ATP-citrate lyase multimerization is required for coenzyme-A substrate binding and catalysis

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Running Title: Role of ATP-citrate lyase multimerization in catalysis

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ABSTRACT

ATP-citrate lyase (ACLY) is the predominant source of nucleocytosolic acetyl-CoA, a fundamental building block of carbon metabolism in eukaryotes. ACLY is aberrantly regulated in many cancers, cardiovascular disease, and metabolic disorders. However, the molecular mechanisms determining ACLY activity and function are unclear. To this end, we investigated the role of the uncharacterized ACLY C-terminal citrate synthase homology domain (CSHD) in the mechanism of acetyl-CoA formation. Using recombinant and purified ACLY and a suite of biochemical and biophysical approaches, including analytical ultracentrifugation, dynamic light scattering, and thermal stability assays, we demonstrate that the C-terminus maintains ACLY tetramerization, a conserved and essential quaternary structure in vitro and likely also in vivo. Furthermore, we show that the C-terminus, only in the context of the full-length enzyme, is necessary for full ACLY binding to CoA, stability and catalysis. Together, we demonstrate that ACLY forms a homotetramer through the C-terminus to facilitate CoA binding and acetyl-CoA production. Our findings highlight a novel and unique role of the C-terminal citrate synthase homology domain in ACLY function and catalysis, adding to the understanding of the molecular basis for acetyl-CoA synthesis by ACLY. This newly discovered means of ACLY regulation has implications for the development of novel ACLY modulators to target acetyl-CoA dependent cellular processes for potential therapeutic use.

INTRODUCTION

All three domains of life utilize acetyl-CoA as an essential metabolite for carbon transport. Acetyl-CoA is necessary for glycolysis, de novo lipogenesis, cholesterol synthesis, and protein acetylation (1). The dysregulation of this metabolite is an omnipresent feature and crucial component of diabetes, hypercholesterolemia, and oncogenesis (2). Among the majority of eukaryotes, nucleocytosolic acetyl-CoA is primarily derived from glucose (3) by the enzyme ATP-citrate lyase (ACLY; E.C. 2.3.3.8). The ubiquity of acetyl-CoA utilization makes ACLY a central node in the intersection of carbon metabolism and cell homeostasis, and is a potent therapeutic target for the control of cell proliferation and metabolic dysregulation (4).

ACLY sequence and function are highly evolutionarily conserved across metazoans. The enzyme sequence has two major superdomains: one that is strongly homologous to the NDP-forming acyl-CoA synthetase superfamily (5), and one that is strongly homologous to the class I citrate synthase (CS; E.C. 2.3.3.1) superfamily (6, 7). Catalysis is thought to occur at the former, spanning ACLY domains 1-5. These domains are structurally and functionally similar to acyl-CoA synthetases, such as succinyl-CoA synthetase (SCS; EC 6.2.1.4) (Fig. 1A). The ACLY catalytic mechanism proceeds through an elaborate series of events that convert MgATP, citrate, and CoA into ADP, acetyl-CoA, and oxaloacetate (8–10). Briefly, the enzyme ATP-grasp domains 4 and 5 bind ATP with Mg2+ and rearrange to autophosphorylate the catalytic histidine 760, which then complexes with citrate at
the junction of two power-helices from domains 2 and 5. The histidine transfers the phosphate to citrate to form a phospho-citryl intermediate. CoA enters into the active site, carries out a nucleophilic attack to form a citryl-CoA intermediate. This reaction also liberates inorganic phosphate. Finally, a general base within the enzyme cleaves the citryl-CoA to produce acetyl-CoA and oxaloacetate.

Although the chemistry is clear, the regulation of these reactions remains poorly understood. Many studies have focused on the regulation of ACLY activity in vitro, such as during insulin stimulation or the Warburg effect (11, 12). This includes regulation by phosphorylation (13–16), acetylation, and ubiquitination (17). These studies have all focused on the regulation of the SCS superdomain. Yet, a significant portion of ACLY remains uncharacterized; the role of the C-terminal citrate synthase homology domain (CSHD) is largely unknown. Improved understanding of the CSHD could present a new dimension to the study of ACLY regulation.

The CSHD is proposed by BLAST (18) to be structurally similar to the citrate synthase (CS) superfamily. CS catalyzes the conversion of oxaloacetate and acetyl-CoA to citrate and CoA, in the reverse of the ACLY mechanism, with the same reaction intermediates. Therefore, it was possible that ACLY utilizes the same chemistry as CS for noncanonical CoA-binding through the CSHD (19). However, this parallel was limited by only partial conservation of the primary sequence. Only some of the CS residues that interacted with CoA and stabilize the CoA intermediates (20) were conserved in ACLY. Thus, ACLY may have evolved to take advantage of another feature of CS, independent of substrate-binding.

CS is present across evolutionary space as a dimeric complex (7). The dimerization of CS is an essential feature, as the citrate synthase dimer interface forms the enzyme active site (20, 21). More broadly, protein self-association may confer increased structural stability and may allow novel chemistry to occur at the association interface (22).

ACLY is also multimeric. Size-exclusion and sedimentation studies of plant and murine homologs suggest that ACLY forms a homotetramer (23, 24), although this has not been confirmed for the human homolog. The conserved multimerization of ACLY parallels the conserved multimerization of CS. Thus, multimerization may play a structural and/or a catalytic role in ACLY function. In this study we hypothesized that ACLY multimerization through CSHD is an essential

**RESULTS**

**Multimerization is mediated by the ACLY C-terminal citrate synthase homology domain**

In order to study the functional role of human ACLY multimerization we first designed and recombinantly prepared ACLY truncations to isolate the SCS and CS superdomains. We produced the protein as full-length (ACLY-FL, residues 1-1101), the SCS-homology superdomain alone (ACLY-Nterm, residues 1-820), and the CHSD alone (ACLY-Cterm, residues 820-1101). Size-exclusion chromatography (SEC) confirmed that the loss of the CSHD collapsed the enzyme complex to a stable monomeric state, in agreement with previous studies of recombinant human ACLY (19). In contrast, when we produced the CSHD alone we observed the migration of the purified species at a significantly earlier retention volume than expected for the 34 kDa CSHD monomer (Fig. 1C). This result indicated that the CSHD was a multimer in solution. However, migration during size-exclusion is dependent on species size and shape (25), and so is an insufficient predictor of molecular weight and stoichiometry. Also, the SEC profiles of the full-length enzyme and the CSHD did reveal the presence of multiple species. The migration profiles were broad, possibly as the result of some instability of these constructs, especially in our buffers that purposefully excluded stabilizing cofactors or other additives. The ACLY-FL and ACLY-Nterm values may have been smaller than predicted due to some degradation over the course of the experiment (Fig. 1B). For this reason, we proceeded with analytical ultracentrifugation and light scattering experiments to better characterize the multimeric state of these constructs.

Sedimentation equilibrium (SE) analysis confirms that the CSHD drives ACLY
tetramerization. A simple, ideal-species global analysis of the SE data for ACLY-FL calculated a molecular weight of 452 kDa, close to the expected tetramer molecular weight of 495 kDa. When linearized, these data aligned closest to the expected distribution for a homotetramer (Fig. 2A). Parallel experiments of ACLY-Nterm provided a global molecular weight of 82 kDa, close to the expected molecular weight of 93 kDa. When linearized, these data aligned well to the expected distribution for a single monomeric state (Fig. 2B). Equally important was the observed molecular weight of the ACLY CSHD, which yielded a single species molecular weight of 138 kDa. Just as with the full-length enzyme, these data aligned closest to the expected distribution for a homotetramer of 138 kDa (Fig. 2C; summarized in Table S1). These trends held across a range of concentrations and centrifugation speeds (Fig. S1). We took these data to support our observation that the CSHD is sufficient for ACLY multimerization.

Yet, there were inconsistencies in the linearization of the ACLY-FL and ACLY-Cterm SE data that, just as with the SEC data, suggested the presence of multiple states. While the SE data described above are indicative of the multimeric state for each of these constructs, SE can only provide a global average molecular weight. Moreover, this approach could mask the presence of an equilibrium among different subspecies. Therefore, we looked into the potential existence of different protein states within each sample.

Dynamic light scattering (DLS) is a fast and non-destructive analysis of protein tumbling in solution (26), which we employed to obtain the ACLY average hydrodynamic radius as well as to detect any sizeable populations of soluble aggregates or breakdown products. We did not observe significant amounts of any such artifacts that may not have separated during SEC (Fig. S2). We next employed Sedimentation velocity (SV) for detection of equilibria among multiple species under cell-free solution conditions. Our SV experiments confirmed that ACLY-FL was predominantly a single species in solution with an average $s_{20,w}$ of 14.6 S, while ACLY-Nterm predominantly sedimented at 4.96 S, and the CSHD at 6.98 S (Fig. 3B, summarized in Table S2). We noticed some broadness and kurtosis of the SV distributions, and so went back to our SE data to test different equilibrium models that might explain these trends.

Upon further analysis of our SE data, the ACLY-FL distribution also fit to a dimer-tetramer equilibrium model, with a predicted $K_d$ of 4.71 μM, although with poorer statistics. The ACLY-Cterm data could also be fit to a dimer-tetramer equilibrium with a predicted $K_d$ of 97.7 μM, although even less well (Table S1). ACLY-Nterm could not be meaningfully fit to any equilibrium model.

Together, these data demonstrate that ACLY-FL exists as a multimer in solution, while ACLY Nterm is monomeric. The ACLY CSHD appears to drive multimerization to a state most consistent with a tetramer. Therefore, we present the CSHD as the means of ACLY multimerization.

The biological relevance of our cell-free studies is supported by the observation of ACLY tetramerization in protein derived directly from plant and murine tissue. However, we endeavored to validate our findings in human cells. To that end, we co-transfected HEK-293T cells with ACLY-FL with an N-terminal FLAG and/or Myc epitope tag. We found that our epitope-tagged ACLY-FL constructs could immunoprecipitate one another (Fig. 4). Consistent with our cell-free data, we conclude that ACLY forms a stable complex with itself in the context of the human cellular milieu.

**Multimerization increases the thermal stability of ACLY**

The formation of homomultimers is a widespread, evolutionarily-conserved feature of eukaryotic proteins with several potential advantages, such as increased stability (22). These same advantages may hold for the ACLY multimer. Therefore, we were curious about the role of the CSHD in ACLY intrinsic stability, substrate binding, and catalytic efficiency. We used differential scanning calorimetry (DSC) to assess the thermal stability of the ACLY-FL multimer (Fig. 5). We observed that ACLY-FL harbored two melting transitions, with average melting transitions ($T_m$) of 53.8°C (referred to as peak 1) and 69.3°C (referred to as peak 2).

Of note, the full-length protein could not be refolded after denaturation. This result suggested that the intramolecular interactions maintaining the various domains of ACLY are stable ones, i.e., we could detect no equilibrium between different conformations of the enzyme. In addition, the unequal $\Delta H_{vH}/\Delta H_{CAL}$ ratio of both peaks 1 and 2 also suggested underlying complexity (27, 28). However, the ACLY-FL peak 1 could only be fit by a single-peak in a non-two state model. We speculate that the hidden, thermodynamically-stable states hinted at by the unequal $\Delta H_{vH}/\Delta H_{CAL}$
Role of ATP-citrate lyase multimerization in catalysis interaction. We next asked what the mechanism of this interaction might be, and what might be the functional consequence of crosstalk between the N- and C-termini.

**ACLY enzymatic activity requires tethered N- and C-terminal cooperation**

Current models of ACLY function are guided by the ACLY homology to acyl-CoA synthetases, with little known about the contribution of the CSHD. The SCS-homology model for ACLY function suggests that the ACLY-Nterm construct should be capable of binding all ACLY cofactors and catalyzing the formation of acetyl-CoA and oxaloacetate. Contrary to this model, we observed that the loss of the CSHD completely nullified acetyl-CoA production (Fig. 7). This observation offered the possibility either that the CSHD contacted the N-terminus to directly interact with cofactors as a part of the molecular mechanism of turnover. In order to test this hypothesis, we assayed if ACLY-Cterm added in trans can restore ACLY-Nterm activity. We observed that incubation of ACLY-Nterm with up to 10-fold molar excess of ACLY-Cterm did not rescue catalysis. Consistent with the DSC data, these titration experiments suggested that the CSHD allosterically regulated the N-terminal domain in cis.

**The ACLY N-terminus optimally binds CoA in the context of the full enzyme**

Sequence analysis of the CSHD suggested that the domain could contain a CoA-binding pocket, and so effect catalysis through direct interaction with CoA. The crystal structures of eubacterial CS with citrate and CoA define an essential histidine and aspartate in the CS active site to stabilize the citryl-CoA enolate intermediate (20), as well as several others that contact the adenine and pantetheine moieties (29). Some, but not all, of these same residues are present in the CSHD. This observation presented the possibility that the CSHD directly binds ACLY cofactors. We used differential scanning fluorimetry (DSF) to assay the potential interaction of our ACLY N- and C-terminal constructs with CoA, citrate, and MgATP in different combinations at 20-fold molar excess (Fig. 8). For ACLY-FL we observed statistically-significant thermal stabilization through Mg$^{2+}$-dependent ATP binding (2.87°C), as well as stabilization through the binding of citrate (1.98°C), and CoA (3.30°C). The stabilization was additive when the cofactors were combined (summarized in Table S3).

We were initially surprised at the consistent bimodal melt profile of the FL enzyme. The similar $T_M$ values of the 2nd peak of the full-length enzyme and the CHSD suggested that the full-length ACLY underwent two distinct unfolding events, one for each superdomain. The CSHD data best fit to a simultaneous unfolding/dissociation model (with the lowest $X^2/DF$ value), as did the ACLY-FL peak 2. Therefore, we propose that the ACLY-FL peak 1 represents the unfolding of the N-terminus. The difference between the two peaks of ACLY-FL and the individual $T_M$ of the truncations may be due to the multimeric context of the SCS superdomain in the full-length. The direction of stabilization relative to ACLY-FL provided a clue to the relationship between the two superdomains in the context of the enzyme complex. These data suggested that the ACLY N- and C-termini interacted with one another. The SCS superdomain is strongly stabilized by the self-association; the SCS superdomain gains approximately 8 degrees of stability when brought into the ACLY tetramer. We would not expect such a change were the SCS superdomain a self-contained molecular unit, isolated from the CSHD. In contrast to the stabilization of the SCS superdomain, ACLY appears to sacrifice some of the stability of the CSHD for the sake of the holoenzyme. That we see this decrease suggests that CSHD may provide more than an inert tether to the other ACLY subunits. However, the communication between the N- and C-termini is not so strong as to produce a single melting event for the ACLY complex. Therefore, the two superdomains may have only a few points of contact.

This conclusion was supported by SEC of ACLY-Nterm mixed with excess ACLY CSHD. This assay demonstrated that the two fragments eluted closer to each other than when ACLY-Nterm and ACLY-Cterm are run separately (Fig. 6). We conclude that in the absence of an intramolecular tether, the two domains may form a weak ratio may be the separate unfolding of the ATP-grasp and citrate-binding regions, separated as they are by the bridging, unstructured domain 5. Further DSC with a slower ramp rate did not help to reveal the likely underlying transitions (Fig. S3).

In contrast to the bimodal ACLY-FL melt profile, ACLY-Nterm and ACLY-Cterm had only single transitions. These constructs had average $T_M$ values of 46.2°C and 73.0°C, respectively (Fig. 5, summarized in Table S3). As expected, multimerization clearly increased the overall stability of the protein.

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Table S4). In line with previous studies, ACLY-Nterm was significantly stabilized by citrate (3.52°C). ATP also had a weakly-significant effect (2.50°C). Although the calculated means suggested a small, potential stabilization of the N-terminus by CoA, this shift was not consistent. For instance, when the N-terminus with MgCl₂/ATP/citrate/CoA was compared against the N-terminus with MgCl₂/ATP/citrate alone, the effect disappeared (p = 0.276). It therefore seemed possible that the synthetase-homology model for CoA binding was inaccurate, with the CSHD instead coordinating with CoA. However, we found that ACLY-Cterm was not bound by any of these cofactors.

To more sensitively probe the effect of CoA binding to ACLY, we carried out a DSC analysis of ACLY-FL, ACLY-Nterm and ACLY-Cterm in the presence and absence of CoA, again at 20-fold molar excess. We observed a clear shift for ACLY-FL upon incubation with CoA (1.0°C), whereas the addition of CoA did not have a statistically significant effect on the thermal stabilities of ACLY-Nterm or ACLY-Cterm alone (Fig. 9). Interestingly, the addition of CoA only affected the stabilization of ACLY-FL peak 1. CoA also sharpened the peak 1 melt transition, producing a better fit of the data. Together with our DSF analysis, we conclude that this shift is indicative of the ACLY N-terminus binding to CoA only when in the context of the intact protein.

DISCUSSION

ACLY is a major node in the transduction of insulin signaling to changes in gene expression. It is a highly-prized target for metabolic diseases, including atherosclerosis, hypercholesterolemia, and also oncogenesis (30, 31). In the latter case, the dependence of tumors on glucose for energy makes ACLY inhibition especially appealing. ACLY knockdown can drastically decrease cancer cell proliferation without significantly affecting nearby healthy tissue (32). ACLY may also be a target for anti-aging studies, as decreased nucleocytosolic acetyl-CoA levels and ACLY inhibition may delay the onset of age-related decline (33, 34).

While ACLY has been the focus of myriad clinical studies (35), many of these efforts have stalled. There are few promising small-molecule ACLY inhibitors. Therefore, there exists a clear need for a better understanding of the catalytic and allosteric biochemistry of the enzyme. Although a great deal of effort has gone into understanding the signaling cascades and post-translational modifications that tune ACLY function in the cell

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(36), limited knowledge about the molecular basis for ACLY function has held back the development of ACLY therapeutic treatments.

In this study, we highlight the importance of the ACLY C-terminal domain. First, we demonstrate that the evolutionarily-conserved CSHD is the driver of ACLY multimerization. Next, we show that ACLY cannot function without the CSHD. Last, we define the CSHD as necessary for CoA binding through coordination with the ACLY SCS superdomain. This is a surprising finding for two primary reasons: the citrate synthase homology domain of citrate synthetase is a dimer and not a tetramer, and although the ACLY N-terminal acyl-synthetase homology domain contains a consensus sequence for binding CoA, it is unable to fully bind CoA in the absence of the C-terminal CSHD.

Each of these findings presents new avenues for ACLY regulation. We connect the evolutionary relationship between ACLY and the CS superfamily to a molecular framework for ACLY function. Our light-scattering, analytical ultracentrifugation, and co-IP experiments show that human ACLY is a stable and consistent homotetramer. To date, we have been unable to isolate stable, monomeric ACLY through point mutagenesis of the CSHD. It stands to reason that structural destabilization through the loss of tetramerization would have severe deleterious effects on catalysis. Thus, multimerization may be as important for ACLY as it is for CS. We posit that a genetic rearrangement event, early in the evolution of citrate utilization for acetyl-CoA production, created the link between SCS and CS as a ready means of increasing enzyme stability and acetyl-CoA output that has endured across millions of years of evolution (37). The potential for a dimer-tetramer equilibrium of ACLY at a biologically-relevant concentration also speaks to this relationship. ACLY tetramerization may be the product of a dimer of CSHD dimers coming together. Citrate synthase dissociation is a powerful regulatory mechanism that responds to protein levels, the local pH, and the local ionic environment to toggle CS between a dimeric active state and a monomeric inactive state, with a Kₜ of 0.2 µM (38). The same could also a means of inactivating ACLY. During periods of high glycolytic flux, cellular ACLY levels also increase several fold (17). Therefore, ACLY may also be controlled by a similar concentration-dependent switch. This connection between the two enzymes can inform on the unknown structure of the CSHD,
offering new frameworks for regulating ACLY levels in vivo.

We conclude from our activity assays that the CSHD is necessary for ACLY function. Moreover, our substrate-binding assays contradict our initial hypothesis that the CSHD directly borrows the substrate-binding residues of CS. Instead, ACLY has adapted the CSHD for a more nuanced role as a facilitator for the activity of the N-terminal SCS superdomain. We observed that the two major regions of the enzyme communicate with one-another. Furthermore, we conclude that the CSHD participates in the chemistry at the active site by facilitating CoA binding.

Ultimately, the ACLY CSHD may be a potent and specific target for rational drug design. Together, these studies highlight a novel and unique role of the C-terminal CSHD in ACLY function and catalysis and a novel avenue for targeting the C-terminal domain of ACLY for regulating acetyl-CoA dependent cellular processes for potential therapeutic use.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant ACLY

The Homo sapiens ACLY isoform 1 (Accession # NM_001096.2) was used in this study. To generate the plasmid DNA constructs for recombinant protein expression, this sequence was PCR amplified with DNA primers adding a C-terminal 6x histidine tag sequence and EcoRI/SalI cut sites for ligation. The PCR product was ligated into a custom-engineered pET Duet-1 vector for Escherichia coli expression or into the pFastBac HT vector (Novagen) for expression in Spodoptera frugiperda (Sf9). ACLY-FL and ACLY-Cterm expressed equally in both systems, whereas ACLY-Nterm could only be produced in the Sf9 system after codon optimization by Genscript (Piscataway, NJ). The cells were harvested by centrifugation at 4000 rpm and lysed via sonication in lysis buffer (25 mM Tris-HCl pH 8.8, 200 mM NaCl, 5 mM MgCl₂, 5 mM potassium citrate tribasic pH 7.5, 10 mM β-mercaptoethanol, 1 μg/mL lysozyme, 1 μg/mL DNase I, 100 μg/mL PMSF, and 1X EDTA-free protease inhibitor [Roche, Basel, Switzerland]). The lysate was clarified by centrifugation at 19000 rpm and purified by Ni-NTA affinity, followed by overnight dialysis into low salt buffer (Tris-HCl pH 8.8, 20 mM NaCl, 5 mM MgCl₂, 5 mM potassium citrate tribasic pH 7.5, and 10 mM β-mercaptoethanol). The dialysate was further purified by HiTrap Q HP (GE Healthcare, Pittsburgh, PA), superdex 200 HiLoad 16/600 (GE Healthcare), and superose 6 increase 10/300 GL (GE Healthcare) into size-exclusion buffer (25 mM HEPES pH 8.0, 200 mM NaCl, 1 mM DTT). Bio-Rad (Hercules, CA) gel filtration standard was used for comparison. Co-migration studies were carried out in size-exclusion buffer with 1 hr pre-incubation at 4°C. All samples were run on a superdex 200 increase 10/300 GL column (GE Healthcare). SDS-PAGE was carried out using ExpressPlus PAGE 12% Tris-MOPS gels (Genscript) with ProSieve prestained protein ladder (Lonza, Walkersville, MD).

Analytical Ultracentrifugation

Sedimentation equilibrium of the recombinant protein was carried out in size-exclusion buffer at concentrations of 0.2, 0.4, 0.8 mgmL⁻¹ at 4°C in a Beckman Optima XL-I analytical ultracentrifuge (Beckman Coulter, Palo Alto, CA). Absorbance optics at 280 nm were taken using quartz windows in six-chamber charcoal centerpiece in an An-60 Ti four-hole rotor (Beckman Coulter) with 6 hr. scans with a radial step-size of 0.001 (four scans per speed, with three independent experiments for ACLY-FL and ACLY-Cterm, two experiments for ACLY-Nterm). The speeds used were 6000, 9000, 12000, and 16000 rpm as recommended based the expected monomer and tetramer molecular weights of the ACLY constructs (39, 40). The molecular weights were calculated by global ideal species analysis by SEDPHAT (41). The error associated with the global analysis was determined by Monte-Carlo simulation with 1000 iterations at a confidence level of 0.95. We only analyzed those data between the sample meniscus (variable) and Absorbance₂₈₀nm ≤ 1.5 AU, to avoid violating Beer-Lambert linearity between concentration and light attenuation. Equilibrium fitting was done with strict mass conservation, rotor stretch correction, as well as sample bottom, baseline, and RI noise fitting. Goodness of fit was determined by global reduced χ². The sample parameters of protein partial specific volume, buffer density, and buffer viscosity calculated by SEDNTERP (42). Protein molar mass and extinction coefficients were calculated by ExPASy ProtParam (43).

Sedimentation velocity experiments were carried out in size-exclusion buffer at 0.3 mgmL⁻¹ for all proteins, at 4°C and 42000 rpm with a radial step-size of 0.001 and a five-minute delay between scans (200 scans total, two independent experiments). Only the minimum number of scans
were analyzed (41), as recommended for the maximum molecular weight by DCDT+ version 2.4.3 by John Philo (44, 45). The data were analyzed by SEDFIT (46) with a continuous c(s20,w) distribution model with mass conservation and a confidence level (F-ratio) of 0.95, where the frictional ratio (f/f0) was allowed to float. The c(s20,w) distribution were plotted using Prism v7.4 (GraphPad, La Jolla, CA).

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**Cell Culture**

HEK-293T cells were maintained in DMEM + 10% FBS. Cells were plated at 1 million cells per plate in 10 cm dishes for 24 hours prior to transfection. 10 µg of pEF6/ACLY-FL/Myc and/or pEF6/ACLY-FL/FLAG was combined with 24 µL of PEI (Sigma-Aldrich, #408727) in 800 µL Opti-MEM and incubated with cells for 6 hours. Following incubation, cells were washed with PBS and placed in maintenance media for 48 hours and harvested.

**Co-Immunoprecipitation**

Harvested cells were lysed in 400 uL of NP-40 buffer containing 1 mM DTT and 1x Proteinase Inhibitor. 1 ng of protein was incubated for 1.5 hours with anti-FLAG beads (Sigma-Aldrich, St. Louis, MO, #M8823). Following incubation, beads were washed 3 times and then boiled in 2x SDS buffer. Boiled IP samples and 30 µLg of input for each sample were run on SDS-page gels, transferred to nitrocellulose membranes and incubated with primary anti-tubulin (Sigma-Aldrich, #T6199), anti-FLAG (Sigma-Aldrich, #F1804) or anti-Myc (Cell Signaling Technology, Danvers, MA, #9B11) antibodies overnight. Blots were washed in PBS-T (3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl, 135 mM NaCl, 0.05% Tween 20, pH 7.4) two times for 10 minutes each and then incubated for 30 minutes with secondary antibodies against mouse or rabbit. Blots were again washed two times for 10 minutes at PBS-T and then imaged on the LI-COR Odyssey 675 fluorescent imager (LI-COR Biotechnology, Lincoln, NE) as previously described (47).

**Dynamic Light Scattering**

Light scattering experiments were performed on a Zetasizer µV (90° back-scattering apparatus, Malvern Panalytical, Malvern, UK) in a 2 µL SUPRASIL quartz cuvette (Hellma Analytics, Müllheim, Germany) at 3.3°C. The protein was tested at 8 µM in size-exclusion buffer in two independent experiments, six replicates (33 scans per replicate). The buffer viscosity and refractive index were calculated with the Zetasizer Software v7.12 and the protein refractive index was set to 1.45. The protein hydrodynamic distribution was calculated by the Malvern Zetasizer DTS software and correlation functions were calculated by SEDFIT. Prism was used to plot the data.

**Kinetic Assays**

The activity of ACLY was measured according to the method of Linn and Srere (48). Briefly, enzyme activity was determined for protein at 20 nM in 1 mL of 200 mM Tris 8.8, 20 mM MgCl2, 20 mM potassium citrate tribasic pH 7.5, 20 mM ATP, and 10 mM DTT with 5U of porcine malate dehydrogenase (Millipore) and 200 µM NADH (Sigma-Aldrich). The reaction was initiated with 400 µM CoA and allowed to proceed for 20 min at during continuous Absorbance detection in a BioMate 3S spectrophotometer (Thermo Fischer Scientific, Waltham, MA). The activity was determined across three independent experiments, n=6 each. Excel (Microsoft, Redmond, WA) was used to select the linear range of the data (where second derivative = 0) and to convert ΔAbsorbance into enzyme velocity using the extinction coefficient of NADH. The data were analyzed for statistical significance and plotted with the median and 95% confidence interval using Prism. The significance of the difference in Vmax was determined by two-way ANOVA with Sidak’s multiple comparisons test.

**Differential Scanning Fluorimetry**

DSF was carried out with 0.1 mgmL⁻¹ protein that had been dialyzed overnight into DSF buffer (25mM HEPES 7.5, 200 mM NaCl, 1 mM DTT). SYPRO Orange protein dye (Thermo Fischer Scientific, Waltham, MA) was added to the protein at a final concentration of 5X, along with additional buffer, 10 mM MgCl2, 10 mM ATP disodium salt, 10 mM potassium citrate tribasic pH 7.5, and/or 1 mM coenzyme A (all reagents from Sigma-Aldrich) to bring the total volume to 20 µL. The concentrations of these additives were chosen to be at least 20X above the published Kd (or Km where the Kd was unavailable) (10, 19), and thus considered to be saturating. The reactions were set up in a 384-well optical plate (Applied Biosystems, Foster City, CA). The plate was read in a 7900HT Real-Time PCR (Applied Biosystems) from 20°C to 95°C at a 2% ramp rate with a 10 min 20°C pre-equilibration step. The raw fluorescence was quantified using the SDS 2.4 software package. The
data were subsequently normalized and taken to the first derivative using Excel, with the inflection point of the melt curve (the max dy/dx value) used as the $T_M$. To account for inflections at the beginning of the PCR stage, data outliers were excluded using ROUT analysis, and the remaining values were tested for significance with a two-way ANOVA with Dunnett’s multiple comparisons test in Prism. Individual tests of significance were determined by t-tests with Sidak’s multiple hypothesis correction. The data were plotted with the median and 95% confidence interval.

**Differential Scanning Calorimetry**

The proteins were dialyzed overnight in DSC Buffer (25 mM HEPES pH 8.0, 200 mM NaCl) to remove any reducing agent, adjusted with the dialysis buffer to 25 µM, and spin-filtered (EMD Millipore, Burlington, MA) at 18k xg to remove particulates. Prior to loading into the cell, the samples were degassed with stirring in an evacuation chamber for 5 min at 10°C. Each sample was analyzed by MicroCal VP-Capillary DSC (Malvern Panalytical, Malvern, UK), with buffer in the reference cell. When DSC was used to assess CoA-mediated stabilization, either CoA or water were added to the protein after dialysis to account for the effect of dilution. DSC buffer with either

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CoA or water was used to provide the buffer-only scan. The samples were scanned from 10.0 °C - 90.0 °C with a 10-min 10.0 °C pre-equilibration and a 30 °C hr$^{-1}$ or 60 °C hr$^{-1}$ ramp rate. Protein refolding (equilibrium) analysis was carried out by cooling the sample from 90 °C-10 °C at 60 °C/hr, with 10 min re-equilibration at 10°C, and a second 60°C hr$^{-1}$ run. The data were analyzed with the ORIGIN 7.0 VP-DSC package. The data were baselined through a linear connect model followed by manual adjustment by cursor. The $T_M$, $\Delta H_{\text{CAL}}$, and $\Delta H_{\text{vH}}$ were calculated through a non-two state Levenberg-Marquardt fitting model and evaluated by reduced $\chi^2$ analysis. The average $T_M$, $\Delta H_{\text{CAL}}$, and $\Delta H_{\text{vH}}$ of the data were calculated using Prism. We report the average weighted estimate $\Delta H_{\text{WA}}$ (49) as a measure of data quality, which corrects for experimental baseline fluctuations and the differences between experiments. The significance of the difference in $T_M$ was determined by an unpaired t-test.

All datasets are available at [http://dx.doi.org/10.17632/m6pz3rh5n4.1](http://dx.doi.org/10.17632/m6pz3rh5n4.1) under a CC 4.0 license.
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Conflict of Interest: The authors declare that they have no conflicts of interest with the contents of this article.

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The abbreviations used are: ACLY, ATP-citrate lyase; CS, citrate synthase; CSHD, citrate synthase homology domain; DSC, differential scanning calorimetry; DSF, differential scanning fluorimetry; IP: immunoprecipitation; SCS, succinyl-CoA synthetase; SE, sedimentation equilibrium; SEC, size-exclusion chromatography; SV, sedimentation velocity
Figure 1. Production of recombinant human ACLY and truncations. (A) Construct map of ACLY. Domains 1-5 are numbered according to domain arrangement in bacterial SCS. (B) SDS-PAGE of ACLY constructs on 12% Tris-MOPS. (C) Single representative SEC traces of ACLY constructs on superose 6 increase chromatography column. The protein concentrations at the time of loading the column were 40 µM for ACLY-FL, 100 µM for ACLY-Nterm, and 40 µM for ACLY-Cterm.
Figure 2. Sedimentation equilibrium of ACLY truncations. Log-linearized radial distribution of SE data obtained through Absorbance$_{280nm}$ optics for samples at 0.4 mgmL$^{-1}$, centrifuged for 24 hrs. at 9000 rpm. The experimental data (purple) were plotted over the square distance of the center of rotation. These were compared to simulated monomer (grey) to tetramer (blue) distributions for ideal globular proteins corresponding to multiples of the monomer molar mass of the corresponding ACLY species.
Figure 3. Determination of multimeric state equilibria of the ACLY constructs in solution. (A) Intensity % distribution of hydrodynamic radii (Rh) measured by dynamic light scattering of ACLY full-length and truncation constructs at 8 µM and 3.3°C across two independent experiments, n=6 replicates each. (B) Sedimentation velocity of 0.3 mgmL⁻¹ of protein at 42000 rpm and 4°C across two independent experiments.
**Figure 4.** Western blotting analysis of ectopic ACLY-FL Co-IP in HEK293T cells. Immunoprecipitation of ACLY-FL with C-terminal Myc epitope, tested with anti-FLAG for evidence of association, with detection by anti-Myc as confirmation of pulldown. Ectopic expression was confirmed through blotting of the pulldown inputs. The levels of β-actin in the input were used a loading control.
Figure 5. Differential scanning calorimetry of ACLY full-length and truncations at 25 µM. Scans were run from 10 °C - 90 °C at a 60°C hr⁻¹ ramp rate. Representative trace of independent experiments, with the average $T_M$ of all experiments reported above each unfolding transition event.
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Figure 6. Co-migration of ACLY-Nterm and ACLY-Cterm. (A) SEC of ACLY-Nterm (5 µM) and ACLY-Cterm (20 µM) individually or after 1 hr. co-incubation on a superdex 200 increase chromatography column. (B) SDS-PAGE of SEC eluate for each SEC run on 12% Tris-MOPS.
Figure 7. MDH-coupled enzyme activity assay of ACLY constructs at 20 nM each, as well as titration of ACLY-Cterm at varying concentrations into ACLY-Nterm at 20 nM. All experiments were carried out under cell-free conditions with three independent experiments, n=6 replicates each. n.s. not significant, * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001.

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Figure 8. Differential Scanning Fluorimetry assay for ACLY affinity for substrates. Each construct was analyzed at 0.1 mgmL\(^{-1}\) with saturating concentrations of citrate, ATP, MgCl\(_2\), and/or CoA. Summary of two independent experiments, n=6 replicates each. Statistical significance was calculated by Dunnett’s multiple comparisons test. n.s. not significant, * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001.
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Figure 9. Differential scanning calorimetry of ACLY full-length and truncations at 25 µM with or without CoA at 500 µM. Representative trace of independent experiments, with average $T_M$ overlaid. Scans were run from 10°C–90°C at a 60 ºC/hr ramp rate.
ATP-citrate lyase multimerization is required for coenzyme-A substrate binding and catalysis
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