ELUCIDATING LIPOIC ACID SYNTHESIS AND ATTACHMENT IN *SACCHAROMYCES CEREVISIAE*

By

AMY MAILAN NGUYEN

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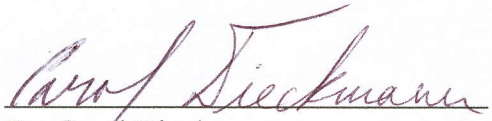

Dr. Carol Dieckmann
Department of Molecular & Cellular Biology

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ABSTRACT

Lipoic acid is an essential cofactor attached to the H protein of the glycine cleavage enzyme (Gcv3) and to the E_2 subunit of enzymes involved in oxidative metabolism (Lat1, Kgd2). Lipoic acid synthesis and attachment to Gcv3 in *Saccharomyces cerevisiae* has been known to occur through the activity of two enzymes, Lip2 and Lip5, but attachment to Lat1 and Kgd2 has been discovered to require an additional Lip3 enzyme, along with an already lipoylated precursor, lip-Gcv3. This finding of a prerequisite for the lipoylation of target proteins is a novel one. The exact structure and relationship of these enzymes is largely unknown, though it was been proposed that they operate within a complex requiring all of the individual components. Attempted rescue experiments with strains of deleted Lip3 and Gcv3 genes and strains of nonfunctional Lip3 and Gcv3 genes were conducted. These have shown little more, though, than that these enzymes work in a manner more complex than already known.

INTRODUCTION

Lipoic acid (6,8-thioctic acid or 1,2-dithiolane-3-pentanoic acid) is a sulfur-containing cofactor present in most prokaryotic and eukaryotic organisms. It can act as a biological antioxidant, and it is required for the function of many enzymes involved in oxidative metabolism, the essential process in which the body converts chemical bonds into usable energy (1). One of the products of these reactions, acetyl CoA, is used to fuel type II fatty acid synthesis (FASII), which is the sole provider of octanoic acid, the necessary precursor for lipoic acid biosynthesis. In this way, lipoic acid synthesis is involved in a positive feedback cycle with fatty acid synthesis and also affects other pathways that intersect with FASII (2).

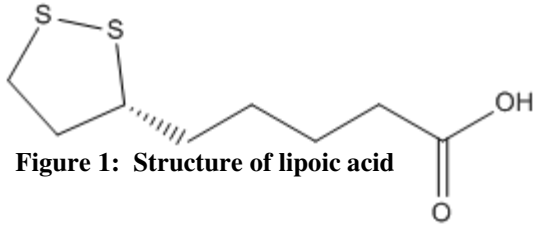

Figure 1: Structure of lipoic acid

The lipoyl moiety is attached to an enzyme by an amide linkage from the carboxyl group of the lipoic acid to the ϵ-amino of a lysine residue on the enzyme subunit. The highly conserved domains in which these lysine residues are located are called lipoyl domains. Protein-bound lipoic acid acts as a "swinging arm" between different active sites of a complex of enzymes. This has been demonstrated in the decarboxylation of 2-oxoacids in aerobic metabolism as well as in glycine cleavage in all three domains of life (3). For instance, in pyruvate dehydrogenase (PDH), a known 2-oxoacid dehydrogenase, two lipoyl moieties are attached to lysine residues on the E2 subunit. In these bound positions, the 14Å lipoamide chains are able to span the physical distance of the E2 subunit and shuttle the substrate from the E1 subunit to the E3 subunit. The lipoyl moieties, then, are responsible for the transfer of the acyl group in the PDH reaction (4).

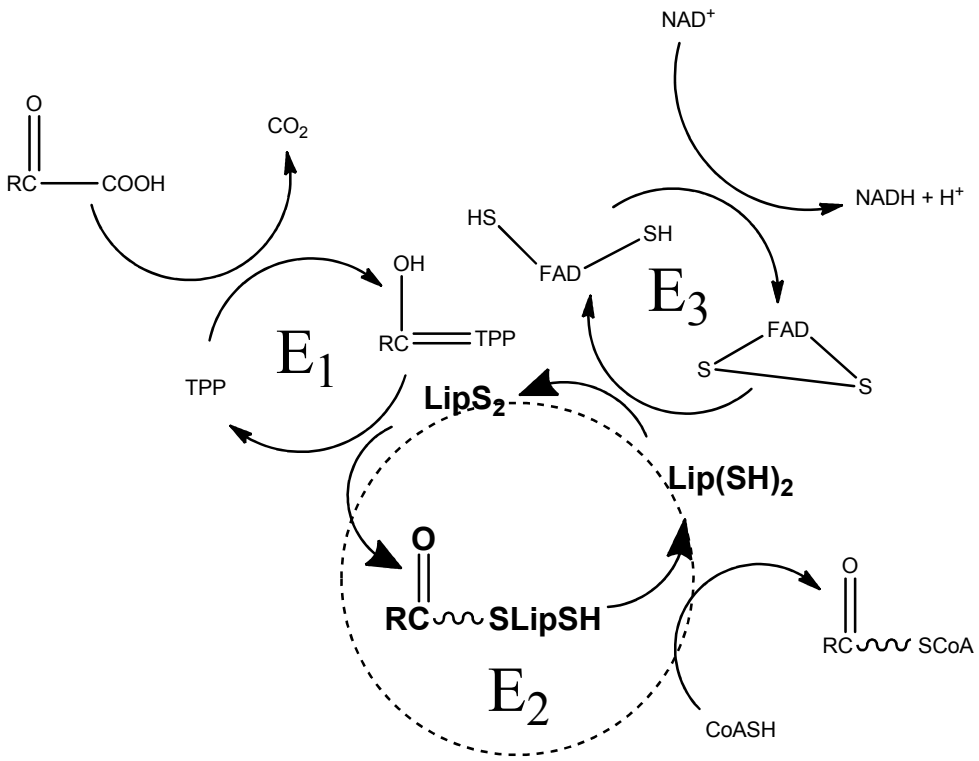

Figure 2: Reaction pathway of pyruvate dehydrogenase. Lipoic acid is a cofactor for the E_2 subunit, shuttling the acyl group across the enzyme.

The mechanisms for lipoic acid synthesis and attachment are still under investigation in eukaryotes but have been well characterized in E. coli. There are two distinct pathways that E. coli is able to undergo: a de novo pathway and a salvage pathway. In the de novo pathway, lipoic acid is synthesized from its precursor, octanoic acid, with the attachment of two sulfur atoms by the lipoyl synthase enzyme (LipA). This reaction may happen before or after attachment to the target protein by a lipoyl (octanoyl)-ACP:protein transferase (LipB). Generally, though, the attachment of octanoic acid to a protein by LipB precedes the addition of sulfur by LipA. The other E. coli pathway, the salvage pathway, requires free lipoic acid from the medium to be scavenged and attached to a target protein by lipoate-protein ligase (LplA). LplA first activates the lipoic acid to lipoyl-AMP and then transfers it to the protein (1,3,5-7).

Aspects of eukaryotic lipoic acid synthesis and attachment have been discovered, although the exact mechanisms of these enzymatic reactions still remain unclear. In

Saccharomyces cerevisiae, a species of budding yeast, a collection of Lip enzymes have been found to be homologous to those in E. coli. Lip2 and Lip5 are the homologs of LipB and LipA, respectively, enabling lipoic acid synthesis, pyruvate dehydrogenase activity, and consequently, respiratory growth of yeast on a glycerol medium. Furthermore, Lip3 is the eukaryotic structural counterpart to E. coli's LplA, although it is involved in the Lip2 and Lip5 pathway, undergoing a mechanism distinct from the salvage pathway of LplA. Lipoic acid synthesis and attachment in yeast is conducted entirely through a single pathway, unlike the two redundant pathways in E. coli. This was determined by two findings: first, that a mutation in any one of the Lip enzymes leads to a deficiency in respiratory function, and second, that growth on lipoic acid does not rescue either the Lip2 or Lip5 mutants. These discoveries point to Lip3's involvement with Lip2 and Lip5, as well as its incapability of attaching lipoic acid from the environment, as LplA does. Since Lip3 cannot fully substitute the function of Lip2 and Lip5, it is clear that while *Saccharomyces cerevisiae* possesses a homolog to LplA, it either cannot utilize exogenously-supplied lipoic acid, or it lacks the equivalent of E. coli's LplA-driven salvage pathway (5).

Lipoic acid in *Saccharomyces cerevisiae* is found covalently bound in the conserved lipoyl domains of the E2 subunit of pyruvate dehydrogenase (Lat1), the E2 subunit of α-ketoglutarate dehydrogenase (Kgd2), and the H protein of the glycine cleavage enzyme (Gcv3). The effects of individual Lip enzyme mutations on the lipoylation of these three proteins were determined through analysis of mitochondrial extracts. A mutation in any of the three Lip enzymes resulted in complete loss of lipoylated Lat1 and Kgd2, while a Lip3 mutant still produced lipoylated Gcv3. This suggests that all three Lip enzymes are necessary for the lipoylation of Lat1 and Kgd2, but only Lip2 and Lip5 are required for the lipoylation of Gcv3.

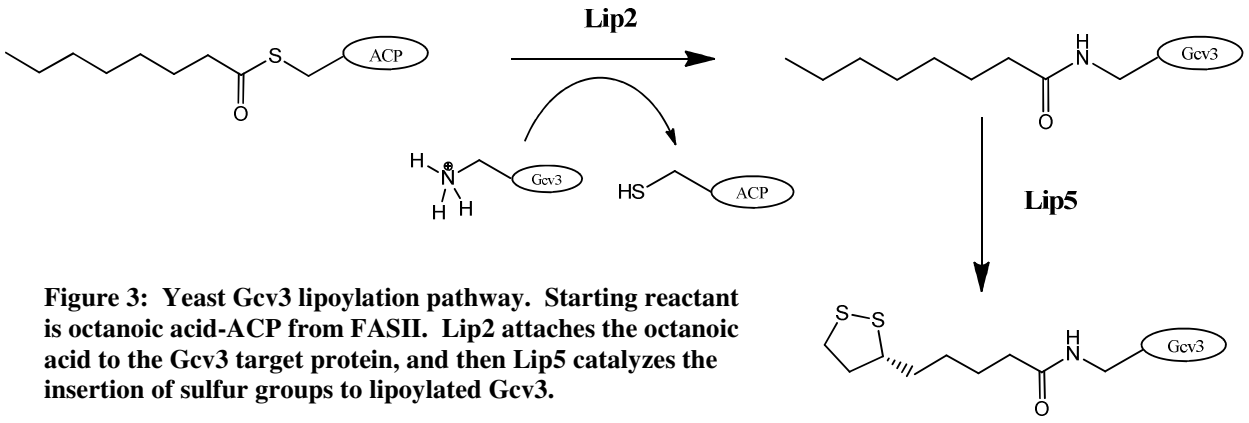

Figure 3: Yeast Gcv3 lipoylation pathway. Starting reactant is octanoic acid-ACP from FASII. Lip2 attaches the octanoic acid to the Gcv3 target protein, and then Lip5 catalyzes the insertion of sulfur groups to lipoylated Gcv3.

In addition, a novel finding for the requirements of protein lipoylation was that a target of lipoylation, Gcv3, is required for further lipoylation of other target proteins, Lat1 and Kgd2. In other words, the presence of lipoylated Gcv3 is absolutely necessary in order for Lat1 and Kgd2 to be lipoylated (5).

The known process of lipoic acid synthesis and attachment in yeast, then, is as follows: Lip2 and Lip5 are sufficient for the lipoylation of Gcv3, but Lip2, Lip5, Lip3, and lipoylated Gcv3 are all necessary for the lipoylation of Lat1 and Kgd2 (5).

While the identities and general functions of the pathway components have been identified, much is still undetermined about these factors. The additional requirements of both Lip3 and Gcv3 in the lipoylation of Lat1 and Kgd2 suggest that they contribute to the process in a central way. One mechanistic possibility is that Lip2, Lip5, Lip3, and lipoylated Gcv3 all work together in a complex to attach octanoic acid to the target protein and transform it to lipoic acid. In this scenario, Gcv3 could assist with complex assembly, help bind the substrate, or act as a positive allosteric activator of the lipoylation pathway. Another possibility is that Lip3 only catalyzes the direct transfer of lipoic acid from Gcv3 to the target protein. Whether these factors function as part of a complex or as individual enzymes is ultimately relevant to mitochondrial

function and its relation to certain disease pathways. The specific aim of this experiment is to elucidate more fully these proteins involved in lipoic acid biosynthesis and the pathways by which they operate.

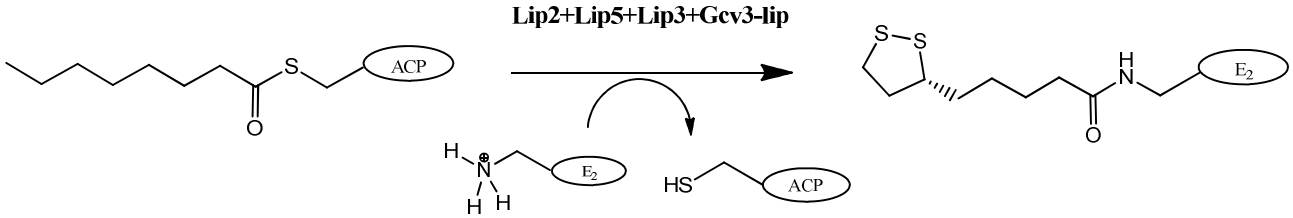

Figure 4: Possible yeast Lat1/Kgd2 E_2 lipoylation pathway. All of the Lip enzymes and lipoylated Gcv3 work in conjunction with one another to lipoylate E_2 using the octanoic acid-ACP precursor from FASII.

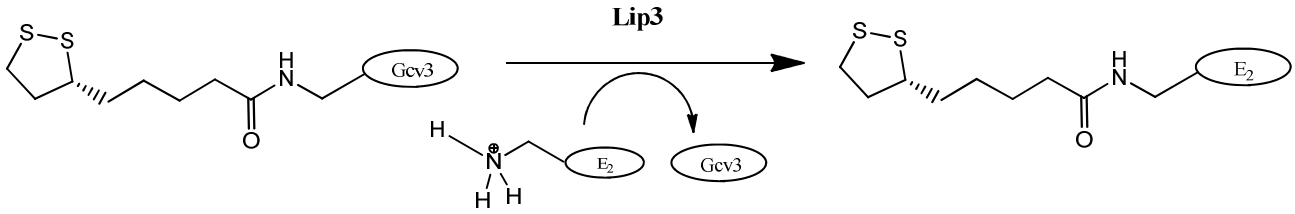

Figure 5: Possible yeast Lat1/Kgd2 E_2 lipoylation pathway. Lip3 only catalyzes lipoic acid transfer from Gcv3 to the E_2 subunit of the target protein.

MATERIALS AND METHODS

Strains and media. Table 1 contains the list of strains used. Distinction was made between gene deletions and loss-of-function point mutations because of the possibility of the enzymes operating in a complex and the range of interactions that might exist. Yeast cells were grown in YPD media (1% yeast extract, 2% peptobactone, 2% glucose). 1 mL of media was inoculated with each strain; cells were cultured in a spinner overnight at 30°C. Cells were then transferred to an additional 9 mL media at an approximately 1:100 dilution and cultured in a water shaker overnight at 30°C. These 9 mL samples were added completely to 2 L of YPD media, and the final samples were cultured overnight in a large shaker at 30°C, 120 revolutions per minute.

Table 1: *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype
BY4741 wild-type	MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0
gcv3Δ	MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, gcv3::kanMX4
lip3Δ	MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lip3::kanMX4
*Gcv3-GFP wild-type	MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, GCV3-GFP::HIS3MX6
*Lip3-GFP wild-type	MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, LIP3-GFP::HIS3MX6

* indicates point mutation resulting in loss of function

Isolation and fractionation of mitochondria. Cells were collected by centrifugation (1L volumes per centrifugation, Beckman J-6B Centrifuge, 5000 revolutions per minute, 5 minutes, 4°C) then washed with 1.2 M sorbitol medium and transferred to 250 mL volumes. These samples were centrifuged again to collect the final pellet (250 mL, Sorvall Superspeed RC2-B, 5000 revolutions per minute, 5 minutes, 4°C). The pellets were weighed and zymolyase mix was made up according to the amount of cells present (60% 2M sorbitol, 15% 0.5 M KPO_4 buffer, 0.04% EDTA, 2.5% EtSH, 3 mg zymolyase per 1 g of cells, H_2O to final volume, 1 mL

total mix per 1 mg zymolyase). The zymolyase mix was added at three times the mass of cells present, and the cells were resuspended in it, resulting in the breaking of the yeast cell walls. Cells were digested in zymolyase at 34°C in a shaker at 120 revolutions per minute, checking for complete digestion at 10 minute intervals starting at 30 minutes. Upon uniform digestion, bottles were filled with cold 1.2 M sorbitol and the pellet was collected by centrifuging twice with a resuspension in 1.2 M sorbitol in between (Sorvall Superspeed RC2-B, 5000 revolutions per minute, 5 minutes, 4°C). A medium of 0.5 M sorbitol, 0.06 M TrisCl pH 7.5, 1mM EDTA, and 0.1% BSA was added to each sample at a volume of 40 mL/L original culture (80 mL /2 L). The pellet was resuspended and blended for 30 seconds in a Waring blender. Mitochondria were collected by centrifuging twice (Sorvall Superspeed RC2-B, 2500 revolutions per second, 10 minutes, 4°C), with the mitochondria located in the supernatant. The supernatant was decanted to Nalgene tubes for further centrifugation (Sorvall Superspeed RC2-B, 16000 revolutions per minute, 10 minutes, 4°C), now with the mitochondria located in the pellet. The pellet was resuspended in 0.5 M sorbitol, 0.05 M Tris pH 7.5, and 1 mM EDTA (no BSA), and the isolated mitochondria were collected by repeating the centrifugation two additional times. Mitochondria were resuspended in 500 µL Tris-EDTA, with addition of 1.5 µL Aprotinin, Leupeptin, and Pepstatin A, and 4µL PMSF protease inhibitors to prevent proteolysis and degradation. The final mitochondria samples were stored in a -80°C freezer.

Breaking of mitochondrial membrane. Sonication has been used in order to break up the mitochondrial membrane and release the proteins needed to perform lipoylation of the target proteins involved. The procedure requires the mitochondria to be suspended in 10mM TrisCl with 1mM EDTA. It is done in three 6-second bursts, with 30 seconds of ice between each burst. At the beginning, a Pasteur pipet should be used to contain whole mitochondria, so that after

each burst, the mitochondria can be tested with more Pasteur pipets to determine if they have broken or not. An alternate method for breaking the mitochondrial membrane is a freeze-thaw, which is gentler and thought to elicit less proteolysis. The samples are frozen at -80°C for at least half an hour and then thawed on ice.

Reaction conditions. A reaction mixture is combined with some differing strains of mitochondria to initiate a combined reaction between them. It consists of HEPES buffer (pH 7.5), which helps maintain a physiological pH in the reaction, $MgCl_2$, KCl, GTP and ATP, providing energy for the reaction, and NADH, in order to further facilitate the reaction as a hydrogen carrier. The components and their concentrations of the 10X reactions mix are as follows: HEPES pH 7.5 (100 mM), $MgCl_2$ (50 mM), KCl (1500 mM), GTP (10 mM), ATP (10 mM), NADH (20 mM). The reaction mix was added to all samples, which were then heated at 30°C for 1 hour, stopped with 4X Laemmli buffer, and boiled for 3 minutes. Proteins tested were Gcv3 (170 amino acids, ~18.5-19.0 kDa), Kgd2 (463 amino acids, ~51 kDa), and Lat1 (482 amino acids, ~53 kDa).

Western blot analysis. Approximately 40 µL of mitochondrial protein was resolved on an SDS-polyacrylamide gel, with a 10% resolving gel and 4.5% stacking gel, according to the protocol given. Each strain was run separately in addition to a combination lane of 1:1 Δlip:Δgcv3. The ladder used was Spectra Multicolor Broad Range Protein Ladder, 20 µL per lane, with an additional 20 µL of Laemmli buffer to balance volumes. Interestingly enough, it was found from previous experiments that with SDS in the transfer buffer, Gcv3 did not transfer to the nitrocellulose membrane, but without the addition of SDS, the protein ladder did not transfer. However, with the novel addition of the Laemmli buffer, the protein ladder did transfer to the nitrocellulose membrane. Protein transfers and antibody incubations were performed

according to Western blot protocol, with an anti-lipoic acid primary antibody and a horseradish peroxidase secondary antibody. Blocking was done with non-fat dry milk solutions in TBS-T.

X-ray visualization. The blot was incubated with equal volumes of SuperSignal West Pico Chemiluminescent Substrate and SuperSignal West Femto Maximum Sensitivity Substrate for 10 seconds. This produced a reaction emitting light for detection by X-ray film. The blot was exposed to X-ray film in a dark room for different time increments, and the film was visualized with the SRX-101A Medical X-ray film developer.

RESULTS

Reaction of ΔGcv3 and ΔLip3. Strains with deleted Gcv3 and Lip3 genes were reacted in an attempt to rescue the wildtype phenotype, but these trials failed to produce the predicted bands. Wildtype lanes were expected to contain lipoylated Lat1, Kgd2, and Gcv3. ΔLip3 lanes were expected to express only lipoylated Gcv3, since Lip3 is presumed to be required for the lipoylation of Lat1 and Kgd2. ΔGcv3 lanes were expected to be empty. The combination of the two, though, was hypothesized to compensate for the deficiencies of both and result in a wildtype phenotype. Through multiple trials, all lanes except those for wildtype were completely blank. The wildtype lanes exhibited bands in the appropriate positions for Lat1 and Kgd2, with possible Gcv3 bands obscured by what appeared to be excessive proteolysis products. The protein ladder also failed to transfer to the nitrocellulose membrane, presumably due to the lack of SDS in the transfer buffer, but reference was made to the original gel.

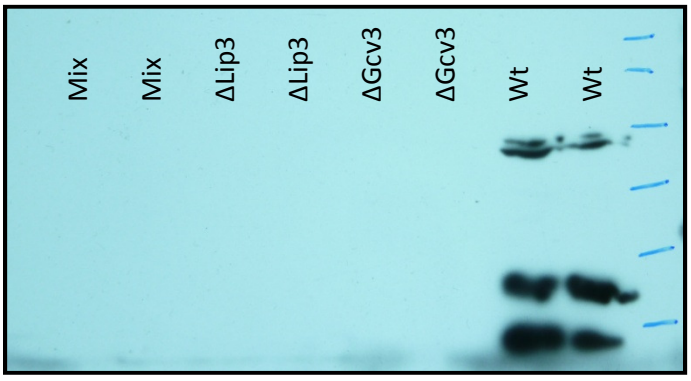

Figure 6: X-ray film of western blot. 10 second exposure. Mix lanes contained 1:1 reaction of ΔLip3 and ΔGcv3.

Reaction of ΔGcv3 and ΔLip3 utilizing different membrane-breaking methods. Attempts were made to obtain more definitive results by reducing the proteolysis evident in the reaction. The same experiment was redone using two different methods to break the mitochondrial membrane and release the mitochondrial proteins for the reaction, sonication and

freeze-thaw, as opposed to only sonication in the previous trials. This produced equally ambiguous results, with an additional anomaly in one of the other lanes for the freeze-thaw experiment. Once again, there was no transfer of the protein ladder to the nitrocellulose membrane.

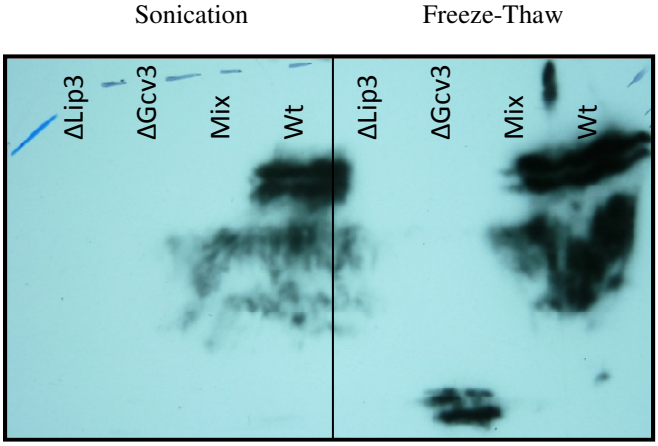

Figure 7: X-ray film of western blot. 5 second exposure. Mix lanes contained 1:1 reaction of ΔLip3 and ΔGcv3.

Reaction of *Lip3 and *Gcv3 utilizing different membrane-breaking methods.

Strains with point mutations rendering the Gcv3 and Lip3 genes nonfunctional were reacted in an attempt to rescue the wildtype phenotype, but these trials failed, as before, to produce the predicted bands. The hypothesis was that if the enzymes Lip2, Lip5, and Lip3 operated in a complex, perhaps with Gcv3, then the structural presence of the enzymes would be necessary, even if the function was missing. Similar to previous experiments, though, only the wildtype lanes produced any visualization. Excessive exposure supports that assertion that the other lanes produced completely blank results. In these trials, Laemmli buffer was added to the protein ladder lanes in order to prevent adjacent lanes from spilling over, and this actually resulted in the unexpected outcome of the transfer of the ladder to the nitrocellulose membrane.

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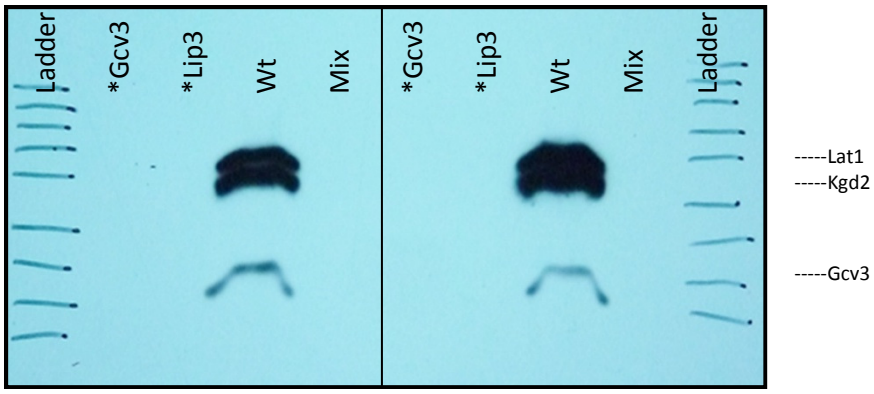

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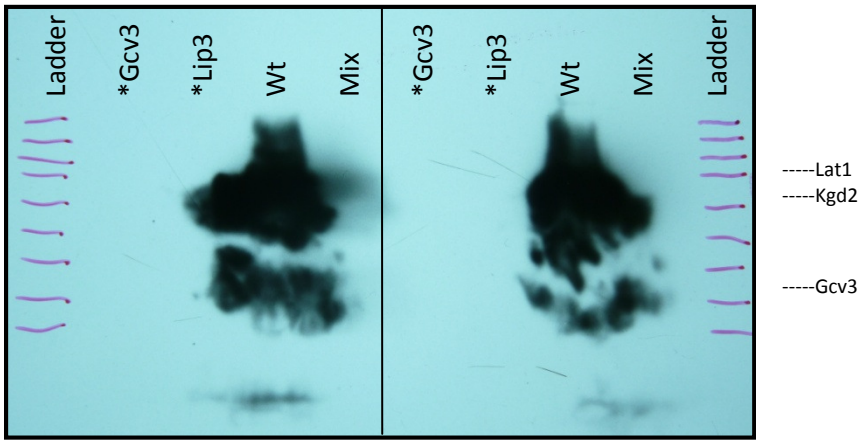

Figure 8: X-ray film of western blot. Mix lanes contained 1:1 reaction of *Lip3 and *Gcv3.
A. 10 second exposure. Bands for Lat1, Kgd2, and Gcv3 are clear in the wildtype lanes, with other lanes apparently empty.
B. 1 second exposure. More high sensitivity substrate used in this trial. Very high signal around the approximate locations of Lat1, Kgd2, and Gcv3 in the wildtype lanes, with other lanes empty with greater certainty.

DISCUSSION

The experiments conducted with the ΔLip3 and ΔGcv3 strains, as well as the *Lip3 and *Gcv3 strains, have been ineffective at elucidating the original points of interest. The objective of these trials was to clarify how the lipoylating enzymes in *Saccharomyces cerevisiae*, Lip2, Lip3, and Lip5, function to synthesize and attach lipoic acid to the target proteins Lat1, Kgd2, and Gcv3.

The failure of the experiments to produce any bands in non-wildtype lanes may suggest a few possibilities. Ultimately, this means that the α-lipoic acid antibody was unable to perceive the presence of lipoic acid on the target proteins. This could be a consequence of the relationship of the enzymes. For the ΔLip3 and ΔGcv3 reactions, this could simply be support that the enzymes operate within a complex and require all components for structural stability. Furthermore, all of the results reflect an inability to substitute exogenous Lip3 or Gcv3 for a deficiency of Lip3 or Gcv3 in the mitochondrial system, implying that the enzymes require very specific conditions in order to function effectively. Whether or not this means that all of the prerequisites for lipoylation work in concert, it is necessary to devise an alternative method for testing their relationship to one another.

There is also, of course, the possibility that the experiment was not successful due to a reason outside of the actual proteins involved. Evidence for this comes from the blankness of the ΔLip3 and *Lip3 lanes. If previous knowledge on the lipoylation reactions in yeast holds true, lipoylated Gcv3 should at least be visualized on the x-ray film in these lanes. Regardless of whether there is a circumstantial reason for this, such as the addition of or lack of SDS in the western blot transfer buffer, this strongly suggests that experimental conditions are flawed, resulting in the lack of expected results.

What seems to be the most likely reason for the blankness in the lanes is an unknown error in the reaction conditions, possibly in the amounts or concentrations of the reaction mix components. The reaction mix medium could be missing a crucial factor, or other conditions relating to this might be less than ideal. If this were the case, though, it would explain why only the wildtype lanes showed signs of lipoylation. Though the reaction mix is added to every sample, it only serves as a control for the wildtype because the strain already contains all the components it needs to carry out the lipoylation reaction. In the combination of Lip3 and Gcv3 mutants, though, the reaction mix is necessary to facilitate the reaction between the two separate strains. This scenario still begs the question of why the mutant Lip3 strains did not produce any Gcv3 lipoylation, however. It was been verified that the protein did not run off the gel, as it was present in the wildtype, and that the α-lipoic acid antibody is functional. Much about this process, however, is completely unknown.

Future experiments would need to either formulate an entirely new procedure to reveal the relationship between the Lip enzymes or discover the flaw in the experiments as conducted in this study. The main difficulty in this is that so little is known regarding lipoic acid synthesis and attachment in *Saccharomyces cerevisiae* that pinpointing the mistakes and/or creating a new scheme that avoids previous mistakes will essentially be a shot in the dark.

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