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# Biochemical investigations of uncharacterized, redox-active enzymes in lipid metabolism

By

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# Chapter 5: A Wide World of Enzymes

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## Introduction

I am delighted to share my work with a broader audience via this special chapter of my thesis supported by the Wisconsin Initiative for Science Literacy (WISL). The Pagliarini lab, where I have spent my career as a graduate student, is committed to discovering new pieces of metabolism and creating a holistic picture of cellular function. I am proud to have been a part of this process by focusing closely on enzymes and enzymatic function, which I believe are at the heart of biochemistry as a discipline now and into the future. My experiences have guided me into my future career path, where I hope to make a difference for individuals affected by rare diseases, as well as their families and caregivers. I thank WISL, including Bassam Shakhashiri, Cayce Osborne, and Elizabeth Reynolds, for allowing me the opportunity to share my work through this initiative.

## Enzymes in Metabolism

When you think of *metabolism*, what do you picture? For many people, this word conjures images of the food we eat and the way it is converted into energy for our bodies. However, metabolism on the molecular level encompasses all of the biochemical processes that allow cells to build and break down biomolecules, process sources of energy, and get rid of waste materials. Everything, from the smallest conversion between two closely related molecules to the large molecular assemblies that make up the structure of the cell, is a part of the broad metabolic picture of the cell. All metabolic activities are at the very core of cellular "life" – without them, the cell would not survive.

On a physics level, metabolism is about mediating and converting energy efficiently. Picture an engine. When you introduce fuel into it, combustion generates a burst of power. However, that power is not well-controlled and most of it is lost to the environment as heat. When the cell breaks down complex molecules, like sugars or fats derived from the diet, through *catabolism*, it cannot allow so much fuel to be lost to the environment. Instead, it stores the energy in carrier molecules such as adenosine triphosphate, or ATP. These "energy currencies" are stored up in the body, just like you would store money in the bank, and can be deployed to fuel *anabolism*, the processes that build complex molecules or other cellular components. The scope of metabolism is broad, limited only by the cell's ability to multitask. Thankfully, the cell is excellent at multitasking: it can derive energy currencies from a variety of fuels all at the same time through separate pathways taking place in different cellular compartments. For example, most people know that "mitochondria are the powerhouse of the cell." This is

because mitochondria house most of the processes that generate energy currency, including cellular respiration.

As a student, I was not particularly interested in nutrition. Learning all the nutrients the body needs for optimal function seemed like an exhaustingly dull exercise in memorization. In reality, the principles of human nutrition, as well as nutrition for other forms of life, such as the food for your pets or the fertilizers for your houseplants, are based on the metabolic fine-tuning necessary for life. One nutrient group that people need in their diet is protein. Proteins are large biomolecules made up of amino acid building blocks. When the body takes in protein through the diet, it digests individual proteins into their constituent amino acids (catabolism) and uses those amino acids to rebuild the human proteins that the body needs to function (anabolism). Many of the body's most important proteins are *enzymes*. Enzymes are biocatalysts; they make a chemical reaction occur more quickly by making it more energetically favorable. Because enzymes are not consumed in a chemical reaction, they can speed up the rate of those reactions over and over again, efficiently processing their *substrates*, or the starting molecules they are capable of acting on, into desirable *products*. There are numerous enzymes responsible for carrying out every kind of reaction that takes place in the human body. Although there are only 20 protein-coding amino acids found in nature, the proteins that can be made from these precursors are incredibly diverse! Over 60,000 protein families have been identified through research (Kunin et al, 2003), grouped according to evolutionary relationship, shared folds (i.e. how a protein's amino acids are shaped in 3D space) or specific functions. Enzymes provide a layer of control to metabolic processes; they turn on or off in response to cellular feedback or process

different reactions in the same region or cellular compartment depending on the needs of the cell.

**“All the world's a stage, and all the men and women merely players. They have their exits and their entrances; and one man in his time plays many parts.”** – William Shakespeare

The “central dogma” of biology describes how proteins are made from genetic transcripts originating from DNA. In this way, genes pass down enzymatic functions from generation to generation. That famous quote of Shakespeare’s – “all the world’s a stage” – may be true about life, but is it true about cellular life? Most enzymes don’t play many parts. Instead, they mediate a single type of reaction on a single class of substrate molecule. Their action is specifically tailored to their role in the cell and the metabolic pathways they take part in. The affinity of an enzyme for its substrate is determined by the inherent qualities of the pocket where the enzyme binds that substrate, which is called its *active site* **(Figure 1A)**. The active site contains unique positively or negatively charged amino acids that couple to *functional groups*, like oxygen or phosphate, present on the substrate molecule. When an enzyme is folded into the correct 3D shape, its active site is loaded with necessary *cofactors*. Cofactors can be simple metals like zinc or iron, or larger complex vitamins like *flavin*; these entities are often directly involved in the catalysis process, likely as a remnant of the way evolutionarily ancient enzymes acted as catalysts (Goldman and Kacar, 2021).

When the correct substrate binds to an enzyme's active site, the substrate induces structural changes in the active site that promote conversion to the product **(Figure 1B).** This is a part of the "lock and key" hypothesis of enzyme-substrate binding: only correctly sized and shaped substrates can bind to the enzyme and activate its function (Nelson and Cox, 2008). After catalysis occurs, the enzyme releases the product and is regenerated; at this point, it is ready to repeat the process with a new substrate molecule. Enzymes can act on a narrow or broad range of substrates depending on the properties of the enzyme itself and the products it needs to generate for the cell. The rest of the protein outside of the active site facilitates other important functions, such as interacting with other proteins or the cell membrane (the protective barrier that surrounds the cell), binding signaling molecules, or receiving feedback from other members of its metabolic pathway. Even though the "job" of each enzyme is very limited, its complex activities allow the cell to organize and deploy its function in an efficient way.

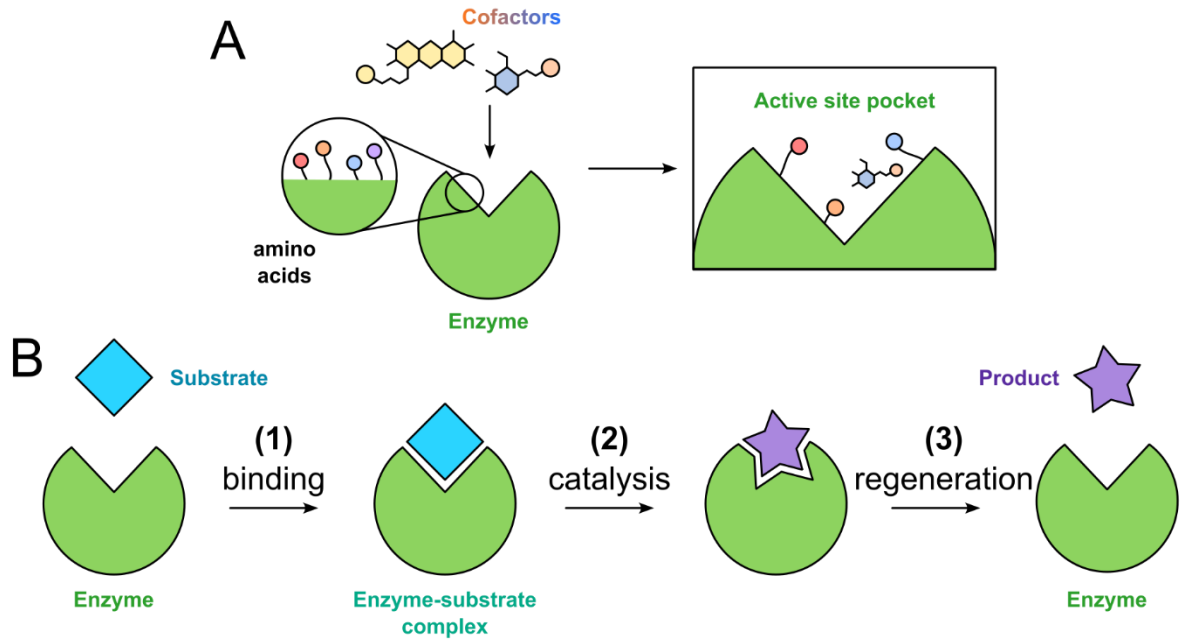


***Figure 1.*** *(A) Enzyme active sites contain functional amino acids and cofactors to facilitate their catalytic activity. (B) Enzymes bind substrates (blue squares) and convert them to products (purple stars). After binding the substrate and catalyzing its conversion, the enzyme is ready to release the product and repeat the process.*

From my very first college biology class, I was interested in enzymes – what they do, how they function, and how we study them. This early introduction to enzymes was my “catalyst” for becoming a biochemist! Enzymes are found at the heart of the beauty and diversity of life. They are instrumental in almost every aspect of biology and the way we understand how our cells and bodies work. While some enzymes have been studied extensively and their functions are very well-understood, there are others that have been the focus of very few studies – or haven’t even been discovered yet! When we characterize enzymes, we fill in the broad brushstrokes of metabolism with incredible

levels of detail. In the end, our goal is not only a more complete picture of the cell, but also a greater appreciation for its beauty and function.

## Enzymology: A Deep Dive into Enzyme Structure and Function

Enzymes have been studied for many decades. In the early days of biochemistry, there was a lot of disagreement among scientists as to whether enzymes could catalyze chemical reactions outside the cell. This relates to “vitalism,” a concept that was popular at the time (Allen, 2005). The first enzymes tested for activity outside of the cell were used in industrial processes – for example, diastase, an enzyme that breaks down starch, was the first discovered enzyme and is a crucial part of malt fermentation (Armstrong, 1933). The discovery of enzymes as proteins was made in 1926, well before the structure of DNA was solved by Watson, Crick, and Franklin in 1952 (Heckmann and Paradisi, 2020). After this revelation, scientists began to puzzle out enzymatic function on a physical, molecular level.

As the name suggests, “enzymology” is the study of enzymes. Enzymology has a rich legacy at UW-Madison; in fact, UW-Madison was home to one of the United States’ first enzyme-focused research centers, the Institute for Enzyme Research, which was founded in the 1940s (van Helvoort, 2002). The idea for the institute came from the famous biochemical research centers found throughout Europe, many of which were destroyed in World War II. The founders of the institute secured funding to construct a building on the western edge of UW-Madison’s campus and recruited talented biochemists from across the country to join the initiative. This included David Green,

who was one of the most well-known enzymologists and later discovered the mechanisms of cellular respiration and other mitochondrial processes (Ernster and Schatz, 1981). The Institute for Enzyme Research remained a crucial part of UW-Madison's research ecosystem and its legacy continues with the enzymology research being conducted in many departments across campus. If you walk down University Avenue on the west side of campus, you can still find the Institute for Enzyme Research's building, housing a few quiet laboratories and administrative offices. When I lived on the west side of campus, I walked past the building every day on my way to the new biochemistry complex. It is hard to believe that such an unassuming place could be at the heart of Nobel-winning discoveries; researchers inside those walls laid the groundwork for so many seminal studies of enzymatic and metabolic function! I could only think of how my fellow researchers and I had carried on the work of that exciting legacy. Enzymology is the core practice of biochemists, and we continue to break ground in this field right up to the present day.

These are a lot of flowery words about the history of enzymology… but what does it look like to DO this type of science? As in all fields, the best practices for studying enzymes have evolved over time. Much of enzymology relates to measuring enzymatic function outside the cell. Experiments done in this manner are *in vitro* – meaning "in glass" in Latin (Lewis and Short, 1879). This paints a vivid picture of mixing enzymes and their substrates in test tubes, but it is not as easy as you might think! Enzymes are maximally active within the cellular environment. Factors like temperature, pH and the presence of intracellular complexes that help with protein folding (aptly called "chaperones") all influence how well an enzyme binds its substrate and converts

it to product. When an enzymologist wants to study a protein *in vitro*, they must first isolate it from all other cellular components and find an appropriate measurement of its function. Enzymes that exist at low concentrations in the cell or have transient, mysterious functions present daunting research challenges. Early biochemists employed what we now call “bucket biochemistry” by grinding up large quantities of cells or tissue to extract less abundant enzymes **(Figure 2).** After separating the cellular components into different fractions, they screened each fraction for the activity of interest. For example, incubating each fraction with glucose might reveal glucose-modifying enzymes. They would track these activities through multiple *purification* steps until, eventually, they had divided the fractions so much that only the enzyme of interest remained. Obtaining pure enzyme allows for more complex *in vitro* testing.

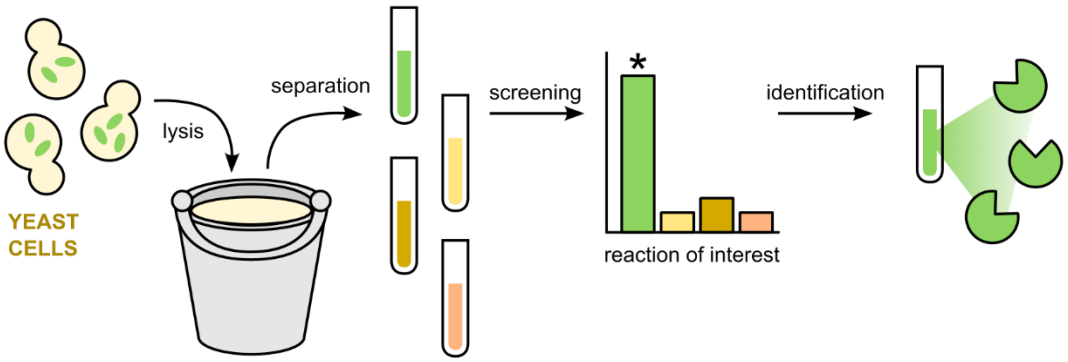


***Figure 2.*** *"Bucket biochemistry" involves the homogenization of large quantities of cells, fractionation, and tracking of an enzyme (green shapes) and its activity through multiple purification steps.*

This process-of-elimination requires massive amounts of both cellular material and effort. The discovery of the bioactive molecule cardiolipin in 1942 required researchers to homogenize fifteen beef hearts and take them through twelve unique purification steps (Pangborn, 1941)! Another common source of cellular material was Baker's yeast, *S. cerevisiae*, possibly due to the relationship between early enzymology and brewing science. Yeast shares many of its metabolic pathways with humans. However, even Baker's yeast, which is easily grown in very large batches, is often not sufficient for measuring enzymes that are produced at very low levels in the cell. Over time, researchers learned more about genetic manipulation, including how to deliver genes into bacteria and induce production of the corresponding proteins. Now, the use of *E. coli* to produce and purify proteins is a staple of enzymology.

Tests that measure enzymatic reactions *in vitro* are called *assays*, and they must strike a balance between finding the right conditions for an enzyme to be functional and being measurable. Most assays are carefully optimized for pH, temperature, and

necessary cofactors to make sure that everything is as close as possible to the cellular environment. If you stray too far from the conditions of the cell, the enzyme may do nothing at all in the reaction mixture! After demonstrating the basic activity of an enzyme, we quantify its activity as *kinetics*. Kinetics gives us concrete numbers about the efficiency of the enzyme and how well it binds a specific substrate. These measurements are helpful for making comparisons between enzymes, especially those that act on the same substrate. Another important area of enzymology revolves around the structural study of enzymes. While proteins have been sequenced since the mid-20$^{th}$ century to find out their amino acid sequences (Sanger and Thompson, 1953), we also have the ability to study their shape in 3D space. This is done through advanced imaging techniques, such as X-ray crystallography or cryo-electron microscopy. Using these tools, we conceptually link the enzymatic functions we observe to its function within the cell. The study of a given enzyme can often be as complex and surprising as metabolic pathways themselves.

Below, I share two exciting enzymology projects I worked on during my graduate research. The first is about COQ4, an enzyme involved in the biosynthesis of coenzyme Q, which I studied using bacterial and yeast model systems. The second is about ACAD10 and ACAD11, two enzymes of unknown function found to help process unusual fatty acid substrates in the cell. Both projects focus on enzymes that act on *lipophilic*, or hydrophobic, substrates, which have a fatty character and are not particularly water-soluble. This makes for challenging *in vitro* study. Using creative experimental design, I made strides in learning more about the functionality of these enzymes and stamped my own small mark on the history of enzymology.

# Project 1: The “Missing Decarboxylase” of Coenzyme Q Biosynthesis

You may have seen coenzyme Q ($CoQ_{10}$, or simply CoQ) supplements at the grocery store and mentally grouped them in with all the other vitamins one can make part of a daily routine. In fact, while CoQ is uniquely important for cellular function, the vast majority must be made inside the cell itself. This is because CoQ is extremely hydrophobic and is not easily absorbed from the gastrointestinal tract (Mantle and Dybring, 2020). CoQ is a lipophilic antioxidant – an antioxidant with fat-like properties. Because it does not mix well with water, it is housed inside the membranes of the cell, especially in mitochondria. CoQ plays a significant role in the respiratory chain, allowing the cell to generate ATP from fuel sources. For this reason, it is crucial for the cell to continuously produce this important molecule.

CoQ is made from two component parts: the lipophilic tail and the headgroup **(Figure 3A).** The tail gives CoQ its lipid-like properties. It is made from small carbon-based building blocks which are used to generate many kinds of bioactive compounds in the body, including steroids or the carotenoids that give carrots their orange color (Poulter and Rilling, 1981). The headgroup is where CoQ’s chemical “action” happens. CoQ collects electrons derived from metabolic processes in its oxygen-rich headgroup. The headgroup is highly modified, complex, and unique; it does not resemble anything the body obtains via diet. Instead, the cell begins with a much simpler ring structure

made from the amino acid tyrosine (Payet et al, 2016). This headgroup precursor is linked to the tail and then modified to transform it into the final, mature CoQ structure.

All of the headgroup modifications needed to create CoQ are catalyzed by a *metabolon* of enzymes called *complex Q* **(Figure 3B)**. Metabolons are complexes of enzymes that work together to accomplish a unified chemical goal (Srere, 1985). If you have ever played with a “marble maze,” constructed of many tunnels, staircases and spirals for a marble to travel down until it reaches the bottom, you might be able to envision how a substrate travels through a metabolon. The grouping of related enzymes helps to concentrate all pathway steps in one place, which greatly increases the efficiency of processing. Because CoQ and its precursors are hydrophobic and stay inside membranes, enzymes cannot easily reach them. Complex Q forms at the membrane surface, so metabolon proteins can more easily access these CoQ intermediates. All members of a metabolon are exceptionally fine-tuned to work together. If even a single member disappears, it can spell disaster for the stability and function of the metabolon as a whole. This is especially true for complex Q. Disappearance of its core members disrupts the cell’s ability to make CoQ altogether (Stefely and Pagliarini, 2017).

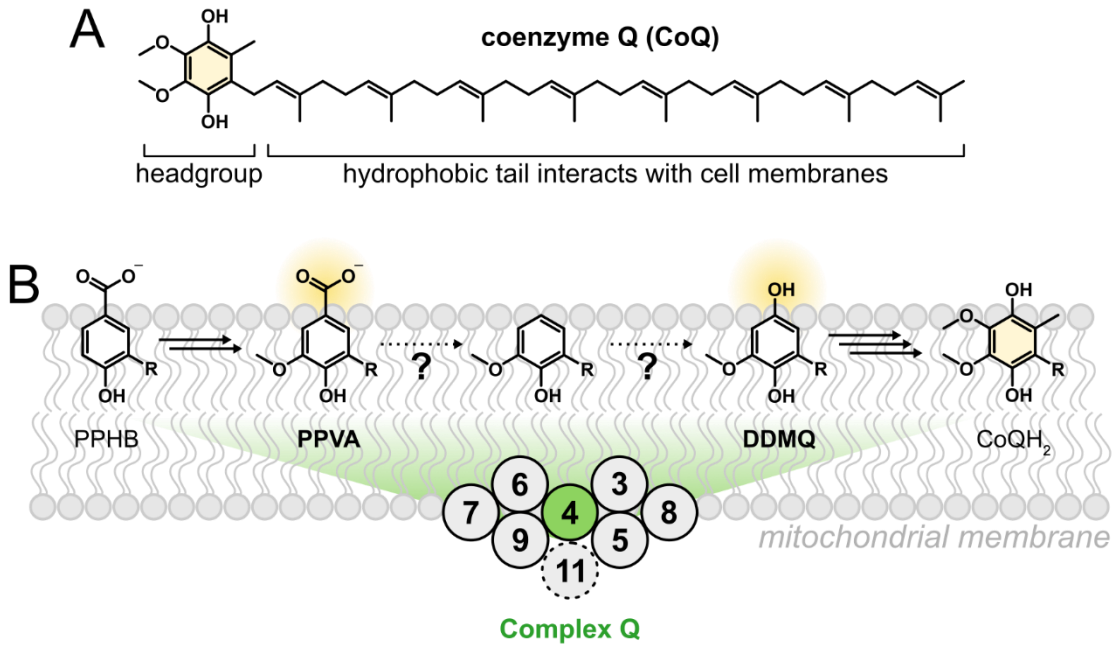


***Figure 3.*** *(A) The structure of coenzyme Q (CoQ), including its lipophilic tail and chemically complex headgroup. (B) A schematic of complex Q, the multi-enzyme entity that mediates CoQ headgroup production. Steps related to the C1 decarboxylation and hydroxylation catalyzed by Coq4 are shown in detail.*

We can understand the steps of CoQ headgroup modification using a concept known as *chemical logic*. Much like cooking, where ingredients often must be added together one at a time, enzymes also must follow the right “recipe” to make CoQ. When highly complex molecules are produced, each unique functional group in their structure must be attached in the most energetically favorable order; through this, the cell conserves energy. By following chemical logic, early coenzyme Q researchers were able to determine the best order of addition for each functional group on the CoQ headgroup. Then, members of complex Q were linked to each specific modification step. Researchers could not find one enzyme for its reaction counterpart: the “missing

decarboxylase.” Decarboxylation reactions remove a $CO_2$ group from the starting substrate and are usually catalyzed by a well-understood class of enzymes known as *decarboxylases*. The headgroup precursor contains a $CO_2$ group, but this group must be removed and replaced by an –OH group to make CoQ. Unfortunately, there is no typical decarboxylase-like enzyme in complex Q. Which enzyme – or *enzymes* – in complex Q can make this exchange? CoQ researchers were stumped.

My research efforts led to a collaborative project with Dr. Fabien Pierrel’s group based in Grenoble, France. Dr. Pierrel’s findings suggested that COQ4, a member of complex Q, might be the “missing decarboxylase” everyone was searching for. COQ4 is a central organizer of complex Q – when its gene is disrupted, complex Q fails to form, other complex members disappear, and CoQ production is completely stalled (Marbois et al, 2009). COQ4 has no clear active site similarity to other enzymes with small molecule substrates. For this reason, it was unexpected that COQ4 could be a decarboxylase. Dr. Pierrel asked me to determine whether COQ4 could support CoQ production in a bacterial model organism. I obtained *E. coli* cells from Dr. Pierrel with disruptions to the decarboxylase and/or hydroxylase genes in bacterial CoQ production. When I gave these cells the gene for yeast COQ4, I observed that they grew the same as *wild type*, or normal, *E. coli* under growth conditions that require CoQ to be produced in the cell. I extracted CoQ and CoQ precursors from these cells and measured their levels. When compared to the cells without COQ4, the COQ4-containing cells had much higher levels of CoQ, explaining their change in growth. This would not have been possible unless COQ4 was able to functionally replace the decarboxylase and

hydroxylase genes from *E. coli* and act as the “missing decarboxylase” in CoQ production. We had found it!

The next step was to observe COQ4’s activity *in vitro*. Unfortunately, studying COQ4 outside the cell was challenging. My aim was to purify COQ4 and perform an assay to observe its activity. However, COQ4 could not be purified despite using a range of approaches. This is because COQ4 is poorly soluble outside the cell. When an enzyme is subjected to unfavorable conditions, it becomes unstable and “crashes out,” resulting in a pellet of insoluble protein. The protein can also form soluble aggregates, which was the case for COQ4; the partially unfolded enzyme becomes “gooey” and sticks to itself. As you might imagine, being a sticky blob of unfolded protein severely compromises the activity of the enzyme! Despite my optimization, the aggregated COQ4 did not show any activity on CoQ headgroup precursors *in vitro*.

With this failed attempt in hand, I went back to the drawing board to find a more innovative way to investigate COQ4’s function. Because COQ4 was so unstable outside of the cell, I decided to stabilize it using conditions that closely mimic its native environment. My idea was what I called a “mito mixing assay.” I purified whole mitochondria from two different batches of Baker’s yeast cells with a disrupted COQ4 gene. One batch had been delivered vanillic acid in the media, which is an alternative precursor of COQ4’s specific substrate (Lopez et al, 2019). These cells accumulated a high concentration of COQ4’s substrate inside mitochondria. We genetically modified the other batch to produce an excess of wild type COQ4 enzyme. By gently breaking open these two groups of mitochondria to release their contents and mixing them together, I could observe whether the substrate from the first group of mitochondria was

consumed and converted to products by COQ4 from the second group. Surprisingly, when I mixed these mitochondria together, I observed a small but detectable increase in CoQ! This did not happen when I mixed the substrate-containing mitochondria with mitochondria that had a “dead” version of COQ4. This suggests active COQ4 enzyme could be responsible for this increase. This was a small but important step towards observing COQ4’s activity *in vitro*. However, using mitochondria, which contain undefined quantities of other proteins and molecules, for an assay raised more questions. Which part of the mitochondrial mixture helped COQ4 become active? Could it be interactions with other complex Q members, or binding an unexpected cofactor? Are there other unknown factors required for COQ4 activity still waiting to be discovered?

There are many exciting research opportunities and future directions for this project. Dr. Pierrel and I published findings from this project in the journal *Molecular Cell*. In our article, we shared our findings about COQ4 and opened the door to future discoveries related to the new function of this enzyme. The identification of the “missing decarboxylase” has big implications for our understanding of how CoQ is produced in the cell. However, CoQ researchers still have a long way to go in learning the details about how COQ4 acts as a decarboxylase, and how complex Q members all must work together to perform their *complex* (pun intended) catalytic functions.

## Project 2: New Tricks for Old Dehydrogenases

When we think of dietary fats, we might think of the *lipid* components of fatty oils such as canola, coconut, or olive oil. Each of these oils has a unique profile of short, medium, and long *fatty acids* – hydrophobic fat precursors which are processed in the body through *fatty acid oxidation*. Lipids broken down by fatty acid oxidation can be used as energy for the cell, creating a clear connection between fatty foods we consume and energy production in the body. The diversity of fat sources in nature means that fatty acid oxidation must be able to process a variety of lipid-like molecules we might consume – short, medium, long, and very long. They do this by employing a group of enzymes known as acyl-CoA dehydrogenases, or ACADs, which control the first step of the fatty acid oxidation pathway (Swigonová et al, 2009). Decades of study have shown us what these ACADs do enzymatically, where they are found in the cell, and which substrates they each prefer.

A class of fatty acids known as 4-hydroxyacids are also known to enter beta oxidation. Unlike other fatty acids, 4-hydroxyacids are defined by an extra –OH group on their carbon chain. This makes them slightly more water-soluble than other lipids, although they still have chain lengths that range from very short to very long. These lipids were first discovered as fatty acid oxidation substrates in mice (Harris et al, 2010). When researchers at Case Western University delivered 4-hydroxyacids to mouse liver cells, they observed a strange and unexpected metabolic process **(Figure 1A)**. In this pathway, 4-hydroxyacids' unusual –OH group is converted to a high-energy phosphate group. Next, the phosphate group is removed to create a product which is capable of entering the fatty acid oxidation process. The researchers understood that this new

pathway was the liver's method for processing 4-hydroxyacids to use them as fuel for the cell.

However, some mysteries remained. There is no part of an ACAD's active site that would allow it to add a high-energy phosphate group to the starting substrate. The ability to add a phosphate group is a quality of a class of enzymes called *kinases*, which are structurally distinct from ACADs. It was hypothesized that a kinase-like enzyme and an ACAD-like enzyme could work together in this new pathway, but the identities of these enzymes were not known. The ultimate goal of processing these 4-hydroxyacid substrates was also unclear.

Through collaborative work with my lab colleague Dr. Eddie Rashan, we discovered the enzymes responsible for this pathway. Our first lead involved a processing pathway for similar compounds in a bacterial species, *Pseudomonas putida*. Bacteria must scavenge ferociously for fuel sources in their environment, and *P. putida* is no exception! Researchers at UW-Madison studied *P. putida* and found levulinate, a type of fatty acid from plants, is a good fuel source for these bacteria. These bacteria used kinase-like and ACAD-like enzymes to process levulinate by adding a phosphate group and then removing it (Rand et al, 2017). Could mice use similar enzymes for the 4-hydroxyacid processing pathway? To find out, we looked for closely related proteins in the mouse genome. Surprisingly, we discovered two enzymes with unknown functions, ACAD10 and ACAD11, matched the enzymes found in the bacteria. These enzymes were discovered previously in mice and humans, but their function was unknown (He et al, 2010). You might think one is a kinase and one is an ACAD, right? However, both enzymes have kinase and ACAD active sites in different regions, or *domains*, of their

structure, allowing them to catalyze two different types of reactions. *Bifunctional* enzymes such as these are like the Swiss army knives of the enzyme world; they can handle multiple steps of a pathway on their own to make sequential reactions even more efficient. For ACAD10 and ACAD11, this would mean they were each capable of performing *both* steps of 4-hydroxy substrate processing without needing any help from other enzymes. To determine whether this was the case, we gave each of them a 4-hydroxy substrate. Surprisingly, both ACAD10 and ACAD11 quickly converted this substrate to the product seen in the pathway! We also detected an intermediate compound containing a phosphate group. These findings strongly suggest that these are the enzymes responsible for introducing 4-hydroxyacids into fatty acid oxidation.

To learn more about these enzymes, I wanted to study the bifunctionality of ACAD10 and ACAD11 through biochemical experiments. The kinase-like and ACAD-like regions of each enzyme work together, but it was unclear how this happens. To learn more, I used cryo-electron microscopy, or *cryo-EM*, to study ACAD11's structure. This form of microscopy is a relatively new tool in a biochemist's toolbox. Since proteins are too small to see under a standard light microscope, ultramicroscopic approaches like cryo-EM are our only way to see the structure of a protein. In cryo-EM, extremely pure protein particles are diluted in a buffer solution. Then, the buffer solution is flash-frozen into a thin layer that can be viewed under a powerful electron microscope. The preparation of the sample must be carefully optimized to get a clear view of each protein particle. Then, snapshots of the individual particles are extracted and merged into a composite image **(Figure 4B)**. This technique is so precise that it is even possible to see individual amino acids in the protein's structure.

Initially, it was unknown whether ACAD11 would be amenable to cryo-EM. The two sections of the protein are connected by a thin, flexible linker region, sort of like a leash holding both domains together. Just like an excited dog out for a walk, the kinase domain can move back and forth around the ACAD domain, making it difficult to create a clear composite image. However, after optimizing the sample preparation conditions, I observed that the kinase and ACAD regions interact and form a stable structure that is very consistent from particle to particle. This interaction is part of a larger configuration of four total ACAD11 molecules interfacing with each other in the active form of the enzyme. When I saw the snapshots of ACAD11's domains interacting for the first time, I was amazed! Each kinase domain interacted with the corresponding ACAD domain at a specific location on the protein structure. If this kinase domain was like a dog out for a walk, it was certainly a very well-behaved one!

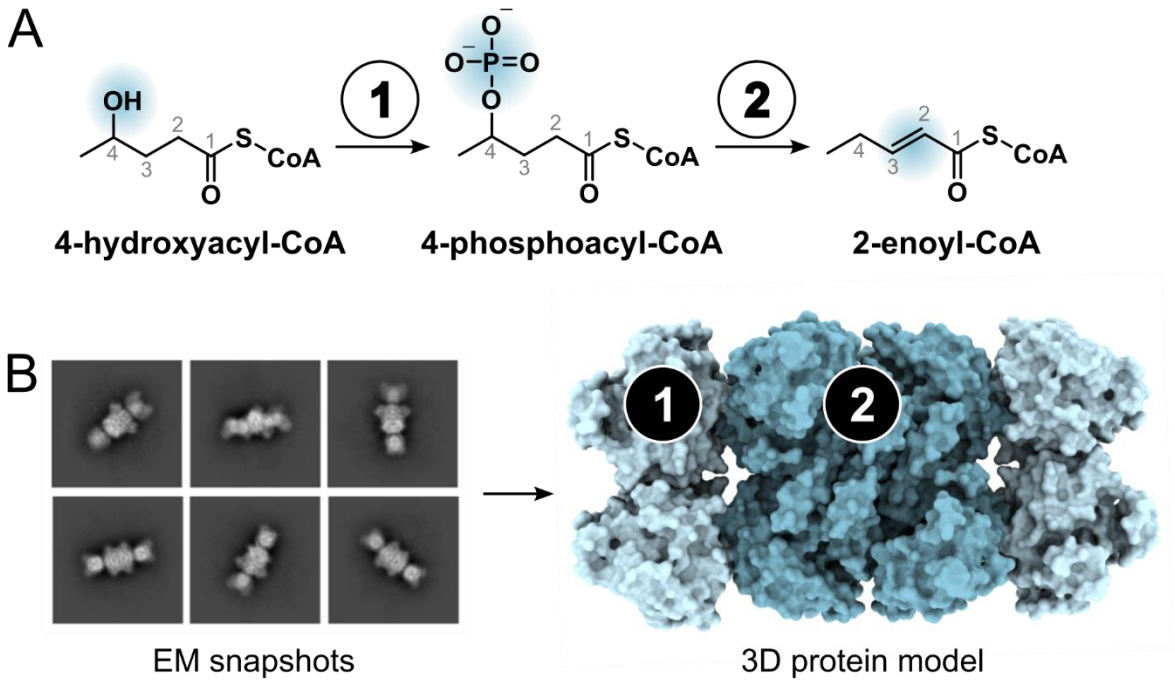


***Figure 4.*** *(A) Proposed pathway for 4-hydroxyacyl-CoA processing in mammals. Carbon numbering is shown surrounding each molecule; functional group atoms*

*attached to the fourth carbon which are modified during the reaction(s) are highlighted in blue. (B) Electron microscope snapshots (left) are composited to make a 3D model of purified mouse ACAD11's folded shape. Kinase regions are shown in light blue and ACAD regions are shown in dark blue. The proximities of the active sites for the two steps of 4-hydroxyacyl-CoA processing are indicated by numbering.*

Creating a detailed structure of ACAD11 allowed me to plan experiments that further investigated its function. The structure of the ACAD active site pocket revealed that ACAD11 also binds a cofactor. This cofactor is a *flavin*, which is a type of vitamin we typically obtain from animal products and nuts in our diet. In other ACADs, flavin has an essential chemical role in converting substrate to product (Thorpe and Kim, 1995). Despite the different functions of ACAD10/11 and other ACADs, I hypothesized that the flavin cofactor must still be used by ACAD10/11 when processing 4-hydroxy substrates. In support of this, I discovered that neither ACAD10 nor ACAD11 is active at all in the absence of flavin. Furthermore, when given a "mock" non-functional flavin to bind instead, they remained inactive. This demonstrates that flavin is also needed for this reaction, just like in the other ACADs. Despite the differences between ACAD10/11 and other ACADs, they still share many similarities, too!

We submitted our findings about ACAD10 and ACAD11 for publication in a major scientific journal. We are eager to share our work with a broader audience of researchers. There are still many questions about the roles of ACAD10 and ACAD11 in broader metabolism. In experiments conducted by my colleague Eddie using samples taken from mice with disrupted ACAD11 genes, we can see changes in the levels of 4-

hydroxyacids, but it is unclear what that means for the mice's overall health. We suspect that 4-hydroxyacids are not taken in through the diet, but produced when cells are under stress. A process called *lipid peroxidation* occurs under stress, wherein lipid chains are attacked and inappropriately modified by reactive species like hydrogen peroxide (Ayala et al, 2014). The cell can correct some of these peroxidation events with antioxidant molecules – however, it is possible that ACAD10 and ACAD11 also help detoxify 4-hydroxyacids that build up. With further research, we will build a broader understanding of why these enzymes do what they do, including their relevance in human health and disease.

## Unlocking Answers for Rare Metabolic Diseases

In both of the research projects above, I describe how genetic changes in the genes encoding COQ4, ACAD10 or ACAD11 can change these enzymes' function in both subtle and unsubtle ways. In the complex systems of a human body, these modulations are not as clear-cut as what we can observe in a test tube. While some changes are harmless, others can have harmful effects. For example, these small, single amino acid changes within proteins are one of the root causes of cancer. When cells *mutate* in a way that allows them to grow unchecked, they can form a tumor or become metastatic. However, when mutations happen early in development, including in an egg, sperm, or zygote, they can also be the causative factor for a lifelong genetic rare disease. Based on the name, you might think that rare diseases are uncommon. However, the rarity of rare diseases comes from their diversity; there are over 7,000 different rare diseases that have been identified, with more being found all the time

(Haendel et al, 2020). This is due to the seemingly endless possibilities for mutations that can occur in the human body. A rare disease induced by changes to a specific gene can also manifest in unexpected ways on the systemic level between different individuals. This leads to unique challenges for treatment, including developing therapies that will benefit the greatest number of patients. Within the broad scope of rare disease, groups that present with similar pathologies may have similar options for treatment, both now and in the future.

Despite the diversity of their root genetic causes, mitochondrial diseases are unified by their shared symptoms. Mitochondria generate energy in the body, and this organelle is home to many other metabolic functions. Traditionally, mitochondrial disease patients have symptoms related to energy consumption. Strenuous activities can further tax already-stressed mitochondrial functions. People with mitochondrial disease can also develop conditions related to the brain, such as mitochondrial epilepsy (Lopriore et al, 2022). These phenomena arise when brain tissues and cell types, such as neurons, are affected by mitochondrial deficiencies. Mitochondrial diseases also impact specific biosynthetic pathways, such as amino acid production, and can cause imbalances (Zschocke and Hoffmann, 2004). Many forms of mitochondrial disease are referred to as *inborn errors of metabolism* due to the mitochondrion's key role in so many metabolic processes. Doctors can use common tests to diagnose mitochondrial disease without relying on genetics. For example, the presence of "ragged red fibers" in a muscle biopsy is a typical marker for some types of mitochondrial disease (Lorenzoni et al, 2014). The "red fibers" in question are actually damaged mitochondria which accumulate near the cell membrane.

A *molecular diagnosis* of mitochondrial disease can be made when DNA sequencing finds a mutation in a gene encoding a mitochondrial protein; this is the gold standard for diagnosing the condition. The increased accessibility of whole genome sequencing (WGS), primarily based on improvements in high-throughput sequencing technologies, has allowed more patients with mitochondrial disease symptoms to receive a molecular diagnosis. This is true across all kinds of genetic rare diseases. However, mitochondrial disease is unique in that mitochondrial genes can also be encoded in mitochondrial DNA. While many proteins found in mitochondria are encoded in the nucleus, mitochondrial DNA houses genes for core mitochondrial machinery. Within the human body – and even within a single cell – multiple distinct populations of mitochondria may exist. A patient may have a mitochondrial mutation in only some regions or tissue types within their body; this concept is known as *mosaicism* and is a challenge for molecular diagnosis. A common misconception about mitochondrial disease is that because mitochondria are inherited maternally, a mother with a mitochondrial disease will always pass the condition on to her children. However, due to mosaicism and the fact that many mitochondrial diseases originate in genomic DNA, this is not always the case.

Despite the fact that mitochondrial disease is diagnosed in around 1 in 5000 births (Thorburn, 2004), there are no known treatments. As with many other rare diseases, treatment approaches have historically focused on approaches that will help broad groups without targeting specific genetic changes. For example, many mitochondrial disease patients take a group of supplements known as the “mito cocktail,” which includes coenzyme Q and other mitochondrial cofactors, to boost

mitochondrial function and compensate for defects in certain pathways (Parikh et al, 2009). However, because mitochondrial diseases range in severity, affected individuals experience a negative impact on their quality of life even with these treatment options in place. Until medical science is advanced enough to create “N-of-1” cures for specific genetic changes, managing mitochondrial disease is a lifelong commitment for the diagnosed individual, their family, and medical providers.

Some of the most meaningful experiences I have had as a scientist involved the mitochondrial disease community. This community encompasses individuals affected by the disease, as well as the clinicians, family and friends who care for and support them day-to-day. In my first year as a graduate student, I was connected via my lab-mates to the United Mitochondrial Disease Foundation (UMDF), an advocacy group focused on supporting people diagnosed with mitochondrial disease. At a UMDF fundraiser event, I realized for the first time how discoveries in the lab are not about making a breakthrough for the next journal article or presentation, or even about stamping a mark on the history of enzymology! When someone has a genetic disease that relates to an enzyme of unknown function, they have no clear treatment options. When a genetic disease is linked to a specific metabolic pathway, a doctor can recommend specific treatments related to that pathway’s function. But where can you start when the scientific community has not discovered which pathway your affected enzyme belongs to? The discoveries I made through my research on COQ4, ACAD10 and ACAD11 will not lead to a “cure” for mitochondrial disease, but the findings may lead to future research and give hope to those seeking information about this disease. My interactions with mitochondrial disease patients and families have revealed how meaningful it is that

researchers take the initiative to study topics that have direct relevance to their condition. This is true for every kind of rare disease, not just mitochondrial disease!

The sum of my experiences in graduate school and at UW-Madison have guided me towards pursuing a career in rare disease after defending my thesis. I am excited to be part of a future where, through focused research efforts, treatments for uncommon and underserved conditions may be discovered, developed, and used in the clinic. Currently, clinical research and medical communities are making efforts to seek feedback from individuals affected by rare diseases when designing and prioritizing therapeutic projects. I find this to be a positive step in this field. For me, there was nothing more meaningful than hearing patient voices speaking about what my work meant to them. They moved me from focusing on the next big enzymatic discovery to thinking about how those discoveries can then be put into action to help others.

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