COVER SHEET

TITLE: Measuring mitochondrial nonenyzmatic lysine acetylation

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Abstract

MEASURING MITOCHONDRIAL NONENZYMATIC LYSINE ACETYLATION

Protein acetylation is a regulatory modification affecting numerous biochemical and cellular processes. Over half of the proteins in mitochondria have been identified as acetylated, however mechanism of acetylation has not been elucidated. This project focuses on understanding the mechanism of mitochondrial protein acetylation, hypothesizing that non-enzymatic acetylation is responsible for the majority of observed acetylation. To understand non-enzymatic acetylation, we are quantifying the rates of the reaction as a function of acetyl-CoA concentrations, and calculating the second order rate constant for individual lysine reactivity in native proteins. This study will focus on the acetylation mechanism for the following mitochondrial proteins: ACAT, PDH, HMGCS2, HMGCL1, and αKGDH, which are reported in scientific literature to be highly acetylated in mouse tissues.

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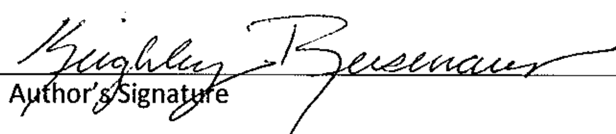
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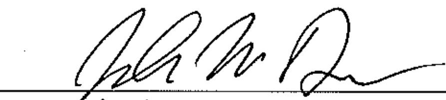
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20 April, 2016
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MEASURING MITOCHONDRIAL NONENZYMATIC LYSINE ACETYLATION

Abstract

Protein acetylation is a regulatory modification affecting numerous biochemical and cellular processes. Over half of the proteins in mitochondria have been identified as acetylated, however the mechanism of how this occurs has not been elucidated. This project focuses on understanding the mechanism of mitochondrial protein acetylation, hypothesizing that non-enzymatic acetylation is responsible for the majority of observed acetylation. In order to understand non-enzymatic acetylation, we must be able to quantify this reaction. Quantifying nonenzymatic acetylation is accomplished by incubating a protein with increasing concentrations of acetyl-CoA. The rate is determined by plotting the change in acetylation over concentration of acetyl-CoA. The slope of the line provides the second order rate constant for individual lysine reactivity in native proteins. This study will focus on the acetylation mechanism for the following mitochondrial proteins: ACAT, PDH, HMGCS2, HMGCL1, αKGDH, and CKMT, which are reported in the scientific literature to be highly acetylated *in vivo*.

Introduction

Mitochondria are highly dynamic organelles that exist within a complex metabolic network to allow for cell-wide adjustments to stresses and energy demands (Osborne, 2014). The critical role of mitochondria in regulating this cellular homeostasis is highlighted by defects in mitochondrial function such as impaired oxidative phosphorylation, excessive reactive oxygen species (ROS), and altered mitochondrial dynamics. These defects can result a wide range of disease including aging diseases, cancer, fragile X, or heart failure (Brock, 2010; Timmermann, 2001; Wager, 2011). Some of these defects can arise from a downstream effect of post-translational

modifications (PTMs) that lead to impaired protein function within the cell (Osborne, 2014; Sack, 2012).

One type of PTM is acetylation, which has been shown to affect protein turnover and intermediate catabolic metabolism (Zhao, 2010; Sadoul, 2008). Acetylation is the process by which an acetyl group is added to an organic macromolecule, such as a protein. When a lysine is acetylated, the acetyl group neutralizes the lysine's positive charge, thus decreasing affinity for negatively charged molecules, which can impair protein structure and stability. This defective protein could then have impaired enzymatic activity, interactions, and localization of target proteins (Glozak, 2005). Several possible functions of acetylation were proposed in Figure 1 (Pazin and Kadonaga, 1997). Because acetylation can occur at many residues, there is no singular effect. The effect of acetylation is dependent on the site of acetylation on the protein and can result in protein conformational changes, increases or decreases in signaling, or protein activity changes.

Historically, acetylation of nuclear histone proteins became the first well-established example of functional protein acetylation when it was discovered more than fifty years ago (Allfrey, 1964). It wasn't until the late 1990s that the first histone acetyltransferases (HATs) and deacetylases (HDACs) were cloned and linked to the regulation of gene expression and chromatin structure (Baeza, 2016; Osborne, 2014). Acetylation acts in part to "open up" chromatin for appropriate transcriptional machinery to access the DNA template (Baeza, 2016). Advances in technology like innovative immunoprecipitation techniques and the development of liquid chromatography coupled mass spectrometry (LC-MS) provided lists of acetylated peptides with their corresponding proteins (Baeza, 2016). Subsequent observations of extra-nuclear deacetylase localization and notable enrichment of mitochondrial metabolic proteins spurred a greater interest in exploring the acetyl-proteome outside the nucleus (Verdin and Ott, 2016).

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Studies have found that 63% of mitochondrially localized proteins contain acetylation sites, both in mouse and human models (Baeza, 2016). Unlike nuclear acetylation, which is catalyzed by several families of lysine acetyltransferases (KATs), mitochondrial acetylation is considered to be largely nonenzymatic. This hypothesis is driven by the lack of direct evidence of KATs in mitochondria, particularly in the high pH environment of the mitochondrial matrix (Baeza, 2015; Osborne, 2014; Pougovkina, 2014; Wagner and Hirschey, 2014). Generally in the mitochondria, protein acetylation typically leads to loss of function in pathways associated with organelle integrity and oxidative metabolism (Baeza, 2016; Weinert, 2015). Lysine acetylation, specifically, has been shown to affect protein turnover and intermediate catabolic metabolism (Zhao, 2010; Sadoul, 2008). A study by Baeza, et. al. reveals that oxidative metabolism is inhibited by higher levels of acetylation among certain metabolic enzymes (Table 1, Baeza, 2016). The study continued to evaluate these enzymes' morphology and observed that lysine sites with the highest acetylation reactivity tend to protrude from the protein surface, while low reactivity sites are protected by electrostatic interactions with neighboring residues (Figure 2, Baeza, 2016). Both surface exposure and local electrostatic interactions influence lysine reactivity toward acetyl-phosphate and acetyl-CoA, but computed pKa values were not a reliable predictor of lysine reactivity (Baeza, 2016).

Sirtuin 3 (SIRT3) acts along these proteins in the mitochondria, but with deacetylation activity (Onyango, 2002). In yeast, "sirtuin proteins are known to regulate epigenetic gene silencing and suppress recombination of rDNA" (NCIB RefSeq 2008). However, unlike typical deacetylases, sirtuins acetylate proteins by consuming NAD^+ and releasing nicotinamide, O-acetyl ADP ribose (OAADPr), and the deacetylated substrate (Fig. 3; Osborne, 2014). Overall, SIRT3 modulates mitochondrial homeostasis and targets proteins involved in mediating energy

metabolism and mitochondrial redox stress (Sack, 2012). In mitochondria, highly reactive sites that exist in clusters of lysine resides are high-affinity substrates of SIRT3 activity, which can lead to enhanced oxidative metabolism by reversing the inhibitory effect of acetylation (Baeza, 2016). Table 1 lists central enzymes that have reversed acetylation by SIRT3 and a rescued function. SIRT3 expression has been shown to increase by fasting and chronic caloric restriction, two conditions that necessitate increased oxidative metabolism (Baeza, 2016; Hirschey, 2010; Sack, 2016; Weinert, 2015). It is not surprising, then, that SIRT3 is involved in the deacetylation of enzymatic proteins that facilitate the conversion of acetate to acetyl-CoA for energy production (Sack, 2012). SIRT3 works to combat and regulate acetylation "carbon stress/ buildup" to ensure metabolic fidelity (Wagner and Hirschey, 2014). Regulated by SIRT3, steady-state mitochondrial acetylation only presents at very low levels, even in response to caloric restriction, suggesting that highly acetylated mitochondria are rapidly turned over to preserve organelle-wide functionality (Baeza, 2016; Weinert, 2015). In contrast, a SIRT3 knock-out results in substantially increased acetylation at target sites that is significantly increased from any changes observed at these same sites in fasted or calorie-restricted conditions (Weinert, 2015). This indicates that SIRT3 has a greater role in acetylation suppression than the effects of just dietary manipulations. Further effects of sirtuin knock-out are an increase of ROS and an alteration of oxidative metabolism, suggesting SIRT3 plays a major role in fine-tuning multiple metabolic programs in response to caloric and redox stressors (Baeza, 2016; Sack, 2012).

The similarities between nuclear and mitochondrial protein (de)acetylation is striking and leads to a need for innovative techniques to study the differences. While nuclear acetylation is enzyme-catalyzed and results in upregulated processes, mitochondrial nonenzymatic acetylation typically results in a loss of function, particularly in proteins involved in oxidative metabolism

(Baeza, 2016). However, a more complete picture of the role of acetylation in mitochondria is required and stoichiometric approaches provide new methods to analyzing mitochondrial lysine acetylation and evaluating the nonenzymatic activity. Stoichiometry is a means to evaluate the fraction of a protein that is modified, instead of measuring fold-change. Figure 4 provides a visual representation between the differences between fold-change and stoichiometric analysis.

Stoichiometry can discern low-level changes in acetylation more accurately, as well as focus on site-specific changes. Across multiple sites, low-level stoichiometry can have additive effects on protein activity. Acetyl sites on the same molecule or acetyl sites on different molecules that share similar function could collectively dampen or enhance protein activity, in accordance with how many sites are occupied (Baeza, 2015). Following the schematic in Figure 5, this study aims to employ stoichiometric analysis to quantify the acetylation reactions of ACAT, PDH, HMGCS2, HMGCL1, and αKGDH mitochondrial proteins. Due to the evidence surrounding the mitochondrial environment and lack of lysine acetyltransferase enzymes, it is hypothesized that majority of mitochondrial lysine acetylation will be result from nonenzymatic reactions, regulated to low stoichiometric levels by SIRT3. Site specific stoichiometry is determined by reacting acetyl-CoA or acetyl-phosphate with native proteins, denaturing the product, and reacting with heavy-labeled acetic anhydride to chemically acetylate all remaining unmodified lysines. The rate is determined by plotting the change in acetylation over concentration of acetyl-CoA. The slope of the line provides the second order rate constant for individual lysine reactivity in native proteins. It is through these approaches and the support from previous literature that this study will focus on the acetylation mechanism for the following mitochondrial proteins: ACAT, PDH, HMGCS2, HMGCL1, αKGDH, and CKMT.

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Methods

Purification of Recombinant proteins

E. coli cells were transfected and grown up in liter cultures to express the protein of interest. Then, the cells were lysed and the protein was purified using fast protein liquid chromatography (FPLC) with nickel affinity resin. (Figure 6). SDS-PAGE gels confirmed purity of the protein. Concentration of protein was then determined via Bradford Assay.

Chemical Acetylation Kinetics

The purified protein was treated with increasing concentrations (0.5 mM, 1 mM, 2mM, 4 mM, and 8mM) of either acetyl-CoA or acetyl-P, causing acetylation at particular lysine residues. Incubation for one hour with an isotopic acetyl group labeled the remaining unmodified residues in vitro (Figure 7). After treatment, the pH was restored to pH 8 using ammonium hydroxide to hydrolyze any O-acetyl esters formed during the reaction. Then, the labeled proteins were spin filtered and digested using trypsin at a 1:100 ratio overnight.

Mass Spectrometry

The sample was cleaned using Stage Tips with C18 carbon activated with methanol. This allows the filter to become "wet" and is used preferentially to water, which would not be able to soak the organic filter material. The filter was then washed with 80% ACN, a nonpolar solvent, which is at the same concentration as the elution solution. A secondary wash with 0.5% acetic acid effectively replaced the CAN and stimulate peptide binding. The sample was then loaded and again washed with 0.5% acetic acid to remove any salts, urea, or small molecules. The acetylated peptides were then stripped from the filter using 80% ACN, which disrupts the peptide's ability to bind. This elution was then dried to be ready for mass spectrometry. After this protein digestion and

desalting, the peptides were injected into the mass spectrometer and the relative abundance of the light and heavy acetyl peptides were measured.

Results / Discussion

The data from the mass spectrometer was analyzed for two proteins. We ran into delays with the remaining proteins due to malfunctions with the MS machine and did not have time to run and analyze the other four purified proteins. We used the Mascot database search engine to determine the lysine residue locations on each protein. We successfully identified one lysine site on pyruvate dehydrogenase (PDH) and the reactivity was significantly higher with Acetyl-CoA than with Acetyl-P (rate = 1.0561 $1*10^5$ $M^{-1}s^{-1}$ and rate = 0.0083 $1*10^5$ $M^{-1}s^{-1}$, respectively). We identified four lysine sites on mitochondrial creatine kinase (CKMT) and all sites were consistently more reactive with Acetyl-CoA compared to Acetyl-P. The most reactive lysine was at K313, 321 (Figure 8). The graphs also suggest that there is a linearly increasing correlation between the acetyl-R concentration and the protein acetylation.

Conclusions / Future Directions

The next steps for data analysis include enzyme activity assays and protein modeling. The assays will help determine the protein's acetylation tolerance as we increase acetyl-R concentration. Each enzyme will be incubated with either Acetyl-CoA or Acetyl-Phosphate using conditions outlined above. The enzyme activity will then be assessed by measuring the increase in absorbance of product formation using steady-state conditions. Secondly, oxidative metabolism is inhibited by higher levels of acetylation among certain metabolic enzymes (Baeza, 2016). Baeza et. al. evaluated the second order rate constant with the crystal structure of metabolic enzymes and observed that lysine sites with the highest acetylation reactivity tend to protrude from the protein

surface, while low reactivity sites are protected by electrostatic interactions with neighboring residues (Figure 9). We intend to complete similar structures for the remaining proteins in the study to capture a three-dimensional and more comprehensive view of the protein's acetylations. Ideally, we will expand our work to include other mitochondrial proteins to more fully understand the acetylation process.

Acknowledgements

I would like to thank Dr. John Denu for the opportunity to complete this research under his guidance and the support of Josue Baeza. I would also like to thank the CALS Honors Program for this opportunity and the privilege of earning Honors distinctions.

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APPENDIX

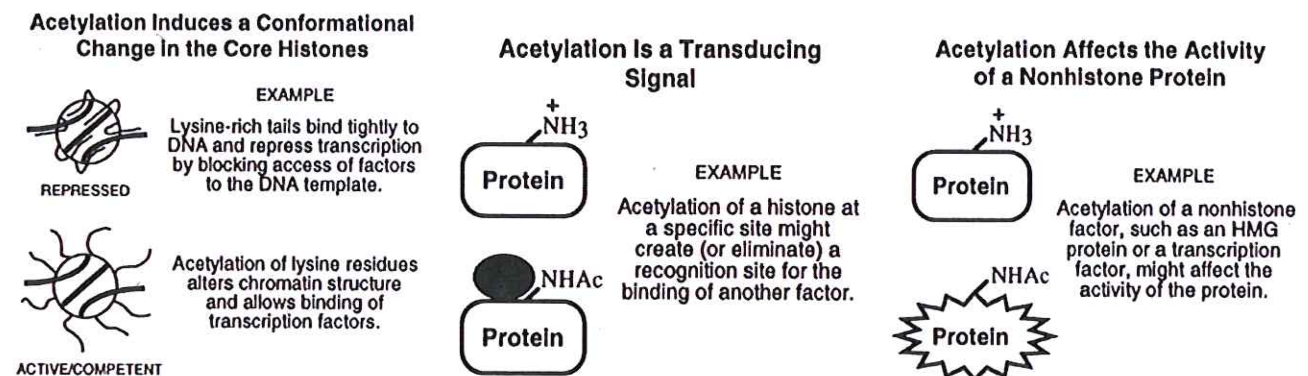

Figure 1. *Examples of possible mechanisms for protein acetylation. Taken from Pazin and Kadonaga, 1997.*

Table 1. (continued)

Gene ID	Gene Name	Organism	Tissue	Site	Effect	Validation	Deacetylase	*In Vitro* Deacetylase Activity with Full-Length Protein?
MDH2	Malate dehydrogenase 2	Hs	N/a	K185, K301, K307, K314	Stimulatory	4K→R	UNK	N/a
		Mm	Liver	K239	Inhibitory	K→Q	SIRT3	No
MRPL10	Mitochondrial ribosomal protein L10	Mm	Liver	N/a	Inhibitory	No	SIRT3	Yes
NDUFA9	NADH dehydrogenase 1a subcomplex 9	Mm	Liver	N/a	Inhibitory	No	SIRT3	No
OGG1	8-Oxoguanine DNA glycosylase 1	Hs	N/a	N/a	Instability	No	SIRT3	No
OPA1	Optic atrophy 1	Mm	Heart	K926, K931	Inhibitory	K→Q, K→R	SIRT3	Yes
OSCP	Oligomycin sensitivity-conferring protein	Hs	N/a	N/a	Inhibitory	No	SIRT3	Yes
OTC	Ornithine transcarbamoyltransferase	Mm	Liver	K88	Inhibitory	No	SIRT3	Yes
OXCT	Succinyl CoA:3-ketoacid-CoA transferase	Mm	Brain	K451	Inhibitory	AcK	SIRT3	Yes
P450scc	P450 cholesterol side-chain cleavage monooxygenase	Hs	N/a	K148, K149	Inhibitory	K→A	SIRT3	No
PDHA	Pyruvate dehydrogenase E1 α subunit	Mm	Muscle	K336	Inhibitory	K→Q, K→R	SIRT3	No
PPID	Peptidylprolyl *cis-trans* isomerase D (cyclophilin D)	Hs	N/a	K145	Binding	K→Q, K→R	SIRT3	No
		Hs	N/a	N/a	Binding	No	SIRT3	No
SDHA	Succinate dehydrogenase complex, subunit A, flavoprotein	Mm	Liver	N/a	Inhibitory	No	SIRT3	No
		Mm	Liver	N/a	Inhibitory	No	SIRT3	Yes

Table 1. *List of identified proteins associated with central metabolism that are acetylated. Taken from Baeza, et. al, 2015*

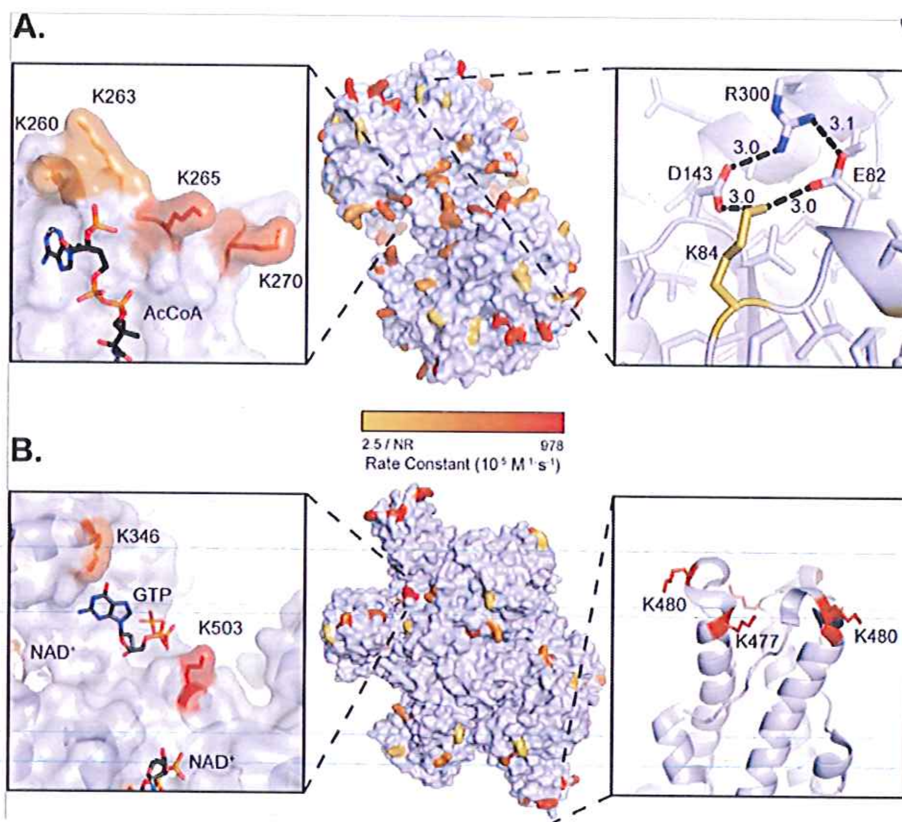

Figure 2. *Diagram of lysine reactivity. Red is high reactivity and yellow is low reactivity. Lysine residues that stick out or are near the acetyl-CoA binding pocket tend to react more strongly than those that are bound internally and are protected by electrostatic interactions with nearby molecules. Taken from Baeza, et. al. 2015.*

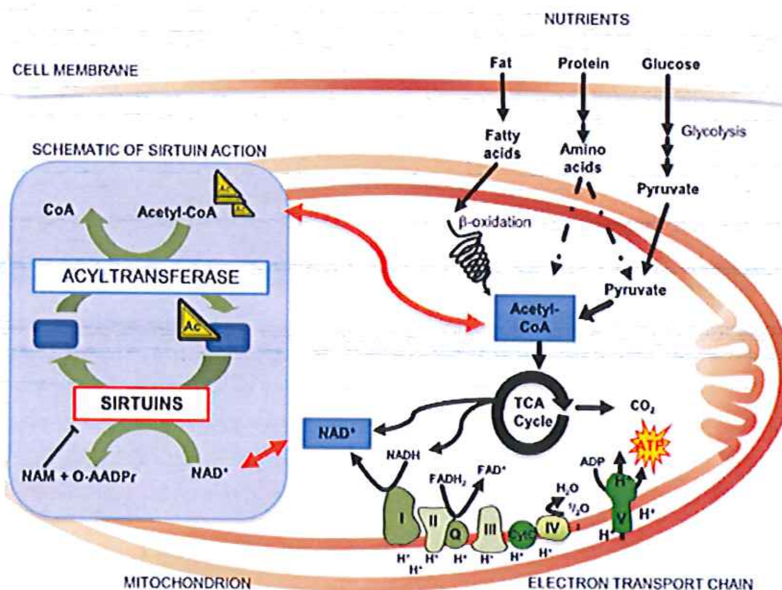

Figure 3. *Diagram of acetyl-CoA consumption pathways. Included is a schematic depicting acetylation and deacetylation of proteins using acetyl-CoA. Taken from Osborne 2014.*

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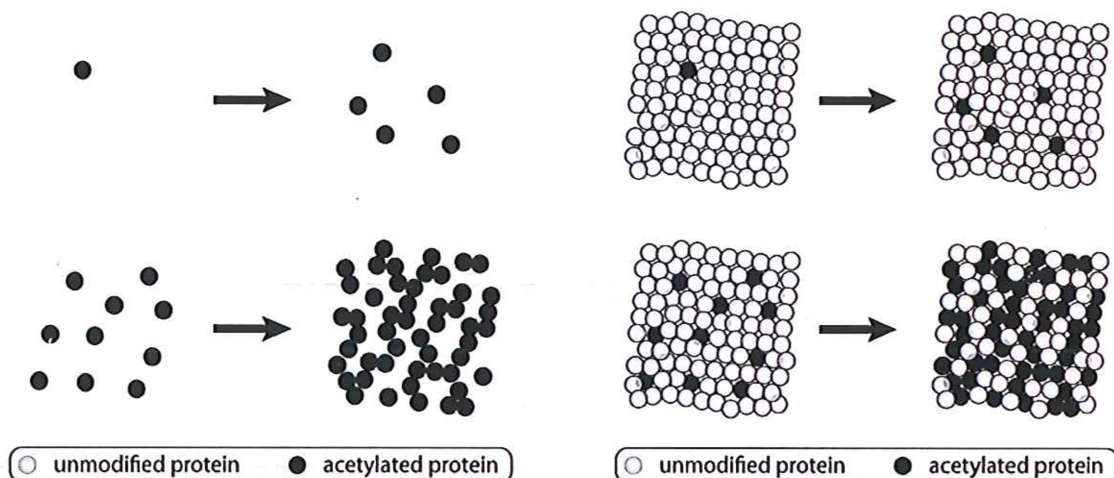

Figure 4. *Diagram describing the difference between fold-change and stoichiometric analysis of protein acetylation. Low-level changes are able to be captured with stoichiometric approaches. Taken from Josh Baeza.*

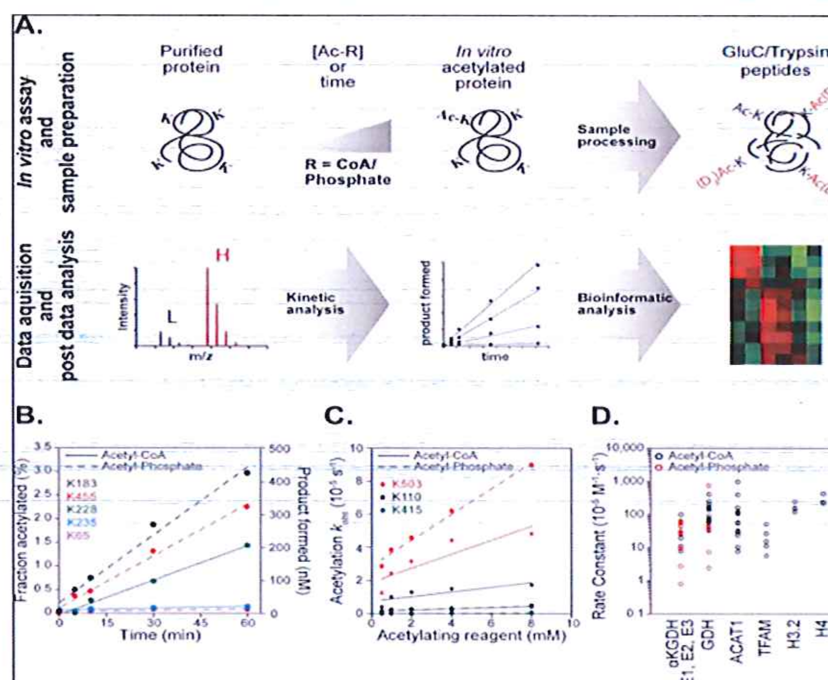

Figure 5. *Kinetic approaches of nonenzymatic lysine acetylation.* ***A.*** *Diagram of methodology used to determine nonenzymatic acetylation. Purified mitochondrial and non-mitochondrial proteins assayed with varying concentrations of acetyl-phosphate or acetyl-CoA.* ***B.*** *Time dependent acetylation of bovine serum albumin showing a linear increase of site-specific lysine acetylation.* ***C.*** *Concentration dependent acetylation of glutamate dehydrogenase, showing a linear increase of rate constants.* ***D.*** *Dot plot of rate constants analyzed in the study. Taken from Baeza, et. al., 2015*

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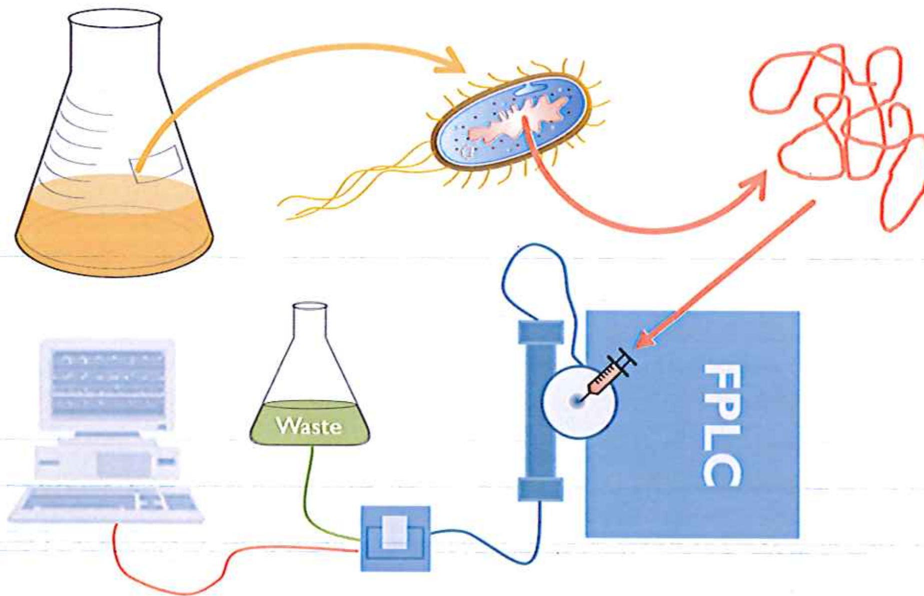

Figure 6. *Visual representation of recombinant protein purification. The E. coli cells are grown in 1L of media, then the protein (represented in pink) is extracted and run on the FPLC over a nickel-affinity column to purify the tagged protein of choice. A spectrometer determines the peak output of the desired protein, which can be analyzed and run on a SDS-PAGE gel for size consistency. This increases confidence that the resulting protein is pure.*

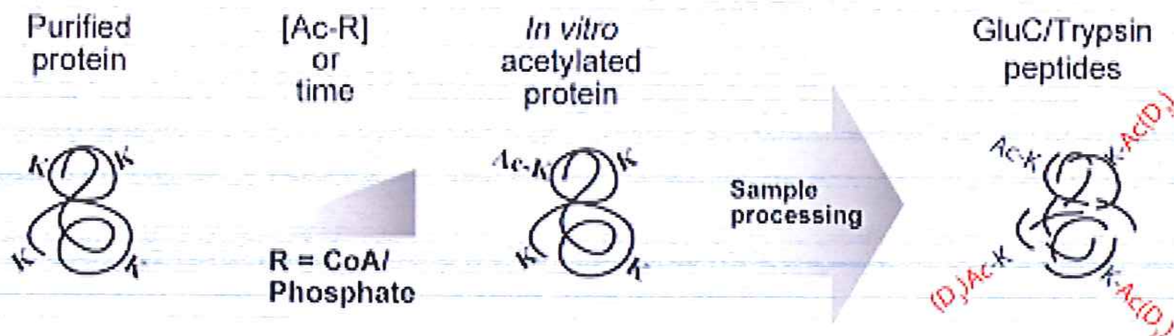

Figure 7. *Visual representation of chemical acetylation kinetics. The diagram depicts the lysine (K) residues on the purified protein, then the incubation with the acetyl-R group in increasing concentrations. Some of the lysine restudies are then acetylated, and further processing labels the remaining lysine residues with a heavy acetyl-R group. Next steps involve running the processed protein on the mass spectrometer and further data analysis. Taken from Baeza, Smallegan, & Denu, 2015.*

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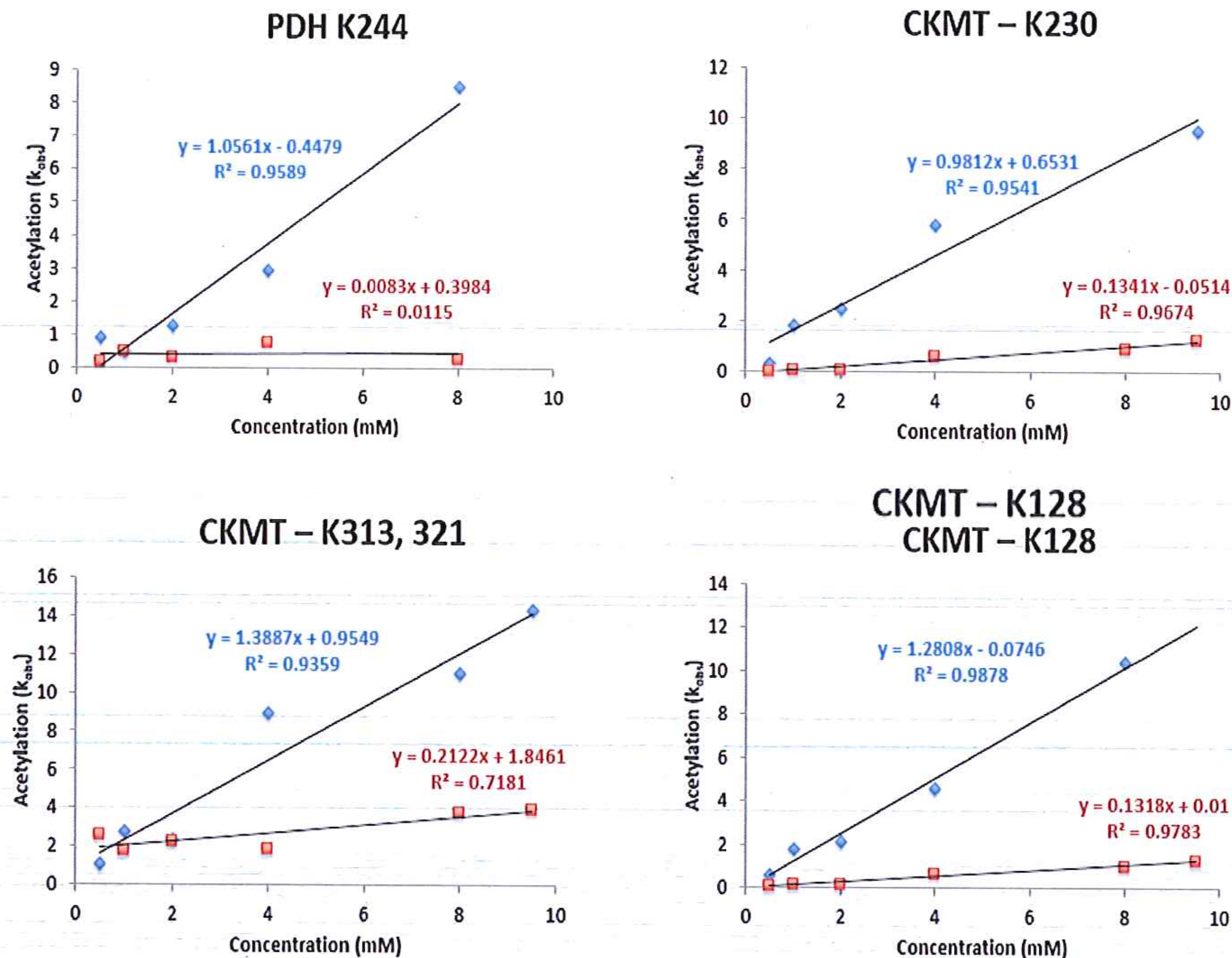

Protein	Site	Reactivity	Acetyl-R
PDH	K244	1.0561	Ac-CoA
PDH	K244	0.0083	Ac-P
CKMT	K230	0.9812	Ac-CoA
CKMT	K230	0.134	Ac-P
CKMT	K313, 321	1.3887	Ac-CoA
CKMT	K313, 321	0.7181	Ac-P
CKMT	K128	1.2808	Ac-CoA
CKMT	K128	0.1318	Ac-P

Figure 8. *In the graphs above, the blue points represent concentrations of acetyl-Co vs acetylation. The red points, acetyl-phosphate (Ac-P). The table describes the reactivity of a specific lysine site on the proteins from mass spectrometry analysis. All sites react more with Ac-CoA than with Ac-P. The graphs also suggest that the relationship between acetyl-R concentration and protein acetylation is linearly increasing.*

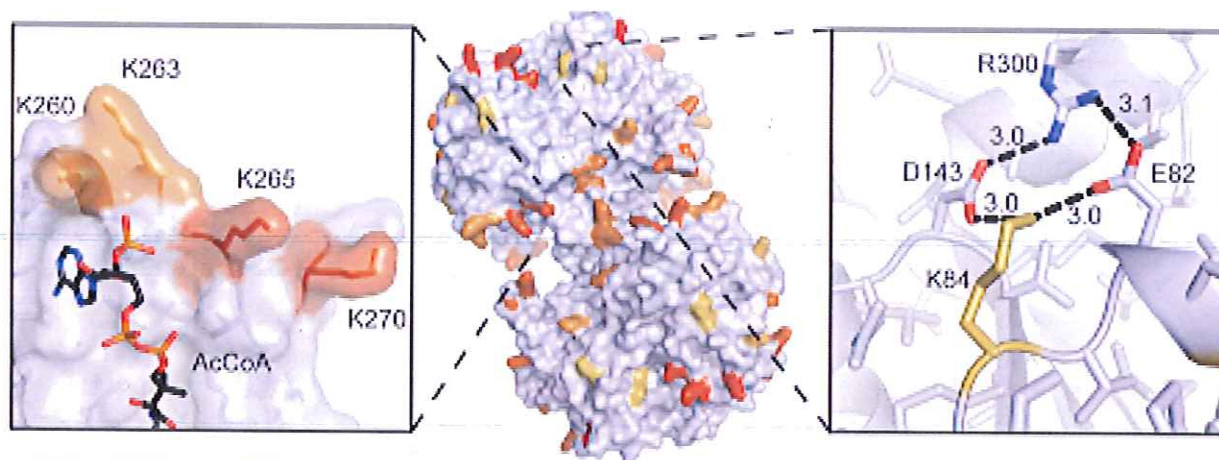

Figure 9. *Diagram of lysine reactivity. Red is high reactivity and yellow is low reactivity. Lysine residues that stick out or are near the acetyl-CoA binding pocket tend to react more strongly than those that are bound internally and are protected by electrostatic interactions with nearby molecules. Taken from Baeza, Smallegan, & Denu, 2015.*