that the Thr^{313}Lys mutation altered the enzyme structure and allowed some acyl-adenylate to escape. Fortunately, our studies of the Trp^{416}Gly MT-ACS1 variant showed no significant activity in the absence of CoASH.

We were also concerned about possible deleterious effects of the *N*-terminal hexahistidine purification tag on enzyme activity. Both the His_6-tagged and native Trp^{416}Gly MT-ACS1 proteins were purified and catalytic activities were assayed in the presence of 40, 0.50 and 20 mM carboxylic acid, CoASH and $MgCl_2$ • ATP, respectively (Table 1).While both enzyme forms showed consistent general trends with respect to acyl chain length preference, activities toward shorter-chain substrates was somewhat higher for the His_6-tagged version. Because the hexahistidine moiety also facilitated purification, all subsequent studies were carried out with the His-tagged form of Trp^{416}Gly MT-ACS1.

Hexahistidine-tagged Trp^{416}Gly MT-ACS1 was then tested with a range of carboxylic acids with various functional groups. In all cases, acids were provided at 40 mM and CoASH and ATP were fixed at 0.50 and 20 mM, respectively. Substrates included α-halogenated acids, benzoic acid, adipic acid, methacrylic acid and α-hydroxyisobutyric acid. Essentially no conversion was observed for any of these substrates (Fig. 3). More success was found for a series of homologous alkenyl- and alkynyl carboxylic acids (Table 2). As expected, both acetic and propionic acids were converted to their CoA thioesters. A conjugated double bond blocked this conversion, however, since acrylic acid was not a substrate for Trp^{416}Gly MT-ACS1.

Apart from the case of acrylic acid, terminal alkenes had little impact on the efficiency of CoA conjugation by Trp^{416}Gly MT-ACS1 and good conversions were found for C_3 to C_7 ω-unsaturated acids

Table 1
Catalytic activities of Trp^{416}Gly MT-ACS1 in the presence and absence of an *N*-terminal His_6 tag.

Substrate		His_6-tagged	Native
C_2	CH_3CO_2H	59%	24%
C_3	CO_2H	30%	9%
C_4	CO_2H	52%	25%
C_5	CO_2H	100%	100%
C_6	CO_2H	145%	90%
C_7	CO_2H	96%	67%
C_8	CO_2H	16%	17%

<5% relative activity was found for:

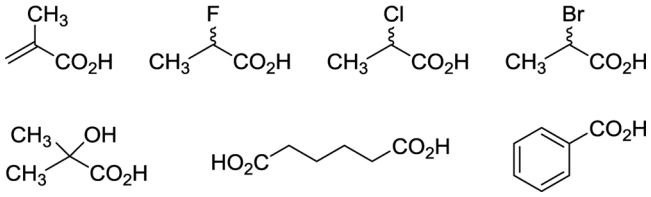

Fig. 3. Carboxylic acids showing ≤5% conversion by Trp^{416}Gly acetyl-CoA synthetase under standard reaction conditions.

Table 2
Substrate range of Trp^{416}Gly MT-ACS1.

Substrate		Relative activity (%)
C_2	CH_3CO_2H	43
C_3	CO_2H	20
	CO_2H	–
C_4	CO_2H	35
	CO_2H	14
	CO_2H	–
C_5	CO_2H	67
	CO_2H	72
	CO_2H	60
C_6	CO_2H	97
	CO_2H	100
	CO_2H	55
	CO_2H	86
C_7	CO_2H	64
	CO_2H	73
	CO_2H	67
C_8	CO_2H	11
C_{16}	CO_2H	–

(Table 2). The relative reaction rates correlated reasonably well with total chain length, suggesting that this is the key parameter for acceptance by the enzyme. We also tested one non-terminal alkenyl acid (*trans*-3-hexenoic acid), which also proved to be a good substrate for the enzyme.

The presence of a terminal alkyne was also well-tolerated by Trp^{416}Gly MT-ACS1, provided that at least two methylenes intervened between the carbonyl and alkyne (Table 2). Thus, while 4-butynoic acid was not accepted as a substrate, higher homologs (C_5 – C_7) were suitable.

All acids showing conversions ≥20% in the initial screening reactions were then tested in preparative-scale reactions with the goal of complete CoASH consumption. Reactions were carried out at pH 7.5 in high concentration Tris buffer to minimize thioester decomposition [20] and the conversions were followed by an HPLC method adapted from Purdon et al. [21] For each acid tested, it was possible to convert all the added CoASH to the corresponding thioester by tuning reaction time (always ≤ 35 min) and quantity of enzyme added (no more than 50 μg). Substrates were divided into three groups (alkyl, alkenyl and alkynyl) and it was possible to separate all of the corresponding thioesters using the same HPLC method (Fig. 4).

In parallel with these trials, a one pot reaction was performed with 5-hexynoic acid in order to functionalize the corresponding CoA thioester. After conjugation with CoA, the addition of sodium NaN_3 and CuCl yielded 4-(1,2,3-triazol-5-yl)butanoyl-CoA (Fig. 5). This result provides proof-of-principle for alkynyl CoA thioester derivatization using Click strategies.

3. Conclusion

We have explored the substrate range of the Trp^{416}Gly variant *M. thermautotrophicus* acetyl-CoA synthetase. While the enzyme did not tolerate α-substitution or an α,β-alkenyl moiety, both alkenes and alkynes further from the carboxylic acid were acceptable. The best substrates were C_5 or C_6 acids, regardless of unsaturation, suggesting that total chain length was the key parameter recognized by the enzyme. A total of eight new substrates for the enzyme were discovered in this study and an HPLC method was developed in order to show quantitative conversion of CoASH to the corresponding thioesters for all acids accepted by the enzyme. We further showed that copper-promoted azide / alkyne cycloaddition could be carried out on a suitable acyl-CoA directly in the enzyme reaction mixture.

4. Experimental section

4.1. HPLC methods

Enzymatic thioesterification reactions were monitored by reversed-phase HPLC using a 150 × 4.6 mm Synergi™ Hydro-RP 80 Å column using 50 mM NaP_i pH 5.0 and 50 mM NaP_i, pH 5.0/40% acetonitrile as Solvents A and B, respectively at a flow rate of 1 mL/min. Initial conditions (3% B) were maintained for 2.5 min, then a linear increase to 18% B over 5 min was immediately followed by a linear increase to 28% B over 2.5 min and a final linear increase to 100% B over 10 min. After an 8 min hold at 100% B, a linear decrease to 3% B over 2 min was followed by a 5 min hold at the initial conditions (3% B). The eluent was monitored by UV absorbance at 220 nm (to detect peptide bonds and adenyl moieties). These conditions allowed baseline separation of all relevant analytes in this study (Coenzyme A, t_r = 8.31 min; acetyl-CoA, t_r = 9.68 min; propionyl-CoA, t_r = 11.66 min; butyryl-CoA, t_r = 12.70 min; pentanoyl-CoA t_r = 13.85 min; hexanoyl-CoA, t_r = 15.41 min; heptanoyl-CoA, t_r = 17.38 min; octanoyl-CoA, t_r = 18.85 min; 3-butenoyl-CoA, t_r = 12.51 min; 4-pentenoyl-CoA, t_r = 13.20 min; 5-hexenoyl-CoA, t_r = 14.45 min; 6-heptenoyl-CoA, *tr* = 16.03 min; *trans*-3-hexenoyl-CoA, *tr* = 14.80 min; 4-pentynoyl-CoA, t_r = 12.35 min; 5-hexynoyl-CoA, t_r = 13.13 min; 6-heptynoyl-CoA, *tr* = 14.25 min).

Click chemistry reaction was monitored by reversed-phase HPLC using a 150 × 4.6 mm Acclaim™ PolarAdvantage II 120 Å column using 50 mM NaPi pH 5.0 and acetonitrile as Solvents A and B, respectively at a flow rate of 1 mL/min. Initial conditions (1.2% B) were maintained for 2.5 min, then a linear increase to 7.2% B over 5 min was immediately followed by a linear increase to 11.2% B over 2.5 min and a final linear increase to 40% B over 10 min. After an 8 min hold at 40% B, a linear decrease to 1.2% B over 2 min was followed by a 5 min hold at the initial conditions (1.2% B). The eluent was monitored by UV absorbance simultaneously at 220 nm (to detect triazole ring, peptide bonds and adenyl moieties). These conditions allowed baseline separation of all relevant analytes in this study (5-hexynoyl-CoA, t_r = 15.420 min; 4-(1,2,3-triazol-5-yl)butanoyl-CoA, t_r = 3.096 min).

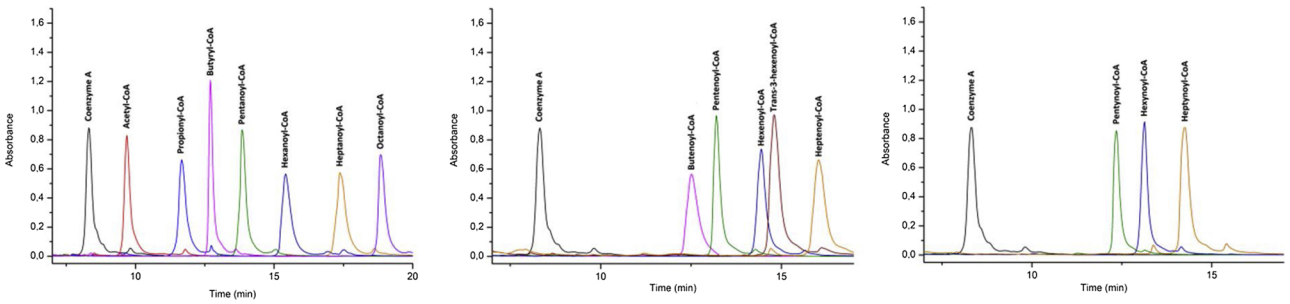

Fig. 4. HPLC characterization of enzymatically-synthesized acyl-CoAs.

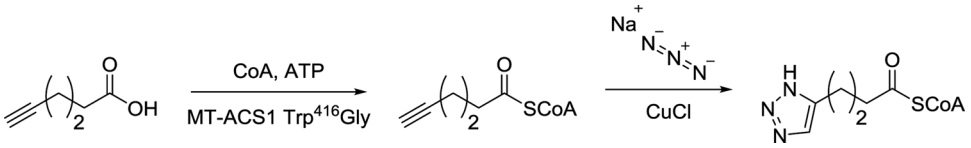

Fig. 5. Post-functionalization of hexynoyl-CoA *via* Click chemistry.

4.2. Acetohydroxamic acid Fe^{III} standard curve at 510 nm

Acetohydroxamic acid was dissolved in water with a final volume of 300 µL (0.25, 0.50, 1.0, 1.5, 2.0, 2.5 and 3.0 mM). To each was added 150 µL of freshly prepared 1 N HCl, 5% trichloroacetic acid, 1.25% $FeCl_3$, after which each turned red/brownish immediately. The A^{510} value for each sample was determined and the slope of the standard curve yielded $\varepsilon^{510} = 634\ M^{-1}.cm^{-1}$.

4.3. Creating an overexpression plasmid for the $Trp^{416}Gly$ acetyl-CoA synthetase gene from M. thermautotrophicusstrainZ245

A linear fragment containing the complete $Trp^{416}Gly$ acetyl-CoA synthetase gene was excised from pLM4 [22] as an *Nde*I, *Bam*HI fragment. A 500 ng portion of this insert was mixed with an equal quantity of *Nde*I, *Bam*HI-digested pET15b vector, then ligated with 1 µL of T4 DNA ligase in a total volume of 20 µL. Successful ligation was confirmed by agarose gel electrophoresis and a sample of the reaction mixture was used to transform *E. coli* ElectroTen Blue cells with selection for ampicillin resistance. Randomly-chosen single colonies were used to inoculate 5 mL portions of liquid LB medium containing 50 µg / mL ampicillin and the cultures were grown overnight at 37 °C and 250 rpm. Plasmid DNA was isolated from each using the Wizard Plus SV Minipreps DNA purification system and the inserts were analyzed by automated fluorescent Sanger sequencing. A plasmid with the predicted sequence was designated pLM5.

4.4. Overexpression and purification of M. Thermoautotrophicus $Trp^{416}Gly$ acetyl-CoA synthetase

Plasmid pLM5 was used to transform *E. coli* BL21(DE3) cells with selection for ampicillin resistance (50 µg / mL) on LB plates. A single colony was used to inoculate a 12 mL portion of liquid LB medium containing 50 µg / mL ampicillin and the culture was grown overnight at 37 °C and 250 rpm. An aliquot (10 mL) was diluted into 1 L of the same medium in a 2 L baffled flask. The culture was grown at 37 °C with shaking at 250 rpm until reaching an $O.D._{600}$ of 0.6. The desired protein was overexpressed by adding sterile isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The culture was incubated for an additional 4 h at 30 °C, then the cells were harvested by centrifugation (6000 × *g* at 4 °C for 15 min). The cell paste was resuspended in 6 mL of cold lysis buffer (50 mM Tris − HCl, pH 8.0) and disrupted by two passages through a French pressure cell at 18,000 psi. The lysate was clarified by centrifugation (18,000 × *g* for 20 min at 4 °C) and applied to a 5 mL Ni–NTA agarose column (GE Healthcare) previously equilibrated with 20 mL of binding buffer (20 mM NaP_i, 500 mM NaCl, 20 mM imidazole, pH 7.4). The column was washed with binding buffer until the A^{280} value returned to its baseline value, then the desired protein was eluted by washing with 20 mM NaP_i, 500 mM NaCl, 500 mM imidazole, pH 7.4.The protein solution was concentrated at 4 °C by ultrafiltration and 1 L culture of culture typically yielded *ca.* 5 mg of purified protein. Purified protein was stored in 20% glycerol 100 µL aliquots at − 20 °C.

4.5. Activity determination

Enzymatic activity was determined by monitoring acyl-CoA formation by the hydroxamate reaction. [16,17] Reaction mixtures contained 100 mM Tris-Cl, pH 7.5, 600 mM hydroxylamine•HCl, pH 7.0, 2 mM reduced glutathione, 40 mM of carboxylic acid, 0.5 mM of CoASH, 20 mM of $MgCl_2$-ATP and 25 µg of $Trp^{416}Gly$ acetyl-CoA synthetase. A standard temperature of 65 °C was used (determined to be the optimal for the enzyme) [14] and after 30 min, reactions were terminated by adding 0.5 vol of stop solution (1 N HCl, 5% trichloroacetic acid, 1.25% $FeCl_3$). Acyl-CoA formation was quantified by Beer's Law using $\varepsilon^{510} = 634\ M^{-1}.cm^{-1}$.

4.6. Preparative conversions

Conversion was determined by HPLC at 220 nm where CoA-containing species show strong absorbance. Reaction mixtures contained 500 mM Tris-Cl, pH 7.5, 11.5 mM carboxylic acid, 0.5 mM CoASH, 1 mM of $MgCl_2$-ATP and 25–50 µg of $Trp^{416}Gly$ acetyl-CoA synthetase in a final volume of 2 mL. A reaction temperature of 65 °C was used [14] and after 35 min, reactions were analyzed by HPLC and the extent conversion evaluated. For all the substrates of interest, total consumption of CoASH was observed.

4.7. Click chemistry assay

Conversion was determined by HPLC at 220 nm where triazoles species show strong absorbance. The reaction mixture contained 500 mM Tris-Cl, pH 7.5, 11.5 mM hexynoic acid, 0.5 mM CoASH, 1 mM of $MgCl_2$-ATP and 25–50 µg of $Trp^{416}Gly$ acetyl-CoA synthetase in a final volume of 2 mL. A reaction temperature of 65 °C was used [14] and after 35 min, 11.5 mM NaN_3 and 1 mg CuCl were added. The resulting mixture was incubated at 65 °C for an additional 30 min prior to HPLC analysis. Total conversion of hexynoate-CoA to the corresponding triazole was observed.

Author agreement

All authors concur with submission and have seen a draft copy of the manuscript and agree with its publication.

The work has not been published and is not under consideration at another journal.

The manuscript does not contain experiments using animals or humans.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.enzmictec.2019.05.005.

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