5-2022

Studying the Lysine Acetylation of Aconitase Isozymes in E. coli

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Studying the Lysine Acetylation of Aconitase Isozymes in *E. coli*

An Honors Thesis submitted in partial fulfillment of the requirements for Honors Studies in Chemistry

By
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Spring 2022

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Acknowledgements

First, I would like to thank Dr. Chenguang Fan for his mentorship and guidance throughout my time doing research at the University of Arkansas. Without the allowance to work in his biochemistry lab, facilitated by his patience and thorough support for my future, I can genuinely say that I would not have the love that I have for research, and my project certainly would not have been possible. I would also like to thank fellow members of our group, Sumana Venkat and Jessica Araujo, for providing supplementary help with techniques in the lab. I am also very thankful for the University of Arkansas Honors College Fellowship Program for providing continuous support in, not only my research endeavors, but my growth as a student and person these last four years. Lastly, I would like to thank members of my thesis committee, Dr. Mack Ivey, Dr. Bill Levine, and Dr. Nan Zheng for taking the time to read and provide insight into my thesis and presentation.

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Abstract

The contents of this thesis have been modified from the publication "Araujo J, Ottinger S, Venkat S, Gan Q and Fan C (2022) Studying Acetylation of Aconitase Isozymes by Genetic Code Expansion. *Front. Chem.* 10:862483". Though studies have found multiple lysine sites in which acetylation takes place in *Escherichia Coli* aconitase, acetylation's effects on the enzyme's activity have yet to be studied. Aconitase is the dehydratase-hydratase found in the citric acid and glyoxylate cycles responsible for the reversible isomerization of citrate to isocitrate via cis-aconitate intermediate. There are two isoforms of aconitase in *E. coli*: AcnA and AcnB. In this study, the genetic code expansion technique was utilized to generate 14 different site-specifically acetylated aconitases, with both isozymes being represented. Following enzyme assays and kinetic analysis of the variants, it was found that the acetylation of two altered aconitases impacted the level of enzyme kinetics from that of their wild-type counterparts, with AcnA K684 decreasing enzyme activity and AcnB K567 increasing enzyme activity.

Introduction

Aconitase Background

Aconitase is an enzyme involved in the citric acid and glyoxylate cycles with its function being composed of two reactions: the conversion of citrate to an intermediate, cis-aconitate, and the subsequent conversion of cis-aconitate to isocitrate. The former is a dehydration reaction, while the latter rehydrates the intermediate (Akram, 2013). The mechanism in which these reactions are carried out is then important to note; between the dehydration and rehydration, cis-aconitate is required to flip 180° about the Cα-Cβ double bond (Lloyd et al., 1999). There are

two genetically variant isoforms of aconitase in *Escherichia coli*: AcnA and AcnB. It has been concluded that while AcnA is an iron and redox stress-induced aerobic stationary-phase enzyme, AcnB acts as the major citric acid cycle enzyme (Cunningham et al., 1997).

When comparing the AcnA and AcnB isozymes of *E. coli* to aconitase enzymes within the eukaryotic cell, mitochondrial aconitase and human iron-responsive-element-binding protein (cytosolic aconitase), it can be noted that the variation between their cellular functions is mainly derived from their respective amino acid sequences; the former having 29% identity and the latter having 53% identity with *E. coli* AcnA (Gruer and Guest, 1994). Upon analyzing the foundation of their structures, however, the three cysteine residues in ligand binding to the iron-sulfur center, are conserved in each variant (Prodromou et al., 1992) (Fig. 1a).

The activity of the enzyme itself is dependent on which of the three states that the central iron-sulfur cluster takes on: active [4Fe-4S], inactive [3Fe-4S], and the apo-enzyme form (Araujo J et al., 2022). It is this conserved center that causes aconitase to be a commonly utilized biomarker for oxidative stress within the cell, for exposure to reactive oxygen species (ROS) such as hydrogen peroxide, superoxide radicals, or hydroxyl radicals, can lead to the release of Fe-α from the active $[4Fe–4S]^{2+}$ state, thus generating the inactive $[3Fe–4S]^{1+}$ composition (Yarian et al., 2006) (Fig. 1b). This effect is evident to such an extent, that *E. coli* mutants lacking superoxide dismutase activity (and therefore the capability to disassemble the ROS superoxide) underwent O_2-dependent aconitase inactivation at a rate estimated to be of the order of 10^9 $M^{-1}s^{-1}$ (Gardner and Fridovich, 1991).

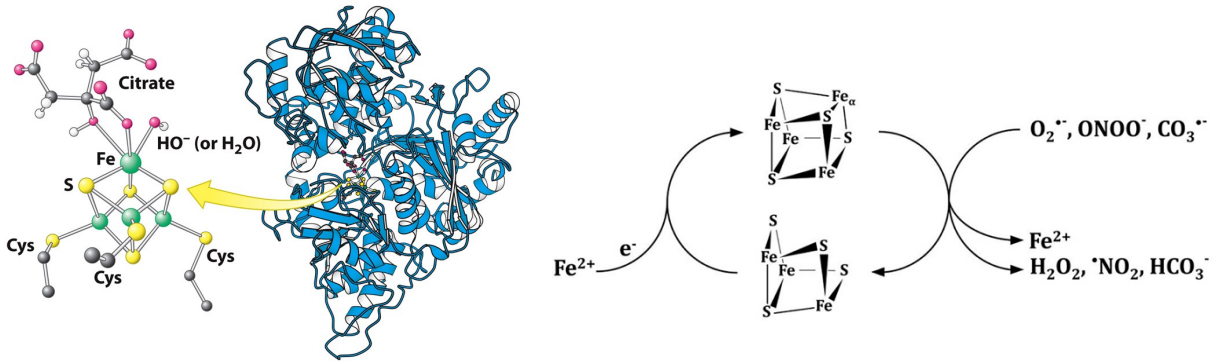

Figure 1a (left). Visual representation of aconitase and its iron-sulfur center (Berg et al., 2015).

Figure 1b (right). Oxygen sensitivity is shown through the activation and inactivation reactions of the iron-sulfur center (Castro et al., 2019).

Post-Translational Modifications

In addition to oxygen dependency, other factors, such as post-translational modifications (PTMs) can be imperative in the regulation of a variable array of cellular processes in eukaryotes, such as acetylation, phosphorylation, ubiquitination, and methylation. These processes then are utilized to regulate cellular activity and protein localization, therefore allowing for the modulation of biological mechanisms such as protein biosynthesis, gene transcription, metabolism, and cellular signaling. In eukaryotes' 'simpler' counterparts, however, the significance of PTMs has been largely neglected as a consequence of varying complications. First, purely acetylated proteins at specialized locations within a polypeptide have exceptionally low stoichiometric levels because bacterial proteins are rapid in nature and, therefore, reactions are often reversible (Macek et al., 2019). Also, these post-translational modifications occur across several sites of any particular protein, which continues to impede upon the identification and classification of a given PTM at a specific site. These complications can be combatted, however, through the use of recent advances in the field of proteomics and mass spectroscopy.

The focal point of this study was acetylation, the process in which an acetyl group is attached to a specified location within a protein. This PTM has been identified as impacting thousands of proteins within the cell across a wide array of protein classes, and therefore, the need for an understanding of its effects on the cell is substantial (Xiong and Guan, 2002). The acetylated groups are usually lysine residues and have been proven to be evolutionarily conserved in many cells from bacterial to human. They have been found to play significant roles involved in the regulation of metabolism in response to nutrient availability and metabolic status of the cell, thus explaining their presence in more than 80 different transcription factors and many other nuclear regulators (Zhao et al., 2010; Yang and Seto., 2008). This evolutionary conservation, therefore, provides evidence of the essentiality of these genes, increasing the importance of their study (Luo et al., 2015).

Approach

To study lysine acetylation, one of the most traditional methods is to use site-directed mutagenesis, utilizing a mimic for acetyllysine, such as glutamine. Noting the sensitivity of the homeostasis within a cell, however, the slight variations in even the most successful analogs can generate consequential behavioral differences. This was found to be the case in our previous study of lysine acetylation of isocitrate dehydrogenase. Though glutamine's side chain was only a seemingly inconsequential ~4Å shorter than its acetyllysine counterpart, this difference caused for contradictory conclusions when comparing the two **(**Venkat et al., 2017). To avoid this error, homogenously acetylated lysine residues could be generated at specific sites, but this approach was unlikely to be successful due to two components: the lack of knowledge when modifying

enzymes for acetylation and lack of regulation to ensure no competition between acetylation and other PTMs. To overcome this issue, we applied the genetic code expansion strategy.

Understanding Genetic Code Expansion

To begin to comprehend the genetic code expansion technique, a grasp of the translation process from messenger RNA (mRNA) into a protein within the cell must first be obtained. First, the codons found on mRNA are displayed in the ribosome, followed by the association of a specific aminoacyl-tRNA (aa-tRNA) anticodon with the mRNA codon that it corresponds to. This aminoacyl-tRNA is composed of both an amino acid and a tRNA segment, therefore through the transitive property, each mRNA codon corresponds to a specific natural amino acid. This process of coordinating mRNA codons with their aa-tRNAs is repeated in the ribosome, thus forming peptide bonds that eventually elongate enough to form a functional protein (Rodnina et al., 2004).

Following this, genetic code expansion allows for the translation process above to be utilized in the incorporation of noncanonical amino acids. In doing so, an aminoacyl-tRNA synthetase (AARS) that has been genetically engineered to recognize the unnatural amino acid, acetyllysine (AcK), is introduced to generate 14 site-specifically acetylated aconitase variants at an assigned codon; this process is represented in Figure 2 (Neumann, 2012). An imperative characteristic of these AARSs and tRNAs was that they did not cross-react with endogenous AARSs and tRNAs in the host cell, thus avoiding aversion from the initial objectives of the study. To ensure that this goal was achieved, the stop codon, “UAG”, was utilized as the site of choice for acetyllysine incorporation, thus indicating that this was the sequence to be recognized by the tRNA used. Though release factors may bind upon tRNA incorporation at UAG, no

natural amino acids could be associated with the tRNA meant to be introduced at this codon, thus making cross-reaction be less likely to occur (Sanders et al., 2022). As of late, this expansion technique has become a vital tool in the ability to incorporate noncanonical amino acids (ncAAs) into specific sites within the genetic code of live cellular proteins and has proven successful in examining the structure and function of the proteins as well. This capability has been extended to the incorporation of many different PTMs.

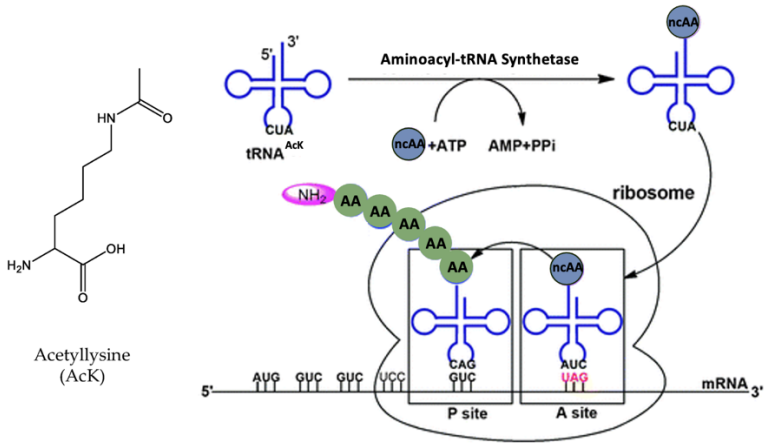

Figure 2. Representation of genetic code expansion technique through the utilization of translation to incorporate our ncAA, acetyllysine, into a stop-codon site (Liu et al., 2010; Chen et al., 2018).

When choosing the strain of *E. coli* in which this enzymatic study would take place, it was crucial to be able to focus on the kinetic activity as impacted by specific sites. To make this possible, non-specific acetylation of other lysine residues was limited by using a strain with wild-type AcnA and AcnB that had been proven to have no or very weak levels of acetylation, even when overexpressed. Thus, BL21 (DE3) strain was chosen as the host cell line, for it had been proven in past studies of other citric acid cycle enzymes (malate dehydrogenase, isocitrate

dehydrogenase, and citrate synthase) to have sparse levels of non-site-specific acetylation (Venkat et al., 2017; Venkat et al., 2018; Venkat et al., 2019; Weinert et al., 2013). Following this, another important factor in regulating our study was the choice of lysine residues identified to be acetylated: K164, K342, K482, and K684 of AcnA and K77, K373, K396, K407, K539, K559, K567, K728, K759, and K835 of AcnB. Chosen sites were recognized as being acetylated in more than three independent acetylomic studies to avoid bias and allow for a broader range of applicability of our findings.

Methodology

General molecular biology and protein analyses

In preparation for the study, chemicals were either obtained from VWR International (Radnor, PA, USA) or Chem-Impex International (Wood Dale, IL, USA). From New England Biolabs (Ipswich, MA, USA), plasmids were assembled utilizing the NEBuilder HiFi DNA Assembly Kit and point mutations were manufactured through the use of the Q5 Site-Directed Mutagenesis Kit. The primary antibody used for western blotting of the purified aconitase isozymes and variants was horseradish peroxidase (HRP)-conjugated acetyllysine antibody (Cell Signaling Technology, Danvers, MA, USA), and Pierce ECL Western Blotting substrates (Thermo Scientific, Waltham, MA, US) were used to produce chemiluminescence to visually analyze the western blot.

Expression and purification of aconitases and acetylated variants

To begin expression, AcnA and AcnB genes and their acetylated variants were inserted into the pCDF-1b plasmid (EMD Millipore, Burlington, MA, USA) with a C-terminal His_6-Tag.

Bacterial transformation of the modified plasmids into BL21 (DE3) cells was then facilitated in addition to our group's routine acetyllysine incorporation system (Venkat et al., 2017). The cells were then allowed to multiply in 400 mL of lysogeny broth, supplemented with streptomycin (100 μg mL^{-1}), chloramphenicol (50 μg mL^{-1}) and acetyllysine (10 mM). Nicotinamine (20 mM) was also added to act as a deacetylase inhibitor. Growth took place at 37°C until the absorbance was between 0.6 and 0.8 when set at 600 nm. Protein expression was then induced through the addition of 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). Following this, cells were incubated at 16°C for 12 hours before centrifugation at 4,000 × g for 20 minutes at 4°C.

The purification process then began through the addition of 12 mL of 50 mM Tris lysis buffer (pH 7.8). This was then allowed to dissolve for 10-15 minutes before the addition of ULTRA cocktail protease inhibitors (0.5 tablet) (Roche, Basel, Switzerland), nicotinamide (20 mM), NaCl (300 mM), imidazole (20 mM), and β-mercaptoethanol (5 mM). The solution was kept on ice, if possible, following this point. Resuspension of the proteins then took place through the use of a vortex mixer until no pellet could be seen, followed by sonication, in which it was set to pulse on and off every 15 seconds for 3 minutes at 70%. The extract was then centrifuged at 20,000 × g for 30 minutes at 4°C. A filtration column containing 2 mL 50% volume Ni-NTA resin (Qiagen, Hilden, Germany) was then prepped by being rinsed DI water to remove excess ethanol and then being washed by a solution containing 25 mL of Tris lysis buffer (50 mM; pH 7.8), NaCl (300 mM), dithiothreitol (DTT;1 mM), and imidazole (50 mM). The supernatant was then filtered using a 20 mL syringe and a 0.45-μm filter before being added to the column in 1 mL increments. Using 2 mL 200 mM imidazole elution buffer (Tris lysis buffer (pH 7.8; 50 mM), NaCl (300 mM), DTT (1 mM), and imidazole (200 mM)), 2 1-mL fractions were collected to be stored at 4°C. Aconitase purity was confirmed through SDS-PAGE

electrophoresis, and correct acetyllysine incorporation was confirmed through western blotting and transfer to PVDF membranes.

Aconitase activity assay and kinetic analyses

Noting the enzymatic function of aconitase, it is understood that the production of downstream components of the citric acid cycle can prove as indicators of the level of aconitase activity (Fig. 3). Therefore, to do so, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was utilized. This was done through the reaction following aconitase's isomerization of citrate to isocitrate: the dehydrogenation of isocitrate to form α-ketoglutarate and NADPH. This NADPH then would have the reductive potential to reduce MTT to form the color-producing MTT formazan (Grela et al., 2018). The optical density (OD) of the violet color (565 nm absorption) could then be quantified to determine kinetic activity of aconitase. Reactivation of purified and acetylated aconitases took place through incubation with $(NH_4)_2Fe(SO_4)_2$ (1 mM) and DTT (5 mM) in Tris (pH 8;50mM) for 30 minutes following previous protocols (Bradbury et al., 1996). The commercial aconitase assay kit from BioAssay system (Hayward, CA) was used to perform enzyme assays, with the OD being measured at 10 and 30 minutes. Steady-state kinetic parameters were calculated utilizing data from varied citrate concentrations (0.1mM-50mM) by non-linear regression software, Grafit (Erithacus Software).

Results & Discussion

Data and analysis

Before beginning to analyze the activity data of the aconitase isozymes and their variants, it was first essential to confirm two factors: enzyme purity and the presence of acetyllysine.

Verification of enzyme purity was executed through SDS-PAGE gel electrophoresis, with the results being shown in Figure 3. Noting that this method separated the proteins by size, the uniform horizontal line followed by each well of modified aconitases allowed for the knowledge that the variants were purely aconitase. It was also acknowledged that utilization of a stop codon as our acetyllysine insertion site would increase the likelihood of truncated, and thus enzymatically inactive, proteins within our sample, so the SDS-PAGE results also allowed for the confirmation that all site-specifically mutated stop codons were paired with an acetyllysine (Bordeira-Carriço et al., 2012).

Following this analysis, western blotting techniques were then carried out, thus allowing for the confirmation of acetyllysine incorporation (Fig. 3). In western blotting, the protein-incorporated PVDF membranes were incubated in two solutions to add the components necessary for acetyllysine detection: horseradish peroxidase (HRP)-conjugated acetyllysine antibodies and Pierce ECL Western Blotting substrates. The former recognized and bound to acetyllysine while the luminol found in the latter was oxidized by HRP to produce chemiluminescence (Marzocchi et al., 2008). Taking this into consideration, the measured chemiluminescence observed in Figure 3 indicated the presence of bound HRP-conjugated acetyllysine antibodies and therefore the definite incorporation of acetyllysines in the site-specific locations of each variant.

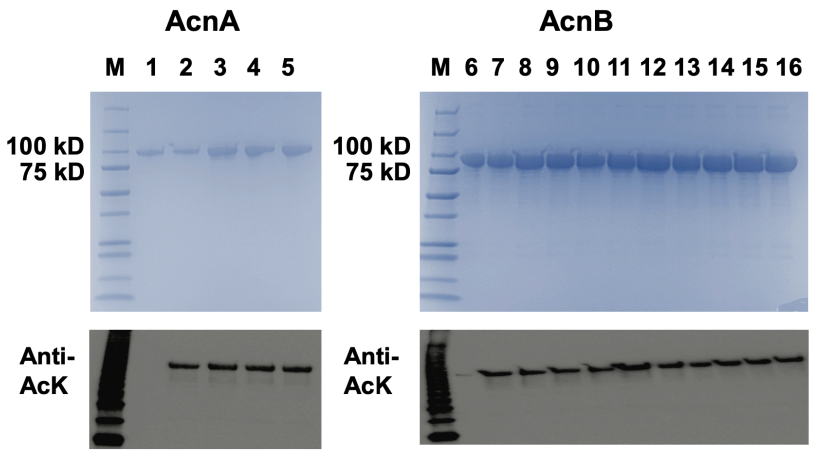

Figure 3. The top set of data is obtained SDS-PAGE analysis, and the bottom set is the chemiluminescence produced by western blotting. Wells 1 and 6 are wild-type AcnA and AcnB respectively. Lane 2, AcnA-164AcK; lane 3, AcnA- 342AcK; lane 4, AcnA-482AcK; lane 5; AcnA-684AcK; lane 7, AcnB- 77AcK; lane 8, AcnB-373AcK; lane 9, AcnB-396AcK; lane 10, AcnB-407AcK; lane 11, AcnB- 539AcK; lane 12, AcnB-559AcK; lane 13, AcnB-567AcK; lane 14, AcnB-728AcK; lane 15, AcnB- 758AcK; lane 16, AcnB-835AcK. For each lane, 2 mg of purified proteins were loaded. Anti-AcK indicates acetyllysine antibody.

Following these verifications, the kinetic activities of each aconitase variant could then be measured, with the data being graphically represented in Figure 4. Upon observation, it was first noted that many lysine sites, when acetylated, did not have a very significant impact on aconitase activities. Furthermore, it was compelling that those that did affect activity did so in opposite ways: lysine acetylation of site 684 decreased AcnA activity while lysine acetylation of site 567 increased AcnB activity (Fig. 4a). Considering these findings, specific enzyme kinetic data was also calculated and documented for the activity levels of these two impactful sites in Table 1. Variables noted were the Michaelis Constant, K_M, the catalytic constant, K_{cat}, and the K_{cat} divided by the K_M. These values represent an inverse measure of enzyme affinity for the substrate, the enzyme turnover number at high substrate concentrations, and enzyme catalytic

efficiency respectively; this information, in combination with data shown in Figure 4, verified our conclusions about AcnA and AcnB activity levels (Cornish-Bowden, 2015).

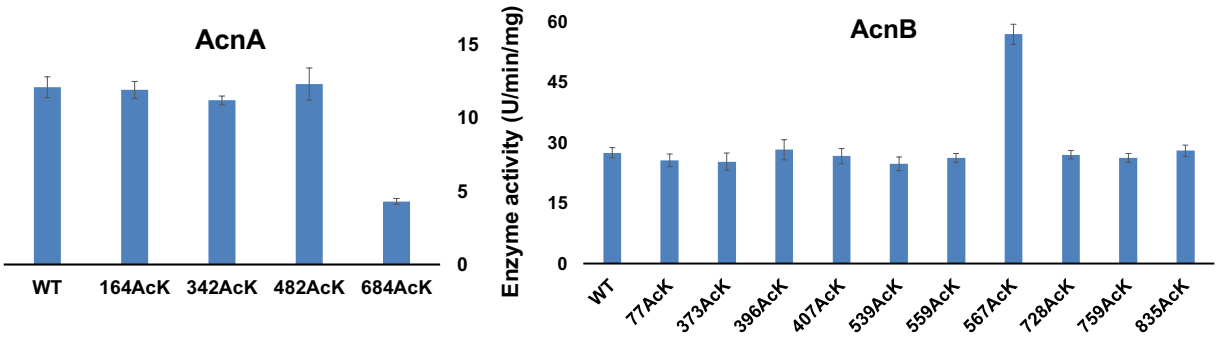

Figure 4. Graphical representation of enzymatic activities of wild-type AcnA and AcnB as compared to their site-specifically acetylated variants. A 50 mM citrate concentration was utilized to obtain this data.

	K_M (mM)	K_{cat} (s^{-1})	K_{cat}/K_M ($s^{-1}mM^{-1}$)
WT AcnA	**1.64 ± 0.21**	**20.6 ± 1.62**	**12.6**
AcnA-684AcK	**3.45 ± 0.23**	**7.33 ± 0.89**	**2.1**
WT AcnB	**9.34 ± 0.72**	**63.5 ± 4.52**	**6.8**
AcnB-567AcK	**9.76 ± 0.67**	**125 ± 9.32**	**12.8**

Table 1. Kinetic parameters of AcnA and AcnB and the two variants that experienced a change in activity levels. Mean and standard deviations were calculated from three replicates.

Though the enzyme-kinetic data did confirm the decrease (AcnA) and increase (AcnB) of catalytic efficiencies as a result of acetyllysine incorporation, it was not evident from this

information alone why the enzyme affinity was more than doubled in the AcnA variant but remained relatively similar in the variant of AcnB. Noting the fact that the only variable component between the activity-altering sites, 684AcK AcnA and 567AcK AcnB, and the other acetylated aconitases was the location of the lysine residue, it was clear that the alteration of behavior was likely to have stemmed from where the site was located relative to the protein's folded conformation.

Taking this fact into account, the two impactful residues were mapped onto their respective crystal structures (Fig. 5). Because the structure of *E. coli* AcnA was unknown, Human cytosolic aconitase (cAcn) was utilized as its homology modeling template, noting their 53% sequence identity, and the crystal structure of AcnB was already known. Observing the locations of the acetylation sites, it was evident that both K684-AcnA and K587-AcnB were found near the active sites of their aconitases, allowing for an understanding of their impacts on catalytic efficiency.

When analyzing their ability to enhance substrate-binding affinity, however, it was noted that despite their proximity to the active sites, K684 of AcnA had a relatively external location compared to K567 of AcnB's presence further within the enzyme. This distance from the outside of the active site then provided a reasonable understanding as to why acetylation of the latter might not be as effective in modifying substrate affinity, for, as was noted when comparing acetyllysine and glutamine (with a ~4Å difference in length between them), even the most minute distances can have substantial effects on affinity.

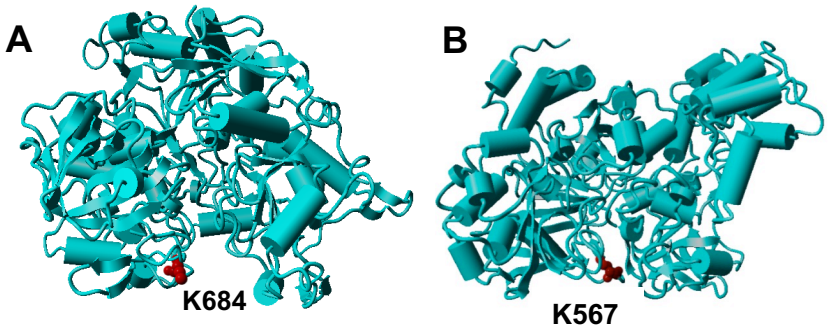

Figure 5. A) The homology model of AcnA's crystal structure based off of human cAcn with K684 labeled. B) The crystal structure of AcnB with K567 labeled.

To dive deeper into this understanding, the structure-function relationships of human acetylation sites proven to affect aconitase activity were then analyzed. Two papers had been published on this topic in mAcn, finding that acetylation of K144 and K258 both alter enzyme activities (Fernandes et al., 2015; Sawant et al., 2021). To map these for comparison, bovine mAcn structure was used in place of human mAcn because there were no reported human mAcn structures; it has 96% sequence identity. Emphasis on overall crystal structure alternative to sequence was also supported by the low identity when human mitochondrial aconitase (mAcn) was compared to both AcnA (28%) and AcnB (24%) (Williams et al., 2002). Analyzing the location of K144, it was found that it was localized to the back of the active site. Interactions between it and Q541 created a hinge affect, thus facilitating enzyme activity through the enhancement of the substrate's accessibility to the active site. K258's placement, on the other hand, was very similar to that of both K684 AcnA and K587 AcnB at the active-site entrance, thus prompting further analysis. Similar to that of K587 AcnB, it was also found that acetylation of K258 enhanced aconitase activities.

Following this analysis, it was then speculated that the variance in acetylation on the activities of AcnA and AcnB are simply mechanisms for cells to use in order to alter activity as needed in different stages of growth. For example, the end of the growth phase of the cell's life cycle is associated with the pinnacle of ATP production by cellular metabolism (Mempin et al., 2013). Coinciding with this increase in levels of metabolism, oxidative stress increases, building up ROS levels within the cell (Davalli et al., 2016). Noting that it would not be in the cell's best interest evolutionarily to continue to facilitate this oxidative stress through metabolism, the aconitase variant that acts as the main citric acid cycle enzyme, AcnB, would want to decrease activity. This then acts essentially as a "circuit breaker," to stop the production of ROS that could damage the cell (Gruer and Guest, 1994). That is why, in adaptation to the phase of life that the cell is currently in (later stage), AcnA will be more prominent than AcnB.

Alternatively, in the earlier, exponential growth stage of a cell's lifespan, metabolism, and therefore AcnB activity, would need to be higher, thus allowing for the energy production necessary for the cells to be able to flourish and multiply. This can further be supported through the comparison of AcnA and AcnB's reaction to oxygenation. Noting the idea that AcnA's oxygen sensitivity is a component that supports the slowing of metabolism in later growth stages, it is reasonable to wonder why this same effect does not occur to such an extent when AcnB is prominent in the cell and metabolism is more active. It is actually not the sensitivity of aconitase that changes, but rather, the capacity for it to recover. In AcnB, oxygen-mediated inactivation is met with a rapid, iron-mediated repair, while on the other hand, AcnA has a much slower recovery. This occurs to such an extent, that the iron-sulfur cluster is likely required to be completely disassembled (Walden, 2002). Coincidentally, up-regulated acetylation at the beginning of a cell's growth would both decrease AcnA K684 activity and increase AcnB K567

activity. This simultaneous, oppositional regulation would not be possible without variation between acetylation's impact on AcnA and AcnB.

Implications

The activity and regulation of aconitase enzymes can be important to note in a multitude of situations. We can begin through the analysis of its biochemical impacts on the citric acid cycle (Fig. 6).

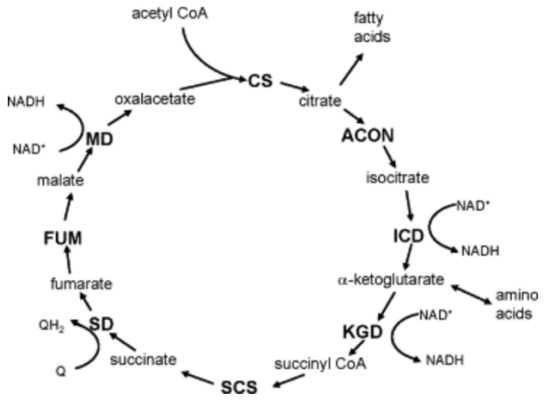

Figure 6. Graphical representation of the Citric Acid Cycle (CAC), depicting the alternative pathways upon the inactivation of aconitase: citrate accumulation can then drive the production of fatty acids and lack of α-ketoglutarate can be remedied through the derivation of more from amino acids (Yarian et al., 2006).

Any component of this cycle has two general fates: to continue through the cycle, or to leave. Upon the decrease in the activity of any given enzyme, the levels of its substrate would increase within the cell and the levels of the components following it would diminish. Applying this to the inactivity of aconitase, a buildup of citrate would ensue, thus increasing the likelihood

that citrate turns to its other fate within the cell, facilitating de novo lipogenesis (O'Neill, 2015) (Fig. 6). Taking this analysis one step further, it is certain that the citric acid cycle would need to keep running despite aconitase inactivity, leading to the utilization of amino acids within the cell to be transformed into α-ketoglutarate.

This information can then be applied to aging and disease in the human body. Beginning with the idea that cellular steady-state level of oxidatively damaged macro-molecules escalates as an organism ages, in addition to the known fact that aconitase is extremely oxygen-sensitive, it can be assumed that this increase in oxidative damage will target aconitase as the human body ages. This has also been backed by the measured accumulation of citrate in the aging housefly, thus indicating a lack of aconitase activity. This interaction between aconitase and oxygen then can further compound upon itself, noting that aconitase inactivation can lead to the blocking of normal electron flow to oxygen, leading to an accumulation of reductive species like NADH, which then would increase the level of ROS in the cell, thus finally leading to more oxidative damage (Yan et al., 1997). The houseflies observed in the study experienced a shorter lifespan when aconitase activities were determined to be low. Could an understanding of how to increase and decrease aconitase activities through lysine acetylation manipulate the effects of aging in humans?

Following this, studies have found evidence that aconitase activity can be impacted by nutritional regulation in addition to that of oxygen and iron levels. This was shown in conditions in which mice were fed high-fat and low-fat diets; upon testing their acetylation levels those with a high-fat diet were found to have significantly increased acetylation levels in their cells when compared to those fed a low-fat diet (Yarian et al., 2006). This is intriguing, noting our findings in which acetylation is more likely to be upregulated while cells are in the exponential growth

stage. Could this mean that a higher-fat diet would directly correlate to a higher level of acetylation and, therefore, fewer effects of aging? Could continuous or increased acetylation be applied in the cell to see how that would impact the duration of its life?

Noting the importance of aconitase regulation and activity in both the human body and *E. coli*, it is then important to address where we can go in the future to continue its study. Moving forward, further insight could be obtained about the acetylation of AcnB 567 through its close relation to the lysine acetylation site 258 in mAcn. It has been found that the mAcn site 258 can be reversibly regulated by sirtuin-3 (SIRT3), an NAD-dependent deacetylase. If the mechanism of SIRT3 could be studied and understood, this could lead to further insight into what aspects of acetylation and deacetylation lead to the activity changes evident in AcnB 567 (Sawant et al., 2021). Rather than simply knowing that activity changes occur, it could be determined why they occur as well.

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