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Enzyme and Biosynthetic Pathway Engineering for Controlling Intracellular Localization
and Chemical Biosynthesis

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Chemical and Environmental Engineering

by

Jyun-Liang Lin

August 2015

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ABSTRACT OF THE DISSERTATION

Enzyme and Biosynthetic Pathway Engineering for Controlling Intracellular Localization
and Chemical Biosynthesis

by

Jyun-Liang Lin

Doctor of Philosophy
Graduate Program in Chemical and Environmental Engineering
University of California, Riverside, August 2015
Dr. Ian Wheeldon, Chairperson

Chemical biosynthesis with enzymes and microorganisms holds great promise for renewable production of chemicals, fuels, and therapeutics. To achieve high productivity, identification of active enzymes and engineering of efficient metabolic or synthetic pathways are essential for massive production. Enzyme co-localization orchestrated by synthetic DNA and protein scaffolds have been shown improvement of pathway yields *in vitro* and *in vivo*. However, an investigation of the effect of DNA scaffold to enzyme activity of enzyme-DNA nanostructure and development of tools for enzyme co-localization on intracellular membranes are limited.

Using a model system of horseradish peroxide and a multi-valent DNA scaffold, we demonstrated that DNA-conjugated horseradish peroxidase (HRP) activity can be enhanced by tuning the binding between substrates and DNA. The concept extracted from this work can be extended to rational designing of enzyme activity toward given substrates.

Biological production of esters can be accomplished by the enzymatic reaction catalyzed by alcohol-O-acetyltransferase (AATase) with condensation of acyl-CoA and alcohol. Ethyl acetate synthesized by Atf1, an AATase, in *S. cerevisiae* relies on the availability of acetyl-CoA metabolized by aldehyde dehydrogenase (Ald6) and acetyl-CoA synthetase (Acs1) from acetaldehyde and ethanol produced by alcohol dehydrogenase (Adh). Both Ald6 and Acs1 have been shown to localize to the cytosol or mitochondria, whereas Atf1 is found on the endoplasmic reticulum (ER) and lipid droplets (LDs). To engineer enzyme co-localization of Ald6, Acs1, and Atf1, we first elucidated molecular transport of Atf1 to the ER and LDs, and essential domains required for membrane association. We then developed an Oleosin-Cohesin-Dockerin based synthetic protein scaffold that functionally localizes Ald6 and Acs1 to Atf1 on LDs. Such intracellular organization of the engineered pathway has improved the yield of ethyl acetate production. In addition, we also developed a spectrophotometric-based high throughput screening assay for determination of AATase activity and discovered a previously unexplored Atf-SI from tomato with high activity toward a diverse set of alcohols and acyl-CoAs. This work not only broadens our understanding of LD biology, but also expands our capabilities to control intracellular localization of enzymes for efficient chemical conversion and to rapidly investigate enzyme activity of AATase.

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Chapter 1. Introduction

1.1 CHALLENGES IN METABOLIC ENGINEERING

The renewable production of chemicals, fuels, and pharmaceuticals is an important societal challenge. Conventional syntheses rely on non-renewable petroleum feedstock and often require heavy demand of energy input and harsh operation conditions. Comparing to petrochemical production, biomass feedstocks and biosynthesis by biochemical processes holds great promise in overcoming this challenge (Figure 1.1). Metabolic engineering, the manipulation of cellular processes and metabolic networks, has made possible the biosynthesis of desirable products at industrial scales [1]. However, achieving high titer, yield, and productivity necessitates engineering at single gene, pathway, and systems level [2]. Enzyme activity and substrate specificity are important for efficient and specific conversion of metabolic intermediates. Metabolic flux balance is important to drive reactions forward along the operated metabolic or synthetic pathways. Ultimately, an overall balance of native metabolic network and cellular processes is essential to achieve industrial production and maintain cell fitness.

Rapid and cheap DNA synthesis and maturation of cloning techniques have greatly improved our capabilities to synthesize mutated genes and express them in microbial hosts. Nevertheless, the development of assays and methods that can aid in screening of enzyme activity has fallen further behind in the design-build-test cycle. Once active enzymes are obtained, the choice of microbial host needs to be taken into account, in

particular a selection between prokaryotes and eukaryotes since organellar structures and post-translational modification of eukaryotes are often vital to enzyme functioning.

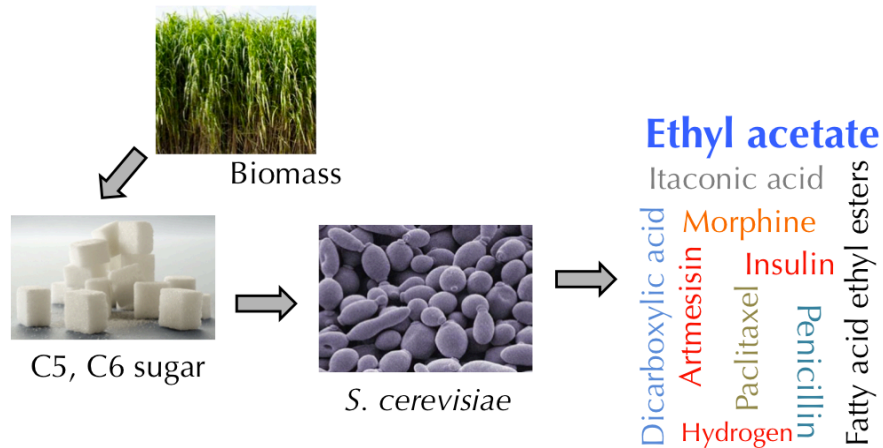


Figure 1.1. Renewable production of chemicals, fuels, and pharmaceuticals from cellulosic biomass. Sugars with five- or six- carbon backbone released from pretreatment and enzymatic digestion of biomass are fed to *S. cerevisiae*, converting to desirable products via metabolic or synthetic pathways in cells.

Development of synthetic biology tools and strategies for controlling metabolic flux has long been a focus of metabolic engineers and synthetic biologists. Efforts on metabolic engineering have been put toward controlling transcription and translation for protein syntheses [3-6]. The strength of transcription can be controlled by well-characterized native and synthetic promoters [3,4]. Translational efficiency can be tuned with ribosome binding, mRNA secondary structure, and terminators [5,6]. The emergence of first work controlling metabolic flux at the post-translational level via synthetic protein scaffolds co-localizing enzymes opened a new era of metabolic engineering [7]. Since then, DNA- and RNA-based synthetic scaffolds were also successfully developed and proved effective to pathways engineering [8,9]. In addition,

pathway flux can also be augmented with compartmentalization of yeast cytosolic upstream enzymes to mitochondria in which downstream enzymes are localized [10]. The so-called “substrate or metabolic channeling” effect observed by enzyme co-localization is generally believed as a consequence of an increased probability of intermediates produced from upstream enzymes being processed by downstream enzymes due to high local enzyme concentrations (Figure 1.2) [11]. Despite the success in these synthetic scaffolds and organelle targeting, synthetic biology tools that can co-localize enzymes on intracellular membrane has yet to be developed.

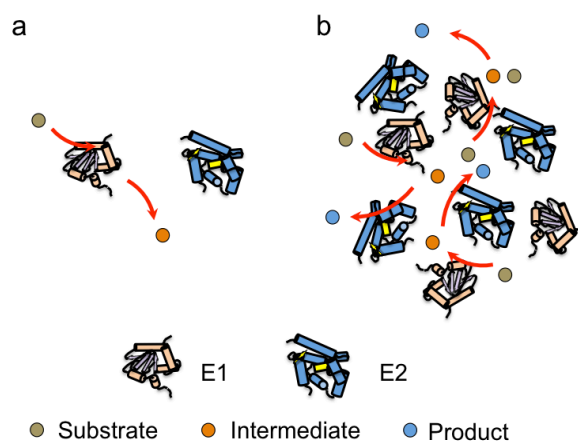


Figure 1.2. Kinetic benefits from enzyme co-localization or clustering. (a) A diluted pathway or cascade reaction. When E1 and E2 are diluted in space, intermediates generated by E1 may escape from access of E2 due to rapid diffusion to the bulk phase. (b) A concentrated pathway or cascade reaction. When E1 and E2 are co-localized or clustered, the probability of intermediates produced from E1 being processed by E2 is increased, preventing intermediates escaping from the reaction agglomerates and enhancing catalytic efficiency.

As a biomolecular material, DNA nanotechnology have been widely used to organize cascade enzymes, reproduce, and interrogate enzyme co-localization effects *in vitro* [12]. Understanding of substrate-DNA interactions and the potential effects of DNA scaffolds on enzyme kinetics will help guide the design of DNA scaffolds avoiding detrimental and

gaining beneficial effects. However, such intermolecular interaction has yet to be explored (Figure 1.3).

Substrate-DNA interaction

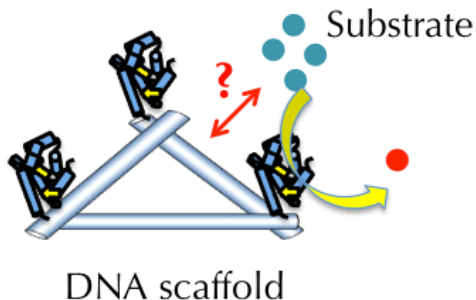


Figure 1.3. Toward understanding of intermolecular interaction between substrate and DNA and potential kinetic effects in the enzyme-DNA nanostructure.

1.2 BIOSYNTHESSES OF ESTERS

The microbial production of ester fragrances, flavor compounds, and biofuels has been a recent focus of metabolic engineering. Long chain waxy esters have potential for use as diesel fuel substitutes and have been produced in engineered *E. coli* and yeast [13-15]. Volatile short and medium chain esters have value as natural food additives (e.g., isoamyl acetate and phenyl ethyl acetate) and industrial solvents (e.g., ethyl acetate), and have been produced in native yeast strains [16,17] and engineered *E. coli* [18-21]. The biosynthetic pathways that produce these compounds share a common terminal reaction step, the condensation of an alcohol with acetyl- or acyl-CoA by alcohol-O-acetyl/acyltransferase (AATase, E.C. 2.3.1.84 and 2.3.1.75, respectively). A number of AATases that produce short and medium chain esters have been identified in various yeast and fruit species and are active during fermentation and fruit ripening [22-28].

Orthologs to the short and medium chain AATases have been identified in *Acinetobacter* and *Marinobacter* species [29,30]. These AATases, also called wax ester synthases, produce waxy esters from long chain acyl-CoAs and fatty alcohols. In this thesis, short chain ester, ethyl acetate was chosen as our targeted chemical.

Identification and evolving of active enzymes are often required for building an efficient pathway. Standard activity assay for volatile esters is headspace gas chromatography (GC) analysis of whole cell lysate reactions [31], while long chain acyl esters are quantified by thin layer chromatography (TLC) and by GC-MS [32]. Such slow and low throughput assays have limited the progress of protein engineering and enzyme screening of AATases. Furthermore, an exploration of a suitable host between prokaryotes and eukaryotes for proper enzyme expression and function is of great importance to metabolic engineering. However, studies pertaining to AATases from other organisms in the most common microbial hosts, *E. coli* and *S. cerevisiae*, have yet under explored.

In *S. cerevisiae*, Atf-1 and -2 are the two AATases responsible for ethyl acetate synthesis, and Atf1 dominates the production [33]. The upstream enzymes, aldehyde dehydrogenase (Ald6) and acetyl-CoA synthetase (Acs1), necessary to synthesis of acetyl-CoA are localized to cytoplasm [34], whereas Atf1 is found to be on the endoplasmic reticulum (ER) and lipid droplets (LDs) (Figure 1.4) [34,35]. Although synthetic protein scaffolds may be applicable to co-localize Ald6 and Acs1 in cytoplasm, a lack of LD localization tool has impeded the channeling effect from Acs1 to Atf1. In addition, the elusive subcellular localization of Atf1 complicates the engineering of

enzyme co-localization of the upstream enzymes to Atf1. A fundamental study of the organelle targeting of Atf1 to the ER and LDs will greatly help engineering membrane localization of the upstream enzymes and contribute our understanding of molecular transport to the field of cell biology (Figure 1.5).

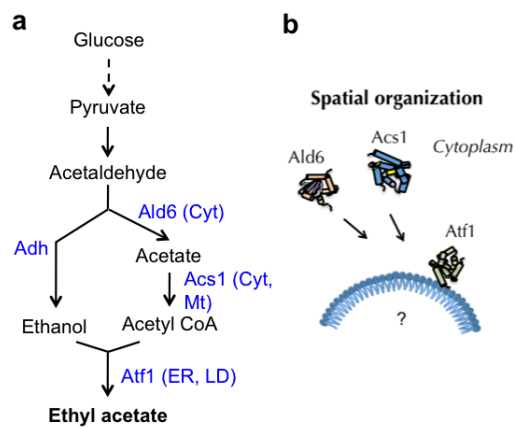


Figure 1.4. Development of synthetic biology tools to organize cytoplasmic enzymes. (a) Metabolic pathway of ethyl acetate synthesis in *S. cerevisiae*. The upstream enzymes required for acetyl-CoA synthesis, Ald6 and Acs1, are in cytoplasm, whereas Atf1 is localized to the intracellular membranes. (b) Spatial organization of Ald6 and Acs1 to the intracellular membrane.

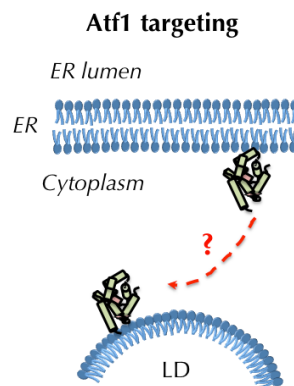


Figure 1.5. Toward understanding of alcohol acetyltransferase (Atf1) molecular transport of membrane targeting and domains required for membrane association.

1.3 DISSERTATION ORGANIZATION

This dissertation covers a range of topics in controlling and understanding enzyme co-localization. These topics include: 1) developing a mechanistic understanding of the kinetic effect of DNA modification to enzymes; 2) microbial host selection of AATases; 3) molecular cell biology of Atf1 membrane transport in *S. cerevisiae*; 4) development of a kinetic assay for AATase activity; and 5) synthetic biology tools for enzyme co-localization on membranes. An interdisciplinary approach integrating principles from biochemistry, cell biology, genetics, computational simulation, and engineering was applied to undertake this research.

Chapter 2 discusses substrate channeling of enzyme complexes, analysis of the effect, and design rules for engineering cascade reactions. Examples of enzyme complexes with channeling capability in nature are included to present the concept. Three distinct methods that evaluate channeling effect are discussed and engineered cascade reactions are incorporated for assessment of the effect. Rules in terms of inter-enzyme distance, active site orientation, and nanostructures for multi-enzyme reactions are addressed.

Chapter 3 investigates the kinetic effect of DNA scaffolds on enzyme-DNA nanostructures. Horseradish peroxidase (HRP) attached to a triangular DNA scaffold was used as the model system. The effects of the DNA scaffold on enzyme kinetics were evaluated with six different substrates. A molecular simulation was applied to estimate binding affinity between substrate and DNA.

Chapter 4 discusses the development of a coupled enzyme spectrophotometric assay for determination of AATase activity of whole cell lysate. The developed assay was

tested with two AATases and shown its robustness across different whole lysate concentrations. Z' factor was analyzed to evaluate the feasibility for high throughput screening on microplate plates. This rapid screening method was then used to explore enzyme activity toward a library of alcohols and acyl-CoAs for six selected AATases.

Chapter 5 explores enzyme localization and function of five selected Atfs in native and non-native hosts. Intracellular localization and activity of enzymes were analyzed in *S. cerevisiae* and *E. coli*.

Chapter 6 interrogates molecular transport of Atf1 from the ER to LDs and the domains required for its membrane association in *S. cerevisiae*. A series of N- and C-terminal truncations were made to narrow down the targeting domains. The detailed binding mechanism was examined by mutating key residues on the identified regions. A time course study was conducted to monitor membrane targeting of Atf1 from the ER to LDs. Due to a structural similarity, the same hypothesis was also tested with Atf2. Five other Atfs from non-*Saccharomyces* yeast and fruits were analyzed for structures and cellular localization in *S. cerevisiae*.

Chapter 7 discusses the development of synthetic biology tools for organization of cytoplasmic enzymes to LDs in *S. cerevisiae*. A series of potential LD localization tags from other organisms were characterized in *S. cerevisiae*. A synthetic scaffold building on the characterized LD tag was developed to maintain activities of cytoplasmic enzymes on LDs. The titer of ethyl acetate was maximized with a combinatorial library aiming to optimizing protein expression and relative enzyme stoichiometry on the scaffold. The

screening of ethyl acetate production was aided by a developed colorimetric assay and verified with GC analysis.

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Chapter 2. Design and Analysis of Enhanced Catalysis in Scaffolded Multienzyme Cascade Reactions

2.1 ABSTRACT

New developments in nucleic acid nanotechnology and protein scaffold designs have enabled unparalleled control over the spatial organization of synthetic multienzyme cascade reactions. One of the goals of these new technologies is to create nanostructured enzyme cascade reactions that promote substrate channeling along the cascade and, in doing so, enhance cascade catalysis. The concept of substrate channeling has a long and rich history in biochemistry and has established methods of evaluation and quantification. In this Perspective, we review the most common of these methods and discuss them in the context of engineered multienzyme systems and natural bifunctional enzymes with known mechanisms of substrate channeling. In addition, we use experimental data and the results of simulations of coupled-enzyme reactions to develop a set of preliminary design rules for engineering multienzyme nanostructures. The design rules address the limitations on interenzyme distance and active site orientation in substrate channeling and suggest designs for promoting enhanced catalysis, specifically, that enzyme orientation should minimize interenzyme distance and that at distances greater than 1 nm between active sites, significant channeling occurs only if diffusion of the intermediate is bounded through interactions with the surface or scaffold between active sites. This field is rapidly developing and promises to create many more new and exciting technologies.

2.2 INTRODUCTION

The multistep reaction cascades of cellular metabolism are highly coordinated, simultaneously processing a battery of biosynthetic and oxidative pathways to build essential biomolecules and catabolize energy sources. One strategy that has evolved to allow for this to occur within the confined environment of a single cell is substrate channeling along spatially organized multienzyme structures. Substrate channeling is the transfer of a reaction intermediate from the active site of one enzyme to the active site of a downstream enzyme without first diffusing to the bulk solution. This is an important phenomenon that can result in sequestering substrates and intermediates along specific pathways, thus increasing pathway flux and minimizing cross-talk between separate pathways.

Substrate channeling has a long history in biochemistry with many interesting discoveries, and like many fields, it is not without controversy in the analysis of experimental evidence and in the existence of different mechanisms [1-3]. The past 5 years have seen a re-emergence of this topic, with a focus on engineering nanostructured assemblies for coupled-enzyme reactions and colocalization scaffolds for biosynthetic pathways [4-7]. At the root of these efforts is the desire to engineer enzyme reaction cascades with enhanced catalysis. Systems are designed to promote substrate channeling and control the local concentration and flux of reaction intermediates and, in doing so, increase reaction rates, prevent undesired side reactions from consuming intermediates, and drive reactions counter to unfavorable thermodynamics in the bulk environment.

Inspired by natural metabolic pathways and bifunctional enzymes, researchers are

developing new strategies to create colocalized and spatially organized multienzyme structures (Figure 2.1). Using protein, nucleic acid, and polymer scaffolds to position

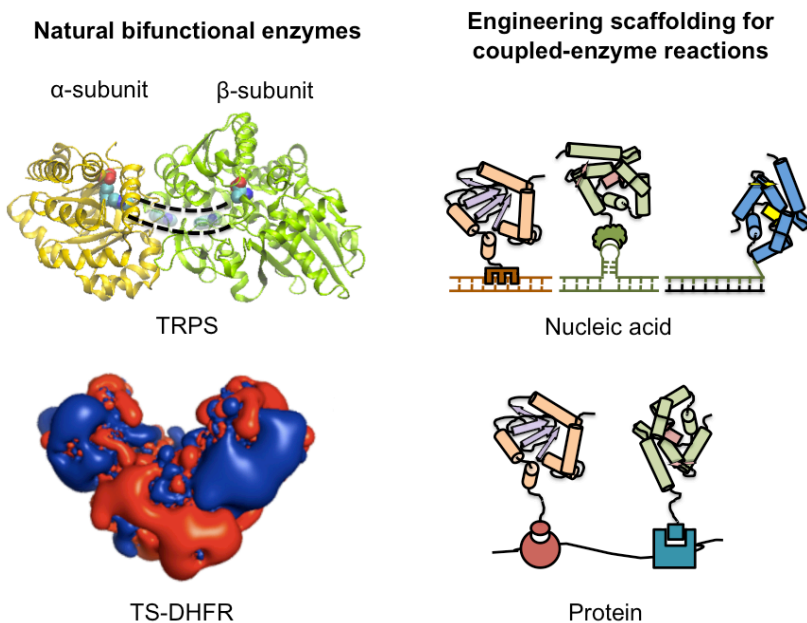


Figure 2.1. Natural strategies of substrate channeling in bifunctional enzymes and engineering approaches to colocalizing enzymes in a cascade reaction. (top, left) Crystal structure of tryptophan synthase (TRPS) with intramolecular tunnel (dashed lines) that connects the active site of the α -subunit to the active site in the β -subunit [20]. (bottom, left) Crystal structure of thymidylate synthase–dihydrofolate reductase (TS-DHFR) bifunctional enzyme. The structure is shown with an overlay of the electrostatic map (negative charge shown in red, positive charge shown in blue). The electrostatic interactions between enzyme and negatively charged cascade intermediate promote diffusion between active sites. Adapted with permission from ref [25]. (top, right) Examples of enzymes attached to nucleic acid scaffolds, including, from left to right, a DNA binding protein fused to an enzyme, aptamer attachment, and chemical conjugation of an enzyme to a nucleic acid strand. (bottom, right) Two enzymes attached to a protein scaffold by unique protein binding domains fused to the enzyme termini.

enzymes in multienzyme structures, enhancements in overall reaction cascade kinetics have been demonstrated both in vivo and in vitro. For example, a modular DNA scaffold expressed in *Escherichia coli* has produced a 5-fold increase in trans-resveratrol yield

from a coupled enzyme reaction [8]; colocalization of a two-enzyme system using protein and RNA scaffolds has produced up to 50-fold increases in the production of biohydrogen in *E. coli* [9,10]; a protein scaffold used to assemble a three-enzyme mevalonate pathway resulted in a 77-fold increase in yield [11]; and two separate examples of DNA scaffolds used to colocalize a model coupled-enzyme reaction in vitro demonstrate a >15-fold increase in the initial pathway reaction rate [12,13]. The prevailing reasoning used to explain enhancements due to colocalization is that when enzymes are assembled in close proximity, enhancements in catalysis are due to an increased local concentration of reaction intermediates. At low intermediate concentration, the second enzyme of a coupled reaction is pseudo-first-order with respect to the intermediate; thus, increases in local concentrations increase the reaction velocity [14]. An alternative mechanism of yield enhancement may be at work in vivo: in pathways with toxic intermediates, it is possible that high local enzyme concentrations ensure that intermediates are largely consumed prior to diffusing from the colocalized pathway, thus conferring a fitness benefit in comparison with microorganisms with freely diffusing pathway enzymes [4].

These new examples of enhanced catalysis in engineered multienzyme structures are important and encouraging, but many challenges remain in developing generalizable systems for assembling multienzyme pathways or cascades with optimized kinetics. For example, accurate positioning and orientation of enzymes at the nanometer scale remains technically challenging, and the diversity of enzyme structures, from primary amino acid sequence to quaternary structure, can prevent broad application of a single strategy for

assembly of arbitrary multienzyme cascades. These and more challenges continue to be defined and are being addressed with new strategies to create spatially organized, multienzyme systems with well-defined nanoscale architectures (reviewed in refs [15,16]).

In this Perspective, we describe recent advances in engineering spatially organized, multienzyme systems in the context of methods of evaluating changes in coupled-enzyme catalysis and, by extension, cascade reactions. We focus on established methods from the biochemistry community to observe and quantify substrate channeling and coupled-enzyme kinetics, and we apply these methods to newly developed spatially organized, multienzyme systems. As a point of comparison, we discuss selected natural examples of bifunctional enzymes with known mechanisms of substrate channeling. Our discussion focuses on in vitro examples because the complexity of the intracellular environment often prevents detailed kinetic analysis and a full understanding of the system architecture is difficult to obtain. On the basis of published experimental data and simulations of such systems, we describe a preliminary set of design rules (in terms of spatial organization, active site orientation, and scaffold design) for engineering multienzyme systems with enhanced catalysis.

2.3 SUBSTRATE CHANNELING IN NATURE

Substrate channeling in natural metabolic pathways is not uncommon. In plant biochemistry, many secondary metabolites, including isoprenoids, alkaloids, and flavonoids, are produced by spatially organized pathways assembled, sometimes

transiently, on lipid membranes [17]. These enzyme complexes create multistep reaction cascades with optimized ratios of rates and overall structures that create optimal local conditions to protect cells from toxic intermediates and drive pathway catalysis [18]. Channeling and spatial organization also occur at the protein level. The canonical example is the bifunctional enzyme tryptophan synthase, whose active sites are linked by an intraenzyme molecular tunnel through which the reaction intermediate can pass (Figure 2.1, top, left). Similar intramolecular channels are also found in carbamoyl-phosphate synthetase and glutamine phosphoribosylpyrophosphate amidotransferase (GPTase) [19,20]. Bifunctional aldolase dehydrogenase complexes are another example of coupling active sites through intraenzyme tunnels [21]. In this case, the open-ended barrels of each enzyme are genetically fused, sequestering the toxic aldehyde intermediate from the bulk solution.

One example that has inspired our research group is the bifunctional enzyme thymidylate synthase dihydrofolate reductase (TS-DHFR). TS methylates the RNA base dUMP to complete the synthesis of the DNA base dTMP. The synthesis requires a methylated tetrahydrofolate cofactor (Figure 2.2).

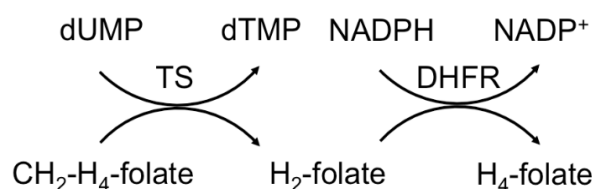


Figure 2.2. The coupled reaction of TS-DHFR. The dihydrofolate intermediate is boxed for emphasis.

Recycling of the cofactor begins with the reduction of the dihydrofolate product from

the TS reaction to tetrahydrofolate at the DHFR active site. The cofactor product of the coupled reaction is subsequently methylated by a third enzyme, serine hydroxymethyltransferase, to complete the cycle. It is in the first step of cofactor recycling that channeling occurs. The TS- DHFR crystal structure of *Leishmania major* reveals a positively charged region on the outer enzyme surface that spans the ~4 nm distance between active sites (Figure 2.1, bottom, left) [22]. This distance is too far for direct transfer of the intermediate from active site to active site to account for the kinetic behavior of the enzyme, and limited conformational change in protein structure prevents intermediate transfer by a dynamic enzyme—substrate complex mechanism. Brownian dynamic simulations show that the negatively charged dihydrofolate intermediate diffuses between the active sites with high efficiency [23]. Under simulated physiological conditions, the electrostatic interactions between intermediate and enzyme create a zone of bounded diffusion, resulting in >50% channeling, increasing to >90% under reduced ionic strength. These simulations are supported by transient and steady-state kinetics that provide evidence of fast intermediate transfer between active sites [24]. A recent work investigating the TS-DHFR bifunctional enzyme from different species reveals that this mechanism of substrate channeling is common among different parasitic protozoa [25].

The TS-DHFR example is an important one for engineering of new spatially organized pathways. Structural and kinetic data come together to create a compelling picture of the extent and mechanism of channeling. Because substrate channeling occurs via a bounded diffusion mechanism, channeling is dependent on the chemical and physical environment,

and careful analysis was required to clearly identify a mechanism of substrate channeling and the kinetic enhancements due to that mechanism.

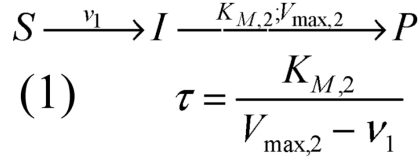
2.4 KINETIC ANALYSIS OF SUBSTRATE CHANNELING

There have been substantial efforts made to understand the kinetics of coupled enzyme reactions, both with and without the potential occurrence of substrate channeling [26-28]. Analysis of channeling often focuses on one or more of the following: (1) transient time of the overall reaction, (2) pathway resistance to a competing side reactions, and (3) enhancement in the initial pathway reaction rate. Isotope dilution/enrichment studies and pre-steady-state kinetic analysis can also be used to evaluate possible channeling but can be technically challenging and may not be applicable to all coupled-enzyme systems. Here, we discuss the more common methods of evaluating coupled- enzyme reactions in the context of bifunctional enzymes and engineered multienzyme systems. We direct readers to a comprehensive review on substrate channeling for a full discussion of all evaluation methods [29].

Transient Time of a Coupled-Enzyme Reaction

Transient time, τ is the time required to reach steady-state flux of an intermediate in a coupled reaction and is an observable lag phase prior to reaching steady state velocity in a coupled reaction. If we consider the simple reaction of substrate, S, to product, P, via intermediate, I, (Scheme 1) when Michaelis—Menten kinetics apply, and the Michaelis constant of the second reaction ($V_{\max,2}$ and $K_{m,2}$, respectively) and the reaction velocity of the first reaction (v_1) in the relationship shown in Scheme 1 [14,29].

Scheme 1



The transient time, τ is of interest because it is readily observable in time course kinetic assays, and decreases in τ can be an indication of increased substrate channeling. To this end, eq 1 has been extended to include a condition of channeling,

$$\tau = \frac{K_{M,2}(1 - p_c p_r)}{V_{\max,2}} \quad (2)$$

This equation was developed with the condition that a relatively small concentration of the intermediate escapes to the bulk in comparing with the value of $K_{m,2}$ [14]. The channeling probability, p_c , is the probability that the intermediate is transferred from active site to active site without first escaping to the bulk. The reaction probability, p_r , is the probability that a reaction occurs once a substrate—enzyme complex is formed at the second active site prior to dissociation of the complex. Figure 2.3 shows the graphical calculation of τ from a time course of a coupled reaction. A linear fit to the data crosses the x-axis at τ , and by eq 1, the concentration of the intermediate at steady state is the negative of the y-intercept.

Analysis of the TS-DHFR reaction cascade shows a decrease in τ from 22 s with monofunction DHFR and TS to a approaching 0 for the bifunctional enzyme under the same experimental conditions (Figure 2.4) [30]. By eq 2, a decrease in τ is indicative of

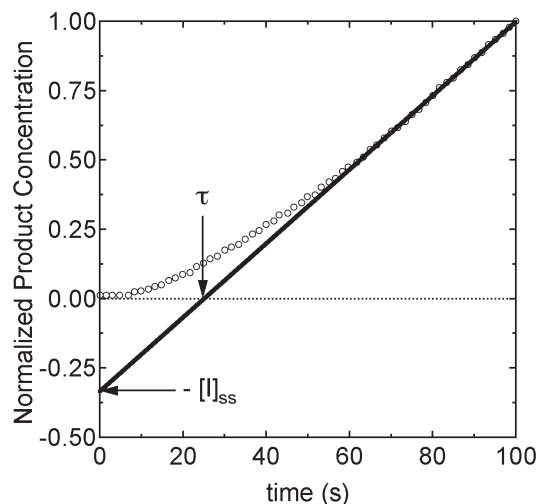


Figure 2.3. Graphical calculation of the transient time, enzyme reaction. A linear fit to the product concentration as a function of time at steady state crosses the x-axis at τ . The y-intercept is equal to the negative of the steady state concentration of the cascade reaction intermediate, $[I]_{ss}$.

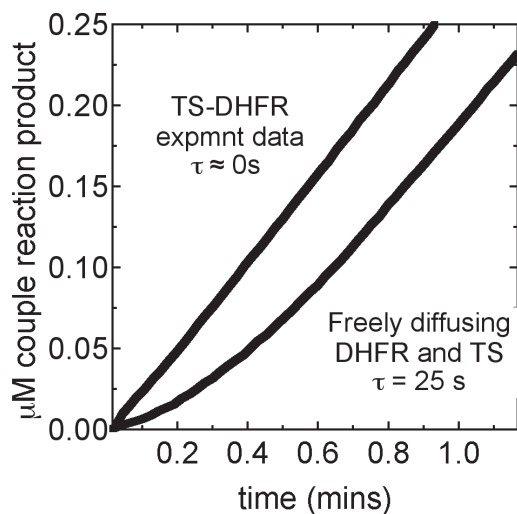


Figure 2.4. Experimental data of the TS-DHFR bifunctional enzyme and freely diffusing DHFR and TS. Adapted with permission from ref [30]. A clear decrease in the transient time can be observed in the bifunctional enzyme in comparison with the freely diffusing enzyme pair.

an increase in the extent of substrate channeling in the bifunctional enzyme in

comparison with the freely diffusing enzyme pair.

Changes in have also been experimentally observed in an engineered system in which glucose oxidase (GOx) and horseradish peroxidase (HRP) were colocalized on a hexagonal DNA scaffold [12]. This model enzyme pair has been used in a number of DNA scaffold technologies that control colocalization of the coupled reaction. Both GOx and HRP are robust, maintain high activity when modified with chemical cross-linking agents, and make for an excellent model cascade for developing new scaffolds and for exploring the effects of spatial organization and scaffold design on the kinetics of coupled- enzyme reactions (Figure 2.5). GOx converts glucose and oxygen to

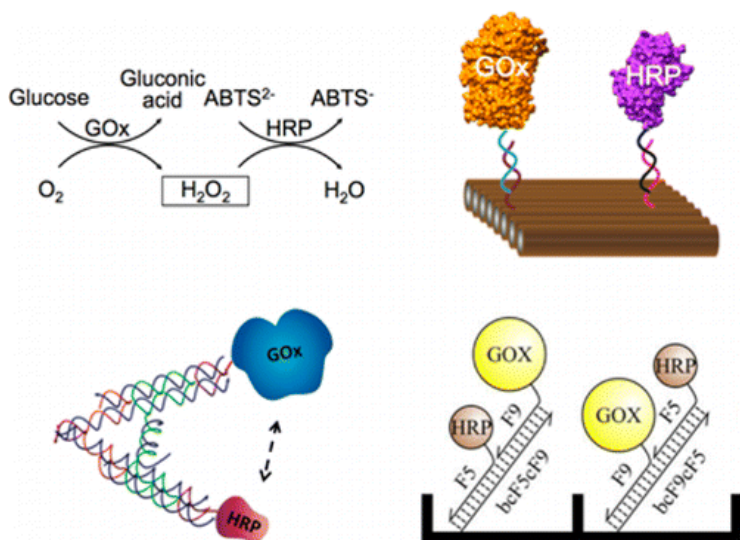


Figure 2.5. Coupled reaction of glucose oxidase and horseradish peroxidase and examples of DNA scaffolding of the GOx–HRP model cascade. (top, left) Reaction scheme of the coupled GOx–HRP reaction. This model cascade is commonly used to explore new scaffold designs for spatially organized multienzyme structures. (top, right) A cartoon representation of the controlled assembly of GOx and HRP on a DNA origami tile [13]. (bottom, left) An active DNA scaffold that, with the addition of a DNA “fuel” strand, assembles and disassembles the GOx–HRP cascade. Adapted with permission from reference [39]. (bottom, right) Immobilization of the GOx–HRP with DNA strands for the controlled assembly of the GOx–HRP cascade. Adapted with permission from reference [38].

gluconolactone and hydrogen peroxide. Hydrogen peroxide is the intermediate of the coupled reaction and is oxidized by HRP with the concomitant reduction of an electron acceptor (in this case, the colorimetric substrate ABTS). A change in the design of the hexagonal DNA scaffold decreases τ from 65 to 45 s. When GOx and HRP are colocalized on the surface of ~ 10 nm diameter micelles, τ is reduced from ~ 25 to <1 s, indicating a substantial increase in peroxide intermediate directly accessing the downstream enzyme prior to diffusing to the bulk [31].

Given well-defined kinetic parameters, for both freely diffusing enzymes and modified enzymes assembled into spatially organized structures, τ -analysis can be a powerful method of evaluating substrate channeling and enhancements in the kinetics of coupled reactions. Equation 2, as well as similar derivations of kinetic expressions of substrate channelings [28,32], can be used to quantify channeling and changes in channeling in a given system.

Competition for Pathway Intermediates from a Competing Reaction

A relatively straightforward and robust method of evaluating coupled-enzyme reactions is challenging the cascade with a parasitic side reaction whose substrate is the intermediate of interest. The observation of any product from the competing reaction indicates the presence of intermediate in the bulk solution and, therefore, less than perfect channeling in the coupled-enzyme system. The effect of the competing reaction can also be observed in τ . Reaction with the competing enzyme decreases the bulk concentration of intermediate and, consequently, the time required to reach steady state concentrations.

These evaluations of channeling (i.e., decreased τ and side product formation) do not require a detailed knowledge of kinetic parameters; however, given such data and an extension of eq 2 to include the third, competing enzyme can be used to quantify the extent of channeling [14].

Challenge from a competing reaction can also affect the overall pathway activity and yield. Figure 2.6 shows the residual activity of a coupled-enzyme reaction when genetically fused into a single bifunctional enzyme in comparison with a freely diffusing enzyme pair [33]. In this experiment, mitochondrial malate dehydrogenase (mMD) and citrate synthase (CS), a coupled reaction from the Krebs cycle that converts malate to citrate via an oxaloacetate intermediate, are fused N- to C- terminus with a three-amino-acid linker. A model of the mMD—CS fusion protein derived from crystal structures of each individual enzyme shows that an electrostatic patch bridges the protein surface from the mMD active site to the CS active site, a distance of ~6 nm. With a high concentration of aspartate transaminase (AAT) competing for the oxaloacetate intermediate, the mMD—CS fusion maintains upward of 60% activity. Under the same conditions, freely diffusing enzymes maintain no more than 20% of the maximum activity, indicating that channeling occurs in the enzyme fusion. Combined with Brownian dynamics simulations showing the importance of electrostatics in mMD—CS channeling [14,34], the structural data and competition assays present compelling evidence of substrate channeling in the fusion protein.

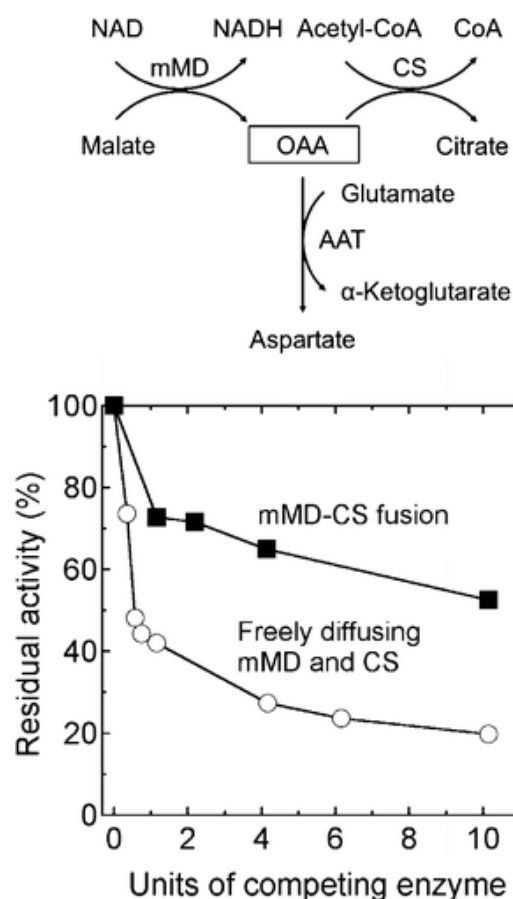


Figure 2.6. Residual activity of the coupled-enzyme reaction of malate dehydrogenase and citrate synthase in the presence of a freely diffusing enzyme that competes for the coupled reaction intermediate. (top) The coupled reaction of mMD and CS with a competing reaction from aspartate transaminase (AAT). (bottom) A fusion between mMD and CS maintains nearly 60% of maximum activity in the presence of 10 units of competing enzyme, whereas the freely diffusing enzyme pair is reduced to ~20% activity. Adapted with permission from ref [33].

The presence of a competing reaction can also affect the yield of the desired cascade. For example, an aptamer-based DNA scaffold that assembles GOx and HRP in close proximity protects the peroxide intermediate from a competing reaction with catalase [35]. In the presence of catalase, the reaction yield of the assembled GOx—HRP structure is decreased by 20%, and the decrease is upward of 50% under the same conditions with freely diffusing, unassembled GOx and HRP. The resistance of the

assembled pathway to the competing reaction is consistent with the concept of channeling by close proximity.

Similarly, the yield of the coupled reaction of bifunctional aldolase—dehydrogenase complexes was used to evaluate substrate channeling. The aldolase cleaves 4-hydroxy-2-oxoacids, producing pyruvate and an aldehyde. The aldehyde intermediate travels through an intramolecular tunnel and is converted to acyl-CoA at the active site of the coupled dehydrogenase. A freely diffusing aldehyde dehydrogenase that converts aldehydes to carboxylates competes for the aldehyde intermediate. The competition assays revealed that upward of 90% of various chain length aldehyde intermediates are channeled from aldolase to the dehydrogenase [36]. This natural bifunctional enzyme is another important example of kinetic and structural data combining to reveal the extent and mechanism of substrate channeling.

Enhancement in Initial Reaction Rate

The overall reaction rate of a coupled-enzyme reaction is the sum of rates from reaction with intermediates that take a direct route from active site to active site (i.e., intermediates that are channeled) and from intermediates that diffuse to the bulk prior to reaching a downstream active site. At steady state, in the absence of side reactions, and when intermediate decomposition is negligible, this overall rate is not affected by substrate channeling [14,28]. Similarly, the overall rate of a multienzyme cascade is not affected by substrate channeling and is limited by the maximum rate of the slowest reaction step. The data presented in Figure 2.4 from the TS—DHFR example demonstrates this point: the slope of the time course data at times $> \tau$ are equal,

corresponding to a steady-state rate of $\sim 0.25 \mu\text{M}/\text{min}$ [30]. Similarly, in analyzing GOx—HRP experimental data, we estimate the rates resulting from two different DNA scaffolds to be equal ($\sim 8 \mu\text{M}/\text{s}$) at times $> \tau$ (46 and 65 s) [12].

The situation is more complex at times $< \tau$ when a coupled-enzyme reaction has yet to reach steady state. At times $< \tau$, the contributions of channeling toward the overall rate can dominate and can result in significant enhancements to the observed overall reaction rate. Modeling and simulation of coupled-enzyme reactions support this idea and demonstrate that the initial enhancement in overall rate can range from $< 1\text{s}$ to tens of minutes, depending on system architecture and reaction volume [37].

Enhancements in overall initial rate have also been experimentally observed. For example, a DNA origami tile that colocalizes GOx and HRP at $\sim 10 \text{ nm}$ interenzyme distance results in a > 15 -fold increase in initial pathway reaction rate (Figure 2.5, top, right) [13]. A 3-fold enhancement in initial rate was demonstrated with the same enzyme pair assembled in close proximity with surface-tethered linear DNA scaffolds (Figure 2.5, bottom, right) [38]. Enhancements in overall rate have also been demonstrated with a dynamic scaffold that repeatedly closes (high activity) and opens (low activity) the GOx—HRP reaction cascade (Figure 2.5, bottom, left) [39]. Finally, the GOx—HRP coupled reaction shows significant enhancement in overall rate when colocalized inside of a DNA nanotube [40].

In the emerging field of DNA scaffolds, the predominant model cascade has been GOx—HRP; however, a number of different multienzyme cascades have been assembled using protein scaffolds (Figure 2.7). For example, three enzymes from the glycolysis

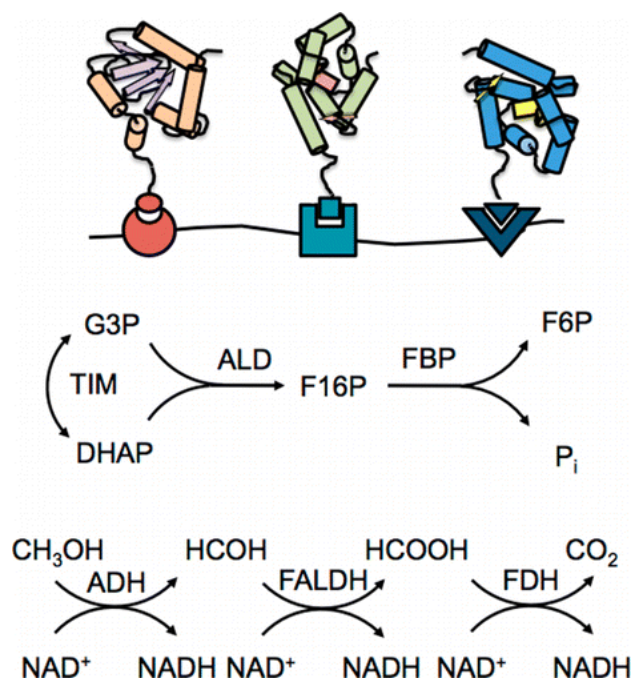


Figure 2.7. Protein scaffolds and examples of engineered multienzyme cascades. (top) Cartoon representation of a three enzyme cascade assembled by protein-protein interactions on a protein scaffold. (middle) Reaction scheme for the 3-step conversion of glucose-3-phosphate to fructose-6-phosphate. (bottom) Reaction scheme for the 3-step conversion of methanol to carbon dioxide.

pathway (triosephosphate isomerase, aldolase, and fructose 1,6-biphosphatase) were assembled with a dockerin/cohesin-based protein scaffold. The assembled multi-enzyme cascade produced a >20-fold increase in initial rate, in comparison with freely diffusing enzymes [41]. A similar scaffold was used to assemble a cascade of dehydrogenases to oxidize methanol to CO₂ [42]. Catalytic enhancement of the assembled cascade was observed by a 5-fold increase in the rate of enzyme cofactor production in comparison with an unassembled control. Finally, a trimeric ring-shaped protein made from proliferating cell nuclear antigens (PCNAs) was used as a scaffold to assemble a redox reaction cascade of cytochrome P450 with P450 electron transfer proteins ferredoxin and ferredoxin reductase. The assembled cascade exhibited a 50-fold increase in initial

activity over the unassembled control [43]. In each of these protein scaffolding examples, the overall structure of the scaffolded cascade is not well-known. Flexible protein scaffolds allow enzymes to aggregate and form a cluster of enzymes in close proximity. Interenzyme distance and orientation are likely variable, thus preventing clear identification of a mechanism of potential substrate channeling.

2.5 PRELIMINARY DESIGN FOR ENGINEERING ENHANCED CATALYSIS IN NANOSTRUCTURED MULTIENZYME CASCADES

Newly developed nucleic acid, protein, and polymer scaffolding technologies have enabled the design and engineering of multienzyme cascade reactions with control over the positioning of cascade enzymes and the overall architecture of the multienzyme structure. From the kinetic analysis of these multienzyme structures and of natural bifunctional enzymes that exhibit substrate channeling, we aim to extract a preliminary set of rules for designing new multienzyme cascades. This set of design rules addresses interenzyme distance, active site orientation, and overall architecture of coupled-enzyme structures for promoting substrate channeling. We present these guidelines and design rules as a preliminary set and do not expect them to account for differences in specific reaction mechanisms. We recognize that there remains significant technical challenges to the precise positioning of multiple enzymes at the $<1 - 10$ nm scale and in the control of active site orientation. As such, we expect that as the set of available tools used to create spatially organized multienzyme structures improves, new experiments will reveal refinements to the design rules as well as reveal new guidelines not proposed here.

Interenzyme Distance

Both experimental observations and molecular simulations of coupled-enzyme reactions show us that the distance between enzymes has a significant effect on the proportion of intermediate that takes a direct path from upstream to downstream active sites. Studies of the TS- DHFR and mMD-CS bifunctional enzymes show that channeling can occur with active site distances of up to 6 nm when aided by electrostatic interactions that promote a diffusional path [14,34]. In the absence of electrostatic interactions in the intervening space between enzymes, Brownian dynamics simulations of two fixed-position enzymes suggest the probability that the products of the first reaction channels to the second active site is >90% at 0.5 nm, decreasing to <10% at a distance of 4 nm, given optimal orientations of the active sites [44]. Many experimental examples that bring enzymes in close proximity (i.e., that are assembled in a structure where upstream and downstream enzymes are essentially in contact) are consistent with these simulation results. For example, when the GOx—HRP cascade is assembled with 10 nm between flexible tethers on a DNA origami tile, a 15-fold increase in the initial rate is observed. The flexibility of the tethers allows for GOx and HRP to come into contact [13]. Similarly, flexible protein scaffolds allow cascade enzymes to cluster, resulting in enhanced initial rates [41-43].

When taken together, the experimental and simulation data describing substrate channeling as a function of the distance between active sites suggests that at fixed distances between active sites greater 1—2 nm, substrate channeling occurs to a significant extent only if diffusion between active sites is promoted by interactions

between the cascade intermediate and the surface or scaffold between the enzymes. This distance can be extended with bounded diffusion between active sites, as is demonstrated in the case of the natural bifunctional enzyme TS—DHFR (~4 nm between active sites) and the fusion of mMD and CS (~6 nm between active sites).

Enzyme Orientation

Intimately coupled to the effects of interenzyme distance on substrate channeling is the orientation of active sites within a cascade. Natural examples of bifunctional enzymes have inherent control over the positioning of active sites, an ability that still remains as a significant technological challenge in engineering multienzyme structures. Recent works have demonstrated the importance of active site orientation in single-enzyme systems immobilized on a surface [45] and in electron transfer reactions with electrode-immobilized redox enzymes [46]. Specific to cascade reactions, Brownian dynamics simulations have been used to describe a relationship between the orientations of the active sites and the probability of the downstream reaction [4]. At a fixed interenzyme distance of 0.5 nm, inward-facing active sites can limit substrate access to the first enzyme in the cascade, thus reducing the overall throughput of the cascade. At 1 nm spacing, direct alignment of the active sites is optimal and leads to the highest probability that the second reaction in the cascade occurs. Experimental descriptions of the effects of the active site and enzyme orientation on cascade reactions has yet to be developed and represents an important area of future research.

Multi-Enzyme Architectures

Newly developed enzyme colocalization techniques have only just begun to enable the analysis of spatially organized coupled reactions. As such, there is not an extensive data set from which we can distill detailed, generalizable design rules for assembling multienzyme structures with optimized channeling. However, from established analysis and from simulations and modeling of an engineered GOx—HRP system, we can draw two preliminary guidelines. First, eq 1 provides the simple principle that the rate of the downstream enzyme must be greater than the velocity of the first reaction. Under these conditions, the concentration of the intermediate will attain a steady state value. If the production of the intermediate is greater than the maximum value of its consumption (i.e., $V_{\max,2}$), then a steady state will not be reached and, regardless of channeling, the concentration of the bulk intermediate will increase. With respect to the design of multienzyme nanostructures, this suggests that the ratio of the upstream enzyme to the downstream enzyme within the cascade should be balanced so that $V_{\max,2}$ is greater than the velocity of the upstream enzyme. Second, modeling and simulation work that describes the kinetic enhancements in an experimental system that couples GOx and HRP in a hexagonal DNA structure shows that the overall structure of the multienzyme systems has a significant effect on system catalysis [37]. Such modeling and simulation efforts are important in understanding these systems and help in our understanding of the kinetics of the systems and the relationships between the system architecture and catalysis.

2.6 CONCLUSIONS

In this Perspective, we aim to connect new progress in engineering nanoscale multienzyme structures to the methods and analysis techniques that the biochemistry community has established over the past 20 or more years to evaluate the kinetics of coupled-enzyme reactions and substrate channeling. Recent progress in the development of DNA nanotechnologies that create programmable, well-defined structures through Watson—Crick base pairing have enabled the creation of multienzyme nanostructures with a high degree of spatial organization. The enzymes of a coupled reaction can be tethered at known distances down to ~ 10 nm [13], organized into aggregate structures with high enzyme density [12], assembled on the inside of nanoscale DNA tubes [40], and dynamically pushed together and pulled apart [39]. Engineerable protein scaffolds that can assemble multienzyme structures with high densities of enzymes and with tunable enzyme ratios compliment these new DNA nanotechnology tools [42,47].

Detailed analysis of the kinetics of coupled-enzyme reactions assembled with these DNA and protein technologies is important and promises to reveal how far we have come in mimicking natural bifunctional enzymes and metabolic cascades that rely on substrate channeling to drive pathway flux and protect cascade intermediates from reaction in alternate pathways. Transient time (τ) analysis along with detailed knowledge of the enzyme kinetic parameters can be used to quantitatively evaluate the extent of substrate channeling in a given system. Challenge from a competing reaction in the bulk solution that uses cascade intermediates as substrates can also be used to evaluate channeling and the extent of protection that a multienzyme structure provides to its cascade

intermediates. Finally, under pre-steady-state conditions, increases in the overall reaction rate can reveal enhanced catalysis and possibly substrate channeling.

A second goal of this Perspective is to develop a preliminary set of design rules or guidelines for promoting substrate channeling in engineered multienzyme systems. To do so, we evaluate published data on natural bifunctional enzyme systems with strong experimental and modeling evidence supporting the existence of substrate channeling as well as data from new engineered multienzyme systems that demonstrate enhanced catalysis. The preliminary design rules are (1) in the absence of bounded diffusion, interenzyme distances in which substrate channeling can occur to a significant extent is limited to approximately 1 nm; (2) significant substrate channeling can be achieved at interenzyme distances of 5—6 nm if diffusion between active sites is promoted through interactions between the surface between the active sites and the cascade intermediate; (3) at close proximity, active site orientation can block substrate access to the coupled reaction; (4) when substrates can access active sites from the bulk solution, active site orientation should minimize interenzyme distance; and (5) to achieve a steady state concentration of cascade intermediate, the ratio of enzymes should be balanced so that the velocity of the first reaction is less than the maximum velocity of the second reaction. These general design rules are preliminary, and we expect that as the capabilities of the engineering tools for multienzyme nanostructures improve, these guidelines will be refined and new rules will be discovered.

This set of guidelines and the data sets from which they were drawn also highlight the technological challenges that remain in engineering generalizable tools for the assembly

of multi- enzyme nanostructures with optimized catalysis. New protein and biomolecular engineering tools are needed to create multienzyme systems with precise interenzyme distances in the $<1-10$ nm range. Similar tools are needed to accurately control the orientations of enzyme active sites. Importantly, many more examples of nanostructured coupled-enzyme reactions are needed so that we can evaluate the effects of new multienzyme architectures as well as the behavior of different enzyme reaction mechanisms in such systems.

Engineering new multienzyme nanostructures with optimized kinetics is a complex and multidisciplinary problem that includes aspects of biophysics and biochemistry, as well as protein, biomolecular, and nanoscale engineering. Recent progress in technology development is encouraging and promises to produce many more interesting discoveries.

2.7 ACKNOWLEDGEMENTS

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Chapter 3. Kinetic Enhancements in DNA-Enzyme Nanostructures

Mimic the Sabatier Principle

3.1 ABSTRACT

Advances in DNA bionanotechnology have lead to the ability to create structures with well-defined chemical and physical features at the nanoscale. Such nanostructures can be used to create spatially organized enzymatic cascades that promote substrate channeling and result in enhanced cascade kinetics. Here, we investigate the potential effects of substrate-scaffold interactions on the catalytic activity of an enzyme-DNA complex using a model enzyme, horseradish peroxidase (HRP), and a nanoscale DNA scaffold with three addressable sites. Kinetic assays with a library of HRP substrates (including charged and neutral phenolics, the colorimetric substrate ABTS, and *o*-phenylenediamine) revealed that DNA scaffolding enhances HRP activity relative to freely diffusing enzymes under similar conditions. The observed trend in kinetic enhancement mimics the Sabatier Principle in heterogeneous catalysis, which states that significant reaction rates are only achieved with intermediate binding energy (i.e., neither too weak or too strong) between substrate and catalyst. Here, the binding of the substrate is to the scaffold and not the catalyst, but the Sabatier trend holds: weak and strong binding substrates showed no enhancement in kinetics, while intermediately bound substrates resulted in greater than 300% increase in enzyme activity. We anticipate that these finding will have broad implications on the design and application of DNA

nanostructures for assembling multi-enzyme pathways and on DNA nanostructures in general.

3.2 INTRODUCTION

Metabolic pathways are often organized in multi-enzyme complexes that promote the efficient transport and processing of substrates and intermediates along the pathway, resulting in enhanced pathways kinetics and high yields [1,2]. When engineering new and re-engineering existing pathways such nanoscale organization and the associated kinetic benefits are often lost. A number of recent efforts to reproduce the kinetic benefits from controlled co-localization in engineered pathways, both *in vitro* and *in vivo*, have focused on nucleic acid [3-7] and protein scaffolding [8-10] of complexes with two or more enzymes of a reaction cascade. However, interactions between enzyme substrates and scaffolds, and their potential effects — beneficial or detrimental — on enzyme kinetics have yet to be explored. As a material, DNA can be used to create precisely defined multi-dimensional shapes with molecular-level control over structural and chemical features [11]. These capabilities are highly useful in designing multi-enzyme cascades or polyvalent enzyme display with well-defined spatial organizations. Here, we investigate a potentially advantageous (or constraining) aspect of these designs, interactions between substrates and DNA scaffolds.

Based on well-known interactions between small molecules and DNA (e.g., DNA stains used in electrophoresis and anti-cancer drugs [12,13]) and DNA templating of aniline monomers for the enzyme-mediated synthesis of polyaniline nanowires [14], we

reasoned that interactions between DNA scaffolds and enzyme substrates may effect local substrate concentrations and alter the kinetics of enzymes assembled in enzyme-DNA nanostructures. To investigate this possibility we used a model system of horseradish peroxidase (HRP) assembled on a triangular DNA scaffold. We elected to use this system as HRP oxidizes a wide range of chemically distinct substrates including phenol, charged and uncharged phenolics, as well as colorimetric substrates such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) that are commonly used in analytical assays. This range of substrates differs in their interactions with DNA, binding with a range of affinity and location thus allowing for experimental investigations of enzyme kinetics with varying substrate-scaffold interactions.

3.3 MATERIAL AND METHODS

Chemicals

Horseradish peroxidase (HRP), phenol (P), *p*-aminophenol (AP), *p*-hydroxybenzoic acid (HBA), *o*-phenylenediamine (OPD), 4-aminoantipyrene (AAP), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (St. Louis, MO). Sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC) was obtained from Pierce (Rockford, IL). Oligonucleotides were purchased from IDT (Coralville, Iowa).

DNA triangle assembly

DNA triangles were assembled by mixing equal molar amount of each strand (50 nM) in TBS buffer (20 mM Tris, 150 mM NaCl, pH 7.4). The mixtures were heated to 90 °C

and subsequently cooled to 4 °C over a 4 hour period. Temperature cycling was performed using a C1000 Touch Thermo Cycler (BioRad).

Synthesis of Enzyme-DNA conjugates

HRP was modified with a 10-base single stranded DNA oligomer by covalent attachment to free lysine side chains. Briefly, 10 mg of HRP (Sigma) was purified by gel filtration chromatography with Superdex 200 10/300 column (GE). To activate DNA, 20 μ L (1 mM) 5'-thiolated oligonucleotides **A1** and **A2** were mixed with 120 μ L (1 M) DTT and incubated for 3 h to reduce any disulfide bond formed upon storage. Purified HRP (90 μ M) was reacted with Sulfo-SMCC (18 mM) in a final volume of 260 μ L for 1 h. Both activated DNA and HRP were purified by gel filtration chromatography. The purified DNA and HRP were combined and incubated for 3 h. All reactions were performed in PBS buffer (pH 7.4) at room temperature. The reaction mixture was concentrated by ultrafiltration (Centricon 10, Millipore) and the buffer was exchanged with Tris buffer (20 mM, pH 7.4). The HRP-**A1** conjugate was purified by anion-exchange chromatography on UNO Q1 column (Bio-Rad) by using a linear gradient from 15 to 25% NaCl (3 M) with Tris buffer (pH 7.4). The conjugate concentration was quantified spectrophotometrically by using $\epsilon_{405} = 102000 \text{ M}^{-1} \text{ cm}^{-1}$).

HRP-DNA assembly and Electrophoretic mobility shift assays

To make HRP-DNA nanostructures, 3 nmol HRP-**A1** conjugate was incubated with 3, 2, and 1 nmol DNA triangle (1-, 2-, and 3- sticky end, respectively). The assembly was performed in TBS buffer at room temperature. The samples were loaded in a precasted 4-15% Mini-PROTEAM TGXTM Gel (Bio-Rad) and 100 bp molecular ladder was used as

the marker (Bio-Rad). Electrophoresis was performed at 4 °C under constant 120 V for 140 min. The bands were visualized by using Sybr Gold Nucleic Acid Gel Stain (Invitrogen).

HRP-DNA assembly yield and kinetic enhancement correction

The assembly yield for HRP-DNA nanostructures were analyzed by quantifying shifted bands with UN-SCAN-IT software. Equation 1 was used to account for the activity of unassembled enzyme-DNA and correct raw activity measurements for assembled structures. Here A_{assem} is the mixture for 1-, 2-, and 3-HRP attached in HRP₃-DNA nanostructure reaction. Y_{assem} is the assembly yield for the mixture.

$$A_{\text{raw}} = A_{\text{assem}} Y_{\text{assem}} + A_{\text{unassem}} (1 - Y_{\text{assem}}) \quad (1)$$

Enzyme kinetic assays. HRP-DNA nanostructures were assembled in 1.5 mL microcentrifuge tubes for 30 min before performing kinetic measurements. Assembly was accomplished as described above. For phenolic substrates (P, AP, HBA) reactions, 0.6 nM HRP-DNA nanostructures, 5 mM P and AP and 30 mM HBA, 0.5 mM AAP, and 1 mM H₂O₂ were used, and reaction rates were monitored by measuring increases in absorbance at 510 nm. For OPD and ABTS reactions, 0.6 nM HRP/DNA nanostructures, 0.5 mM substrate, and 0.05 mM H₂O₂ were used, and reaction rates were monitored by measuring increases in absorbance at 418 and 405 nm for OPD and ABTS respectively. For TMB reaction, 0.6 nM HRP/DNA nanostructures, 0.2 mM substrate, and 0.05 mM H₂O₂ were used, and the reaction rate was monitored by measuring increases in absorbance at 450 nm. Reactions for P, HBA, ABTS, and TMB were performed in sodium phosphate buffer (50 mM, pH 7.4). For AP and OPD, reactions were performed

in phosphate/citrate buffer with pH 5.0 and 4.2, respectively. The volume of reaction mixtures was 500 μ l, and reactions were initiated by adding hydrogen peroxide. The absorbance of products was monitored by Nanodrop 2000. A minimum of three replicates of each assay was performed.

Substrate-DNA Docking Simulations

Double stranded DNA macromolecules of oligonucleotides (CCTGC, CCTGCCTGCC, CCTGCCTGCCCCCTGC) were prepared by 3D-DART [1]. Polar hydrogen atoms were added and Kollman charges were assigned to the DNA molecules by AutoDock 4 [2]. All small molecules were drawn by using Symyx Draw 4.0, and hydrogen atoms and Gasteiger charges were assigned for DNA docking by VEGA ZZ. All the torsional bonds of small molecules were set to rotate and the grid dimensions were 60 x 60 x 100 with points separated of 0.375 Å. The Lamarckian genetic algorithm (LGA) was used as search method. Each LGA job consisted of 30 runs, using initial population of 150 individuals, with 2.5×10^6 energy evaluations, a maximum number of 2.7×10^4 iterations, a mutation rate of 0.02, a crossover rate of 0.80. After docking, all docking poses were clustered into groups and the pose with the lowest binding energy for each small molecule was chosen and analyzed for hydrogen bonding. Docking results against a 10 base pair double stranded DNA oligomer are reported; however, no significant differences in the docking results were found when docking against the 5 and 15 base pair sequences listed above.

3.4 RESULTS AND DISCUSSION

One, two, and three HRPs were assembled on a triangular DNA scaffold (Figure 3.1a). The scaffold design was based on a previously demonstrated DNA nanostructure [15] and was modified in this work to reduce each side of the structure to ~25 nm in length. This scaffold was selected as its tensegrity design maintains a rigid structure, the structure has high assembly efficiency, and it has the potential to be used as a scaffold for the controlled assembly of reaction cascades with two and three enzymes. HRP was first conjugated with the thiol-terminated 10-base single stranded DNA oligomer, A1, with the bi-functional crosslinker Sulfo-succinimidyl-4-(*N*-maleimido-methyl)-cyclohexane-1-carboxylate (Sulfo-SMCC). Purified HRP-A1 conjugates were mixed with triangular DNA scaffolds with one, two, or three complementary sticky-ends (un-hybridized 10-base segments) to produce enzyme-DNA nanostructures with one, two, and three HRPs on the points of the triangular scaffold (HRP₁-, HRP₂-, and HRP₃-DNA, respectively). For detailed preparation of DNA scaffold and enzyme-DNA assemblies, see Materials and Methods, SFigures 3.1 and 3.2 and STable 3.1 in the Supporting Information. Figure 3.1b shows the successful assembly of HRP₁-, HRP₂- and HRP₃-DNA by electrophoretic mobility shift assay. Lanes 5, 7, and 9 (numbered from left to right) show distinct bands of increasing molecular weight that correspond to HRP₁-, HRP₂-, and HRP₃-DNA, respectively. No shift in DNA bands were observed in the absence of HRP-A1 or when the scaffold did not display sticky-ends (Figure 3.1b, Lane 3). The shift assay reveals that assembly of each structure results in a mixed population of scaffolds with no attached enzymes and scaffolds with one, two, and three HRPs attached when applicable.

Assembly yield HRP₁-DNA, determined by image analysis of the shift assays, was found to be 0.57 (lane 5), while HRP₂- and HRP₃-DNA were assembled with yields of 0.34 and 0.30 (lanes 7 and 9), respectively.

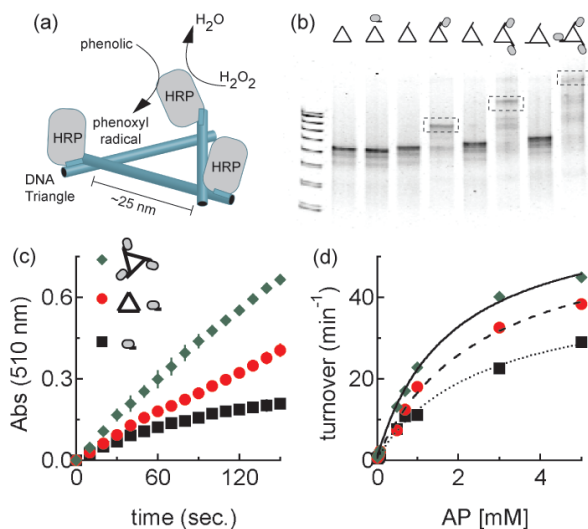


Figure 3.1. Kinetic enhancement of HRP-DNA nanostructures. (a) Illustration of HRP-DNA nanostructures, indicating that structures with 1, 2, and 3 HRP may be present. (b) Electrophoretic mobility shift assay of HRP_n-DNA nanostructures with 4-15% polyacrylamide native gel (lane 1. DNA ladder (increments of 100 bp from 200 to 1000), 2. DNA triangle (DNA), 3. DNA with unassembled HRP-A1, 4. DNA with 1-sticky end, 5. HRP₁-DNA, 6. 2-sticky end DNA, 7. HRP₂-DNA, 8. 3-sticky end DNA, 9. HRP₃-DNA. (c, d) Product formation as a function of time and turnover number as a function of substrate concentration for HRP_{1,2,3}-DNA nanostructures and freely diffusing and unassembled enzyme controls.

To explore whether HRP kinetics are affected by substrate—scaffold interactions, we first used *p*-aminophenol (AP) as a substrate. AP has potential for electrostatic and hydrogen bonding interactions with the negatively charged DNA scaffolds and is readily oxidized by HRP at pH below the pK_a of the primary amine para to the phenolic hydroxy group (pK_a = 5.29) [16]. The activity of HRP—DNA nanostructures as well as unbound HRP—A1 in the presence of the scaffold and freely diffusing HRP—A1 controls were

determined by measuring the absorbance of the quinoneimine dye product of oxidized AP and 4-aminoantipyrine at 510 nm at pH 5 [17]. In kinetic assays of equal enzyme concentrations, HRP3—DNA showed a clear increase in initial activity over freely diffusing HRP—A1 and unassembled HRP—A1 and DNA scaffold (Figure 3.1c). The initial rate of AP oxidation with HRP3—DNA (uncorrected for yield) was found to be $1182 \pm 166 \text{ s}^{-1}$ (mean $\pm$ SD), whereas initial rates of HRP—A1 in the presence and absence of DNA scaffold were 775 ± 18 and $493 \pm 42 \text{ s}^{-1}$, respectively. The initial rates of HRP1— and HRP2—DNA were found to be equal to that of HRP3—DNA (Figure 3.1c).

Since similar specific activities were observed in all HRP—DNA nanostructures, HRP3—DNA was selected for all subsequent studies, including the determination of the apparent Michaelis—Menten constant (K_M) and turnover number (k_{cat}). The apparent K_M of HRP3—DNA was $1.8 \pm 0.1 \text{ mM}$, smaller than both the unbound and freely diffusing HRP—A1 with K_M of 2.6 ± 0.4 and $2.5 \pm 0.3 \text{ mM}$, respectively, suggesting that DNA scaffolds increased the affinity of AP to HRP. In addition, the k_{cat} of HRP3—DNA was significantly greater than the freely diffusing control (HRP3—DNA, $k_{cat} = 2100 \pm 60 \text{ s}^{-1}$; freely diffusing HRP—A1, $k_{cat} = 1420 \pm 90 \text{ s}^{-1}$). Although initial rates of the HRP3—DNA are greater than the unassembled control, fits to the rate data yield k_{cat} values that are statistically similar. Increased turnover of HRP with phenolic substrates at concentrations above and below their respective K_M values have also been observed in the presence of excess calf thymus DNA in solution [18]. Changes in K_M and k_{cat} for

DNA modified heme centers in HRP has also been reported [19]. The increase in turnover in the presence of DNA was also observed in our system (i.e., the unassembled control), possibly as a result of interactions between the positively charged HRP ($pI = 8.8$) and negatively charged DNA .

If the observed enhancement in specific activity was due solely to electrostatic interactions between the negative charged phosphate backbones of DNA and the positively charged amino group of AP, the enhancement should be suppressed in the presence of salt. Here, we define the percent enhancement of enzyme activity as the ratio of the specific activity of the HRP_n-DNA nanostructure over the specific activity of HRP-A1 with AP and H₂O₂ concentrations 2-3 times the expected K_M values (see ref [17] and STable 3.2). Figure 3.2 (left) shows the kinetic enhancement decreased to 153 ± 21 , 131 ± 11 , and $158 \pm 24\%$ in the presence of 50, 150, and 300 mM NaCl, respectively (Supporting Information SFigure 3.3), suggesting that electrostatics is in part responsible for the effect and that other interactions may also be present. In support of nonelectrostatic interactions, an increase in activity of $244 \pm 6\%$ was observed with phenol (P; Figure 3.2, right, Supporting Information SFigure 3.4). In addition, the phenolic substrate p-hydroxybenzoic acid (HBA), which has a negatively charged carboxylic acid group in place of the positively charged amino group on AP, showed a $197 \pm 6\%$ enhancement in activity. With two positively charged amino groups, the enhancement in activity ($130 \pm 16\%$) with the nonphenolic substrate, o-phenylenediamine (OPD), was much smaller than that of AP, also suggesting a partial role of electrostatics in the enhancement. The kinetic enhancement of TMB, which is also

positively charged, was found to be $138 \pm 12\%$. Finally, the common HRP substrate ABTS resulted in a slightly reduced activity ($88 \pm 7\%$), possibly due to its negative charge. A control sample with a 50 bp double-stranded DNA oligomer conjugated to HRP also yielded enhanced activity ($174 \pm 12\%$; Supporting Information SFigure 3.5), indicating that the scaffold structure was relevant to the enhanced activity.

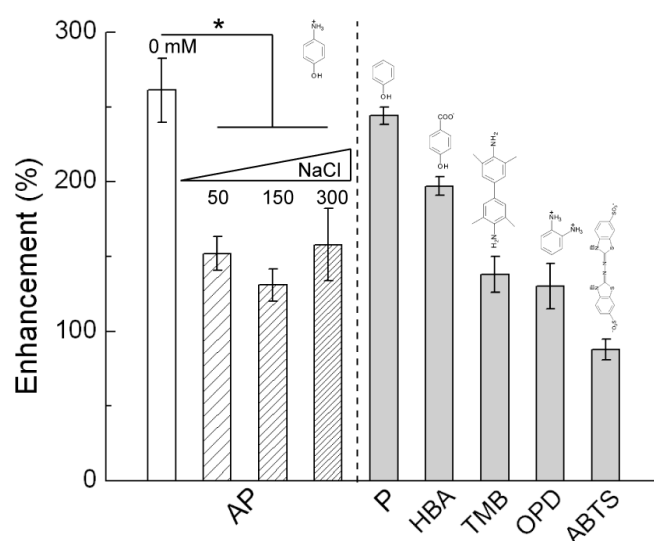


Figure 3.2. Kinetic enhancement of DNA scaffolded HRP is salt concentration (left) and substrate dependent (right). Percent enhancement is defined as the ratio of the specific activity of the HRP₃-DNA nanostructure over the specific activity of freely diffusion HRP modified with a 10-base oligomer. Error bars are standard deviation ($n \geq 3$, * $p \leq 0.05$).

Substrate binding to the DNA scaffold was analyzed by molecular docking simulations with each substrate and the 10-bp double stranded DNA fragment closest to the immobilized enzyme (i.e. DNA oligomer A1). Simulations with AutoDock software predicted stable docking poses and corresponding binding energy (comprised of van der Waals, hydrogen bonding, desolvation, electrostatic, and torsional energies) [20]. With the exception of OPD, all substrates were predicted to bind preferentially in the minor

groove of the DNA double helix (Figure 3.3a and SFigure 3.6). OPD bound to the phosphate backbone bridging the major and minor grooves and bound with higher affinity than all other substrates (OPD, $\Delta G = -7.42$ kcal/mol, $k_d = 4$ μ M). TMB also bound strongly to the double-stranded DNA segment ($\Delta G = -6.21$ kcal/mol, $k_d = 28$ μ M), whereas ABTS bound weakly in the minor groove ($\Delta G = -2.72$ kcal/mol, $k_d = 10$ mM). The phenolic substrates also bound in the minor groove with the rank order from strongest to weakest of HBA, AP, and P (HBA, AP, and P, $\Delta G = -5.43$, -4.96 , and -4.00 kcal/mol and $k_d = 104$, 231 , and 1160 mM, respectively). Interestingly, the substrates with the highest and lowest affinities showed little, if any, enhancement in activity.

A well-known concept in heterogeneous catalysis is the Sabatier Principle, which states that binding between substrate and catalyst should be “just right”, i.e. substrate binding to the catalyst should be neither too weak or too strong [21]. Weakly binding substrates fail to associate with the catalyst resulting in no reaction, while strongly bound substrates are slow to dissociate and prevent reaction by blocking active sites. This concept is graphically shown by plotting reaction rate as a function of heat of formation of the adsorbate. The result is a volcano plot, so called as the maximum of the inverted parabola (or triangle) fit to the data peaks at the optimum binding energy and maximum reaction rate [22].

This trend is mimicked here with the enhancement of enzyme activity resembling a volcano plot. Figure 3b shows the kinetic enhancement for each substrate (corrected for HRP₃-DNA assembly yield; see Materials and Methods in the SI) as a function of the substrate-DNA binding energy. Substrates that bind weakly and strongly to the scaffold

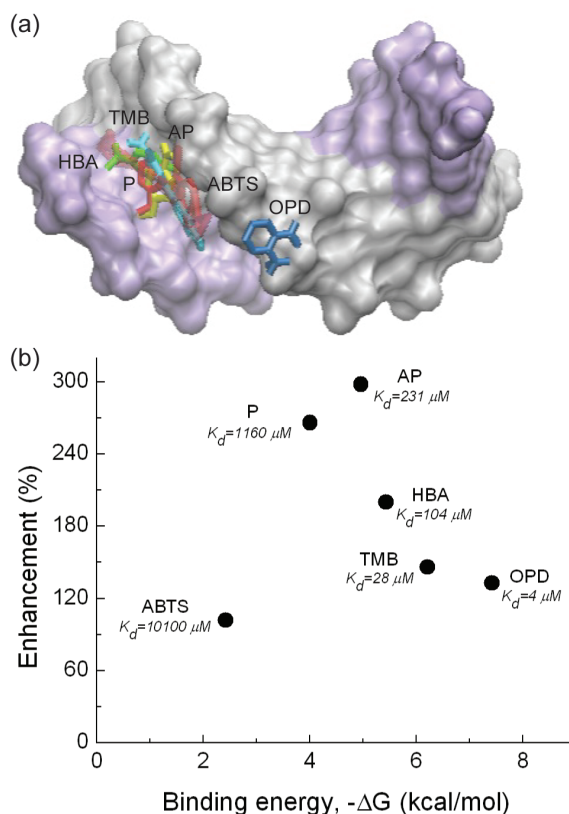


Figure 3.3. Kinetic enhancements in HRP-DNA nanostructures mimic the Sabatier principle. (a) Molecular model of putative binding sites for each substrate on DNA: phenol (P), red, *p*-aminophenol (AP), yellow, *p*-hydroxybenzoic acid (HBA), green, *o*-phenylenediamine (OPD), blue; 3,3',5,5'-tetramethylbenzidine (TMB), cyan; and ABTS, faint red. Molecular docking to the 10 base pair sequence closest to the immobilized enzymes was performed by AutoDock. (b) The enhancement of HRP activity (corrected for HRP₃-DNA assembly yield) as a function of the predicted substrate-DNA binding energy. Calculated binding constants for each substrate are shown as data labels.

showed little to no enhancement. The maximum enhancement resulted from an intermediate binding energy between scaffold and substrate. It is important to emphasize that the Sabatier Principle is mimicked and not reproduced. The effect is one of kinetic enhancement in enzyme-DNA nanostructures and not absolute reaction rate as is traditionally shown in volcano plots. We ascribe the observed effect to a potential increase in concentration of the substrate localized around the DNA scaffold in close

proximity to the attached enzyme, an effect that is supported by the decrease in apparent K_M of AP (Figure 3.1d)

Regardless of the mechanisms that bring about the trend in kinetic enhancement (Figure 3.3), the kinetic data of multiple substrates oxidized by HRP assembled on a DNA scaffold clearly shows that scaffold—substrate interactions can affect the overall performance and functioning of the system. In the case presented here, the interactions between enzyme substrate and scaffold are mostly beneficial: enzyme turnover was increased, and the apparent Michaelis binding constant (K_M) was reduced. This observation has potential implications in designing multienzyme reaction cascades with nucleic acid scaffolds [3-7,11], and will also affect the design of addressable DNA scaffolds for protein nanoarrays [23,24], DNA origami-based drug delivery systems [25], and the design and application of DNA-based materials in general. More specifically, our observations that the apparent turnover and binding constant may be altered when enzymes (or binding proteins) are assembled on nonchemically inert scaffolds suggest that (i) the kinetics and ligand binding should be characterized after assembly of the system, (ii) the changes may be substrate- and scaffold-dependent, and (iii) it may be possible to exploit the binding properties of the scaffold to increase local concentration of substrates and, in the case of couple reactions, increase the local concentration of reaction intermediates.

In this work, we have designed and created a nanoscale triangular DNA scaffold to polyvalently display up to three HRP enzymes. We used this system to investigate potential effects on enzyme kinetics due to substrate—scaffold interactions. Phenolic

compounds (P, AP, and HBA) were oxidized at enhanced rates over unassembled HRP modified with a 10-bp, single-stranded DNA oligomer. Other substrates, including ABTS and OPD, and TMB showed little to no enhancement in rate with DNA-scaffolded HRP. Plots of the kinetic enhancement as a function of the predicted binding energy of the substrate to the 10-bp closest to the immobilized enzyme revealed a trend that mimics the Sabatier Principle in heterogeneous catalysis. These observations have implications on the design and application of multienzyme nanostructures assembled with DNA scaffolds and with DNA bionano materials in general.

3.5 ACKNOWLEDGEMENTS

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3.7 SUPPLEMENTARY INFORMATION

STable 1. DNA strands used in this study.

Sticky end	Strand	Sequence
0	P1	CAGAGCTTAGATACCTGTAATAATGTTGACTTACCGTACTAAGCAGGAC AATGAGTAGCCGT
	P2	CCGATAACTAGCGCCTGTGTACCTCACTGACAGAGACGCAGTATGGAC GTAGATACTGTGC
	P3	ACGGCTACTCATTGTGGCACGTTTTTCGTGCCTGTCGAGTAGATCGTATGA TGGCATCGCTCGGACGACGTTTTTCGTGCGTGGCGCTAGTTATCGG
	P4	AGTCAACATTATTACACCATACTGCGTCTCTGTCAGTGAGGTGACACACC GAGCGATGCCATCATAACGATCTACTCGACACCTGCTTAGTACGGTA
	P5	GCACAGTATCTACGTGGTATCTTAGCTCTG
1	P5	GGCAGGCAGGG CACAGTATCTACGTGGTATCTTAGCTCTG
2	P1	CAGAGCTTAGATACCTGTAATAATGTTGACTTACCGTACTAAGCAGGAC AATGAGTAGCCGT GGCAGGCAGG
3	P2	GGCAGGCAGGCC GATAACTAGCGCCTGTGTACCTCACTGACAGAGAC GCAGTATGGACGTAGATACTGTGC
	A1	ThioMC6/CCTGCCTGCC
	A2	ThioMC6/CCTCCCTCCCTAGATTAGATTGTGTTGTGTTAGATTAGATCCTGCCTGCC
	cA2	GGCAGGCAGGATCTAATCTAACACAACACAATCTAATCTAGGGAGGGAGG

STable 2. Reference kinietic parameters for HRP substrates.

Substrate	K_M (mM)	k_{cat} (1/s)
AP [3]	2.36	1272
P [3]	3.59	691.7
HBA [3]	18.76	311.7
OPD [4]	0.38	528
ABTS [5]	0.18	736
TMB [4]	0.089	172

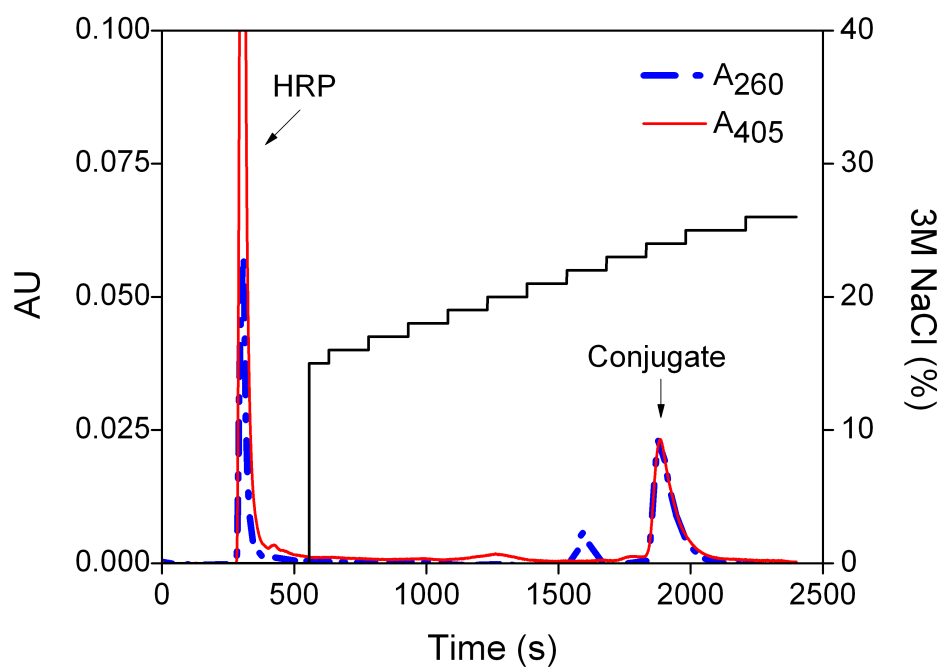


Figure 3.1. Anion exchange purification of HRP-DNA conjugates with sSMCC crosslinker.

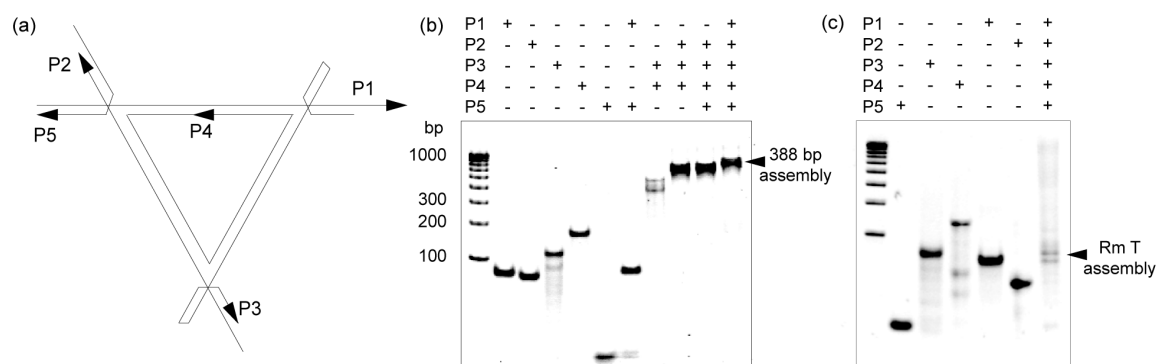


Figure 3.2. Assembled and unassembled triangular DNA scaffolds. (a) The design of DNA triangle (P1-5 are oligonucleotides, sequences given in STable 1). Native gel analysis of DNA triangle assembly (b) and the unassembled structure (c).

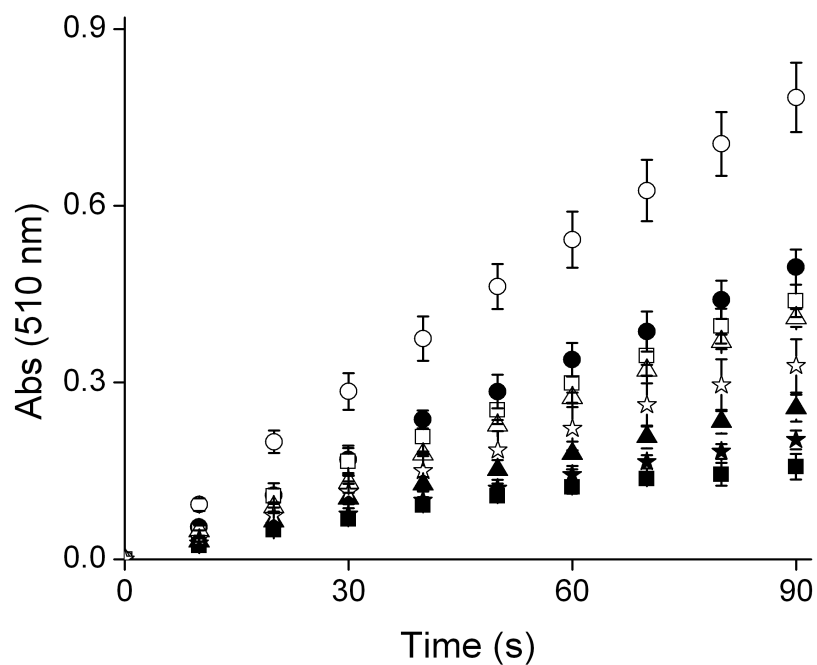
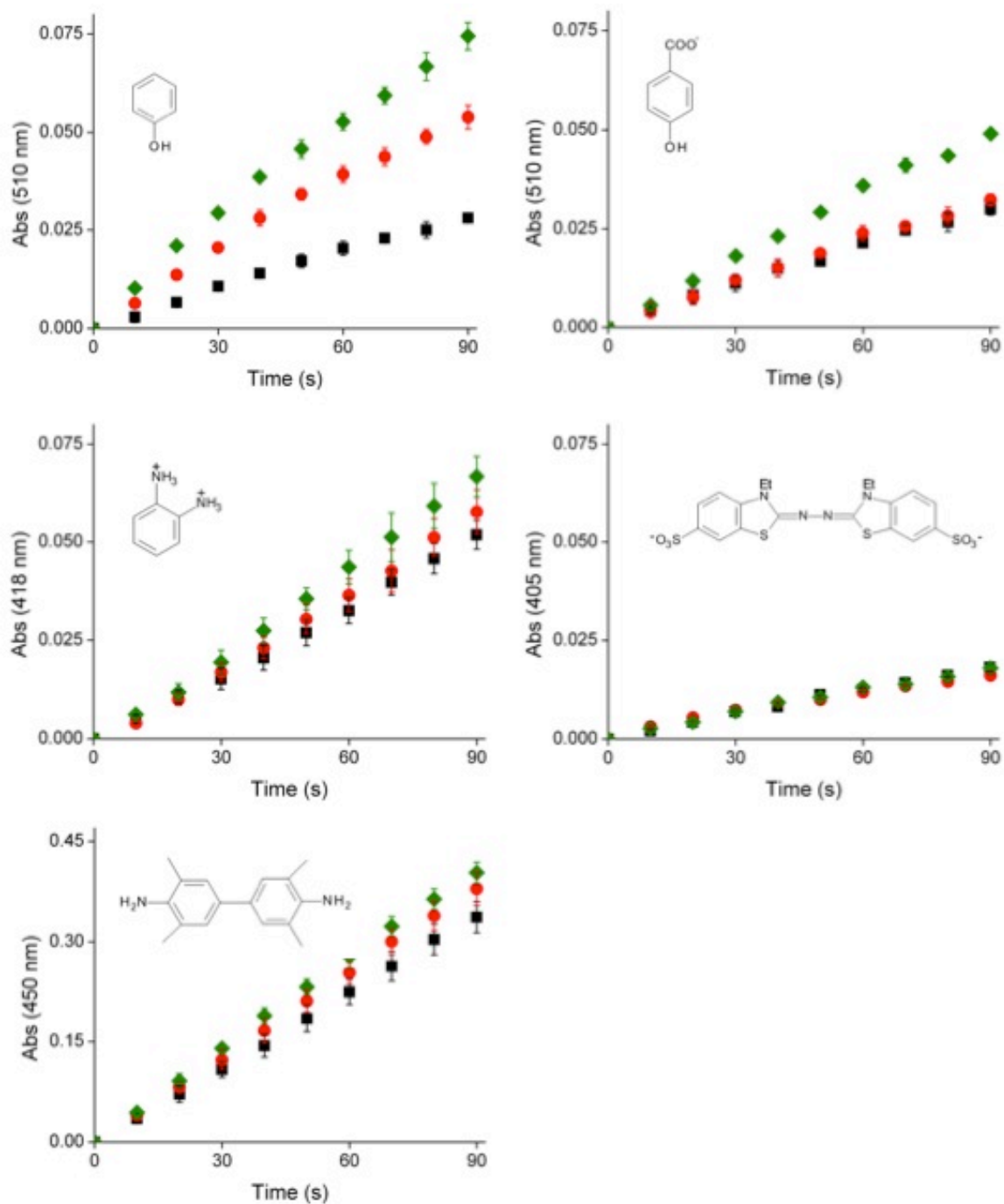
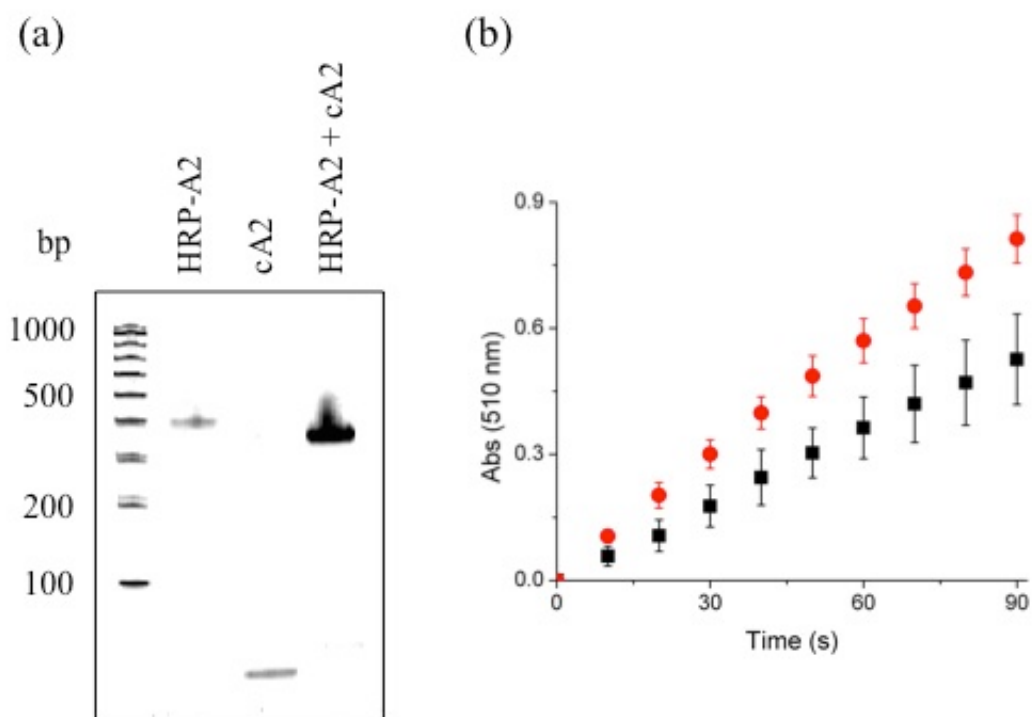


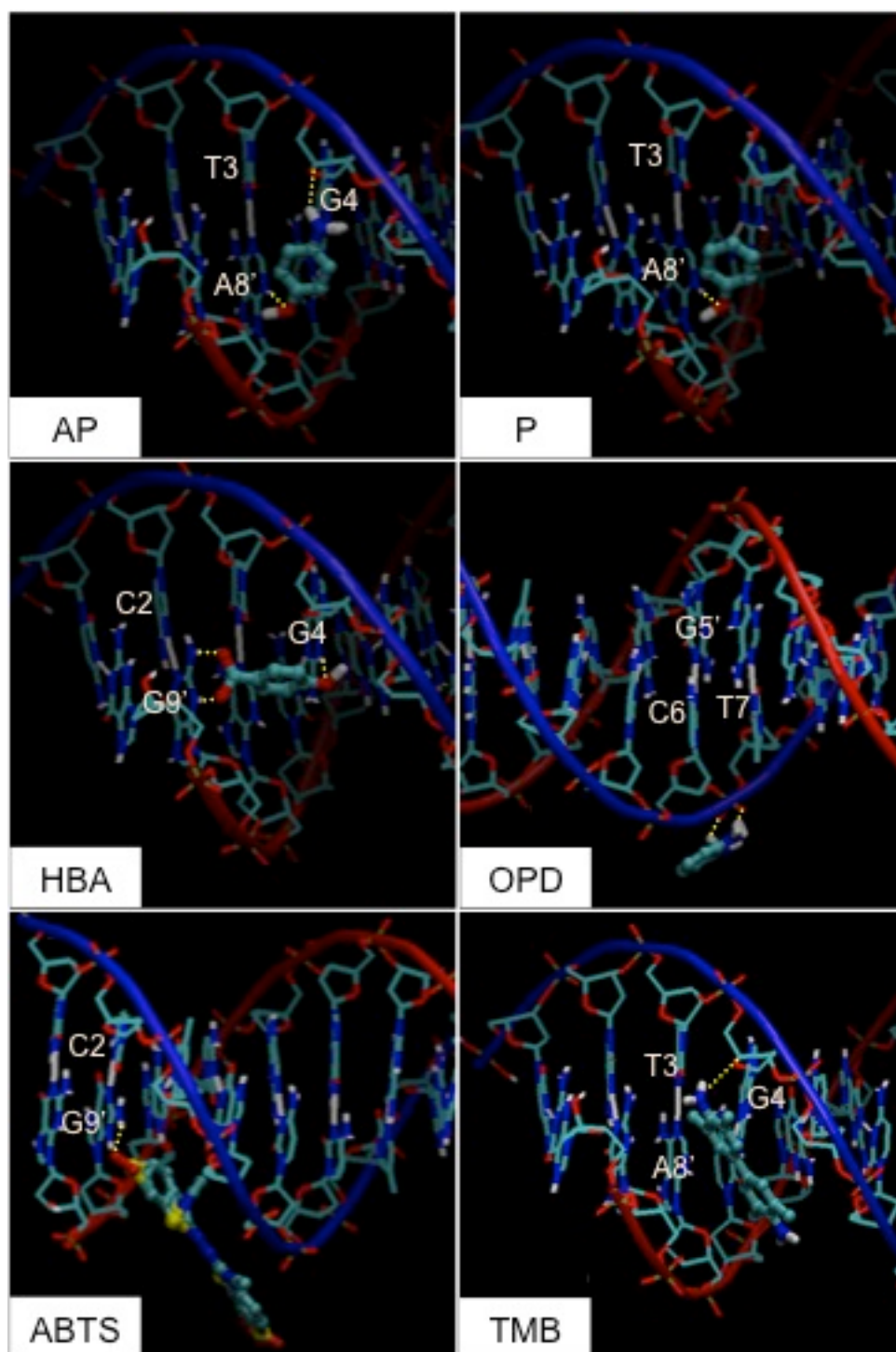
Figure 3.3. Salt-dependent enhancement of HRP₃-DNA nanostructures: 0 mM for HRP₃-DNA (empty square) and HRP-A1 (solid square); 50 mM for HRP₃-DNA (empty circle) and HRP-A1 (solid circle); 150 mM for HRP₃-DNA (empty triangle) and HRP-A1 (solid triangle); and 300 mM for HRP₃-DNA (empty star) and HRP-A1 (solid star).



SFigure 3.4. Kinetics of HRP/DNA nanostructures with different substrates. (Upper left) Phenol. (Upper right) *p*-hydroxybenzoic acid. (Middle left) *o*-phenylenediamine. (Middle right) 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid). (Lower left) 3,3',5,5'-tetramethylbenzidine. HRP₃-DNA (Green diamond); HRP-A1 with free diffusing scaffold (red circle); and HRP-A1 (black square).



SFigure 3.5. (a) Electrophoretic mobility shift assay for the assembly of HRP-A2 and cA2 (complementary strand). (b) Kinetic enhancement of 50 base-pair conjugated HRP. HRP-A2/cA2 (red circle); and HRP-A2 (black square).



SFigure 3.6. Docking models for potential hydrogen bonding between substrates and double-stranded DNA (CCTGCCTGCC). Only best binding poses with the lowest binding energy were shown for the analysis.

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Chapter 4. Rapid Ester Biosynthesis Screening Reveals a High Activity Alcohol-O-acetyltransferase from Tomota Fruit

4.1 ABSTRACT

Long-, medium-, and short-chain ethyl and acetate esters are naturally produced in various yeast, plant, and bacteria species. The biosynthetic pathways that produce these esters share a common terminal reaction step, the condensation of acetyl/acyl-CoA with an alcohol by alcohol-O-acetyl/acyltransferase (AATase). Recent metabolic engineering efforts have exploited AATase activity to produce fatty acid ethyl esters as potential diesel fuel replacements as well as short- and medium-chain volatile esters as fragrance and flavor compounds. These efforts have been limited due to the lack of a rapid screening method to effectively quantify ester biosynthesis. Enzyme engineering efforts have also been limited by the lack of a high throughput screen for AATase activity. As a result, only a small set of well-characterized AATases have been used for ester biosynthesis. In this work, we developed a robust high throughput assay for AATase activity and used this assay to discover a high activity AATase from tomato fruit, *Solanum lycopersicum* (Atf-S.l). This new AATase exhibited broad specificity towards acyl-CoAs with acyl chain length from C4 to C10 and was found to be specific towards 1-pentanol. Atf-S.l was one of six AATases screened, including Atf1, Atf2, Eht1, and Eeb1 from *Saccharomyces cerevisiae*, and Atf-C.m from melon fruit, *Cucumis melo*. In addition to the identification of Atf-S.l, the AATase screen revealed new acyl-CoA substrate specificities of each enzyme, thus increasing the pool of characterized AATases

that can be used in the biosynthesis of ester-based fragrance and flavor compounds as well as fatty acid ethyl ester biofuels.

4.2 INTRODUCTION

The microbial production of ester fragrances, flavor compounds, and biofuels has been a recent focus of metabolic engineering. Long chain waxy esters have potential for use as diesel fuel substitutes and have been produced in engineered *E. coli* and yeast [1-3]. Volatile short and medium chain esters have value as natural food additives (e.g., isoamyl acetate and phenyl ethyl acetate) and industrial solvents (e.g., ethyl acetate), and have been produced in native yeast strains [4,5] and engineered *E. coli* [6-9]. The biosynthetic pathways that produce these compounds share a common terminal reaction step, the condensation of an alcohol with acetyl- or acyl-CoA by alcohol-O-acetyl/acyltransferase (AATase, E.C. 2.3.1.84 and 2.3.1.75, respectively). A number of AATases that produce short and medium chain esters have been identified in various yeast and fruit species and are active during fermentation and fruit ripening [10-16]. Orthologs to the short and medium chain AATases have been identified in *Acinetobacter* and *Marinobacter* species [17,18]. These AATases, also called wax ester synthases, produce waxy esters from long chain acyl-CoAs and fatty alcohols.

While there have been successes in metabolic engineering of ester biosynthesis via AATase activity, enzyme engineering and enzyme screening have been limited. Rational design to alter substrate specificity and improve turnover is challenging because there are no known crystal structures and a lack of high similarity templates results in low

confidence homology models [19,20]. Directed evolution and enzyme screening have been limited by the lack of a suitable high throughput assay. Part of the challenge is that many AATases are membrane associated, localizing to the endoplasmic reticulum (ER) and lipid droplets [21-23]. Loss of localization during heterologous expression in *E. coli* results in aggregation and low enzyme activity [24]. Purification is also challenging requiring non-ionic detergents or protein solubility tags to maintain activity [19,25,26]. As a result the standard activity assay for volatile esters is headspace gas chromatography (GC) analysis of whole cell lysate reactions [24], while long chain acyl esters are quantified by thin layer chromatography and by GC-MS [27]. Such assays are not ideal for the rapid and high throughput screening necessary for effective enzyme discovery and engineering.

Here, we developed a coupled enzyme assay to spectrophotometrically quantify ester synthesis by AATase activity. The assay was developed for whole cell lysate reactions to enable high throughput screening of AATase libraries and directed evolution experiments. The yeast *S. cerevisiae* was used as a host to allow for native localization of homologous enzymes and lipid droplet and ER localization of heterologous enzymes. To validate and apply the assay we screened a library of six AATases: two homologs from *S. cerevisiae*, Atf1 and Atf2, with specificity towards acetate esters; Eht1 and Eeb1 from *S. cerevisiae* with specificity towards medium chain ethyl esters; a medium chain acetate ester specific AATase from *Cucumis melo* (melon); and an uncharacterized AATase from *Solanum lycopersicum* (tomato fruit).

4.3 MATERIAL AND METHODS

Strains, plasmids, enzymes, and cell culture

The strains and plasmids used in this work are shown in STable 4.1. The plasmid pRS426-pPGK1 containing a phosphoglycerate kinase I (PGK) expression cassette was used for enzyme overexpression in *S. cerevisiae* [21]. Six AATase genes, including *ATF1*, *ATF2*, *EHT1*, and *EEB1* from *S. cerevisiae*, an AATase from *S. lycopersicum* (*Atf-s.l*), and an AATase from *C. melo* (*Atf-c.m*), with encoded C-terminal myc tags were cloned into the SacII and NotI sites of pRS426-pPGK1. Genes from *S. cerevisiae* were amplified by PCR from genomic DNA, whereas genes from *S. lycopersicum* and *C. melo* were created by gene assembly as previously described [21]. Primers used to amplify genes are provided in STable 4.2. Molecular cloning was accomplished in *E. coli* cells, while *S. cerevisiae* was used for overexpression. Cells harvesting expression plasmids were grown in synthetic minimal (SD) medium containing 0.67% yeast nitrogen base (Becton-Dickinson), amino acid supplement without uracil, and 2% glucose.

Preparation of whole cell lysate

Yeast cells harboring expression plasmids were cultured to early stationary phase in 50 ml SD media without uracil in 250 ml baffled flasks. Cells were harvested by centrifugation at 3,500 rpm for 5 min at 4 °C and washed twice with 100 mM potassium phosphate buffer (pH 7.4) containing 2 mM magnesium chloride. Equal volumes of wet cell pellets and 425-600 µm diameter acid-washed glass beads (Sigma-Aldrich) were added to a 15 mL tube and resuspended in 1 mL ice-cold lysis buffer (100mM potassium phosphate buffer, 2mM magnesium chloride, 2mM DTT, and protease inhibitor cocktail

(Roche), pH7.4). Cells were disrupted by vortexing 10 times for 30 s. After each vortexing the suspension was kept on ice for 30 sec. The beads were removed by centrifugation at 500g for 5 min at 4 °C and the supernatant was transferred to a pre-cooled 1.5 mL tube. Whole cell lysate protein concentrations were determined by a commercially available assay (Pierce 660 nm Protein Assay, Thermo Fisher).

AATase activity assay and screening

Alcohol-O-acetyl/acyltransferase activity was determined by monitoring NADH production from a coupled reaction with α -ketoglutarate dehydrogenase (α -KGDH). The reaction was performed at room temperature in 100 mM potassium phosphate buffer, pH 7.4. The assay solution contained final concentrations of 2 mM α -ketoglutarate (α -KG), 10 mM cysteine, 2 mM NAD^+ , 10 mM MgCl_2 , and 0.03 U α -KGDH (Sigma-Aldrich). Whole cell lysate samples, pyrazole (15 mM final concentration), and alcohol (20 mM final concentration) were added to the assay solution. It has previously been reported that the order of substrate addition affects long chain AATase activity [1]. To minimize potential variations in measured activity due to the reaction protocol, it was determined that the order of substrate addition did not affect catalysis (SFigure 4.1). The coupled enzyme reaction was initiated by adding 0.5 mM acetyl/acyl-CoA to the reaction solution. The assay was monitored by measuring the absorbance of NADH at 340 nm ($\epsilon_{340} = 6.22 \text{ mM}^{-1}\text{cm}^{-1}$) for 4 minutes. Assay development experiments were performed in 1 cm pathlength cuvettes with measurements made using a Nanodrop 2000 (ThermoFisher). Screening experiments were performed in 96-well microtiter plates with a final volume of 100 μL and the rate of NADH production was monitored with a

BioTek Synergy H4 hybrid multi-mode microplate reader. The library of six AATases (Atf1, Atf2, Eht1, Eeb1, Atf-C.m, and Atf-S.l) was screened for ethyl ester synthesis with acetyl-, butyryl-, hexanoyl-, octanoyl-, decanoyl-, and lauroyl-CoA. Atf-S.l was also screened for acetate ester synthesis with methanol, ethanol, 1-propanol, 1-butanol, 2-butanol, isobutanol, 1-pentanol, and isopentanol.

Z' factor analysis

To evaluate the assay's response and its appropriateness for high throughput screening, control assays were performed in three individual microplates and the resulting Z' factor was determined according to equation (1), where μ is the mean and σ is the standard deviation [2]. Cells overexpressing Atf1 and Eth1 were used as the positive controls (c^+) and cells harboring an empty vector were used as negative controls (c^-).

$$Z' = 1 - \frac{(3\sigma_{c^+} - 3\sigma_{c^-})}{|\mu_{c^+} - \mu_{c^-}|} \quad (1)$$

Western blot analysis

Whole cell lysates were prepared from cells grown to early stationary phase and 1 μ g total protein was used for western blot analysis. Mouse anti-myc (Invitrogen), mouse anti-GAPDH (Thermo Fisher Scientific), goat anti-mouse IgG-HRP (Thermo Fisher Scientific) and chemiluminescent HRP substrate (Immobilon Western, Millipore) were used for protein analysis and signal detection.

4.4 RESULTS AND DISCUSSION

A coupled-enzyme reaction for high throughput AATase activity screening

The strains, plasmids, and AATase genes used in this study are listed in STable 4.1. The genes encode AATases from *Saccharomyces* yeast (Atf1, Atf2, Eht1, and Eeb1), an AATase from melon (*C. melo*; Atf-C.m), and an AATase from tomato fruit (*S. lycopersicum*; Atf1-S.l). Each gene was overexpressed in *S. cerevisiae* from a 2 μ plasmid under the control of the constitutive PGK promoter.

AATases condense an alcohol with acetyl- or acyl-CoA to produce the corresponding ester. Breaking of the acetyl/acyl-CoA thioester bond produces free CoA-SH that can be quantified through a coupled reaction with NAD⁺ co-factor dependent α -KGDH (Figure 4.1A). In the presence of CoA-SH, α -KG, cysteine, and NAD⁺, α -KGDH produces succinyl-CoA, CO₂ and reduced co-factor, NADH, which can be quantified by absorbance at 340 nm. The free thiol group of CoA-SH can also be quantified by Ellman's reagent [18,28] and an α -KGDH based assay has previously been used to quantify acetyltransferase activity [30,31]; however, these assays require purified enzyme samples to minimize background and non-specific activity. As shown in Figure 4.1B, whole cell lysates from cells harboring empty vectors exhibited rapid NAD⁺ reduction in the presences of AATase substrates (ethanol and acetyl-CoA) and α -KGDH and its substrates (α -KG, cystein, and NAD⁺).

The ethanol fermentation capacity of *S. cerevisiae* is well known and there are seven native NAD⁺-dependent alcohol dehydrogenases (ADHs) that could contribute to the observed NADH background (Figure 4.1C) [32]. As such, we explored the use of pyrazole, an ADH chemical inhibitor, to suppress the background activity. A previously

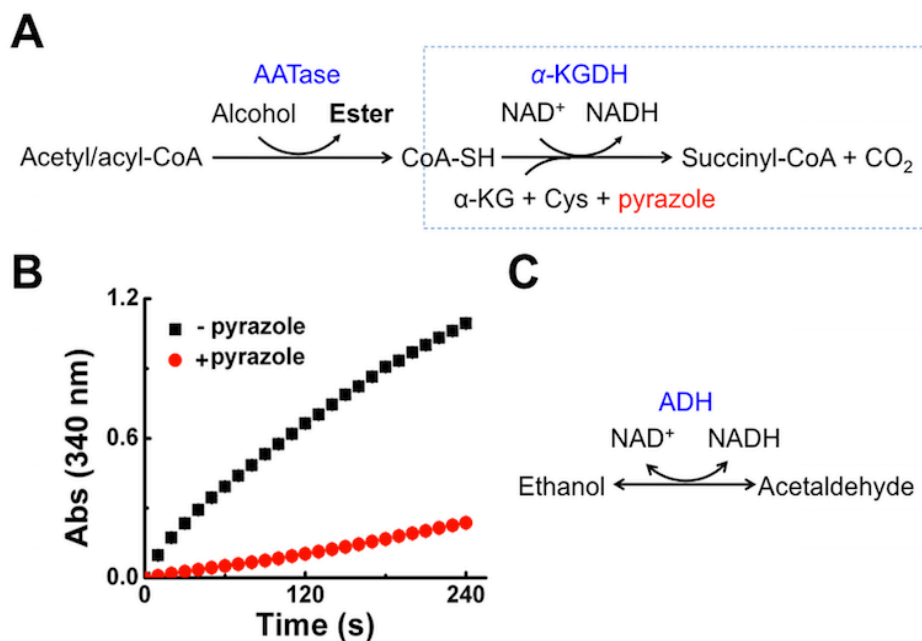


Figure 4.1. A coupled-enzyme reaction for quantifying alcohol-O-acetyl/acyltransferase (AATase) activity. **(A)** α -ketoglutarate (α -KG), cystein (Cys), and NAD^+ are substrates of α -ketoglutarate dehydrogenase (α -KGDH). Pyrazole is a chemical inhibitor of alcohol dehydrogenase (ADH) and is included in the assay to suppress background NAD^+ reduction activity from *S. cerevisiae* whole cell lysate samples. The rate of ester synthesis by AATase was measured by the generation of NADH ($\epsilon_{\text{NADH},340\text{nm}} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$). **(B)** NAD^+ reduction from *S. cerevisiae* whole cell lysates in the presence and absence of 15 mM pyrazole (Reaction conditions: 20 mM ethanol, 0.5 mM acetyl-CoA, 2 mM α -KG, 10 mM cystein, 2 mM NAD^+ , 10 mM MgCl_2 , and 0.03 U α -KGDH, and 100 mM phosphate, pH 7.4). **(C)** Alcohol dehydrogenase activity (ADH) reduces NAD^+ to NADH in the presence of ethanol.

developed assay for acetylaldehyde dehydrogenase activity in *S. cerevisiae* used 15 mM pyrazole to suppress ADH activity in lysates [33]; therefore, we tested background NAD^+ reduction in the presence of 15, 30, and 50 mM pyrazole. The background rate of NAD^+ reduction was reduced from 3.76 ± 0.05 to $0.97 \pm 0.01 \text{ mmol min}^{-1} \text{ mg}^{-1}$ of whole cell lysate protein with 15 mM pyrazole and decreased an additional 2.5 and 4.7 % with 30 and 50 mM, respectively (Figure 4.1B and SFigure 4.2). To rule out the possibility that

pyrazole alters AATase activity, Atf1 synthesis of ethyl acetate was measured by standard headspace gas chromatography. Ester synthesis rates of overexpressed Atf1 in the presence and absence of 15 mM pyrazole were nearly equal (0.129 ± 0.001 and 0.119 ± 0.01 mmol min⁻¹ mg⁻¹ of whole cell lysate protein; SFigure 4.3), suggesting that pyrazole at this concentration does not significantly effect AATase kinetics. As such, all subsequent assays included 15 mM pyrazole.

To test the feasibility of the coupled enzyme assay for acyl-CoA substrate screening, we tested the assay with Atf1, an AATase with known activity towards acetate ester synthesis, as well as Eht1, a medium chain ethyl ester AATase [14,24]. With background NAD⁺ reduction suppressed by pyrazole, ethyl acetate synthesis by Atf1 resulted in the coupled enzyme production of NADH at a rate significantly higher than the background (Figure 4.2A). The observed rate of Eht1 synthesis of ethyl butyrate was also higher than the empty vector control (Figure 4.2B). Importantly, the measured activities of Atf1 and Eht1 increased linearly with whole cell lysate concentrations from 0.25 to 1.0 mg, thus providing evidence of a robust assay (Figure 4.2C, D).

Z' factor analysis

Z' factor analysis provides a quantitative test of the suitability of an assay for high throughput screening: Z' values greater than 0.5 are acceptable, while values less than 0.5 indicate that the difference between positive and negative controls is insufficient for high throughput screening [29]. Atf1 was used as a positive control for short chain ethyl ester synthesis and Eht1 was used as a positive control for medium chain ethyl ester synthesis. In both cases, lysates from empty vector cells were used as negative controls. Atf1

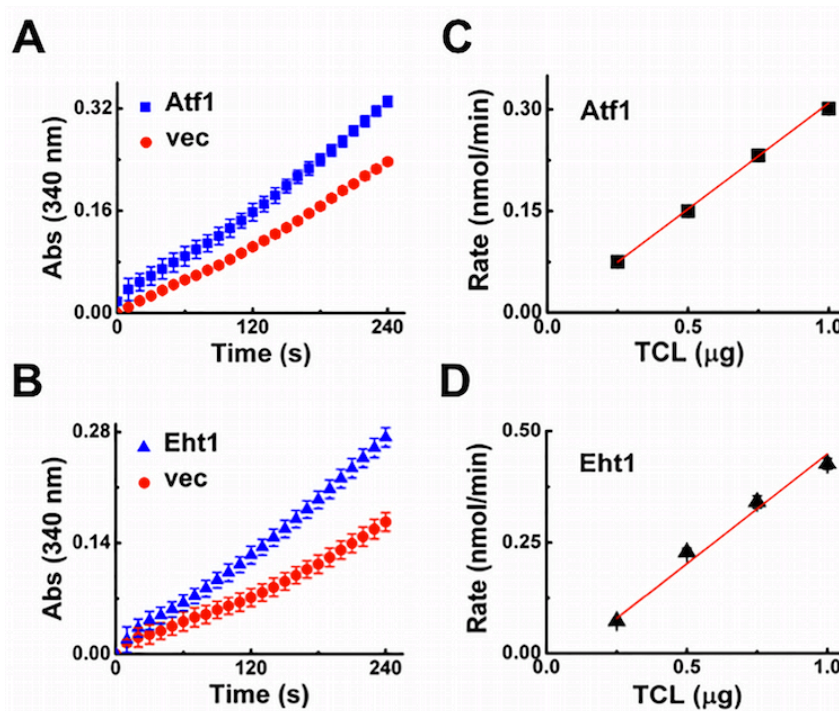


Figure 4.2. A coupled-enzyme assay for quantifying short and medium chain ethyl ester biosynthesis. **(A)** NADH production from the coupled α -KGDH reaction with Atf1 and an empty vector control. **(B)** Ester biosynthesis assay of Eht1 with ethanol and butyryl-CoA. **(C, D)** Atf1 and Eht1 ethyl ester synthesis rates measured by the α -KGDH coupled reaction with increasing concentration of total whole cell lysate. All assays were performed in triplicate with the following reaction conditions: 20 mM ethanol, 0.5 mM acetylacetyl-CoA, 2 mM α -KG, 10 mM cystein, 2 mM NAD^+ , 10 mM MgCl_2 , and 0.03 U α -KGDH, and 100 mM phosphate, pH 7.4. One-mg of whole cell lysate protein was used in A and B.

synthesis of ethyl acetate resulted in a Z' of 0.57 and Eht1 synthesis of ethyl butyrate produced a Z' of 0.67, indicating that the coupled enzyme assay was suitable for high throughput screening (Figure 4.3).

Rapid acyl-CoA substrate screening of an AATase library

The development of a coupled enzyme assay valid for high throughput screening of whole cell lysates allowed for the rapid testing of a library of AATases including Atf1,

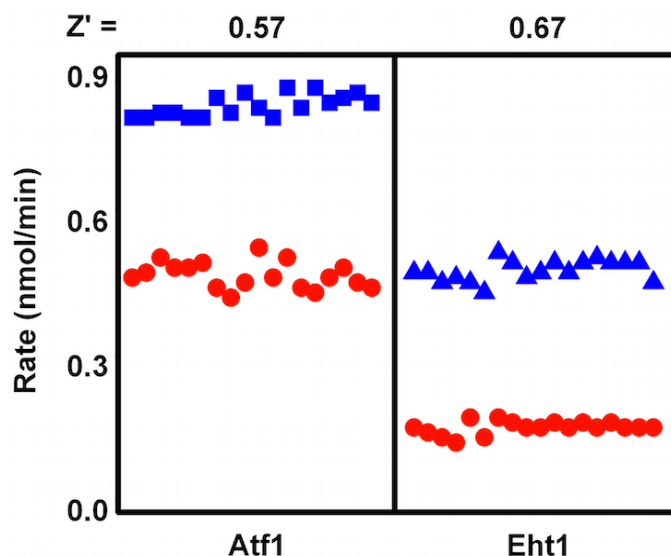


Figure 4.3. Z' factor analysis of the α -KGDH coupled-enzyme assay for AATase activity screening: Aft1 synthesis of ethyl acetate from ethanol and acetyl-CoA (blue squares), Eht1 synthesis of ethyl butyrate from ethanol and butyryl-CoA (blue triangles), and empty vector controls (red circles).

Atf2, Eht1, and Eeb1 from *S. cerevisiae*, Atf-C.m from melon, and Atf-S.l from tomato fruit. The library was screened for ethyl ester synthesis with a series of acyl-CoA substrates with acyl chain lengths of C2, C4, C6, C8, C10, and C12. The library included two enzymes, Eht1 and Eeb1, with known substrate specificity that were used as internal controls to validate the screen. Recent reports found that Eht1 and Eeb1 have activity towards butyryl-, hexanoyl-, and octanoyl-CoA (C4, C6, and C8 acyl chain lengths, respectively) with high activity towards octanoyl-CoA [12,30]. In addition to validating the coupled enzyme assay, the screen was used to generate new data on the acyl-CoA specificity of Atf1, Atf2, Atf-C.m. and Atf-S.l, as well as the alcohol substrate specificity of Atf-S.l.

AATase screening results are shown in Figure 4.4A as a heat map of enzyme activity in terms of nmol of ethyl ester produced per min. Enzyme activity from each assay is provided in STable 4.3. Accompanying the screening results is a western blot analysis of enzyme expression (Figure 4.4B). The western blots show that the expression levels of native *S. cerevisiae* enzymes Atf1, Atf2, Eht1, and Eeb1, were significantly higher than the expression levels of the heterologous AATases from tomato (Atf-S.l) and melon (Atf-C.m). A coarse comparison between *S. cerevisiae* enzymes revealed that Atf1, Atf2, Eht1, and Eeb1 had measurable activity towards the set of screened acyl-CoAs. Also of note is that Eht1 exhibited the highest average activity towards ethyl ester synthesis and Atf1 exhibited the highest acetylation activity. Despite low expression levels, the measured activities of Atf-C.m and Atf-S.l were comparable with both Atf1 and Atf2. Atf-C.m and Atf-S.l also exhibited promiscuous activity, as both Atf-C.m and Atf-S.l were active with acyl-CoA with acyl length up to C10.

Eht1 and Eeb1 have previously been characterized as medium chain fatty acid ethyl ester AATases with activity towards butyryl-, hexanoyl-, and octanoyl-CoA [12]. Eht1 is also known to have activity with acyl-CoAs with acyl chain length up to C14 and exhibits a preference for octanoyl-CoA [30]. Our results are consistent with the previous studies: Eht1 was active with acyl-CoAs with acyl length between C2 and C12 and the highest activity was observed with octanoyl-CoA ($2.22 \pm 0.19 \text{ nmol min}^{-1}$); and, Eeb1 had maximum activity with octanoyl-CoA ($1.48 \pm 0.14 \text{ nmol min}^{-1}$) and exhibited broad acyl-CoA specificity. Our screen also revealed that Eht1 and Eeb1 are relatively poor enzymes

for acetylation, as both enzymes showed relatively low activity with acetyl-CoA (0.044 ± 0.014 and 0.037 ± 0.004 nmol min⁻¹, respectively).

The screening results shown in Figure 4.3A and STable 4.3 revealed broad acyl-CoA specificity for Atf1. Coupled enzyme reactions with Atf1 resulted in measurable activity towards ethyl esters from acyl-CoA with C2 - C12 acyl chain lengths. Consistent with previous reports, Atf1 exhibited high activity with acetyl-CoA and the synthesis of ethyl acetate [24,34]. This screen evaluated acyl-CoA specificity with ethanol as a co-substrate, but alcohol substrate specificity has also been explored with high Atf1 activity reported for isobutyl alcohol [6] and isopentyl alcohol [2,25,35].

Atf2 is known to localize to lipid droplets in *S. cerevisiae* [21] and exhibits specificity towards sterol acetylation [36,37]. This acetylation activity has previously been exploited to produce ethyl, butyl, isobutyl, and isopentyl acetates by whole cell catalysis and fermentation [7,24,35]. Similar to Atf1, our screen revealed broad acyl-CoA substrate specificity with octanoyl-CoA as the preferred substrate. Atf2 produced 0.23 ± 0.03 nmol min⁻¹ of ethyl octanoate, a rate 10-fold higher than the observed rate of ethyl acetate synthesis (0.020 ± 0.002 nmol min⁻¹). This high rate of ester synthesis with medium chain acyl-CoAs suggests that Atf2 may have biological function beyond the acetylation of sterols.

Characterization of AATase activity in cell-free extracts from ripe melon fruit identified acetylation activity towards a range of alcohols including 1-butanol, 1-hexanol, isopentyl alcohol, and phenylethyl alcohol, among others [38]. Recombinant expression of AATases from melon have also demonstrated broad alcohol substrate specificity with

acetyl-CoA [20]. The acyl-CoA substrate screening of Atf-C.m conducted in this work confirmed acetylation activity, but in this case with ethanol as a co-substrate. Atf-C.m also exhibited activity towards longer chain acyl-CoAs with the highest measured activity towards decanoyl-CoA ($1.46 \pm 0.15 \text{ nmol min}^{-1}$). The relatively low expression level of Atf-C.m in comparison to the native *S. cerevisiae* enzymes indicates that the specific activity may be significantly higher than Atf1, Atf2, Eht1, and Eeb1. These results suggest that Atf-C.m could be useful for the metabolic engineering of medium chain esters including butyrates [8].

An AATase gene was previously identified in the genome of *S. lycopersicum*. The protein sequence corresponding to this gene was deposited into the NCBI protein data bank under the accession number NP_001234496.1 [39]. The gene encodes for an enzyme 442 amino acids in length with the putative AATase active site, H-X-X-X-D, beginning at residue 221 [21]. In previous characterizations of this enzyme, our group has demonstrated that Atf-S.l does not localize to the ER or lipid droplets [21] and that it has activity towards ethyl acetate synthesis [24]. AATase activity has also been identified in tomato fruit extracts, but the activity of Atf-S.l with acyl-CoAs and various alcohols has not been characterized [40]. The acyl-CoA screen conducted in this work revealed that Atf-S.l is active towards acyl-CoAs with chain length up to C10. The highest activity was observed with hexanoyl-CoA ($0.326 \pm 0.042 \text{ nmol min}^{-1}$), but low expression levels (Figure 4.4B) suggest that its specific activity may be higher than the *S. cerevisiae* enzymes including Eht1, which showed the highest measured activity in the current acyl-CoA screen.

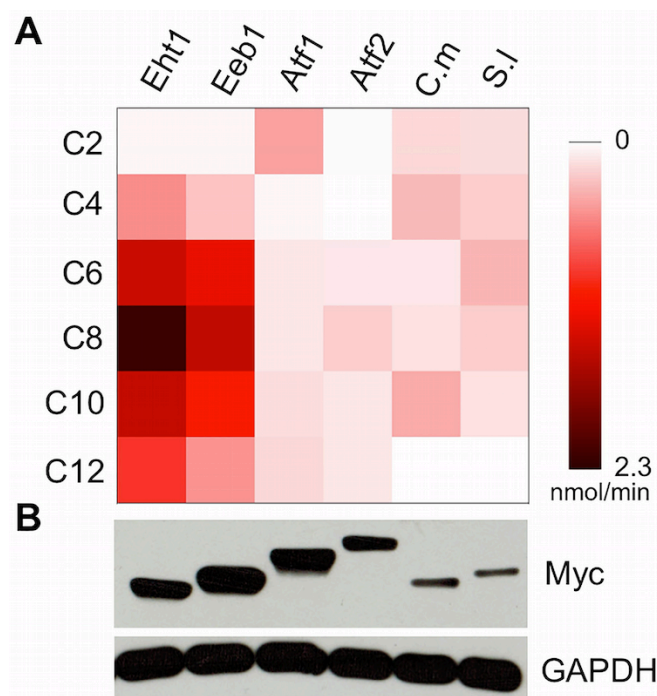


Figure 4.4. AATase library screening. (A) Heat map of ethyl ester synthesis rates from Eht1 (*S. cerevisiae*), Eeb1 (*S. cerevisiae*), Atf1 (*S. cerevisiae*), Atf2 (*S. cerevisiae*), Atf-C.m (*C. melo*), and Atf-S.l (*S. lycopersicum*). All assays were performed with the following reaction conditions: 20 mM ethanol, 0.5 mM acetylacetyl-CoA, 2 mM α -KG, 10 mM cysteine, 2 mM NAD^+ , 10 mM MgCl_2 , and 0.03 U α -KGDH. Reactions were buffered with 100 mM phosphate, pH 7.4 and 1 mg of whole cell lysate protein was used in each assay. The mean value of triplicate reactions is shown. (B) Western blot analysis of protein expression. Anti-myc antibody was used to against AATases while anti-GAPDH was used as the loading control.

Alcohol substrate screening of an AATase from tomato fruit

The alcohol substrate specificities of Atf1, Atf2, Eht1, Eeb1, and Atf-C.m have previously been characterized, but a similar characterization of Atf-S.l has not yet been accomplished. As such, the coupled enzyme assay with α -KGDH was used to screen the activity of Atf-S.l with various primary and secondary alcohols with carbon chain lengths up to C5 (Figure 4.5). The screen revealed no activity towards methanol and the secondary alcohol 2-butanol. Ethanol, 1-propanol, and 1-butanol were found to be

acceptable substrates with activities of 0.11 ± 0.04 , 0.12 ± 0.04 , and 0.13 ± 0.04 nmol min⁻¹. Isobutanol and isopentyl alcohols resulted in higher acetate ester synthesis rates with measured activities of 0.29 ± 0.03 and 0.15 ± 0.02 nmol min⁻¹, respectively. However, the synthesis of pentyl acetate from 1-pentanol and acetyl-CoA proved to have the highest activity (0.55 ± 0.05 nmol min⁻¹).

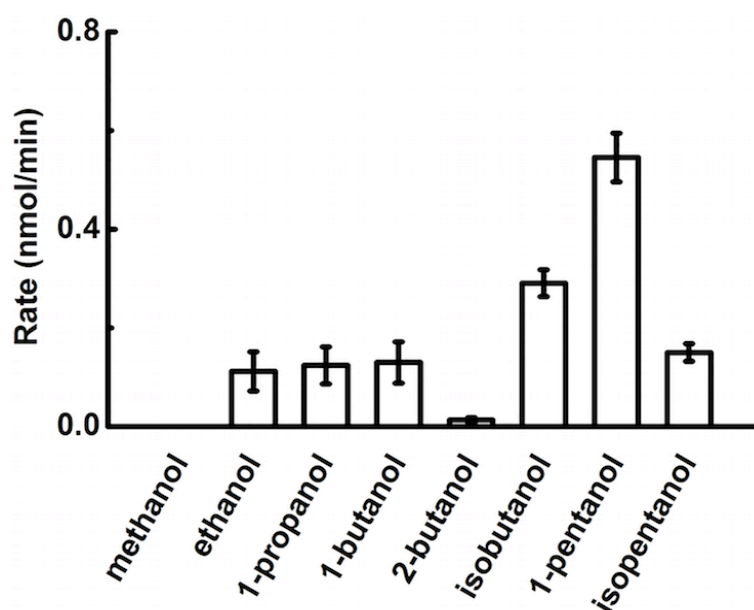


Figure 4.5. Alcohol substrate screening of alcohol-O-acyltransferase from tomato fruit (Atf-S.1). Acetyl-CoA was used as a co-substrate. All reactions were performed in triplicate. Bars are mean $\pm$ standard deviation.

4.5 CONCLUSIONS

High throughput screening of AATase activity for enzyme discovery and enzyme engineering has been limited due to the lack of a suitable screen. Typically, activity towards short and medium chain volatile esters is determined by headspace GC, while activity towards longer chain ester synthesis is accomplished by GC-MS. In this work, we developed a spectrophotometric-coupled enzyme assay that is suitable for high

throughput screening of ester synthesis by AATase activity. The coupled assay relies on the conversion of AATase produced CoA-SH to succinyl-CoA by α -KGDH. The coupled α -KGDH reaction reduces NAD^+ to NADH, thus enabling the spectrophotometric measurement of AATase activity. Suppression of background NAD^+ reduction by adding the chemical inhibitor pyrazole to the assay solution allowed for the quantification of AATase activity in whole cell lysate samples. This new assay was used to rapidly screen a library of six AATases with acyl-CoA substrates with acyl chain length ranging from C2 to C12. One member of the library, a previously uncharacterized AATase from tomato fruit (Atf-S.I), was found to be highly active towards ethyl ester synthesis with acyl-CoAs up to decanoyl-CoA and was specific to 1-pentanol as a co-substrate. The developed high throughput assay was used to screen short and medium chain ethyl ester synthesis as well as short and medium chain acetate ester synthesis, and we anticipate that it will be suitable for measuring long chain ester synthesis rates as well as high throughput enzyme engineering

4.6 ACKNOWLEDGEMENTS

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4.8 SUPPLEMENTARY INFORMATION

Material and Methods

Gas chromatography

Produced ethyl acetate was quantified by a headspace gas chromatography with a flame ionization detector (Agilent Technologies 7890A GC with CTC-PAL headspace mode injector). The separation of volatile compounds was carried out by Rtx®-1 column (30 m, 0.32 mmID, 5 mm film thickness; RESTEK) with helium as carrier gas. GC oven temperature was initially at 75 °C for 7 min and increased with a gradient of 30 °C/min until 175 °C, followed by a gradient of 50 °C/min until 275 °C. The injector and detector were held at 275 °C. 1 mL headspace gas was injected to the GC and 1-pentanol was used as internal standard.

STable 4.1. Strains, plasmids, and enzymes used in this study.

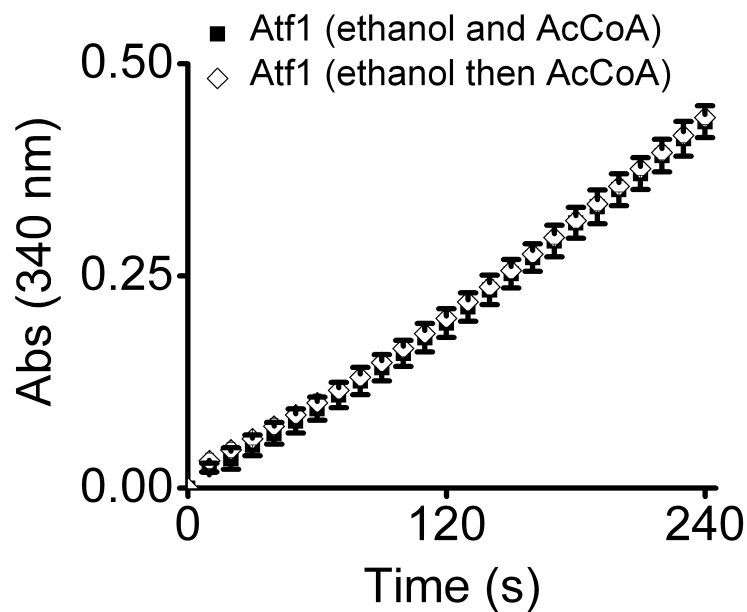
Name	Description	Source
Strains		
<i>S. cerevisiae</i> (BY4742)	<i>MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	GE Healthcare
<i>E. coli</i> (DH5α)	<i>F– Φ80lacZAM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1</i>	Life technologies
Plasmids		
pRS426		GE Healthcare
pRS426-pPGK1	Constitutive expression driven by PGK1p-PGK1t	Lin and Wheeldon
pRS426-pPGK1-Atf1(Sc)-myc	Atf1 from <i>S. cerevisiae</i> cloned into SacII and NotI sites of pPGK1	this study
pRS426-pPGK1-Atf2(Sc)-myc	Atf2 from <i>S. cerevisiae</i> cloned into SacII and NotI sites of pPGK1	this study
pRS426-pPGK1-Atf1(Sl)-myc	Atf1 from <i>S. lycopersicum</i> cloned into SacII and NotI sites of pPGK1	this study
pRS426-pPGK1-Atf1(Cm)-myc	Atf1 from <i>C. melo</i> cloned into SacII and NotI sites of pPGK1	this study
pRS426-pPGK1-Eht1(Sc)-myc	Eht1 from <i>S. cerevisiae</i> cloned into SacII and NotI sites of pPGK1	this study
pRS426-pPGK1-Eeb1(Sc)-myc	Eeb1 from <i>S. cerevisiae</i> cloned into SacII and NotI sites of pPGK1	this study
Enzymes		
Atf1 (Sc)	NCBI accession no.: NP_015022	
Atf2 (Sc)	NCBI accession no.: NP_011693	
Atf1 (Sl)	NCBI accession no.: NP_001234496, codon optimized for <i>S. cerevisiae</i>	
Atf1 (Cm)	NCBI accession no.: BAB78588, codon optimized for <i>S. cerevisiae</i>	
Eht1(Sc)	NCBI accession no.: NP_009736	
Eeb1 (Sc)	NCBI accession no.: NP_015230	

STable 4.2. Primers used in this study.

Primers	DNA sequence	
Atf1(Sc)-myc	Forward: CAAACCGCGGATGAATGAAATCGATGAGAAAAAT C Reverse: CAAAGCGGCCGCCTAGAGGTCTTCTTCGGAAATCAACTTCTGTTC AGGGCCTAAAAGGAGAGC	this study
Atf2(Sc)-myc	Forward: CAAACCGCGGATGGAAGATATAGAAGGATACGAAC Reverse: CAAAGCGGCCGCTTAGAGGTCTTCTTCGGAAATCAACTTCTGTTC AAGCGACGCAAATTCGCC	this study
Atf1(Sl)-myc	Forward: CAAACCGCGGATGGCTAATATTTTGCCAATTCT Reverse: CAAAGCGGCCGCTTAGAGGTCTTCTTCGGAAATCAACTTCTGTTC AATATTAGAAATAATAACTCTGAAAGTCAT	this study
Atf1(Cm)-myc	Forward: CAAACCGCGGATGGAAACTATGCAAATATTGATTT Reverse: CAAAGCGGCCGCTTAGAGGTCTTCTTCGGAAATCAACTTCTGTTC CAAAGCAGACAAAATAGTTTGCA	this study
Eht1(Sc)-myc	Forward: CAAACCGCGGATGTCAGAAGTTTCCAAATGGC Reverse: GAAAACTAGTCTAGAGGTCTTCTTCGGAAATCAACTTCTGTTC TACGACTAATTCATCAAACCTTAGTGAAA	this study
Eeb1(Sc)-myc	Forward: CAAACCGCGGATGTTTCGCTCGGGTTACTAT Reverse: GAAAACTAGTCTAGAGGTCTTCTTCGGAAATCAACTTCTGTTC TAAAACTAACTCATCAAAGCTGCC	this study

STable 4.3. AATase library screening. Reaction rates of ethyl ester synthesis from Eht1 (*S. cerevisiae*), Eeb1 (*S. cerevisiae*), Atf1 (*S. cerevisiae*), Atf2 (*S. cerevisiae*), Atf-C.m (*C. melo*), and Atf-Sl (*S. lycopersicum*). Rates reported in nmol/min.

nmol/min	acetyl-CoA	butyryl-CoA	hexanoyl-CoA	octanoyl-CoA	decanoyl-CoA	lauroyl-CoA
Eht1	0.044±0.014	0.518±0.090	1.415±0.083	2.220±0.186	1.463±0.154	0.933±0.280
Eeb1	0.037±0.004	0.267±0.021	1.248±0.095	1.482±0.144	1.079±0.118	0.478±0.080
Atf1	0.428±0.022	0.039±0.210	0.106±0.021	0.111±0.017	0.144±0.055	0.174±0.032
Atf2	0.020±0.002	N/A	0.102±0.051	0.229±0.031	0.115±0.060	0.119±0.018
C.m	0.161±0.027	0.311±0.086	0.099±0.064	0.140±0.071	0.388±0.043	N/A
S.l	0.156±0.041	0.216±0.106	0.326±0.042	0.222±0.015	0.136±0.039	N/A



SFigure 4.1. The order of substrate addition does not affect AATase activity. The assays were performed with the following reaction conditions: 20 mM ethanol, 0.5 mM acetyl-CoA (AcCoA), 2 mM α -KG, 10 mM cysteine, 2 mM NAD^+ , 10 mM MgCl_2 , and 0.03 U α -KGDH, and 100 mM phosphate, pH 7.4. In Atf1 (ethanol and AcCoA), ethanol and AcCoA were added together. In Atf1 (ethanol then AcCoA), ethanol was added first followed by the initiation of the reaction with AcCoA after one minute. 1-mg of whole cell lysate protein was used and the reactions were performed in triplicate.

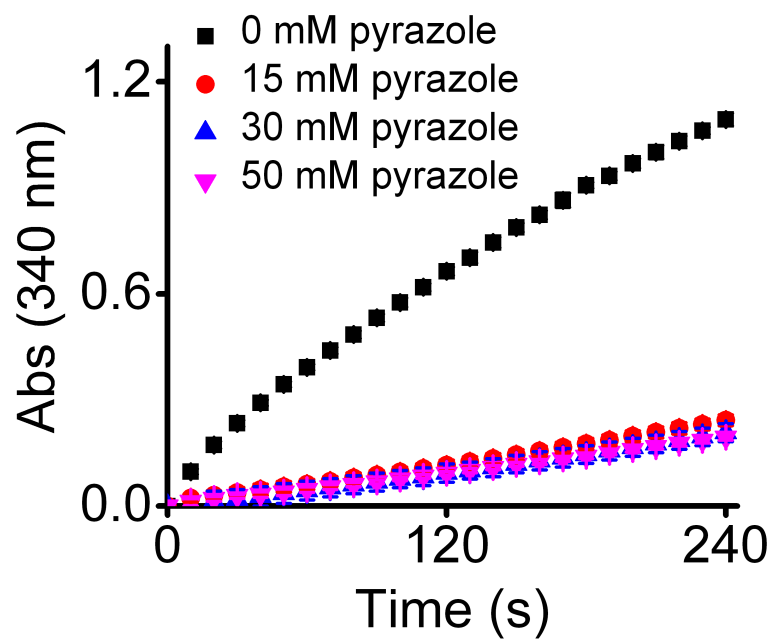


Figure 4.2. Background reduction of NAD^+ in the presence of pyrazole. 0, 15, 30, and 50 mM pyrazole were used to suppress background activity of whole cell lysates from empty vector samples. Reaction conditions: 20 mM ethanol, 0.5 mM acetyl-CoA, 2 mM α -KG, 10 mM cysteine, 2 mM NAD^+ , 10 mM MgCl_2 , and 0.03 U α -KGDH, and 100 mM phosphate, pH 7.4). 1-mg of whole cell lysate protein was used and the reactions were performed in triplicate.

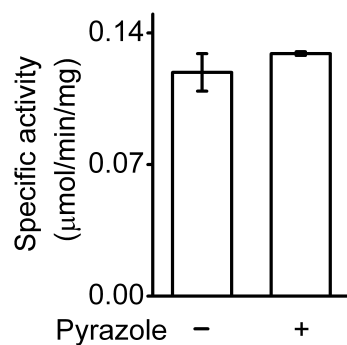


Figure 4.3. GC analysis of the effect of pyrazole addition to Atf1 activity. 15 mM pyrazole was used to test the effect to Atf1 activity. The assays were performed with the following reaction conditions: 200 mM ethanol, 0.5 mM acetyl-CoA, 10 mM MgCl₂, and 100 mM phosphate, pH 7.4. 100-mg of whole cell lysate protein was used and the reactions were performed in triplicate.

Chapter 5. Microbial Host Selection Affects Intracellular Localization and Activity of Alcohol-O-acetyltransferase

5.1 ABSTRACT

A key pathway for ester biosynthesis in yeast is the condensation of an alcohol with acetyl-CoA by alcohol-O-acetyltransferase (AATase). This pathway is also prevalent in fruit, producing short and medium chain volatile esters during ripening. In this work, a series of six AATases from *Saccharomyces* and non-*Saccharomyces* yeasts as well as tomato fruit were evaluated with respect to their activity, intracellular localization, and expression in *Saccharomyces cerevisiae* and *Escherichia coli* cell hosts. The series of AATases includes Atf1 and Atf2 from *S. cerevisiae*, as well as AATases from *S. pastorianus*, *Kluyveromyces lactis*, *Pichia anomala*, and *Solanum lycopersicum* (tomato). When expressed in *S. cerevisiae*, Atf1, Atf2, and an AATase from *S. pastorianus* localized to lipid droplets, while AATases from non-*Saccharomyces* yeasts and tomato fruit did not localize to intracellular membranes and were localized to the cytoplasm. All AATases studied here formed intracellular aggregates when expressed in *E. coli*, and western blot analysis revealed that expression levels in *E. coli* were upwards of 100-fold higher than in *S. cerevisiae*. Fermentation and whole cell lysate activity assays of the two most active AATases, Atf1 from *S. cerevisiae* and an AATase from tomato fruit, demonstrated that the aggregates were enzymatically active, but with highly reduced specific activity in comparison to activity in *S. cerevisiae*. Activity was partially recovered at lower expression levels, coinciding with smaller intracellular aggregates. In

vivo and in vitro activity assays from heterologously expressed Atf1 from *S. cerevisiae*, which localizes to lipid droplets under homologous expression, demonstrates that its activity is not membrane dependent. The results of these studies provide important information on the biochemistry of AATases under homologous and heterologous expression with two common microbial hosts for biochemical processes, *S. cerevisiae* and *E. coli*. All studied AATases formed aggregates with low enzymatic activity when expressed in *E. coli* and any membrane localization observed in *S. cerevisiae* was lost in *E. coli*. In addition, AATases that were found to localize to lipid droplet membranes in *S. cerevisiae* were found to not be membrane dependent with respect to activity.

5.2 INTRODUCTION

During yeast fermentation and fruit ripening short chain linear and branched alcohols are converted to their corresponding acetate esters by alcohol-O-acetyltransferase (AATase; EC 2.3.1.84; Figure 5.1). These volatile esters produce sweet and fruity fragrances: phenyl ethyl acetate smells of flowers, isoamyl acetate (isopentyl acetate) smells of bananas, and ethyl acetate smells of sweet pears. In plants, these and other esters function as attractors to pollinating species and as a defense mechanism, attracting predators to animals feeding on their leaves and fruit [1,2]. The function of microbial ester biosynthesis is not as well understood. AATase activity in *Saccharomyces cerevisiae* is repressed by oxygen and unsaturated fatty acids [3-5] and it has been suggested that this activity functions as a means of CoA recycling with the co-production of organic acids [6,7], possibly as a response to stress conditions [8].

We have previously shown that Atf1 and -2 from *S. cerevisiae* localize to lipid droplets (LDs) via N- and C-terminal amphipathic helices [19]. The AATase ortho- log from *S. pastorianus* also localizes to LDs by a similar mechanism, while AATases from non-*Saccharomyces* yeasts and fruit species, including *Cucumis melo* (melon), and *Solanum lycopersicum* (tomato) that do not have the conserved terminal helices from *S. cerevisiae* and do not localize to LDs. Early biochemical studies of Atf1 and -2 suggest that enzyme activity is membrane dependent. Purification in the presence of non-ionic detergents (e.g., heptyl thioglucoside, octyl thioglucoside, and Triton- X100) resulted in measurable enzyme activity, while purification in the absence of such detergents resulted in inactive samples [6,20-22]. Due in part to this apparent membrane dependency as well as the hydrophobic nature of the AATase family, the standard activity assay has evolved to include Triton-X100 above the critical micelle concentration [23].

The apparent membrane dependency of Atf1 and -2 activity is interesting in the context of heterologous expression in *E. coli* or other microbial hosts for ester biosynthesis. Reported activities of homologously expressed Atf1 and -2 are moderate, ranging from 0.01 to 10 nmol min⁻¹ per mg of protein of whole cell lysate [18,21,22,24], while the activity of orthologs from *Pichia anomla* and *Klyuveromyces lactis* are low (<1 nmol min⁻¹ per mg of protein) [25]. Reported activities of strawberry AATases range from 8 – 75 nmol min⁻¹ mg⁻¹ of enzyme [26,27]. The activities of Atf1 and -2 were measured in whole cell lysates that contain native LDs to which the enzymes can associate or in the presence of suitable membrane substitutes during purification. The successful metabolic engineering of *E. coli* to produce esters via an AATase pathway

indicates that Atf1 and -2 maintain some activity in heterologous environments. In the absence of LDs Atf1 and -2 may associate with the plasma membrane, but the intracellular localization of Atf1 and -2 and other AATases heterologously expressed in *E. coli* and the effects of this localization on activity are not known.

In this work, a series of six AATases from *Saccharomyces* and non-*Saccharomyces* yeasts as well as tomato fruit (*S. lycopersicum*) were overexpressed in *S. cerevisiae* and *E. coli* and compared in terms of their intracellular localization, enzymatic activity, and expression level. The studies revealed that some AATases localize to LDs in *S. cerevisiae* and all studied AATases form enzymatically active aggregates in *E. coli*. Aggregate formation resulted in significantly reduced activities in comparison to activities measured in *S. cerevisiae*.

5.3 MATERIAL AND METHODS

Strains, plasmids, and culture conditions

Strains and plasmids used in this work are shown in Table 1. *E. coli* strains were grown in LB medium containing 30 µg/mL kanamycin. *S. cerevisiae* strains were prepared as previously described [19], and were grown in synthetic minimal (SD) medium containing 0.67% yeast nitrogen base (Becton-Dickinson), amino acid supplements (Sunrise), and 2% glucose. Expression in *E. coli* was induced by adding IPTG at OD₆₀₀ of 0.4.

Preparation of whole cell lysate

Cells were harvested by centrifugation at 3,500 rpm for 5 min at 4°C and washed twice with 100 mM potassium phosphate buffer (pH 7.4) containing 2 mM magnesium

chloride. Equal volumes of wet cell pellets and 425–600 μm acid-washed glass beads (Sigma-Aldrich, G8772) were added to a 15 mL tube and resuspended in 1 mL ice-cold lysis buffer (100 mM potassium phosphate buffer, 2 mM magnesium chloride, 2 mM DTT, and protease inhibitor). The cells were disrupted by vortexing 10 times for 30 s. After each vortexing the suspension was kept on ice for 30 s. The beads were removed by centrifugation at 500 g for 5 min at 4°C, and the supernatant was decanted to a cold 2 mL tube. The protein concentrations of whole cell lysates were determined by Thermo Scientific Pierce 660 nm Protein Assay.

Enzyme activity assays

AATase activity was measured using ethanol and acetyl-CoA as substrates. A reaction mixture contains 100 mM potassium phosphate (pH 7.4), 500 mM ethanol and 0.5 mM acetyl-CoA and 100 μg lysate was used. After incubation at 30 °C for 0.5 hours, the reaction was stopped by the addition 60 μmol H_2SO_4 . 100 μg of 1-pentanol was added as an internal standard and 1 g NaCl was added to reduce the solubility of ethyl acetate. The concentration of ethyl acetate produced was measured by headspace gas chromatography. To determine the activity of soluble and insoluble cell fractions, whole cell lysates were centrifuged at 15,000 rpm for 20 min at 4 °C. The supernatant was isolated and taken as the soluble fraction. The pellet was washed twice with lysis buffer before re-suspending in lysis buffer. The activity of the re-suspended pellet and soluble fraction were measured as described above.

Fluorescent microscopy and image analysis

Cells were observed as described in [19]. Briefly, an Olympus BX51 microscope

(UPlanFL 100X 1.30 oil-immersion objective lense, mercury lamp) with Q-Imaging Retiga Exi CCD camera was used to capture images. CellSens Dimension 1.7 software (Olympus) was used to process images. Image J software was used to measure protein aggregate size. Quantitative values of aggregates size and number are from a minimum of 100 cells. Statistical analysis of aggregate size was accomplished by one-way analysis of variance. A p value <0.05 was applied for statistical significance.

Western blot analysis and quantification of protein expression

Western blots were performed using standard procedure. Protein extracts were loaded on Any kDTM Mini-PROTEAN® TGXTM Gel (Bio-Rad) and run at 150 V for 1 hour. Samples were electrophoretically transferred to a PVDF membrane at 25 V overnight. Membranes were blocked with 5% non-fat milk in TBST buffer for 1 hour at room temperature and incubated with GFP Rabbit Serum Polyclonal Antibody (Life Technologies) diluted to 1: 20000 in TBST buffer with 1% non-fat milk. Goat Anti-Rabbit IgG-HRP (Life Technologies) diluted to 1: 10000 was added as secondary antibody and incubated at room temperature for 0.5 hours. After washing with TBST, HRP substrate (Bio-Rad) was used for signal detection. Image-J software was used to quantify band intensity.

5.4 RESULTS

The strains, plasmids, and AATase genes used in this work are listed in Table 5.1. The genes include encoded AATases from *Saccharomyces* yeasts (Atf1-S.c, Atf2-S.c, and Atf1-S.p), and the non-*Saccharomyces* yeasts *P. anomala* (Atf-P.a), and *K. lactis* (Atf-

Table 5.1. Strains, plasmids, and primers used in this study.

Name	Description	Source
Strains		
<i>E. coli</i> BL21(DE3)	F ⁻ ompT gal dcm lon hsdSB(rB ⁻ mB ⁻) λ (DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	New England Biolabs
<i>E. coli</i> BL21(DE3)-RIL plus	F ⁻ ompT hsdS(rB ⁻ mB ⁻) dcm ⁺ Tetr gal λ (DE3) endA Hte [argU ileY leuW Camr]	Agilent Technologies
<i>S. cerevisiae</i> BY4742	MAT α his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0	GE Healthcare
Plasmids		
pGFP	pET-28b(+) derivative with gfp insertion	this study
pATF1G	pET-28b(+) derivative with atf1(S.c)-gfp insertion	this study
pATF2G	pET-28b(+) derivative with atf2(S.c)-gfp insertion	this study
pSPG	pET-28b(+) derivative with atf(S.p)-gfp insertion	this study
pKLG	pET-28b(+) derivative with atf(K.l)-gfp insertion	this study
pPAG	pET-28b(+) derivative with atf(P.a)-gfp insertion	this study
pSLG	pET-28b(+) derivative with atf(S.l)-gfp insertion	this study
pYPGK	pRS426 derivative; PGK1p-PGK1t	Lin, Wheeldon (2014)
pYATF1G	pYPGK derivative with atf1(S.c)-gfp insertion	Lin, Wheeldon (2014)
pYATF2G	pYPGK derivative with atf2(S.c)-gfp insertion	Lin, Wheeldon (2014)
pYSPG	pYPGK derivative with atf(S.p)-gfp insertion	Lin, Wheeldon (2014)
pYKLG	pYPGK derivative with atf(K.l)-gfp insertion	Lin, Wheeldon (2014)
pYPAG	pYPGK derivative with atf(P.a)-gfp insertion	Lin, Wheeldon (2014)
pYSLG	pYPGK derivative with atf(S.l)-gfp insertion	Lin, Wheeldon (2014)
Primers		
<i>gfp</i>	5'GCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACC ATGGCTAGCATGACTGGTG3'	this study
<i>atf1-S.c-gfp</i>	5'CCGCTCGAGTTATTTGTATAGTTCATCCATGCCATG3' 5'GCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACC ATGAATGAAATCGATGAGAAAAATCAG3'	this study this study
<i>atf2-S.c-gfp</i>	5'CCGCTCGAGTTATTTGTATAGTTCATCCATGCCATG3' 5'GCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACC ATGGAAGATATAGAAGGATACGAACCATATCACTC3'	this study this study
<i>atf-S.p-gfp</i>	5'ACGCGTCGACTTATTTGTATAGTTCATCCATGCCATG3' 5'GCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACC ATGGAAACAGAAGAAAGCCAATTAGCAGTATAAC3'	this study this study
<i>atf-K.l-gfp</i>	5'CCGCTCGAGTTATTTGTATAGTTCATCCATGCCATG3' 5'GCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACC ATGGGTTTCGGTGTGTTTATCATCAAAAAAGTTAG3'	this study this study
<i>atf-P.a-gfp</i>	5'CCGCTCGAGTTATTTGTATAGTTCATCCATGCCATG3' 5'GCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACC ATGGTTGTAAATTCAAAAGCAAAATCAATAACAAAGG3'	this study this study
<i>atf-S.l-gfp</i>	5'CCGCTCGAGTTATTTGTATAGTTCATCCATGCCATG3' 5'GCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACC ATGGCTAATATTTTGCCAAATTC3'	this study this study
	5'CCGCTCGAGTTATTTGTATAGTTCATCCATGCCATG3'	this study

K.l), as well as tomato fruit, *S. lycopersicum* (Atf-S.l). Preliminary activity screening from *S. cerevisiae* whole cell lysates with overexpressed AATases revealed that Atf1-S.c has the highest activity towards C2 to C5 alcohols with acetyl-CoA (STable 5.1). As such, initial experiments focused on determining the intracellular localization and the enzymatic activity of Atf1-S.c towards ethyl acetate when overexpressed in *S. cerevisiae*

and *E. coli*. Importantly, *E. coli* BL-21 without chloramphenicol acetyltransferase (CAT) activity was used, as CAT has been shown to exhibit AATase activity toward ethyl acetate synthesis (Figure S1 and [16]).

In *S. cerevisiae* Atf1-S.c is known to localize to ER in early exponential phase and sort to LDs as cells progress in to stationary phase [19]. Atf1-S.c with a C-terminally fused GFP reporter co-localized with a fluorescently tagged LD marker, Erg6-DsRed, indicating LD localization in *S. cerevisiae* (Figure 5.2A, left). An overexpressed GFP control localized to the cytosol and fluorescent imaging did not indicate LD localization (Figure 5.2A, right). When overexpressed in *E. coli*, fluorescence microscopy revealed that GFP is cytosolically expressed and did not form visible aggregates or punctate structures (Figure 5.2B, right). In contrast, Atf1-S.c. formed aggregates when overexpressed in *E. coli* (Figure 5.2B, left). Nine percent of observed *E. coli* cells expressing Atf1-S.c. had one aggregate, 75% had two aggregates, and 16% had three aggregates, while no cells were observed with zero aggregates or four or more aggregates (Figure 5.2B, bottom). No *E. coli* cells expressing Atf1-S.c were observed to have an expression pattern similar to the GFP control, which showed fluorescence throughout the cell. Western blot analysis of protein expression in *S. cerevisiae* and *E. coli* showed that Atf1-S.c with a C-terminally fused GFP reporter expressed upwards of 100-fold more in *E. coli* than in *S. cerevisiae* (Figure 5.2C). Whole cell lysate activity assays of Atf1-S.c expressed in *S. cerevisiae* reached $58 \pm 8 \text{ nmol min}^{-1} \text{ mg}^{-1}$ of protein. Despite the difference in expression levels, *E. coli* whole cell lysate activity assays were limited to $11.6 \pm 0.1 \text{ nmol min}^{-1} \text{ mg}^{-1}$ of protein (Figure 5.2D).

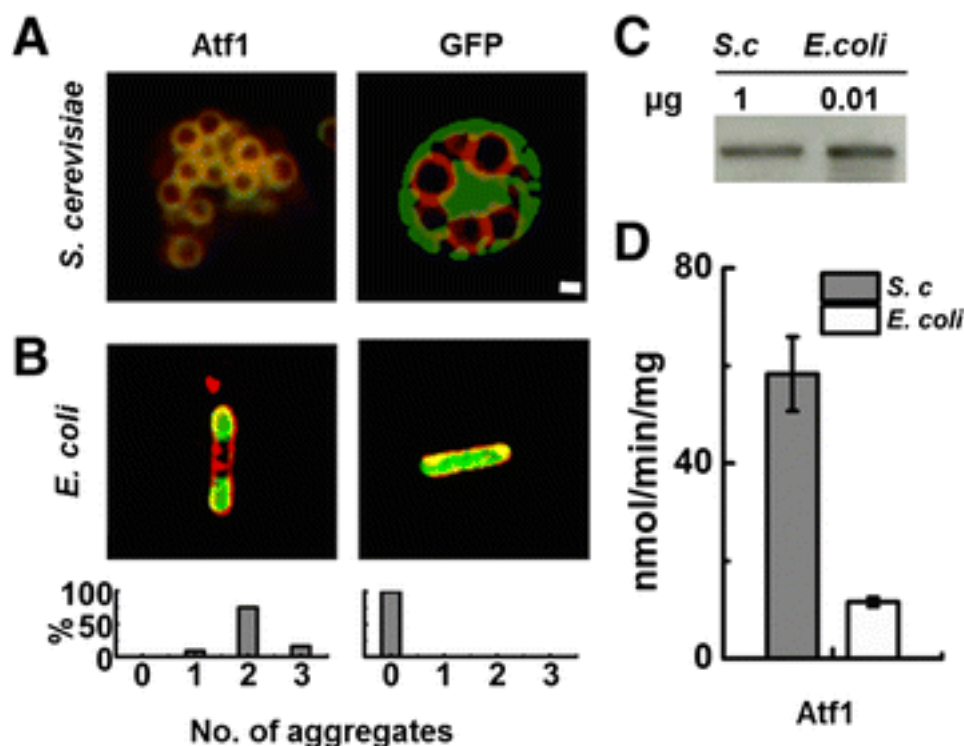


Figure 5.2. Expression, intracellular localization, and activity of Atf1 from *S. cerevisiae* (Atf1-S.c). A) Fluorescence microscopy images of *S. cerevisiae* co-expressing Atf1-GFP, GFP, and the LD marker Erg6-DsRed. Fluorescence from Erg6-DsRed is shown in red and fluorescence from Atf1-GFP is shown in green. Overlapping Erg6-DsRed and Atf1-GFP signals, indicating co-localization, are shown in yellow. GFP controls show cytosolic localization. Scale bar (1 μ m) applies to A) and B). B) Fluorescent microscopy images of *E. coli* expressing Atf1-GFP and GFP. CellMaskTM Orange plasma membrane staining is shown in red and fluorescence from Atf1-GFP and GFP is shown in green. Graphs below the fluorescence images indicate the number of aggregates observed in *E. coli* cells. A minimum of 100 cells were counted from three independent experiments. C) Western blot analysis of Atf1-GFP expression in *S. cerevisiae* (S.c) and *E. coli*. D) In vitro ethyl acetate production from whole cell lysates of Atf1-GFP expressed in *S. cerevisiae* and *E. coli*. Error bars represent standard deviation (n = 3).

Similar intracellular localization results were observed with other studied AATases (Figure 5.3A,B). Atf2-S.c and Atf-S.p localized to LD in *S. cerevisiae* and formed aggregates when overexpressed in *E. coli*. Fluorescent microscopy imaging revealed that Atf-K.1 with C-terminally fused GFP was soluble in *S. cerevisiae*, but formed aggregates in *E. coli*. Punctate structures were observed with Atf-P.a in *S. cerevisiae* and,

similar to the other yeast AATases, formed aggregates in *E. coli*. Finally, Atf-S.l from tomato fruit appeared to localize homogenously throughout the cytosol of *S. cerevisiae*, but formed aggregates in *E. coli*. In each case, the majority of *E. coli* cells contained one or two aggregates of a given AATase with less than 8% of cells containing three aggregates (Figure 5.3B, bottom). All observed *E. coli* cells contained at least one aggregate and no cells were observed to contain more than three aggregates.

Whole cell lysate assays revealed that all AATases exhibited measurable activity when expressed in *S. cerevisiae* and *E. coli* (Figure 5.3C). Atf2-S.c activity in *S. cerevisiae* lysate was $1.2 \pm 0.6 \text{ nmol min}^{-1} \text{ mg}^{-1}$, lower than the $3.1 \pm 0.9 \text{ nmol min}^{-1} \text{ mg}^{-1}$ observed in *E. coli* lysate. Atf-S.p had activities of 0.3 ± 0.3 and $0.4 \pm 0.1 \text{ nmol min}^{-1} \text{ mg}^{-1}$ in *S. cerevisiae* and *E. coli*, respectively. The activities of Atf-K.l and Atf-P.a reached 0.3 ± 0.2 and $0.4 \pm 0.3 \text{ nmol min}^{-1} \text{ mg}^{-1}$ in *S. cerevisiae*, but only reached 0.02 ± 0.02 and $0.001 \pm 0.001 \text{ nmol min}^{-1} \text{ mg}^{-1}$ in *E. coli*, respectively. Atf-S.l activity in *E. coli* lysate was limited at $0.5 \pm 0.2 \text{ nmol min}^{-1} \text{ mg}^{-1}$; however, in *S. cerevisiae* lysate Atf-S.l exhibited activity of $21 \pm 3 \text{ nmol min}^{-1} \text{ mg}^{-1}$, second only to the activity of Atf1-S.c. Western blot analysis revealed that in all cases AATase expression in *E. coli* was at least 100-fold greater than expression in *S. cerevisiae* (Figure 5.3D). In this context, normalization of activity to *S. cerevisiae* expression levels revealed that AATase activity in *E. coli* whole cell lysates for all AATases studied here is less than or equal to $0.08 \text{ nmol min}^{-1} \text{ mg}^{-1}$ of protein.

The ethyl acetate activities of Atf1-S.c and Atf-S.l when expressed in *S. cerevisiae* were significantly greater than the activities of all other studied orthologs. As such,

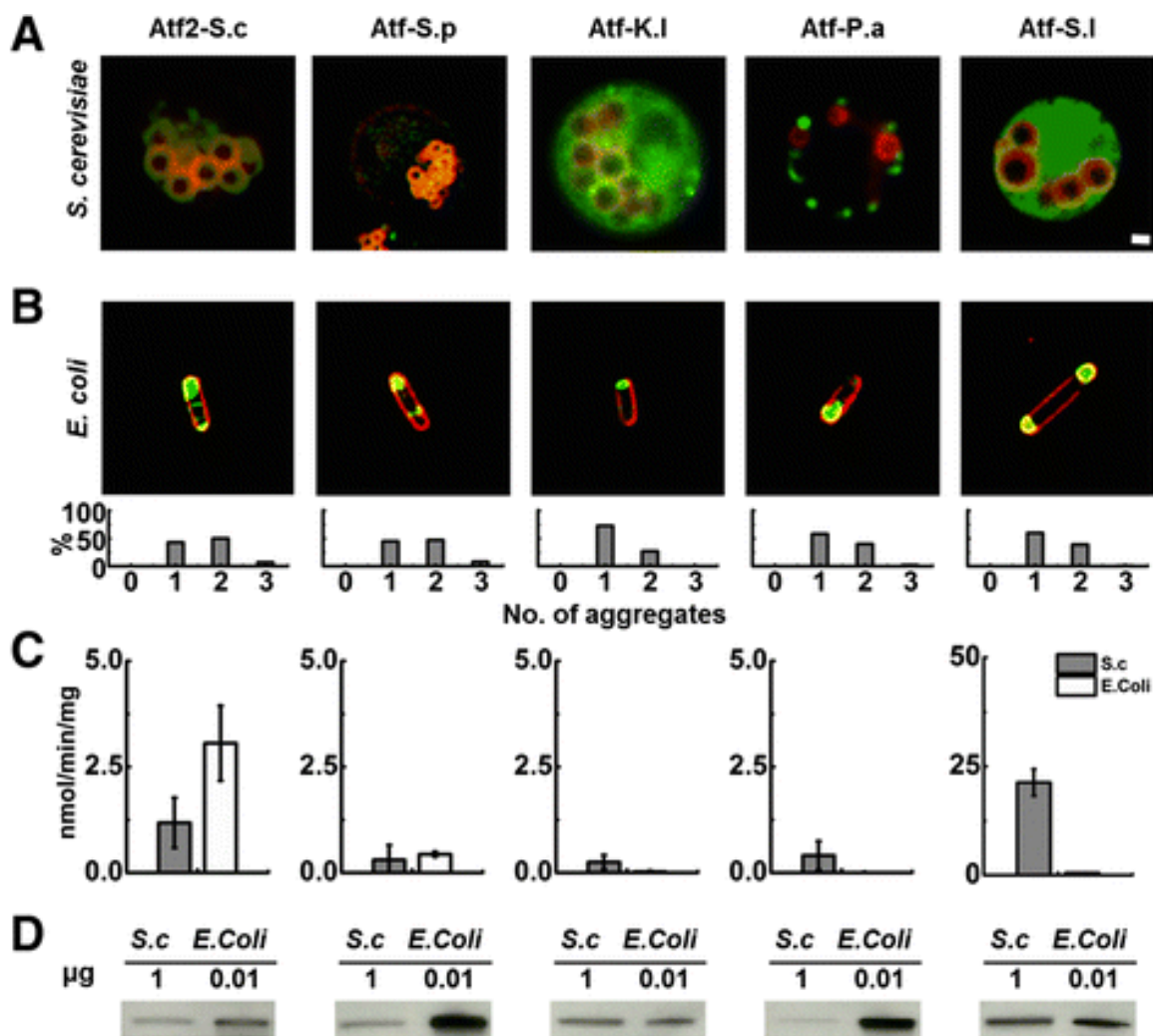


Figure 5.3. Expression, intracellular localization, and activity of AATases. A) Fluorescence microscopy images of *S. cerevisiae* co-expressing AATase-GFP and the LD marker Erg6-DsRed. Expressed AATases include Atf2 from *S. cerevisiae* (Atf2-S.c), and AATases from *S. pastorianus* (Atf-S.p), *K. lactis* (Atf-K.l), *P. anomala* (Atf-P.a), and *S. lycopersicum* (Atf-S.l). Fluorescence from Erg6-DsRed is shown in red and fluorescence from AATase-GFP is shown in green. Overlapping Erg6-DsRed and GFP signals, indicating co-localization, are shown in yellow. Scale bar (1 μ m) applies to A) and B). B) Fluorescent microscopy images of *E. coli* expressing AATases. CellMaskTM Orange plasma membrane staining is shown in red and fluorescence from AATase-GFP is shown in green. Graphs below the fluorescence images indicate the number of aggregates observed in *E. coli* cells. A minimum of 100 cells were counted from three independent experiments. C) In vitro ethyl acetate production from whole cell lysates of AATase-GFP expressed in *S. cerevisiae* (S.c) and *E. coli*. Error bars represent standard deviation (n = 3). D) Western blot analysis of AATase-GFP expression in *S. cerevisiae* and *E. coli*.

subsequent experiments focused on these two enzymes. To determine if the AATase aggregates formed in *E. coli* were insoluble and active, we measured the activity of soluble and insoluble protein fractions after fractionation by centrifugation. Figure 5.4A shows that soluble lysate fractions maintained high activity for both Atf1-S.c and Atf-S.l in both hosts (Atf1-S.c: 45 ± 18 and 10 ± 3 nmol min⁻¹ in *S. cerevisiae* and *E. coli*, respectively; Atf-S.l: 14 ± 4 and 2.2 ± 0.6 nmol min⁻¹ in *S. cerevisiae* and *E. coli*, respectively). Atf1-S.c showed higher activity in the insoluble fractions of both *S. cerevisiae* and *E. coli* (50 ± 17 and 42 ± 6 nmol min⁻¹, respectively). In contrast, the insoluble fractions containing overexpressed Atf-S.l showed measurable, but minimal activity (0.25 ± 0.12 and 1.3 ± 0.6 nmol min⁻¹ mg⁻¹ in *S. cerevisiae* and *E. coli*, respectively). The comparison of activity from *S. cerevisiae* and *E. coli* lysates is not complete without a similar comparison of expression levels in each host (Figure 5.4B). Western blots of *S. cerevisiae* lysates showed that Atf1-S.c separated equally between the soluble and insoluble fractions, but Atf-S.l separated strongly to the insoluble fraction. When expressed in *E. coli*, both Atf1-S.c and Atf-S.l were largely insoluble. A 100-fold dilution of the insoluble fraction of *E. coli* overexpressing Atf1-S.c and Atf-S.l resulted in western blot bands of near equal intensity to the soluble fractions with no dilution.

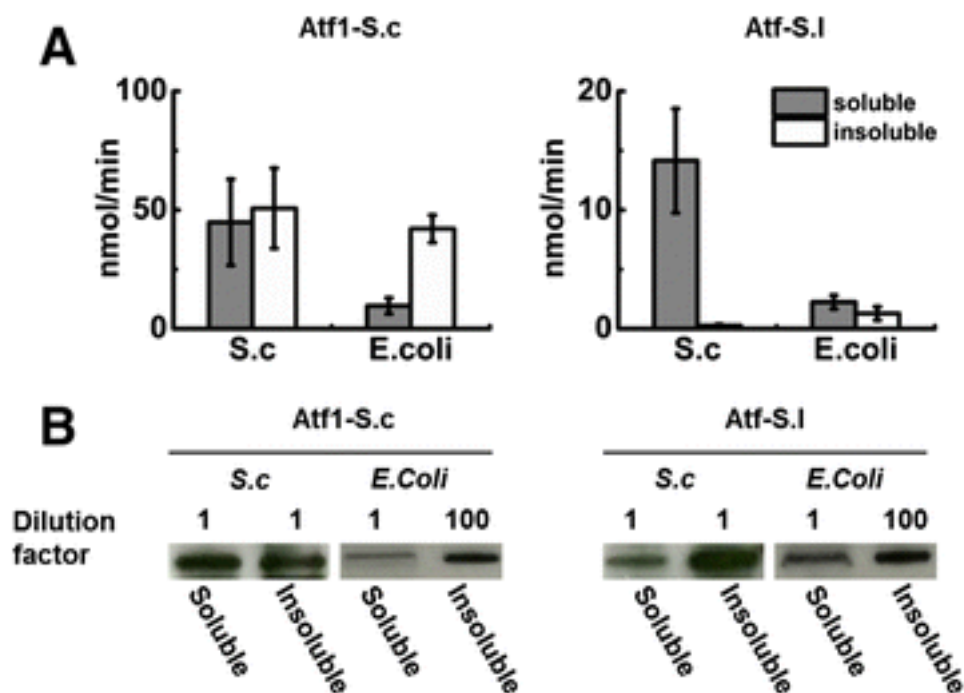


Figure 5.4. Atf1-S.c and Atf-S.l activity in soluble and insoluble cell fractions. A) Comparison of AATase activity in soluble and insoluble fractions from *S. cerevisiae* (S.c) and *E. coli*, Atf1-S.c (left) and Atf-S.l (right). Error bars represent standard deviation (n = 3). B) Western blot analysis of soluble and insoluble fractions of Atf1-S.c and Atf-S.l from *S. cerevisiae* and *E. coli* expression.

5.5 DISCUSSION

A major challenge in engineering metabolic pathways, including ester biosynthesis, is pathway optimization including the alleviation of kinetics bottlenecks and balancing the kinetic capacity of pathway steps. One approach to maximizing flux is pathway engineering to alter the expression levels of each step [28,29]. Enzyme engineering to improve the kinetics of key steps is also critical [30]. The difficulty of these approaches is compounded when native intracellular localization, activity, and expression of key enzymes are effected by heterologous expression. Flux analysis and pathway modeling are also negatively affected because experimentally determined kinetic parameters used

in modeling do not accurately translate to those exhibited *in vivo*. The AATases studied here are prime examples of these problems as heterologous expression in *E. coli* resulted in losses in specific activity and in some cases a loss of intracellular localization.

Homologous expression of Atf1-S.c in *S. cerevisiae* revealed high activity in whole cell lysates and native localization to LDs. Heterologous expression in *E. coli* was approximately 100-fold higher than in *S. cerevisiae*, but whole cell lysate assays revealed significantly reduced specific activity and *in vivo* fluorescence imaging revealed a loss of membrane localization (Figure 5.2). These results were consistent with Atf2-S.c from *S. cerevisiae* and Atf-S.p from *S. pastorianus*. Enzymes localized to LD in *S. cerevisiae*, but membrane localization was not observed in *E. coli* (Figure 5.2A,B). Despite high expression in *E. coli*, specific activities of Atf2-S.c and Atf-S.p in *E. coli* cell lysates were significantly less than observed in *S. cerevisiae* lysates. AATases from *K. lactis*, *S. lycopersicum*, and *P. anomala* did not localized to LD or other membranes when expressed in *S. cerevisiae* and did not localized to membranes in *E. coli*. Again, expression in *E. coli* was high and specific activities measured in whole cell lysates were significantly reduced in comparison with activities measured in *S. cerevisiae* lysates. Previous work investigating the localization of AATases in *S. cerevisiae* has shown that minimal modifications to the C-terminus did not affect the membrane localization of Atf1-S.c, Atf2-S.c, or Atf-S.p and that the C-terminal GRF reporter did not prevent localization [19]. Conserved N- and C-terminal amphipathic helices were identified as necessary for AATase membrane localization, motifs that are lacking in Atf-K.l, -P.a, and -S.l. In this context, it is unlikely that the C-terminal GFP reporter alters membrane

localization; however, this remains as a possible explanation for the lack of AATase localization in *S. cerevisiae*.

A contributing factor to the loss of activity was AATase aggregation in *E. coli*. Fluorescence imaging of *E. coli* cells revealed that in all cases expressed AATase formed one or more cytosolic aggregates (Figure 5.2B, 5.3B). Importantly, the aggregates were functional. We expected that loss of tertiary structure in inclusion bodies would destroy protein function, but in all cases C-terminally fused GFP maintained fluorescence and whole cell lysates exhibited AATase activity suggesting that AATases expressed in *E. coli* were not misfolded [31]. Moreover, analysis of fractionated *E. coli* lysates containing Atf1-S.c or Atf-S.1 demonstrated measurable activity in both the soluble and insoluble fractions (Figure 5.4). Common to the AATase family are short stretches of hydrophobic amino acids that are a possible source of protein aggregation [8,18]. Specific to AATases from *S. cerevisiae* and *S. pastorianus*, the N- and C-terminal amphipathic helices that function as ER and LD membrane anchors may also be a source of aggregation [19]. Regardless of mechanism(s), the results of heterologous expression in *E. coli* were clear, the formation of AATase active aggregates. The loss of activity was most likely due to a combination of diffusion limitations and blocked active sites. It is also possible that activity in aggregates was due to the disruption of active protein conformation, an effect that is also likely decreased with aggregate size. An alternative explanation is that AATase solubility was increased at lower induction levels, an effect that has previously been demonstrated with single chain antibodies expressed in an *E. coli* host [32]. An additional means of decreasing expression and increasing solubility is culturing at lower

temperatures; however, fluorescence microscopy revealed that expression in *E. coli* at 20 and 30°C produced aggregates (SFigure 5.2).

Biochemical studies of Atf1 from *S. cerevisiae* (Atf1-S.c) have suggested that activity is membrane-dependent [6,20-22]. The studies presented here confirm that Atf1-S.c, Atf2-S.c and Atf-S.p localize to LDs in *S. cerevisiae*, but do not localize to membranes in *E. coli*. The loss of membrane localization did not eliminate activity towards ethyl acetate suggesting that these AATases are not strictly membrane dependent with respect to activity (Figures 5.2 and 5.3). Published purification protocols for Atf1-S.c use surfactants or non-ionic detergents and in their absence activity is significantly reduced. In our hands, nickel- affinity chromatography purification of Atf1-S.c resulted in AATase active samples (SFigure 5.3), thus supporting the claim that membrane localization is not necessary for enzymatic activity. This lack of strict membrane dependency provides an explanation as to why metabolic engineering of *E. coli* to synthesize short chain volatile esters has been successful, and our analysis of AATase expression and activity in *S. cerevisiae* and *E. coli* hosts provides important information for future metabolic engineering of ester biosynthesis.

5.6 CONCLUSIONS

Ester biosynthesis is a promising new target for metabolic engineering. The market price of ethyl and butyl acetates are upwards of \$1500 per tonne ([http://www. icis.com/](http://www.icis.com/)), and the total market value for flavor and fragrance compounds is greater than \$16 billions (<http:// www.ialconsultants.com/>). The high volatility of shorter chain esters such as ethyl

acetate is also attractive from a separation perspective, as high volatility facilitates separation from fermentation broths [33]. The results presented here demonstrate that microbial host selection is critical to ethyl acetate biosynthesis through AATase activity. Heterologous AATase expression in *E. coli* resulted in significantly decreased specific activity in comparison to activity measure in *S. cerevisiae*. All studied AATases formed aggregates when expressed in *E. coli* and any membrane localization in *S. cerevisiae* was lost in *E. coli*. One solution to minimizing the loss of activity is reduced expression levels in *E. coli*, which resulted in smaller aggregate size and increased specific activity in comparison to high overexpression. The effects of host selection on AATase expression and activity described here are interesting in that they provide evidence that the AATases studied here are not strictly membrane dependent with respect to activity and are important when considering metabolic engineering strategies for ester biosynthesis.

5.7 ACKNOWLEDGEMENTS

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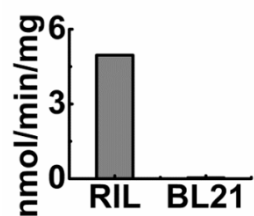
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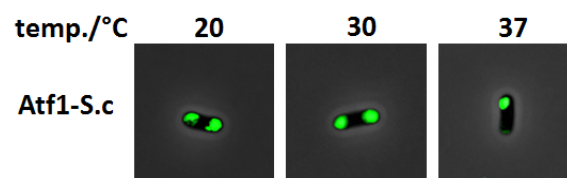
5.9 SUPPLEMENTARY INFORMATION

STable 5.1. AATases activity towards different alcohols and acetyl-CoA. Data shown is mean (n=3). Not detected (n.d.).

Alcohol	Alcohol Acetyltransferase Activity ($\mu\text{mol}/(\text{min}\cdot\text{mg})$)						
	ATF1 (sc)	ATF2 (sc)	ATF1 (sl)	ATF1 (cm)	ATF1 (sp)	ATF1 (pa)	ATF1 (kl)
Methanol	0.0311	n.d.	0.0007	n.d.	n.d.	n.d.	n.d.
Ethanol	0.8795	0.0245	0.2406	n.d.	n.d.	n.d.	0.0040
Propanol	0.0522	0.0049	0.0104	0.0005	0.0006	0.0003	0.0004
Isopropanol	0.0130	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Butanol	0.0143	0.0011	n.d.	n.d.	n.d.	n.d.	n.d.
Isobutanol	0.0067	0.0105	0.0003	n.d.	0.0008	n.d.	n.d.
Pentanol	0.0030	0.0012	0.0011	n.d.	n.d.	0.0006	0.0023
Isopentanol	0.0041	0.0012	0.0004	0.0121	n.d.	n.d.	0.0016



SFigure 5.1. Background ethyl acetate synthesis from *E. coli* strains BL21(DE3)-RIP plus and BL21(DE3). The RIL strain expresses chloramphenicol acetyltransferase, which exhibits AATase activity towards ethanol and acetyl-CoA.



SFigure 5.2. Fluorescent microscopy imaging of *E. coli* expressing Atf1-S.c at culture temperatures (temp.) of 20, 30, and 37 °C.

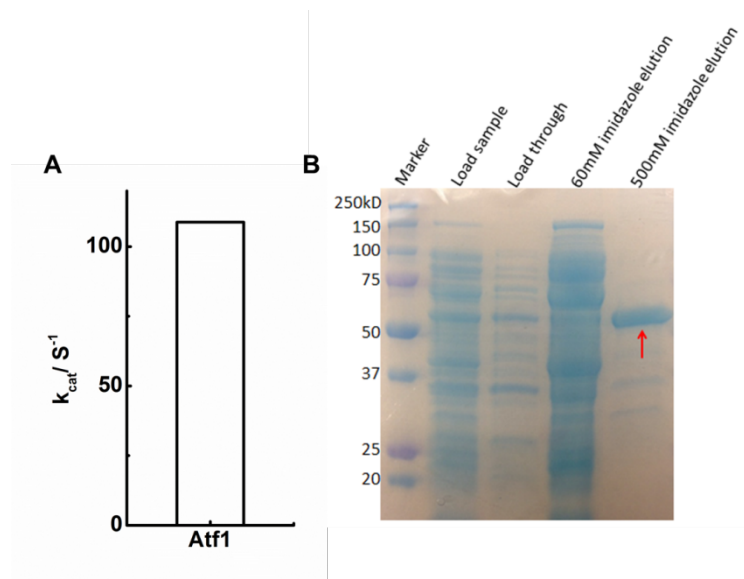


Figure 5.3. A) Specific activity of purified Atf1-S.c with C-terminal his tag. B) SDS-PAGE gel of purified Atf1-S.c. The red arrow points to Atf1.

Chapter 6. Dual N- and C- Terminal Helices Are Required for Endoplasmic Reticulum and Lipid Droplet Association of Alcohol Acetyltransferases in *Saccharomyces cerevisiae*

6.1 ABSTRACT

In the yeast *Saccharomyces cerevisiae* two alcohol acetyltransferases (AATases), Atf1 and Atf2, condense short chain alcohols with CoA to produce volatile acetate esters. Such esters are, in large part, responsible for the distinctive flavors and aromas of fermented beverages including beer, wine, and sake. Atf1 and Atf2 localize to the endoplasmic reticulum (ER) and Atf1 is known to sort from the ER to lipid droplets (LDs). The mechanism and function of these localizations are unknown. Here, we investigate potential mechanisms of Atf1 and Atf2 membrane association. Segments of the N- and C-terminal domains of Atf1 (residues 24-41 and 508-525, respectively) are predicted to be amphipathic helices. Truncations of these helices revealed that the terminal domains are essential for ER and LD association. Moreover, mutations of the basic or hydrophobic residues in the N-terminal helix and hydrophobic residues in the C-terminal helix disrupted ER association and subsequent sorting from the ER to LDs. Similar amphipathic helices are found at both ends of Atf2, enabling ER and LD association. As was the case with Atf1, mutations to the N- and C-terminal helices of Atf2 prevented membrane association. Sequence comparison of the AATases from *Saccharomyces*, non-*Saccharomyces* yeast (*K. lactis* and *P. anomala*) and fruits species (*C. melo* and *S. lycopersicum*) showed that only AATases from *Saccharomyces* evolved terminal

amphipathic helices. Heterologous expression of these orthologs in *S. cerevisiae* revealed that the absence of terminal amphipathic helices eliminates LD association. Combined, the results of this study suggest a common mechanism of membrane association for AATases via dual N- and C-terminal amphipathic helices.

6.2 INTRODUCTION

Alcohol acetyltransferase (AATase; E.C. 2.3.1.84) produces acetate esters through the condensation of an alcohol and acetyl-coenzyme A (CoA). Owing in large part to the aromas and flavors of volatile aliphatic and alicyclic esters the biochemistry of AATase in wine and brewer's yeasts has been extensively studied [1-4]. For example, during fermentations 2-phenyl ethanol and CoA condense to phenyl ethyl acetate to produce a flowery aroma reminiscent of roses. Similarly, synthesized ethyl and isoamyl acetates produce scents of sweet pear and banana, respectively. In *Saccharomyces cerevisiae* these reactions are catalyzed by two AATases, Atf1 and Atf2 [5,6]. Double knockouts of these enzymes eliminated isoamyl ester synthesis and reduced ethyl acetate synthesis by 50% [7]. Single knockouts of the two homologs demonstrated that Atf1 is primarily responsible for acetate ester synthesis, but broad substrate specificities lead to the production of a range of linear and branched esters that add to the complexity of aromas during fermentations [7-9]. With respect to expression, it has been shown that the transcription of *ATF1* is negatively regulated by oxygen and unsaturated fatty acids [10,11] and that fermentations conditions (temperature, nitrogen content and glucose

concentration) can significantly alter Atf activity and the resulting volatile ester profiles [12,13].

While the biochemistry of AATases in *S. cerevisiae* is well studied, comparatively little is known about their structure. There are no crystal structures of Atf from *Saccharomyces* or *non-Saccharomyces* yeast and there are no suitable templates to generate high confidence homology models. Atf1 from *S. cerevisiae* and large Atf1 (lgAtf1) from *S. pastorianus* have high sequence similarity (83% similarity, 77.5% identity), but there is little sequence similarity between Atf1 and Atf2 (48.9% similarity, 34.5% identity). Despite a conserved H-X-X-X-D putative active site, there is even less similarity between Atf1 from *S. cerevisiae* and from Atfs from *non-Saccharomyces* yeast and various fruit species [14-16].

It is known that Atf1 localizes to the endoplasmic reticulum (ER) and to lipid droplets (LDs). Early biochemical studies isolated Atf activity from microsomal cell fractions [4,17] and C-terminal GFP-tagged Atf1 confirmed LD localization by Nile Red co-staining [18]. High throughput screening suggests ER localization of both Atf1 and -2 [19]. Atf2 activity has been isolated in cell fractions that possibly contained LDs and in cell membrane fractions [20]. In addition, fluorescence imaging of GFP-tagged Atf2 and fractionation with the ER marker Wbp1 are evidence of ER localization [21].

LDs are dynamic organelles that serve as neutral lipid storage depots and hubs for lipid metabolism [22]. Across eukaryotes, evidence suggests that the monolayer phospholipid membrane of LDs and their neutral lipid contents emanate from the ER [23,24]. In *S. cerevisiae*, LDs are composed of near equal molar ratios of triacylglycerols (TAGs) and

sterol esters (SE) and proteomic studies reveal that upwards of 40 different proteins can localize to LDs, many of which also localize to the ER [25-27]. A number of LD association mechanisms are known [23]: amphipathic helices can directly bind to the monolayer LD membrane; terminal hydrophobic domains can embed into LDs; and, internal hydrophobic hairpin loops can anchor to the LD membrane. It is also possible that post-translation lipidation provides a lipid anchor and that LD associated proteins provide protein-protein interactions for anchoring. For many LD proteins the mechanism of targeting remains an open question. LD proteins such as perilipins are known to traffic from the cytosol to LD and associate by hairpin loops; however, the mechanisms of sorting proteins from the ER to LD have yet to be determined [28,29].

While there is strong evidence for Atf1 localization to the ER and LDs, the mechanism of this association remains unclear. Atf2 is also membrane-associated and has been shown to localize to the ER; however, the extent of this localization and the possibility of sorting to LDs in a similar manner as Atf1 has not been explored. There are no obvious lipidation sequences within Atf1 or -2, and there are no predicted transmembrane domains [30,31]. Helical domains at the N- and C-termini of both Atf1 and -2 that we predicted to be amphipathic in nature suggest possible membrane-association domains and are the origin of our hypothesis: the N- and/or C-terminal helical domains of Atf1 and -2 may be required for membrane association. Here, we use a series of N- and C-terminal deletions and selected point mutations to test our hypothesis. We demonstrate that the predicted N- and C-terminal AHs of Atf1 are necessary for LD localization and that membrane association originates at the ER. In addition, we discovered that Atf2 also

localizes to LDs, and that the conserved helical structures of Atf2 are also essential for ER and LD localization. The absence of such evolved structures in *non-Saccharomyces* yeast and certain fruit disables membrane association to the ER and LDs in *S. cerevisiae*.

6.3 MATERIAL AND METHODS

Strains and culture conditions

The *S. cerevisiae* strain BY4742 was used in all experiment of this study. Cells harvesting expression plasmids were grown in synthetic minimal (SD) medium containing 0.67% yeast nitrogen base (Becton-Dickinson), amino acid supplements (Sunrise), and 2% glucose. To promote LD synthesis, cells were cultured in oleic acid medium containing 0.67% yeast nitrogen base, 0.1% yeast extract, 0.2% Tween 40, and 0.1% oleic acid according to [32]. Cells were first cultured in SD medium to late log phase prior to washing and subsequent culturing in oleic acid medium for an additional 48 h.

Plasmids

An overexpression cassette with C-terminal GFP tag driven by the constitutive yeast promoter phosphoglycerate kinase 1 (PGK1p) and terminated by PGK1t was constructed. The promoter and terminator were amplified from genomic DNA of BY4742 and cloned into pRS426. PGK1p was inserted using SacI and SacII sites. PGK1t was inserted using SpeI and KpnI sites, while GFP was inserted at NotI and SpeI sites. All *ATF* genes were cloned into PGK1 overexpressing cassette using SacII and NotI sites. *ATF1*, *ATF2* and truncations to *ATF1* were amplified from BY4742 genomic DNA. *ATF1* and -2 variants

with point mutations were designed and synthesized by a gene assembly method [33]. Briefly, the N-terminal 195 base pairs (bp) and C-terminal 201 bp gene fragments with mutations were assembled and fused to the rest gene using overlap extension PCR. *ATF* genes from *S. pastorianus* (AY242066), *S. lycopersicum* (NP_001234496), and *C. melo* (BAB78588) were also assembled by gene assembly and codon optimized for yeast expression. *ATFs* from *K. lactis* (XM_455762), and *P. anomala* (JN701429) were amplified from genomic DNA. pERGmDsRed (a gift from J. Goodman, University of Texas Southwestern Medical School) was used to express an LD marker Erg6 [34]. A cassette consisting DsRed-PGK1t-LEU2 for C-terminal tagging of chromosomal genes was constructed by sequential cloning. DsRed, amplified from pERGmDsRed, was first inserted into pRS426 containing PGK1p-PGK1t at NotI and SpeI sites. LEU2, amplified from pRS315, was subsequently inserted between KpnI and EcoRV sites. This cassette was used to fuse DsRed to the 3' end of the ER marker Sec61 and the LD marker Erg6 by PCR-based homologous recombination as previously described [35].

Oil red staining

Cells grown to stationary phase were washed twice with phosphate buffer saline (PBS), fixed with 3.7% formaldehyde for 45 min, and washed twice more with PBS. Fixed cells were stained with Oil Red O solution (Sigma) for 2 min and washed twice with PBS before fluorescent microscopy imaging. Oil Red O solution was prepared by diluting the stock solution with two volumes of deionized water and filtered with a 0.45 μ m syringe filter.

Fluorescent microscopy and quantification of LD or ER localization.

Cells were observed with an Olympus BX51 microscope (UPlanFL 100X 1.30 oil-immersion objective lens, mercury lamp) and fluorescent images were captured by a Q-Imaging Retiga Exi CCD camera. Images were processed using CellSens Dimension 1.7 software (Olympus). For the quantification of LD localization, cells displaying nascent, premature, and mature LD are considered as LD localized cells. For ER localization, only cells exhibiting ER localization exclusively are scored in a population. For detail classification, please see SFIGURE 6.3.

Subcellular fractionation.

LD isolation was carried out as previously described [36] with slight modifications. Cells were cultivated to stationary phase, harvested, and transformed into spheroplasts by zymolyase 20 T (Seikagaku Corporation, Japan). Spheroplasts were resuspended in LD-A buffer (12% Ficoll, 10 mM MES/Tris pH 6.9, 0.2 mM EDTA, 1mM PMSF) and homogenized with 30 strokes in a Dounce Homogenizer (Sartorius). Cell debris was removed by centrifugation at 6000 rpm for 10 min using a Sorvall SS34 rotor. The collected homogenate was overlaid with buffer LD-A in an Ultra-Clear centrifuge tube (Beckman) and centrifuged at 25,000 rpm for 1 h using a SW-28 swing bucket rotor (Beckman). The supernatant was saved as the cytosolic fraction, and the floating white layer on top (the crude LD fraction) was collected and homogenized gently with 7 strokes in a Dounce Homogenizer. The resulting homogenate was overlaid with buffer LD-B (8% Ficoll, 10 mM MES/Tris pH 6.9, 0.2 mM EDTA, 1 mM PMSF) in a new centrifuge tube and centrifuged at 25,000 rpm for 30 min. The resulting white layer on top was used as the purified LD fraction. Five- μ g, determined by Bradford reagent (Sigma), of each

subcellular fractionation sample was resolved by SDS-PAGE and western blot analysis. To detect proteins, the following antibodies were used: rabbit anti-GFP (Invitrogen), goat anti-rabbit IgG-HRP (Invitrogen), goat anti-DsRed (Santa Cruz Biotech), donkey anti-goat IgG-HRP (Santa Cruz), mouse anti- β actin (mouse), and goat anti-mouse IgG-HRP (Thermo Fisher Scientific).

6.4 RESULTS

N- and C-terminal domains are required for membrane association of Atf1

Deletions to the N- and C-termini of Atf1 were made to investigate the possible role of each terminus in membrane association (Figure 6.1A). Wild type Atf1, and D2-48 and D503-525 truncates tagged with GFP were constitutively overexpressed from a high copy number plasmid under the control of a PGK1 promoter. Cells were co-transformed with a single copy number plasmid expressing the LD marker Erg6. Fluorescent microscopy images of cells overexpressing Atf1-GFP and Erg6-DsRed cultured in oleic acid medium revealed co-localization of GFP and DsRed fluorescence, indicating that Atf1 localized to LDs (Figure 6.1B). Atf1-GFP also localized to LDs at stationary phase when expression was driven by a single copy plasmid (Supporting Information, SFigure 6.1). Deletions of residues 2-48 and 503-525 disrupted LD targeting resulting in dispersed Atf1-GFP fluorescence in the cytosol. These results were further substantiated by culturing cells in glucose medium. Fluorescent imaging of Atf1-GFP showed GFP fluorescence surrounding oil red stained LDs (Figure 6.1C). Corresponding to the results from the

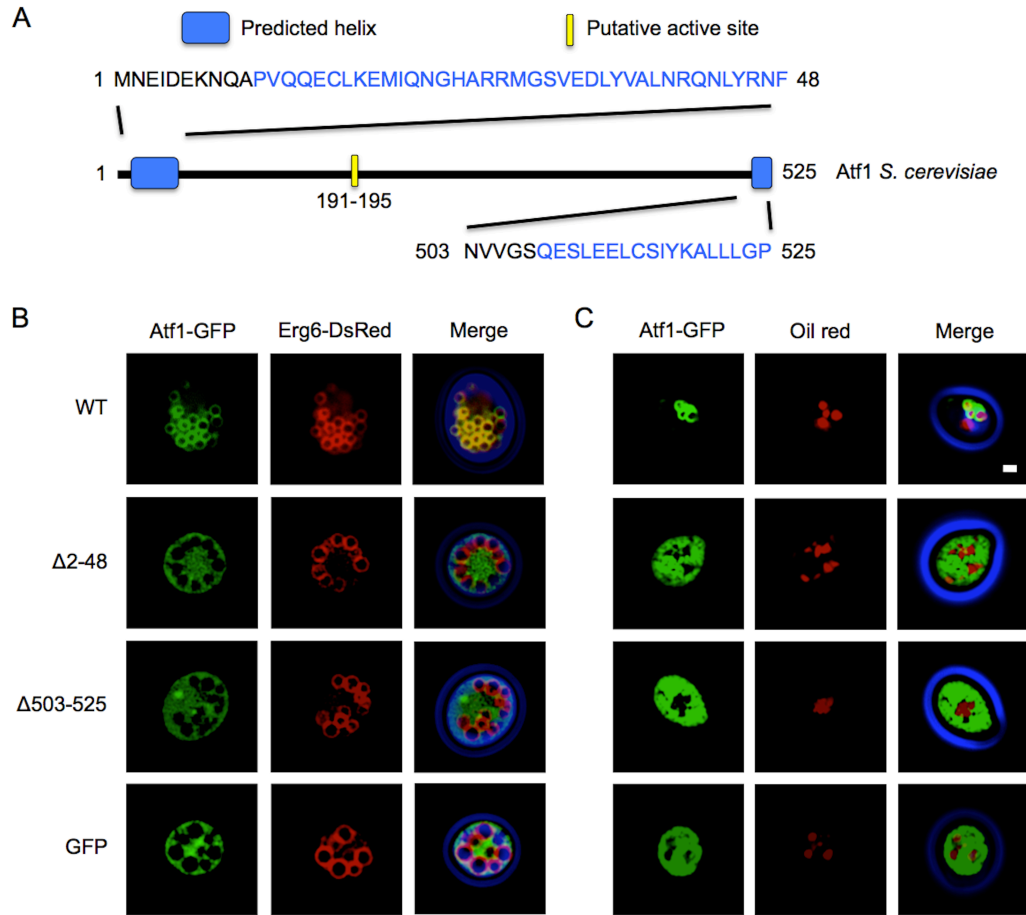


Figure 6.1. The N- and C-terminal domains of Atf1 are necessary for lipid droplet localization. (A) Atf1 from *S. cerevisiae* with the location of the putative active site and predicted N- and C-terminal helices. The amino acid sequence of the terminal helices are shown. (B) Fluorescent microscopy images of selected stationary phase cells cultured in oleic acid media co-expressing Atf1-GFP and the LD marker Erg6-DsRed (Ex. 400650, Em. 500625). Atf1-GFP signal is shown in green and Erg6-DsRed is shown in red. The merged image shows the phase contrast image false colored in blue. Co-localization of green and red is indicated by yellow and demonstrates Atf1-GFP localization to LDs in the wild type (WT) Atf1. A GFP control shows cytosolic localization. (C) Fluorescent images of stationary phase cells cultured in glucose media overexpressing Atf1-GFP with LD oil red staining (red). Localization of WT Atf1-GFP around stained LDs indicates LD localization. Quantification of fluorescence microscopy was performed by counting a minimum of 300 cells from three independent experiments. LD localization was observed in $91 \pm 1\%$ of cells expressing wild type Atf1 and 0% for D2–48, D503–525, and the GFP control. In both oleic acid and glucose media WT Atf1-GFP associates with LDs. N- or C-terminal truncations (D2–48 and D503–525, respectively) result in cytosolic GFP signal indicating a loss of LD localization. Scale bar, 1 μ m.

oleic acid cultures, the N- and C-terminal deletions resulted in cytosolic localization, thus confirming the importance of these two regions in membrane association.

The predicted N-terminal amphipathic helix is required for membrane association of Atf1

To examine the possible molecular mechanism of Atf1 LD and the ER association, we predicted the secondary structure of the N-terminus [37]. The results strongly suggested that residues 11-48 form an α -helix. Analysis of this region by helical wheel projection [38] demonstrated that residues 24-48 were predicted to be an amphipathic helix (AH) (Figure 6.2A,B). The hydrophobic patch comprising Met29, Val32, Leu35, Tyr36, and Leu39 resides on one side of the helix, while polar residues Arg27, Arg41, Glu33, and Asp34 reside on the opposite side. To investigate the importance of the predicted helical structure, deletions including or excluding the predicted helix and point mutations on the AH were made. Deletion of residues 2-9 resulted in localization similar to the wild type, co-localization with Erg6 (Figure 6.2C). Deletion of N-terminal 11-23 attenuated protein localization with a fraction of the D11-23 Atf1-GFP mutant dispersed in cytosol and some co-localized on LDs. Deleting residues 24-48 of the N-terminal AH resulted in Atf1-GFP fluorescence accumulating in the cytosol. These results infer that the AH structure has an important role on LD localization.

To confirm that LD association of Atf1 depends on the amphipathic character of the N-terminal helix (residues 24-48), point mutations were made to disrupt the helix. Basic residues Arg27, Arg28, and Arg41 were all mutated to alanine (Nterm-3A-GFP), resulting in cytosolic localization. Hydrophobic residues Met29, Val32, Tyr36, and Leu39 were mutated to alanine (Nterm-4A-GFP), resulting partial LD localization

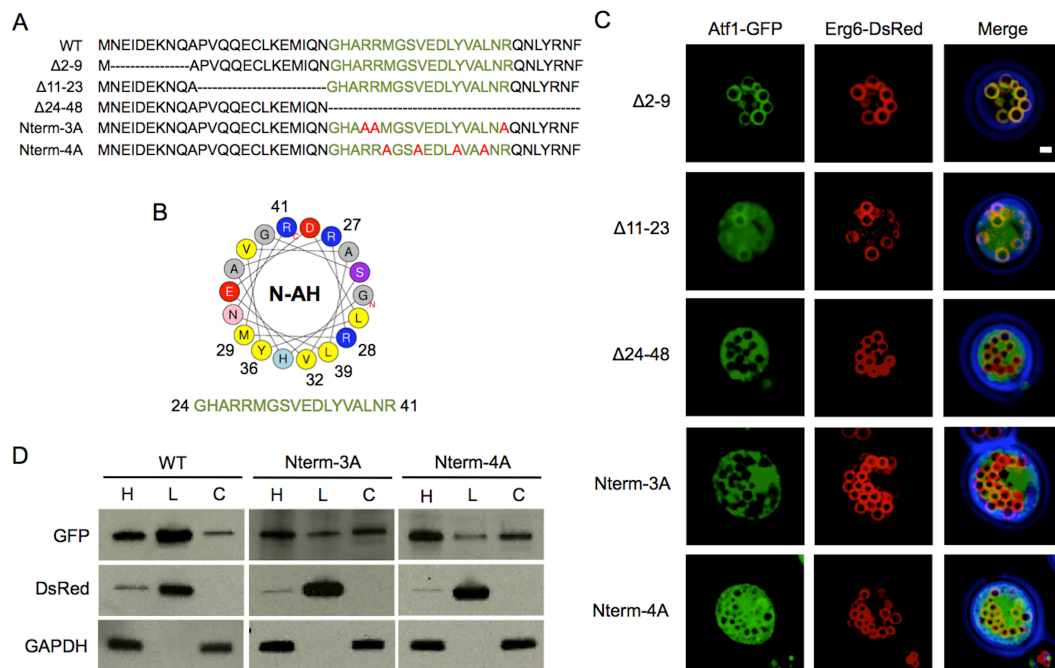


Figure 6.2. The N-terminal amphipathic helix of Atf1 is necessary for LD localization. (A) N-terminal sequences studied. The amino acid sequences of the predicted amphipathic helix are shown in green with point mutations to alanine shown in red. (B) Helical wheel projection of the N-terminal helix. Hydrophobic residues are colored yellow, neutral in gray, basic in blue, and acidic in red. The numbered basic residues were mutated to alanine in Nterm-3A and the numbered hydrophobic residues were mutated to alanine in Nterm-4A. (C) Fluorescent images of stationary phase cells cultured in oleic acid media overexpressing Atf1-GFP, truncates, and mutants with co-expression of the LD marker Erg6-DsRed. Truncation of residues 2–9 has no effect on LD localization while deletion of 11–23 and 24–48 attenuate LD localization. Mutation of residues in Nterm-4A and Nterm3A also attenuate LD localization. Scale bar, 1 μ m. Quantification of fluorescence microscopy was performed by counting a minimum of 300 cells from three independent experiments. LD localization was observed in $89 \pm 4\%$ of D2–9 expressing cells and $25 \pm 8\%$, 0% , 0% , and $4 \pm 1\%$ of cell expressing D11–23, D24–48, Nterm-3A, and Nterm-4A, respectively. (D) Western blot analysis of cell homogenates (H), and LD (L) and cytosolic (C) cell fractions. Erg6-DsRed was used as a LD marker and GAPDH was used as a cytosolic protein marker.

(Figure 6.2C). These results were confirmed by subcellular fractionation (Figure 6.2D).

The majority of wild type Atf1-GFP was detected in LD fraction; however, a small fraction of the protein was also detected in cytosolic fraction, indicating that the cellular localization of Atf1 is not exclusively to LDs. AH disruption in Nterm-3A-GFP resulted a

significantly reduced LD fraction and increased cytosolic fraction. Comparatively, Nterm-4A-GFP resulted in a reduction, but not complete elimination, of LD localization. Combined, these results suggest that the N-terminal AH is necessary but not sufficient for complete Atf1 LD association.

The predicted C-terminal amphipathic helix is required for membrane association of Atf1

Similar to the analysis of the N-terminal sequence of Atf1, C-terminal residues 503-525 were analyzed for secondary structure. Residues 508-525 were predicted as an AH (Figure 6.3A, B). The hydrophobic residues Leu511, Leu514, Cys515, Tyr518, Leu521, and Leu522 reside on one side of the helical wheel, while polar residues Lys519, Glu509, Glu512, and Glu513 reside on the opposite side. To investigate the significance of the AH at the C-terminus, deletions and mutations were made to key hydrophobic and acidic residues of the AH as well as to various parts of the C-terminus (Figure 6.3A). Deletion of the last two residues resulted in no change to LD association as judged by fluorescent microscopy (Figure 6.3C). Deletion to residues 515-525, which includes the hydrophobic residues within the predicted AH, resulted in cytosolic localization. In contrast, deletion of residues 508-513 could still localize the protein on LD. These results suggest that the AH structure at the C-terminus serves an essential role in LD localization.

To confirm that the amphipathic property of C-terminal helix (residues 508-525) is important to membrane association, point mutations were made to eliminate the amphipathic character. The acidic residues Glu509, Glu512, and Glu513 were mutated to alanine (Cterm-3A-GFP). Surprisingly, the mutated proteins were still able to localize to LDs. The discrepancy between deletion of 508-513 and mutations to the acidic residues

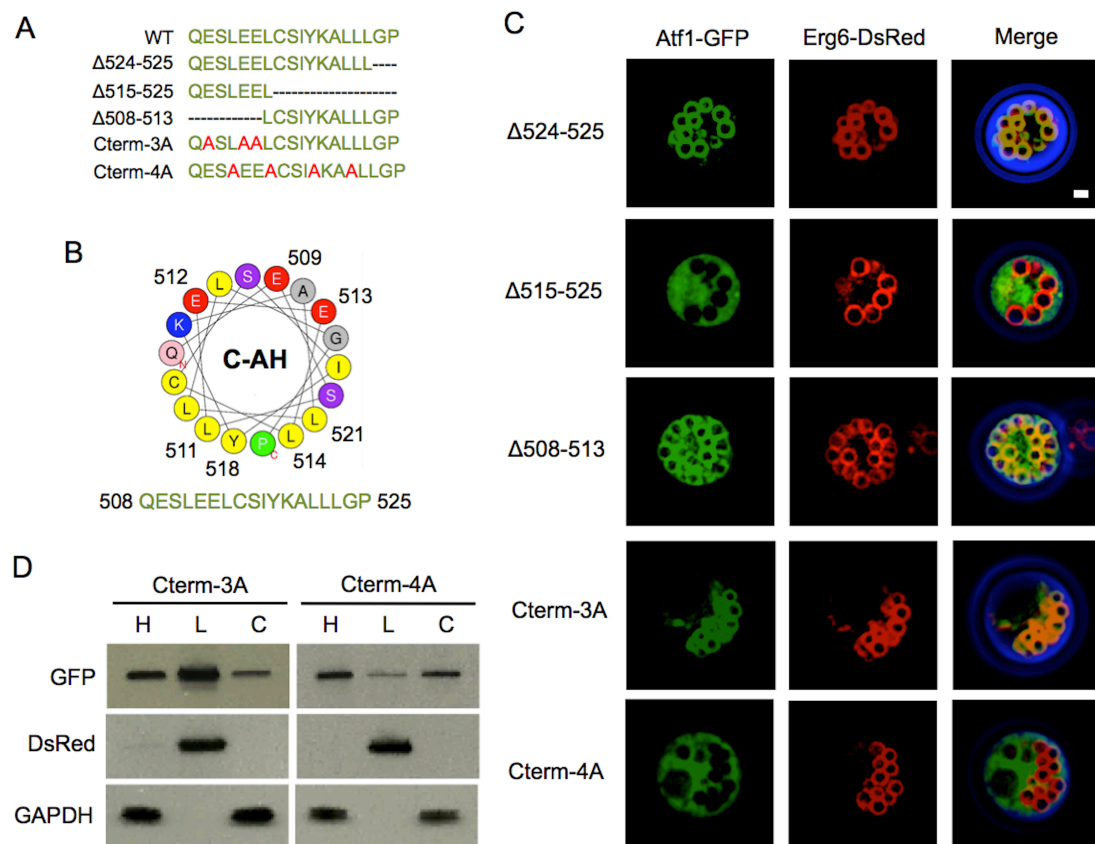


Figure 6.3. The C-terminal amphipathic helix of Atf1 is necessary for LD localization. (A) C-terminal sequences studied. Mutated residues shown in red and the amphipathic sequence shown in green. (B) Helical wheel projection of the C-terminal helix. Hydrophobic residues are colored yellow, neutral in gray, basic in blue, and acidic in red. The numbered basic residues were mutated to alanine in Nterm-3A and the numbered hydrophobic residues were mutated to alanine in Nterm-4A. (C) Fluorescent images of stationary phase cells overexpressing Atf1-GFP, truncates, and mutants with co-expression of the LD marker Erg6-DsRed. Truncation of residues 524–525 has no effect on LD localization while truncation of 515–525 and deletion of 508–513 eliminate LD localization. Mutation of residues in Nterm-4A attenuates LD localization, while Cterm-3A has little effect on LD localization. Scale bar, 1 μ m. Quantification of fluorescence microscopy was performed by counting a minimum of 300 cells from three independent experiments. LD localization was observed in $89 \pm 1\%$ of Δ 524–525 expressing cells, while cells expressing Δ 515–525, Δ 508–513, Cterm-3A, and Cterm-4A showed LD localizations of 0%, $8 \pm 2\%$, $84 \pm 4\%$, and 0%, respectively. (D) Western blot analysis of cell homogenates (H), and LD (L) and cytosolic (C) cell fractions. Erg6-DsRed was used as a LD marker and GAPDH was used as a cytosolic protein marker.

of C-terminal AH may be due to a rotation of the helix resulting from the deletions. We next mutated four hydrophobic residues Leu511, Leu514, Tyr518, and Leu512 into alanine (Cterm-4A-GFP), resulting in a loss of membrane association (Figure 6.3C). To verify the results of Cterm-3A-GFP and Cterm-4A-GFP subcellular fractionation and analysis of the fractions by western blot were performed (Figure 6.3D). Cterm-3A-GFP was found in LD and cytosolic fractions in similar ratios to wild type Atf1-GFP, most protein were distributed in LD fractions and only a small amount of proteins was localized in cytosolic fraction indicating that the acidic residues were not essential for LD localization. In contrast to Cterm-3A-GFP, LD localization of Cterm-4A-GFP was strongly reduced.

N- and C-terminal amphipathic helices are required for membrane association of Atf2.

To determine whether the structural homolog, Atf2, can localize to LDs, we analyzed the sequence of Atf2 in a similar manner as Atf1. Both the N- and C-termini of Atf2 were predicted as AHs (Figure 6.4A) and Atf2-GFP was able to localize to LD (Figure 6.4B). The similarity in terminal sequences and LD localization suggested that Atf2 may share the same LD localization mechanism as Atf1. To explore this possibility polar residues Arg22, Arg23, and Arg36 were mutated into alanine on the predicted N-terminal AH and the hydrophobic residues, Trp518, Phe521, Ile529, and Phe532 were mutated to alanine on the predicted C-terminal AH. Disruption to both the N- and C-terminal AHs resulted in a loss of LD localization as both Atf2-GFP mutants were found to be diffusely expressed in the cytoplasm (Figure 6.4B). These results suggested that the targeting mechanism of LD localization of Atf1 and Atf2 are conserved in *S. cerevisiae*.

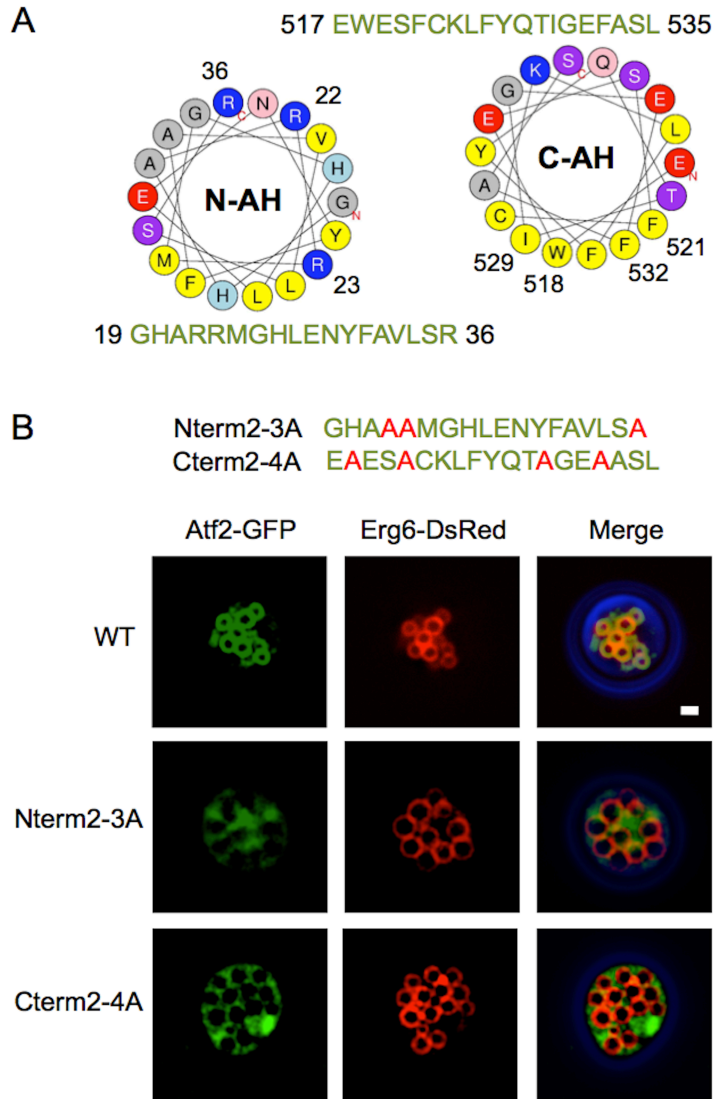


Figure 6.4. Atf2 from *S. cerevisiae* localizes to LDs. (A) Helical wheel projections of the N- and C-terminal domains of Atf2. Hydrophobic residues are colored yellow, neutral in gray, basic in blue, and acidic in red. The numbered basic residues were mutated to alanine in Nterm2–3A and the numbered hydrophobic residues were mutated to alanine in Cterm2–4A. (B) Fluorescent images of stationary phase cells overexpressing Atf2-GFP and mutants with co-expression of the LD marker Erg6-DsRed. Mutation of the basic residues in the N-terminal amphipathic helix (Nterm2–3A) and the hydrophobic residues in the C-terminal amphipathic helix (Cterm2–4A) eliminates LD localization. Scale bar, 1 μ m. Quantification of fluorescence microscopy was performed by counting a minimum of 300 cells from three independent experiments. LD localization was observed in $80 \pm 3\%$, 0%, and 0% of cells expressing Atf2-GFP, Nterm2–3A, and Cterm2–4A, respectively.

To rule out the possibility that mutations to Atf2 produced mis-folded or truncated protein, we analyzed expression by western blot. There was no apparent degradation of overexpressed Atf2 or its mutants, suggesting that the fusions of Atf2 and GFP are well-folded and not susceptible to proteolysis (SFigure 6.2). Similar results were found with wild type and mutant Atf1. In addition, fluorescence images of Atf1, -2 and their corresponding mutants did not exhibit significant aggregation, suggesting that fusion proteins are well folded.

N- and C-terminal amphipathic helices of Atf1 and -2 are necessary for ER association prior to LD sorting

Our results thus far (Figures 6.1–4) demonstrate the necessity of terminal AH domains for LD targeting of Atf1- and 22, but they do not indicate a trafficking mechanism. Figure 6.5 shows a time course study of the intracellular localization of Atf1 and 22 at time points of 0, 4, 10, 18, and 24 hours. For the initial time point (0 hours), cells were grown to stationary phase prior to dilution in sterile media. In lag and early log phases (4 and 10 hours, respectively), 80% GFP tagged Atf1 and 22 co-localized with the DsRed tagged ER marker Sec61 (Figure 6.5B). Atf1 and 22 were observed to shift from the ER to LDs as cell progressed from late log phase to stationary phase, suggesting that the transport is growth dependent (Figure 6.5B and SFigure 6.3). In addition, the formation of nascent and premature LDs observed by GFP tagged Atf1 and 22 suggests the process is ER associated, consistent with current models of LD formation [22–24]. Disruption of either terminal AH in Atf1 and 22 (Nterm-3A, Cterm-4A, Nterm2-3A, and Cterm2-4A, respectively) prevented association with the ER in log phase, thus resulting in

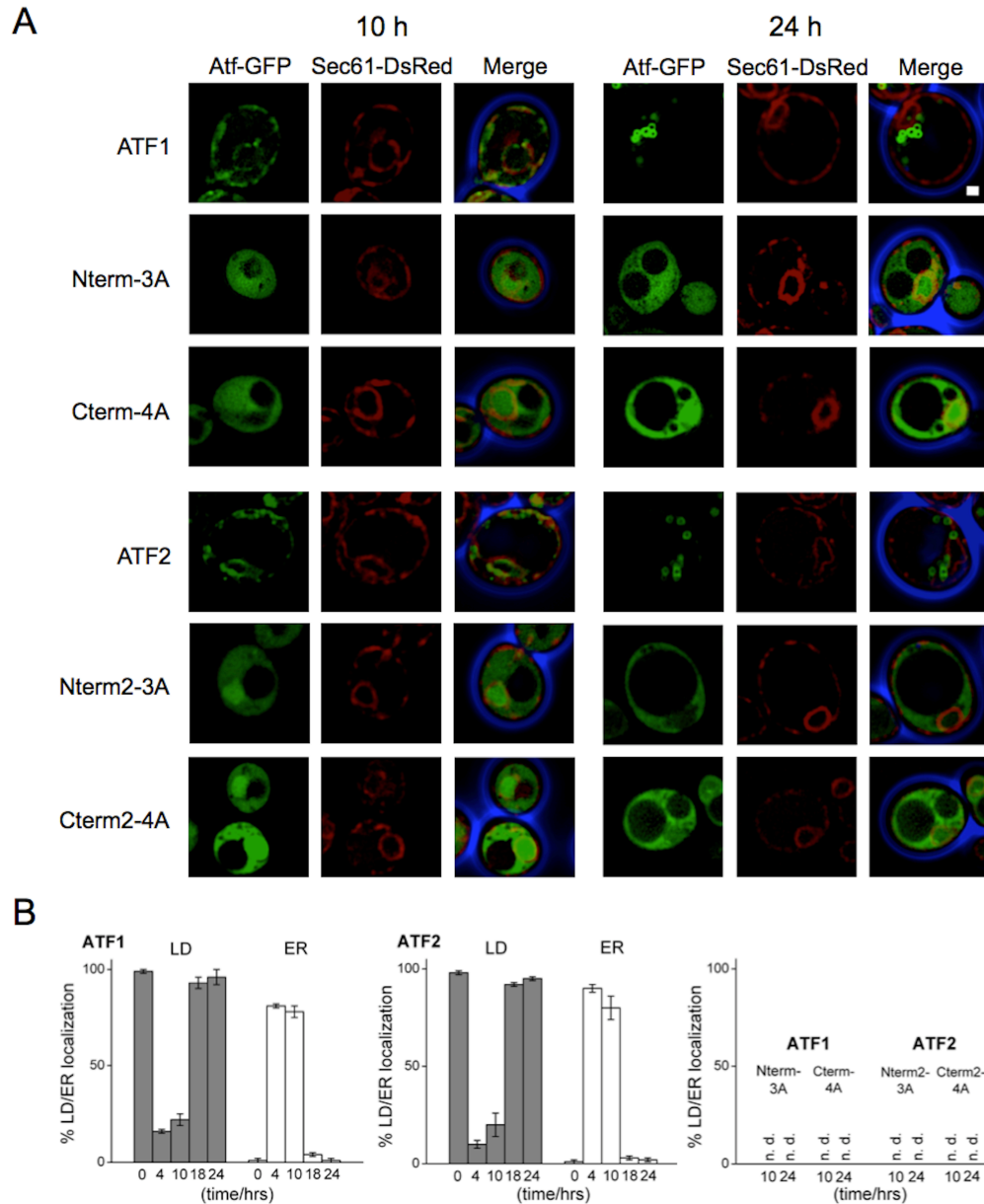


Figure 6.5. Atf1 and Atf2 from *S. cerevisiae* localize to LDs via the ER. (A) Cells overexpressing wild type and mutant Atf1 and 22 were cultured in synthetic uracil dropout media with 2% glucose to log phase and stationary phase and analyzed by fluorescence microscopy. The ER is identified by chromosomal tagging of the ER marker Sec61 with DsRed. Wild type Atf1 and 22 associate with the ER during log phase and preferentially sort to LDs during stationary phase. Disruption of the N- or C-terminal amphipathic helices in both Atf1 and 22 prevents ER association and subsequent localization to LDs. Scale bar, 1 μ m. (B) Quantification of LD and ER localization at different time points. Data are plotted as mean $\pm$ SD. A minimum of 300 cells were counted from three independent experiments.

localization to the cytosol. Localization to the ER or LDs is not recovered in stationary phase, suggesting that the inability of the mutants to localize to LDs is a consequence of disrupted ER targeting.

Terminal AH domains of Atf1 are necessary but not sufficient for ER and LD localization

While the N- and C-terminal AHs of Atf1 are responsible for ER targeting and eventual sorting to LDs, fusion of one or both helices to GFP fails to produce the same function (Figure 6.6). N-terminal fusion of residues 1-48 and, separately, C-terminal fusion of residues 503-525 to GFP results in cytosolic expression. Similarly, N-terminal fusion of both helices (1-48 and 503-525) and N- and C-terminal fusions of 1-48 and 503-525, respectively, resulted in cytosolic expression. All fusions of one or more terminal AH domains from Atf1 to GFP were dispersed in cytosol, thus indicating that these fragments are not sufficient for ER and LD targeting.

*N- and C-terminal amphipathic helices are essential for membrane association of Atfs in *S. cerevisiae**

Several AATases from yeast and fruit species have been identified and enzymatic activity towards short linear and branched chain alcohols has been demonstrated. A bioinformatic analysis comparing fungal orthologs of Atf1 and Atf2 shows a conserved putative active site, H-X-X-X-D (Figure 6.7A) [14]. Using the active site as a common point of alignment, the sequences of Atf orthologs from *Saccharomyces pastorianus*, the non-*Saccharomyces* yeasts *Kluyveromyces lactis* and *Pichia anomala*, and the fruit species *Solanum lycopersicum* and *Cucumis melo* were compared with Atf1 and -2. From

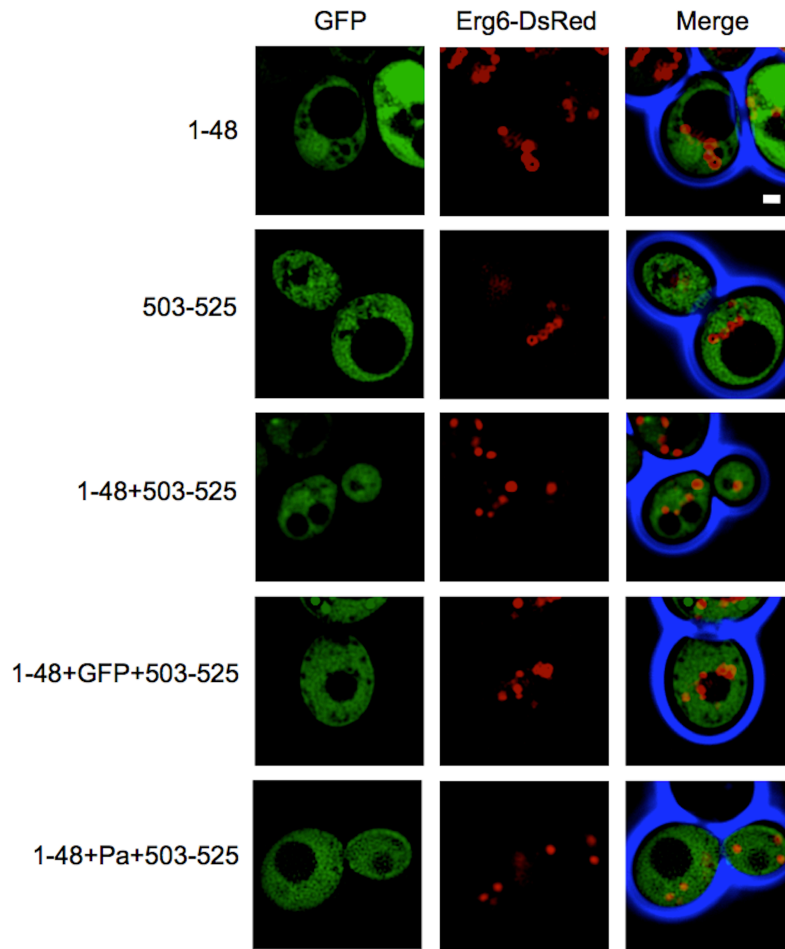


Figure 6.6. The N- and C-terminal amphipathic helices of Atf1 are not sufficient to localize GFP to LDs. Fluorescence images of stationary phase cells overexpressing Atf1 amphipathic helix-GFP fusions, including N-terminal (1–48), C-terminal (503–525), both N- and C-terminal helices fused to the N-terminus of GFP, and 1–48 fused to the N-terminus of GFP with 503–525 fused to the C-terminus of GFP. Fusion of N- and C-terminal helices of Atf1 (*S. cerevisiae*) to Atf from *P. anomala* is also shown. Lipid droplets are identified with chromosomally tagged Erg6-DsRed. Scale bar, 1 μ m. Quantification of fluorescence microscopy was performed by counting a minimum of 300 cells from three independent experiments. No significant localization to LDs was observed (0% for all protein constructs).

the alignment it was apparent that Atf orthologs differ, among other things, in total length (by as many as 98 amino acids) and differ in the length of the terminal domains. Atf1 from *S. pastorianus* (Atf1-Sp) is 545 amino acids in length and is predicted to have

conserved amphipathic structures at the termini. Atf from *K. lactis* (Atf-Kl) is similar in length to Atf1 from *S. cerevisiae* but the predicted terminal amphipathic helices are missing. Atfs from *P. anomala*, *S. lycopersicum*, and *C. melo* (Atf-Pa, -Sl, and -Cm, respectively) are shorter in length and also do not have predicted AHs at the termini. To examine whether the presence of terminal AHs can localize enzymes to LD, we heterologously expressed these Atfs in *S. cerevisiae*. Similar to Atf1-GFP and Atf2-GFP from *S. cerevisiae*, Atf1-Sp-GFP co-localized with Erg6-DsRed (Figure 6.7B). In contrast, the absence of terminal AHs disabled enzyme localization to LDs: Atf-Kl-GFP was unevenly distributed in cytoplasm; Atf-Pa-GFP tended to form punctates in cytoplasm; Atf-Sl-GFP was evenly dispersed in cytoplasm; and, Atf-Cm-GFP was prone to aggregate in cytoplasm. These results support the notion that terminal AHs are important for Atf LD localization *S. cerevisiae*.

To understand how these AHs evolved, a phylogenetic tree was constructed (Figure 6.7C). The selected Atfs segregated into two distinct evolutionary groups—yeast and fruits. The terminal AHs appeared to have developed along the *Saccharomyces* branch of the yeast Atfs. While similar helices and the corresponding function of LD localization did not develop in non-*Saccharomyces* yeast or in fruit species.

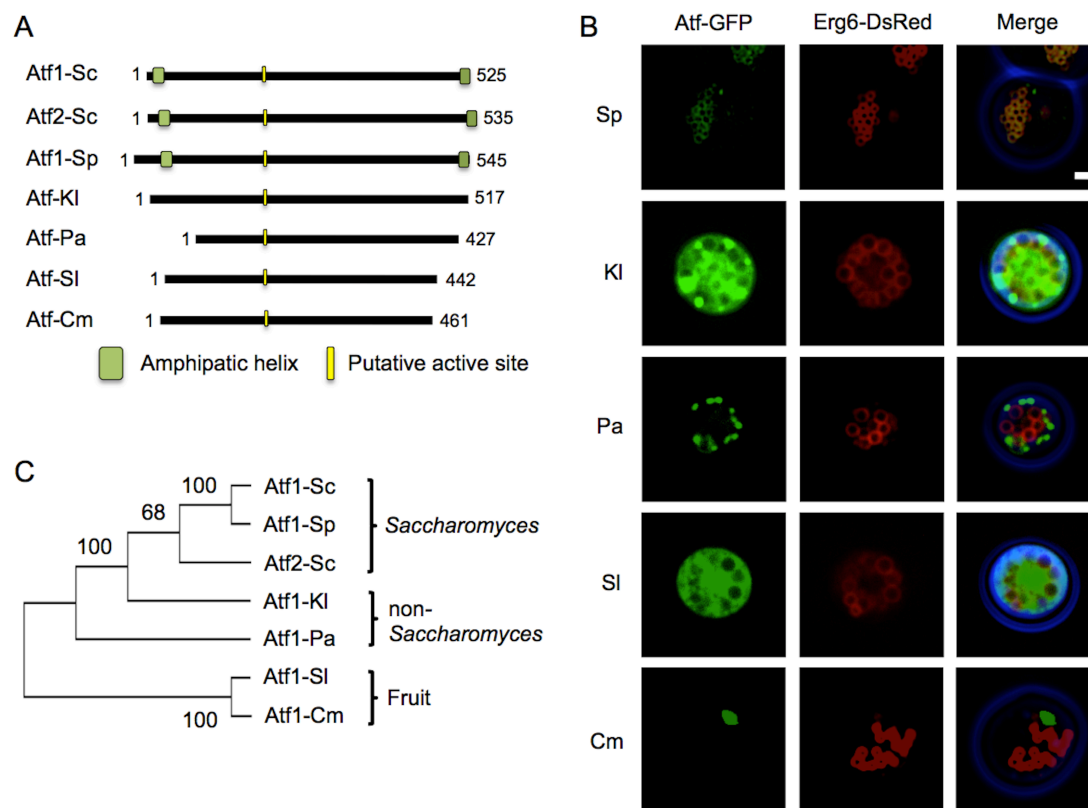


Figure 6.7. N- and C-terminal amphipathic helices are necessary for LD localization of Atfs. (A) Schematic representation of the amino acid sequences of Atf orthologs from *S. cerevisiae* (Sc) *S. pastorianus* (Sp), the non-*Saccharomyces* yeast *Kluyveromyces lactis* (Kl) and *Pichia anomala* (Pa), and the fruit *Solanum lycopersicum* (Sl) and *Cucumis melo* (Cm). Predicted amphipathic helices and the putative active site (HXXXD) are shown in green and yellow, respectively. The length of the primary sequence is given at the end of the schematic for each enzyme. (B) Fluorescent images of stationary phase cells co-expressing Atf-GFP and the LD marker Erg6-DsRed. Atf1 from the *Saccharomyces* yeast *S. pastorianus* localizes to LDs, while orthologs that do not have predicted amphipathic helices at the N- and C-termini, including *K. lactis*, *P. anomala*, *S. lycopersicum*, and *C. melo* do not localize to LDs. Scale bar, 1 μ m. (C) Phylogenetic tree of the studied Atfs. Amino acid sequences were obtained from National Center for Biotechnology Information and aligned by Clustal W [57]. Phylogenetic analysis was implemented in Mega 5.2.2, and trees were constructed by neighbor-joining algorithm [58]. Bootstrap values are displayed at the nodes.

6.5 DISCUSSION

Atf1 and Atf2 from *S. cerevisiae* have been the focus of considerable research from fermented beverage industries and from metabolic engineers. Brewers and wine makers are interested in Atf activity and its role in creating aromas and flavors in their products [4,7-9], while metabolic engineers have goals of industrial production of short chain esters [39-43]. The biochemistry of Atf2 has also been investigated for its ability to produce sterol esters [20] and its role in the export of sterols and steroids [21]. Here, we explored the intracellular localization of these enzymes and formulate a potential mechanism for such spatial organization. We demonstrated that membrane association of Atf1 requires dual amphipathic helices at the enzyme's termini (Figures 6.1, 6.2 and 6.3). The necessity of the N- and C-terminal amphipathic helices for membrane association is also demonstrated in the Atf2 homolog (Figure 6.4). In both cases, disruption of the terminal helices prevents association with the ER, thereby eliminating LD localization (Figure 6.5). Finally, a comparison of seven Atfs from yeasts and fruits revealed this mechanism of LD localization is common to *S. pastorianus* and that only those Atfs with dual amphipathic helices localize to LDs (Figure 6.7).

Deletion studies reveal that N-terminal residues 2-48 and C-terminal residues 503-525 of Atf1 are necessary for LD localization (Figure 6.1). We predicted that sections of these terminal sequences form AHs and our data demonstrates their involvement in LD and ER membrane association. The absence of multiple proline residues within these sequences suggests that it is highly unlikely that either termini form hairpin loops, a motif that is known to mediate membrane association [44]. In addition, it is unlikely that Atf1

associates with LD or ER membranes via a lipid anchor. It has been reported that Rab18 may target LDs with a prenylated lipid anchor conjugated to a CaaX motif at the C-terminus [45,46], where C is cysteine, “a” are aliphatic residues, and X is any amino acid. Sequence analysis of C-terminal AH revealed no such motif in Atf1. The possibility that a protein mediates membrane association remains to be investigated; however, our deletions and point mutations to Atf1 that remove or disrupt AHs at the termini suggest a mechanism of AH to membrane association (Figures 6.1-6.5). Fusions of Atf1 AHs to GFP are not able to localize the fluorescent protein to LDs or the ER (Figure 6.6); therefore, we proposed that the terminal AHs of Atf1 are necessary but not sufficient for ER dependent, LD localization.

AH binding to phospholipid membranes depends on interactions between hydrophobic residues and the lipid membrane as well as electrostatic interactions between basic residues and negatively-charged phosphate groups of the phospholipid layer [47]. Our results show that amphipathic character of the N- and C-termini of Atf1 and -2 are necessary for LD localization and association with the ER. Mutation of the hydrophobic residues of each AH disrupts localization (Figure 6.2, 6.3, 6.4 and 6.5). These results are consistent with previously identified AH-dependant LD proteins including the antiviral protein, viperin, where reduced localization was observed with mutation to the hydrophobic residues of an N-terminal AH [48]. Similar to our results with the C-terminal AH in Atf1, mutations to acidic residues in the viperin AH did not influence membrane binding, indicating that the acidic residues are dispensable (Figure 6.3C).

One significant difference between Atf1, Atf2, viperin and other LD proteins that associate by an AH mechanism is the necessity of dual AH domains. In many cases the identified LD localizing AH is sufficient to target GFP to LDs [48-50]. In the case of Atf1 and -2, disruption of one of the two helices attenuates localization. Similar type behavior is observed in a Hepatitis C virus (HCV) core protein where two AHs connected by a hydrophobic loop are needed for LD localization [51]. Moreover, fusions of Atf1 AHs to GFP do not localize to the ER or LDs, leading to the conclusion that N- and C-terminal AHs of Atf1 are necessary but not sufficient for LD targeting. A similar conclusion has been drawn for murine diacylglycerol acyltransferase-2 (DGAT2), a transmembrane protein transiently localized to LDs [52]. Two putative LD localization domain, a proline knot motif and an 18 amino acid hydrophobic region, are necessary for sorting from the ER to LDs, but fusion to a fluorescent protein demonstrates that the domains are not sufficient for LD localization. In the case of Atf1, it is possible that one or more hydrophobic patches within the protein may also be involved with ER association and subsequent sorting to LDs.

It has previously been reported that Atf2 localizes to the ER during exponential growth [21]. It has also been reported that 85% of Atf2 activity from *S. cerevisiae* purifies from lysates with cell fractions found in the supernatant of 25,000 rpm centrifugations [20]. While it was not explicitly shown, these fractions likely contain LDs [53,54]. These two reports and our data demonstrating Atf2 LD localization are mutually supportive. The prevailing model of LD genesis is one of LDs budding from the ER with LD proteins originating on the cytosolic side of the ER [23]. Both Atf1 and -2 exhibit this behavior,

associating with the ER during exponential phase growth sorting to LDs as they bud from the ER in stationary phase (Figure 6.5).

In reporting Atf2 localization to the ER, Tiwari et al. [21] showed that the N- and C-termini of Atf2 were resistant to proteinase K digestion, suggesting that these two regions are protected in the ER lumen. Our results show that the terminal AHs are necessary for the membrane association of Atf2 and -1 through a putative mechanism of AHs binding to the ER and LDs (Figure 6.5). This is consistent with the proteinase protection result as the AH domains are partially embedded in the membrane and protected from proteinase activity.

Our data and a previous report [18] clearly demonstrate Atf1 LD localization, and for the first time we demonstrate that Atf2 also localizes to LDs in *S. cerevisiae*. The biological function of this localization remains unclear. One possible explanation for Atf2 localization is its activity towards acetylation of sterols [20,21]. Steryl ester hydrolases that release sterols from LDs also localize the LD membrane [55], thus presenting the possibility of transient metabolon formation between Atf2 and steryl ester hydrolases. In such a case, released sterols would be locally accessible for acetylation by Atf2. It is also possible that transient metabolons form with Atf1. It has been suggested that biological role of Atf1 is the regeneration CoA without concomitant production of toxic acids [4,56]. The regenerated CoA would then be locally accessible to LD localized acyl-CoA synthetases that activate free fatty acids mobilized from LD TAGs [25]. Studies exploring possible protein-protein interactions between Atf2 and LD bound hydrolases or

Atf1 and LD bound acyl-CoA synthetases have not yet been conducted and represent an interesting new avenue of research.

6.6 ACKNOWLEDGEMENTS

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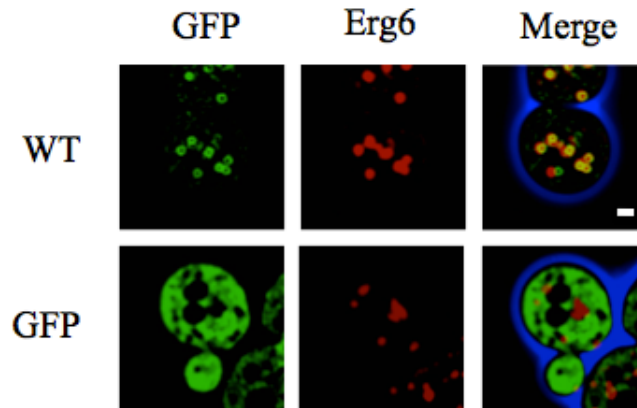
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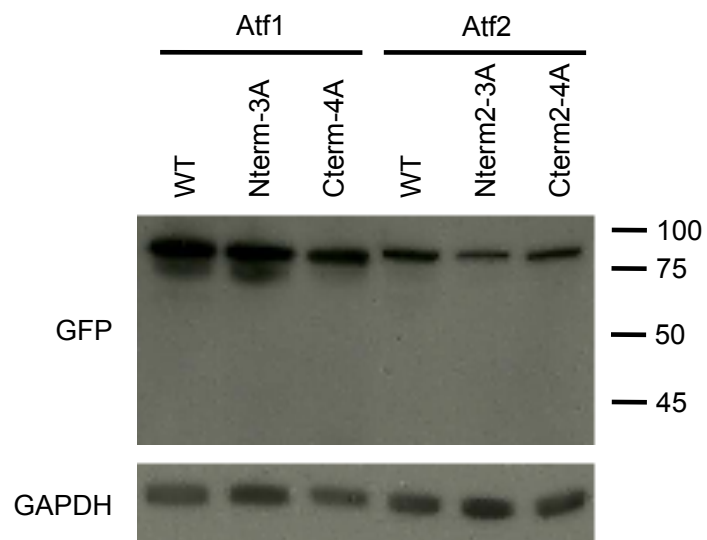
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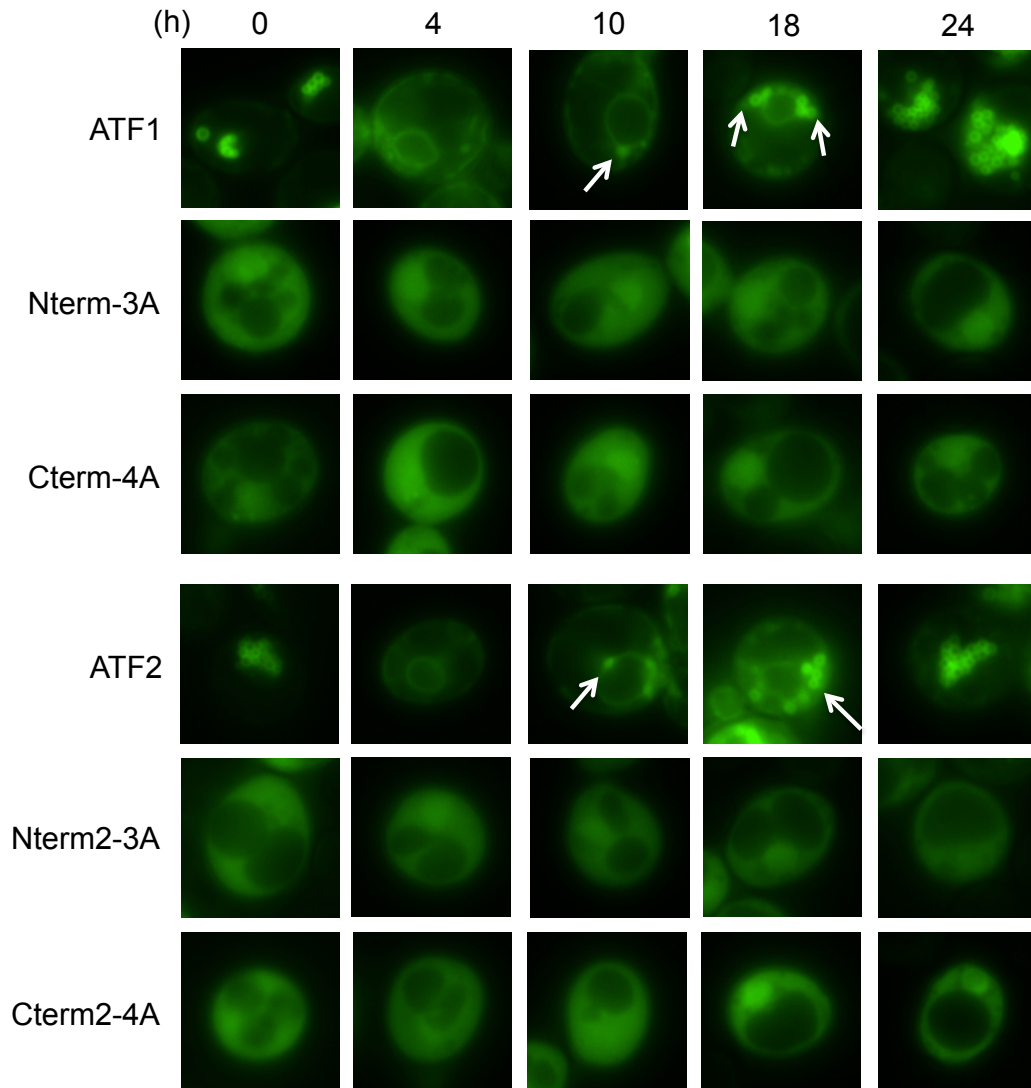
6.8 SUPPLEMENTARY INFORMATION



SFigure 6.1. Fluorescence microscopy analysis of cellular localization of Atf1p under low expression conditions. Protein expression was driven by PGK1 promoter in the single copy number plasmid CEN/ARS. Erg6 is the lipid droplet marker and the fluorescence signal is from DsRed tagged to C-terminal of Erg6 on the chromosome. The cells were grown on 2% glucose and cultured to stationary phase. Scale bar 1 μ m.



SFigure 6.2. Western blot analysis of Atf1, -2 and their mutants. All enzymes are C-terminally tagged with GFP.



SFigure 6.3. Time course study of cellular localization of Atf1, Atf2, and their mutants. Cells were first grown to stationary phase followed by dilution in fresh media (t = 0 hrs.). Cells were harvested and analyzed at different time points (t = 4, 10, 18, and 24 hrs.). Nascent and premature LDs are indicated by white arrows.

Chapter 7. Relocalization of ester biosynthesis to lipid droplet membranes in yeast improve pathway flux

7.1 ABSTRACT

Substrate channeling effect benefited from enzyme co-localization or clustering in intracellular space has been a goal that bioengineers strive to achieve. Here, we developed synthetic membrane scaffolds that functionally co-localize two upstream enzymes from the cytoplasm to the ultimate enzyme on lipid droplets for ethyl acetate biosynthesis in *S.cerevisiae*. To prove co-localization effect, we demonstrated 1.9-fold kinetic enhancement of the co-localized pathway *in vitro* when the overall catalysis from the last two reactions was evaluated. A combinatorial library aiming to tune gene expression and enzyme stoichiometry on scaffolds was created for pathway optimization. Aided by the developed colorimetric assay, the library was rapidly screened and top five productions were further confirmed by gas chromatography analysis. With these strategies, we improved 83.0- and 2.0-fold in yields of ethyl acetate production as compare to wild type and the unstructured pathway, respectively, resulting in a titer of 51.6 mg/L. This work opens up new strategies for pathway engineering and chemical screening.

7.2 INTRODUCTOIN

Metabolic engineering holds great promise to enable a renewable production of chemicals, fuels, and pharmaceuticals [1-3]. To produce target molecules with microbial

hosts, a series of consecutive reactions are often required to convert feedstocks to end products. When constructing metabolic or synthetic pathways in these microorganisms, discontinued enzymatic reactions imparted by spatial fractionation of enzymes in cellular compartments may lead to undesirable effects, including accumulation of toxic intermediates, competition of diffused substrates with side pathways, and consequently, compromised yield. Engineering substrate or metabolic channeling that overcomes this challenge has become an attractive route to the field of metabolic engineering. Upon co-localizing or clustering enzymes, owing to high local enzyme concentrations, the probability of intermediate being processed by subsequent enzyme is increased, thereby preventing intermediate diffusion and resulting in improvement of pathway yield [4].

Various synthetic biology tools or strategies that aim to co-localize or compartmentalize enzymes for the channeling effect have been developed, and the pathway yields were improved upon enzyme co-localization. For examples, mevalonate, hydrogen, and resveratrol titers have been improved in engineered *E. coli* when pathway enzymes were co-localized via protein [5], RNA [6], and DNA [7] scaffolds, respectively. An improvement of isobutanol synthesis was also obtained in engineered *S. cerevisiae* as cytoplasmic enzymes of the pathway were compartmentalized in mitochondria [8]. Despite the success described above, the strategy co-localizing cytoplasmic enzymes to membrane associated enzymes on organelles for efficient chemical biosyntheses has yet to be demonstrated.

Numerous enzymes involved in metabolic pathways are membrane bound. Alcohol-O-acetyl/acyltransferase (Atf1, Atf2, and Eht1) responsible for short- and medium- chain ester syntheses in *S. cerevisiae* are found to be the endoplasmic reticulum (ER) or lipid droplet (LD) associated [9,10]; additionally, acetyl-CoA carboxylase (Acc1) participated in fatty acid synthesis was also reported on the ER membrane [11]. Strictosidine b-D-glucosidase (SGD) [12], cytochrome P450 monooxygenase [13], DOPA oxidase (Dueber) [14], and salutaridine synthase (SalSyn) [15] involved in alkaloid biosynthetic pathways in plant were suggested a localization of the ER. Cyclosporin synthetase synthesizing immunosuppressive cyclosporin in *T. inflatum* was discovered as a vacuolar membrane protein [16].

Ethyl acetate, a volatile ester, is widely used as industrial solvent to manufacture inks and adhesives. Because of the fruity flavor, it is included as food additive in beverages and as fragrance in perfume. Biological synthesis of ethyl acetate can be accomplished by an enzymatic reaction catalyzed by alcohol-O-acetyltransferase (AATase) with condensation of acetyl-CoA and ethanol, and leaving a side production of CoA. A screening of enzyme activity of six AATases from yeasts and fruits reveals that the most active AATase for synthesis of ethyl acetate is the endogenous Atf1 in *S. cerevisiae* [17]. Cytological studies of Atf1 indicate that subcellular localization of the enzyme is growth phase dependent, originated from the ER at lag and log phase and transported to lipid droplets (LDs) at stationary phase [9]. By contrast, aldehyde dehydrogenase (Ald6) and acetyl-CoA synthetase (Acs1) converting acetaldehyde to acetyl-CoA are naturally segregated to the cytosol or mitochondria [18-20] (**Fig. 7.1a**). Although synthetic protein

scaffolds may be applicable to co-localize Ald6 and Acs1 in cytoplasm, a lack of LD localization tool has discontinued the channeling effect transferring from Acs1 to Atf1.

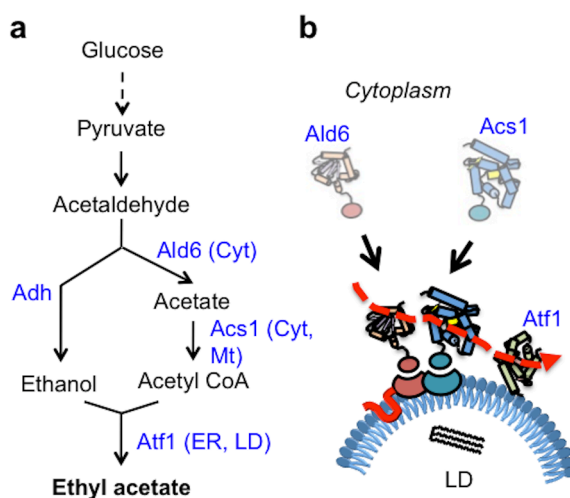


Figure 7.1. An enzyme relocalization strategy to increase metabolic flux to ethyl acetate production in *S. cerevisiae*. (a) The metabolic pathway of ethyl acetate synthesis and subcellular localization of Ald6, Acs1, and Atf1. Cyt: cytosol; Mt: mitochondrion; ER: endoplasmic reticulum; and LD: lipid droplet. (b) The concept and strategy used to enzyme relocalization. Ald6 and Acs1 from the cytoplasm are colocalized to lipid droplets (LDs) via the synthetic membrane scaffold, giving rise to an increase of metabolic flux of acetyl-CoA to Atf1.

Here we developed a protein-based synthetic membrane scaffold that can functionally localize cytoplasmic enzymes to LDs (**Fig. 7.1b**). We demonstrated the strategy co-localizing enzymes on the organellar membrane was effective with an *in vitro* kinetic assay examining potential channeling effect from Acs1 to Atf1. We optimized the pathway for ethyl acetate production by creating a combinatorial library tuning Acs1 stoichiometry on the scaffold and expression of Acs1 and the scaffolds. A colorimetric assay that can quantify ethyl acetate from cultures was developed to suggest high titer producers from the library screening. We verified the assay with results measured by gas

chromatography (GC) and found it was comparable to the screening. With strategies of enzyme co-localization and the screening of combinatorial library, we improved ethyl acetate titer by 83-fold with 51.6 mg/L production.

7.3 MATERIAL AND METHODS

Strains and culture conditions

All *E. coli* and yeast strains used are summarized in **Supplementary Table 7.1**. *E. coli* strains used for molecular cloning and protein expression were DH5 α and BL21 (DE3), respectively. *E. coli* was grown in Luria Broth (LB) (Sigma) medium containing 50 μ g/ml ampicillin (Fisher) for cloning experiment and 50 μ g/ml kanamycin (Fisher) for HA-myc-GFP-(His)₆ expression. All modified yeast strains used in this study were derived from BY4742. The gene tagging strains, *ERG6::DsRed* and *SEC61::DsRed*, were constructed in our previous study [9]. To construct a yeast strain with Atf1 constitutive expression, pRS316 containing *LEU2-P_{TDH3}-ATF1-myc-T_{TEF2}* integration cassette was constructed with Gibson assembly [21], and PCR mediated gene integration method [22] was applied to insert the cassette on *yprcA15* site of Chr 16. Parental and modified BY4742 transforming with plasmids were grown in synthetic complete medium (SC) containing 0.67% yeast nitrogen base (Bacton-Dickinson), amino acid supplements (Sunrise and Sigma), and 2% glucose (Sigma).

Plasmid and yeast strain construction

The plasmids used in this study are listed in **Supplementary Table 7.2**. All heterologous genes were codon optimized for expression in *S. cerevisiae* by IDT codon

optimization tool and synthesized by a gene assembly method [23]. DNA sequences of heterologous genes were shown in **Supplementary Table 7.3**. Yeast genes, promoters, and terminators were amplified from genomic DNA. The peptide tags used for western blot were incorporated into proteins by designing DNA sequence on primers for PCR amplification.

To analyze the cellular localizations of potential LD targeting motifs, heterologous genes synthesized by a gene assembly method (ref) were fused with CFP gene at 3' end via overlap extension PCR (OE-PCR) [24], and the resulted gene fusions were cloned into pRS426-*P_{PGK1}-T_{PGK1}* [9] with NotI and SpeI sites. To analyze the cellular localization of Oleosin, Ald6 and Acs1, and Oleosin-tagged Ald6 and Acs1, pRS426-*P_{PGK1}-CFP-T_{PGK1}* was first created as the entry vector, where CFP was cloned into NotI and SpeI sites of pRS426-*P_{PGK1}-T_{PGK1}*. Oleosin, Ald6, and Acs1 amplified by PCR, and Ald6/Acs1-Oleosin synthesized by OE-PCR were inserted between SacII and NotI sites of pRS426-*P_{PGK1}-CFP-T_{PGK1}*. To construct the synthetic scaffolds, Oleosin, X82 (C₁), and Cohesin (C₂) were amplified by PCR and assembled via Gibson assembly. To construct enzyme-tag-dockerin, DNA fragments of enzymes, peptide and fluorescent tags, and dockerin domains were assembled via OE-PCR. The resulted fragments were cloned into either pRS426-*P_{PGK1}-T_{PGK1}* at SacII and SpeI sites or p413-*P_{GPD}* [25] at SpeI and XhoI sites. pRS426-*P_{PGK1}-Oleosin-Cohesin (C₂)-YFP-T_{PGK1}* was constructed with an insertion of YFP at NotI and SpeI sites and Oleosin-Cohesin (C₂) at SacII and NotI sites. pRS426-*P_{PGK1}-ATF1-DsRed-T_{PGK1}* was constructed with an insertion of *ATF1* at SacII and NotI sites and DsRed at NotI and SpeI sites of pRS426-*P_{PGK1}-T_{PGK1}*. Plasmids for

overexpression of the pathways were assembled to either pRS316 or pRS426 by transforming promoters, genes, and terminators to *S. cerevisiae* through DNA assembler [26].

To make the combinatorial library for pathway optimization, the synthetic scaffolds varied with 2- and 3-Cohesin (C_2), and the additional copy of Acs1 and the scaffolds differed from promoters were first adapted to pRS426. Briefly, Oleosin-X82 (C_1)-Cohesin (C_2) was extended with 1- or 2-copies of Cohesin (C_2) at 3' end by Gibson assembly. Genes including the additional copy of Acs1 and the scaffolds flanked by promoters and the terminator were assembled to pRS426 via Gibson assembly. The resulting gene cassettes and cassettes of the original copy of Acs1 and Ald6 were transformed into *S. cerevisiae* for pathway assemblies through DNA assembler. Plasmids constructed by yeast homologous recombination were isolated by ZymoprepTM Yeast Plasmid Miniprep I kit. The resulted plasmids were transformed into *E. coli*, amplified, and isolated for verification of DNA sequence. To construct an additional copy of *ATF1* on chromosome, an integration cassette comprising *LEU2* marker amplified from pRS315, *P_{TDH3}*, *ATF1*, and *T_{TEF2}* were assembled to pRS316 through DNA assembler. To express epitope tagged GFP, a forward primer containing HA and myc tags and a reverse primer containing His tag were used amplify GFP. The resulted fragment was cloned into pET29 between XbaI and XhoI sites.

Fluorescence microscopy

Cells were observed by an Olympus BX51 microscope (UPlanFL 100X 1.30 oil-immersion objective lens, mercury lamp) and fluorescent images were captured by a Q-

Imaging Retiga Exi CCD camera. Images were processed using CellSens Dimension 1.7 software (Olympus).

Preparation of whole cell lysate

Yeast cells harboring expression plasmids were cultured to early stationary phase in 50 ml SC -U in 250 ml baffled flasks. Cells were harvested by centrifugation at 3,500 rpm for 5 min at 4 °C, and washed twice with 100 mM potassium phosphate buffer (pH 7.4) containing 2 mM MgCl₂. Equal volumes of wet cell pellets and 425-600 µm diameter acid-washed glass beads (Sigma-Aldrich) were added to a 15 mL tube and resuspended in 1 mL ice-cold lysis buffer (100mM potassium phosphate buffer, 2mM MgCl₂, 2mM dithiothreitol (DTT), and protease inhibitor cocktail (Roche), pH7.4). Cells were disrupted by vortexing 10 times for 30 s. After each vortexing, the suspension was kept on ice for 30 s. The beads were removed by centrifugation at 500g for 5 min at 4 °C and the supernatant was transferred to a pre-cooled 1.5 mL tube. Whole cell lysate protein concentrations were determined by a commercially available assay (Pierce 660 nm Protein Assay, Thermo Fisher) and quantified with Nanodrop 2000.

Enzyme and pathway activity assays

The assays for determination of aldehyde dehydrogenase and acetyl-CoA synthetase activities were modified as described previously [27]. To determine the activity of aldehyde dehydrogenase, the reaction mixture containing 0.4 mM DTT, 10 mM MgCl₂, 0.4 mM NADP⁺, 15 mM pyrazole, and 100 mM potassium phosphate buffer (pH 8.0) was used, and 1 ~ 5 µg whole cell lysate was used for kinetic measurement. The reaction was initiated with 0.1 mM acetaldehyde. To determine the activity of acetyl-CoA

synthetase, reaction cocktail 1 consisting of 10 mM malate, 8 mM ATP, 1 mM NAD⁺, 10 mM MgCl₂, 3 U malate dehydrogenase (Sigma), 0.4 U citrate synthase (Sigma), and 100 mM potassium phosphate (pH 7.4) was used, and 1 ~ 5 µg whole cell lysate was used for kinetic measurement. The reaction was initiated with reaction cocktail 2 containing 0.2 mM CoA, and 10 mM potassium acetate.

To evaluate pathway efficiency from acetyl-CoA synthetase (Acs1) to alcohol acetyltransferase (Atf1), the reaction mixture containing 10 mM MgCl₂, 500 mM ethanol, 8 mM ATP, 1 mM CoA, and 100 mM potassium phosphate (pH 7.4) was used, and 50 µg whole cell lysate was used for kinetic measurement. The reaction was initiated with 100 mM potassium acetate, and reacted for 5 min. 1 g NaCl was used to stop the reaction, and the produced ethyl acetate was quantified by headspace gas chromatography (GC). The experiment was performed in 10 ml GC vial (Fisher).

Western blot

Western blots were performed using standard procedure. 5 µg whole cell lysates were loaded on Any kDTM or 7.5% Mini-PROTEAN® TGXTM Gel (Bio-Rad) and run at 150 V for 30 to 60 min. Samples were electrophoretically transferred to a PVDF membrane at 25 V overnight. Membranes were blocked with 5% non-fat milk in TBST buffer for 1 h at room temperature, washed, and incubated with primary antibody in TBST with 1% non-fat milk for 1 h. Secondary antibody in TBST with 1% non-fat milk was applied, and incubated at room temperature for 0.5 h. After washing with TBST, Immobilon Western HRP Substrate (Millipore) was used for signal detection, and UN-SCAN-IT gel 6.1 software was used to quantify band intensity. To detect proteins, the following antibodies

were used: rabbit anti-HA (71-5500; Invitrogen), mouse anti-His (A00186-100; Genescript), rabbit anti-V5 (V8137, Sigma), rabbit anti-myc (C3956; Sigma), mouse anti-GAPDH (MA5-15738; Thermo Fisher Scientific), goat anti-rabbit IgG-HRP (65-6120; Invitrogen), goat anti-mouse IgG-HRP (31430; Thermo Fisher Scientific).

Colorimetric assay

The colorimetric assay was developed based on the reactions as described previously [28]. 2.5% (w/v) hydroxylamine•HCl (Fisher) and sodium hydroxide solutions were prepared in 95% ethanol separately. To prepare alkaline hydroxylamine solution, equal volumes of hydroxylamine•HCl and sodium hydroxide solutions were mixed, left on bench for 15 min, and filtered to remove salt. Ferric chloride was first dissolved in 75% perchloric acid (Sigma) and an equal volume of deionized water was then added to form 0.8% (w/v) ferric perchlorate stock solution. To prepare ferric perchlorate working solution, the stock solution was diluted 20 times in absolute ethanol. The colorimetric assay was performed by mixing 150 μ l hexane-dissolved ethyl acetate and with 50 μ l alkaline hydroxylamine solution, and the mixture was left on bench for 10 min. The produced hydroxamic acid was reacted with ferric perchlorate working solution on bench for 5 min to form ferric chelate complex. The amount of ethyl acetate was measured by quantifying ferric chelate complex at 520 nm with DU 800 spectrophotometer.

Screening for the combinatorial library

Single colonies were cultured in 2 ml SC –U with 2% glucose in 14 ml culture tube (Falcon), and the cultures were grown overnight at 250 rpm and 30 °C. Overnight cultures were transferred to 3 ml SC –U with 10% glucose in 15 ml conical tubes

(Fisher). The cultures were started with OD₆₀₀ of ~0.05 cells, and fermented for 24 h at 250 rpm and 30 °C. 50 µl cells was withdrawn for OD₆₀₀ measurement, and ethyl acetate was extracted from cultures with 3 ml hexane. The extraction was conducted on a rotary shaker (MAXQ 2508) for 10 min with vigorous mixing. 1.4 ml of the mixtures was transferred to microcentrifuge tubes and 13,000 r.p.m. was used to separate hexane and the cultures. Ethyl acetate extracted in the top layer was withdrawn and assayed by the developed colorimetric assay on 2.0 ml 96-well microplates (PlateOne®). The formed ferric chelate complex was quantified at 520 nm with DU 800 spectrophotometer.

Fermentation

Single colonies were inoculated into 2 ml SC -U and with 2% glucose in 14 ml culture tubes (Falcon), and the cultures were grown overnight at 250 rpm and 30 °C. Overnight cultures were transferred to 20 ml SC -U with 2% glucose in 50 ml conical tubes (Fisher). The cultures were initiated with OD₆₀₀ of 0.05 cells, and fermented for 24 h at 250 rpm and 30 °C. 1.4 ml culture of fermentation was transferred to a microcentrifuge tube, centrifuged at 13,000 r.p.m., and 1 ml supernatant was harvested and transferred to a 10 ml glass vial (Fisher) for GC analysis. 1 g NaCl was added to the sample, and 50 mg/L pentanol was used as the internal standard.

Gas chromatography analysis

Ethyl acetate was quantified by a headspace gas chromatography (GC) with a flame ionization detector (FID) (Agilent Technologies 7890A GC with CTC-PAL headspace mode injector). The separation of volatile compounds was carried out by Rtx®-WAX column (30 m, 0.32 mmID, 5 mm film thickness; RESTEK) with helium as carrier gas.

The GC oven temperature was initially held at 30 °C for 7 min and increased with a gradient of 50 °C/min until 200 °C, and the held for 4 min. The FID was held at 250 °C. 1 ml headspace gas was injected to the GC with 10:1 split ratio, and 1-pentanol was used as internal standard.

7.4 RESULTS

Identification and characterization of lipid droplet targeting tags

Amphipathic helix (AH) attachment and hairpin loop insertion are two mechanisms by which proteins localize to LDs [29]. To organize cytoplasmic proteins to LDs, we focused on searching proteins and protein motifs with AH or hairpin loop structure from literatures. One protein and five protein motifs from other organisms were found and characterized in *S. cerevisiae*: (i) an entire Oleosin containing 186 amino acid (aa) with acyltransferase/phospholipase activity and hairpin loop structure from Zea may [30], (ii) N-terminal 1-38 of AAM-B, a putative methyltransferase, with a stretch of hydrophobic residues from human [31], (iii) C-terminal 118-164 of hepatitis C virus core protein (HCV cp) with AH-loop-AH structure [32], (iv) N-terminal 1-42 of viperin, an antiviral protein, with AH structure from mouse [33], (v) internal 1004-1048 of GBF1, a guanine nucleotide exchange factor (GEF), with AH structure from mammal [34], and (vi) N-terminal 1-28 of 17 β HSD11, a hydroxysteroid dehydrogenase, with unknown structure from human [35]. Among the selected potential tags, oleosin and AAM-B (1-28) have been shown capable of targeting fluorescent proteins to LDs during aerobic growth in *S.*

cerevisiae. However, the cellular localization of these tags at different growth phase of aerobic culture and anaerobic condition is yet to be explored.

To characterize cellular localization of the six potential tags, genes tagged with cyan fluorescent protein (CFP) at C-terminal ends were constitutively overexpressed by PGK promoter on a high copy number plasmid. The molecular transport of proteins from the ER to LDs has been shown to be growth phase dependent. (ref) As such, yeast strains overexpressing tag-CFP were cultured for 10 and 24 h, corresponding to the exponential phase and stationary phases of aerobic growth. At 10 h, fluorescence microscopy images of cells overexpressing oleosin-CFP showed vesicle-like structures attaching to the signal from Sec61-DsRed (an ER marker), suggesting a fast trafficking of Oleosin to prematured LDs. Fluorescent imaging of AAM-B (1-38)- and HCV cp (118-164)-CFP showed overlapped fluorescence with Sec61-DsRed, suggesting localization of tags to the ER (**Fig. 7.2a**). Fluorescent imaging of Viperin (1-42)-, GBF1 (1004-1048)-, and 17 β HSD11 (1-28)-CFP suggested cytosolic localization of tags, as indicated by dispersion of CFP fluorescence in the cytosol, even though some fusion proteins appeared to form distinct patterns (**Supplementary Fig. 7.1**). At 24 h, fluorescent signals of Oleosin-, AAM-B (1-38)-, and HCV cp (118-164)-CFP showed similar patterns to those of Erg6-DsRed, indicating LD targeting of tags at stationary phase (**Fig. 7.2a**). By contrast, fluorescence of Viperin (1-42)-, GBF1 (1004-1048)-, and 17 β HSD11 (1-28)-CFP was observed in whole cells, suggested cytosolic localization of these potential tags (**Supplementary Fig. 7.1**). Although oleosin, AAM-B (1-38), and HCV cp (118-164) could localize to LDs under aerobic condition, limited supply of oxygen may render

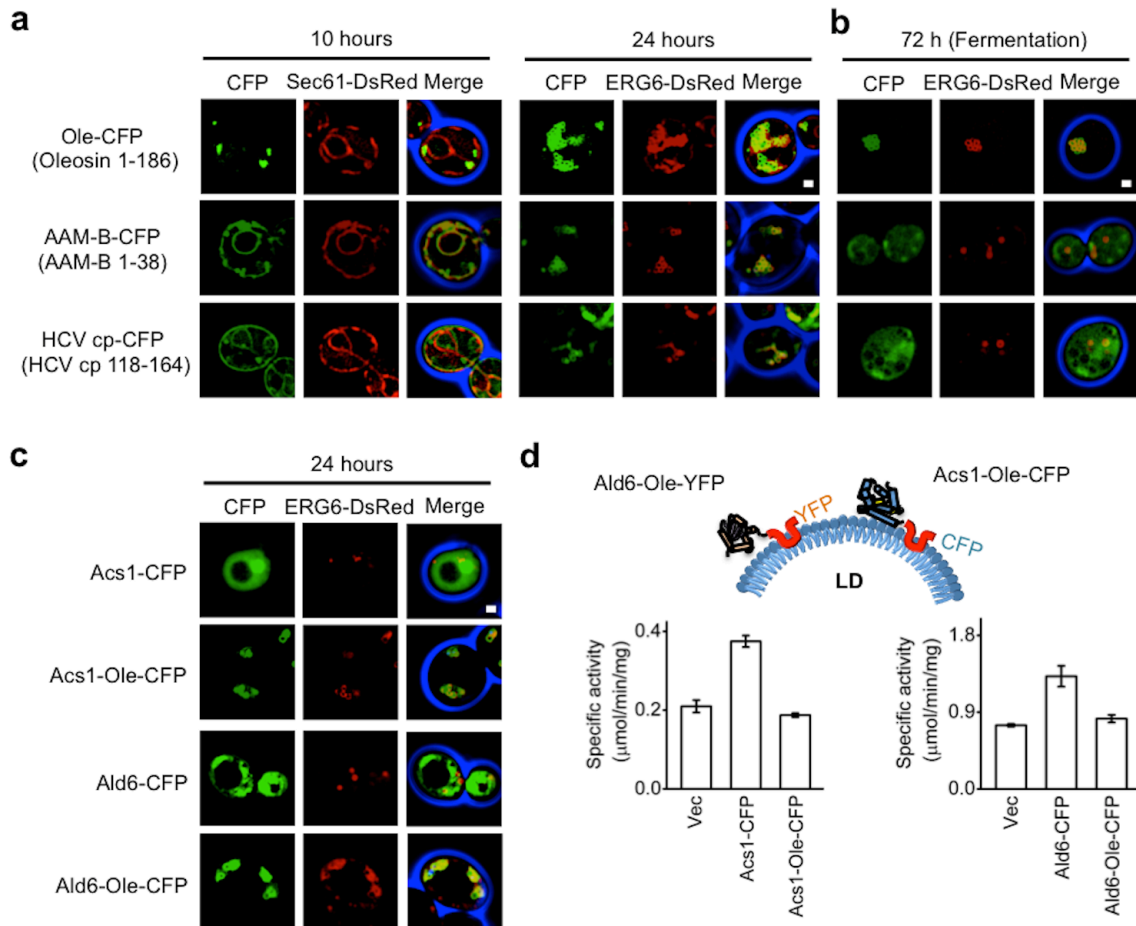


Figure 7.2. Characterization and implementation LD targeting tags. Fluorescence microscopy analysis of subcellular localization of three potential LD tags including Oleosin, N-terminal 1-38 of AAM-B, and C-terminal 118-164 hepatitis C virus core protein (HCV cp) (**a**) at different time course of aerobic growth, and (**b**) fermented condition after 72 h. Sec61 and Erg6 are the ER and LD markers, respectively. CFP: cyan fluorescent protein; and DsRed: red fluorescent protein. Scale bar: 1 μm . (**c**) Fluorescence microscopy analysis of subcellular localization of wild type and oleosin-tagged Acs1 and Ald6 after culture of 24 h in aerobic condition. Scale bar: 1 μm . (**d**) Enzyme activities of Acs1 and Ald6. Cells overexpressing fusion constructs were cultured to stationary phase in aerobic condition, and whole cell lysate were prepared for kinetic assays. Empty vector served as the vector control.

alteration of membrane curvature, resulting in de-localizing these tags. As such, cellular localization of these potential tags was analyzed after 72 h fermentation. Only Oleosin localized to LDs, whereas AAM-B (1-38) as well as HCV cp (118-164) were dispersed in

cytosol (**Fig. 7.2b**). As the robustness of Oleosin was observed, the protein was chosen as the LD tag for subsequent spatial organization of Acs1 and Ald6 to LDs.

Functional localization of cytoplasmic enzymes to lipid droplets

We examined whether oleosin can drag Acs1 and Ald6 from cytoplasm to LDs. Expression of wild type Acs1 and Ald6 tagged with CFP at C-termini on high copy number plasmids were localized to cytoplasm. Both enzymes were targeted to LDs when Oleosin was added to redirect protein localization (**Fig. 7.2c**). Remarkably, no mitochondria localization of Acs1-Oleosin was observed even though the enzyme has been reported mitochondrial localization. One possible explanation is that a tight association and rapid transport of Oleosin to LDs lead to bypass mitochondria targeting pathway of Acs1. We next investigated the activities of Oleosin tagged Acs1 and Ald6. Comparing to an empty vector control, both Acs1 and Ald6 remained to be active when CFP was added to C-termini of enzymes. 0.21 ± 0.02 $\mu\text{mol}/\text{ming}/\text{mg}$ and 0.37 ± 0.01 $\mu\text{mol}/\text{ming}/\text{mg}$ were detected for the vector control and Acs1-CFP, respectively, when assayed with acetyl-CoA synthetase reaction. 0.75 ± 0.02 $\mu\text{mol}/\text{ming}/\text{mg}$ and 1.32 ± 0.12 $\mu\text{mol}/\text{ming}/\text{mg}$ were measured for the vector control and Ald6-CFP, respectively, when assayed with aldehyde dehydrogenase reaction (**Fig. 7.2d**). However, the functions of Acs1 and Ald6 were affected as we re-directed enzymes to non-native localization. A lost activity of Acs1-Oleosin-CFP with 0.19 ± 0.01 $\mu\text{mol}/\text{ming}/\text{mg}$ and a compromised activity of Ald6-Oleosin-CFP with 0.82 ± 0.04 $\mu\text{mol}/\text{ming}/\text{mg}$ were observed when enzymes were localized to LDs (**Fig. 7.2d**). We then examined the structure of Acs1, and discovered that the active site is surrounded by positively charged residues [36]. We

hypothesized that the negatively charge LD membrane may attract the active site of Acs1 by electrostatic interaction, resulting in a block of substrate access. As a consequence, a new design that cushions the membrane and enzymes on LD surface was incorporated to overcome this bottleneck.

Various protein-protein interaction domains were characterized and engineered to co-localize enzymes *in vivo* [5] and *in vitro* [37]. For example, interaction domains of GBD, SH3, and PDZ were applied to organize heterologous enzymes (HMGR and HMGS) and native enzyme (AtoB) intracellularly in *E. coli* for improvement of mevalonate production [5]. Cohesin and dockerin from *Clostridium* and *Ruminococcus* species were used to recruit three dehydrogenases in a cascade reaction on cell surface of *S. cerevisiae* for enhancement of NADH generation [37]. We selected cohesin and dockerin protein domains as the orthogonality to *S. cerevisiae* and species-specific interaction, avoiding potential binding of competitors intracellularly and proving modularity for pathway architecture. We proved our hypothesis by cushioning the enzyme and the LD membrane with this plug-and-play design. Acs1 was first tested as its important role of acetyl-CoA production in the pathway and a complete loss of its enzyme function. Cohesin and Dockerin from *Clostridium thermocellum* (Ct) [38] were first tested in *S. cerevisiae*. We constructed a synthetic membrane scaffold comprising Oleosin and Cohesin (Ct), and Acs1 was fused with Dockerin (Ct). Upon expression of Oleosin-Cohesin (Ct) tagged with yellow fluorescent protein (YFP) at C-terminal end, fluorescent result revealed LD localization of the scaffold (**Supplementary Fig. 7.2**). We then co-expressed the scaffold and Acs1-CFP-Dockerin (Ct) into yeast. In the absence of the scaffold, as expected,

Acs1-CFP-Dockerin (Ct) was dispersed in the cytoplasm. The enzyme was successfully organized to LDs once the scaffold was expressed (**Supplementary Fig. 7.2**). We went on to search an additional pair of cohesin and dockerin for LD localization of Ald6. X82, a cohesin-like protein, and dockerin from *C. perfringens* [39] were incorporated to the scaffolding system. The scaffold, Oleosin-Cohesin (Cp)-Cohesin (Ct), was constructed to co-localize Ald6 and Acs1 on LDs. LD localization of Ald6 was enabled by tagging with dockerin (Cp) at the C-terminus. To demonstrate enzyme localization of Ald6, Acs1, and Atf1, YFP, CFP, and DsRed were used for fluorescence microscopy analysis, respectively. The pathway containing multiple gene cassettes was assembled to a single copy number plasmid by DNA assembler. Yeast strains were cultured to stationary phase aerobically and analyzed for enzyme localization. In the absence of the scaffold, Ald6 and Acs1 were observed localizing to the cytoplasm; by contrast, Atf1 was targeted to LDs (**Fig. 7.3b**). Both Ald6 and Acs1 were co-localized to LDs upon expression of the scaffold. LD localization of Ald6 and Acs1 was also observed when the yeast strain fermented for 24 h (**Supplementary Fig. 7.3**). These results clearly demonstrate success of spatial organization of cytoplasmic enzymes on LDs.

We examined whether the addition of cushion to enzymes and the LD membrane rescues enzyme activities. All fluorescent proteins used for fluorescence microscopy and previous kinetic assay were replaced by peptide tags for protein detection. For simplicity, peptide tags are not displayed in the text and figures. Detailed information of tag usage is included in **Supplementary Table 7.2**. Comparing to vector controls, expression of Acs1-Doc (Ct) and Ald6-Doc (Cp) showed activities of 0.31 ± 0.01 and 1.64 ± 0.06

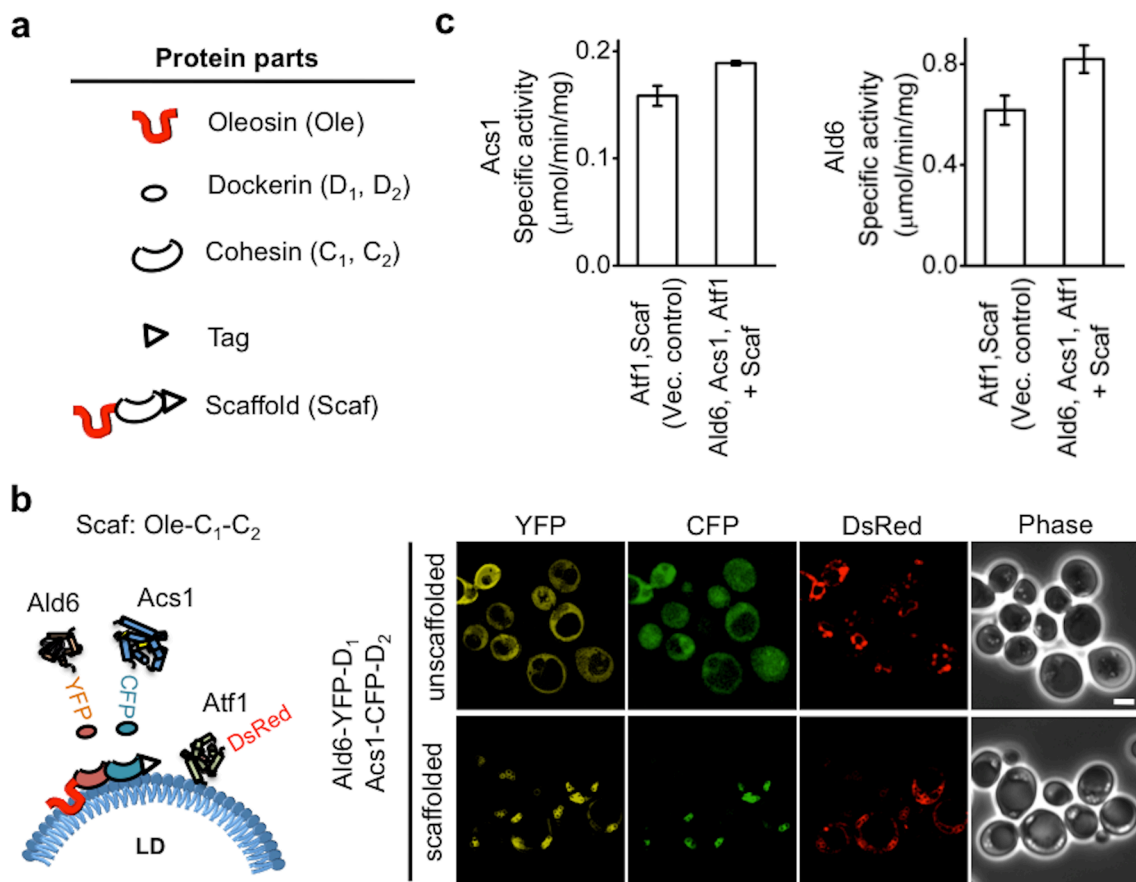


Figure 7.3. Functional relocation of the upstream enzymes to LDs via synthetic membrane scaffolds. (a) Protein parts of the synthetic membrane scaffold and abbreviations used in the figure. C_1 and D_1 are X82 (a cohesin-like protein) and dockerin from *C. perfringens* while C_2 and D_2 are cohesin and dockerin from *C. thermocellum*. A V5 tag was fused to C-terminus of the scaffold for detection of protein expression. (b) Fluorescence microscopy analysis of subcellular localization of Ald6, Acs1, and Atf1 in the absence and presence of the scaffold. Cells were cultured to stationary phase in aerobic condition. YFP: yellow fluorescent protein. V5 peptide tag was used for western blot analysis of the expressed scaffold. Scale bar: 3 μm . (c) Enzyme activities of Acs1 and Ald6. The whole cell lysate overexpressing Ald6, Acs1, Atf1, and the scaffold were analyzed for enzyme kinetics of Acs and Ald. Since overexpression of the genes in the pathway resulted in alteration of whole cell lysate activity, overexpression of Atf1 and the scaffold was used as a vector control.

$\mu\text{mol/min/mg}$, respectively, suggesting active enzymes in the cytoplasm. To evaluate activities of cytoplasmic enzymes on LDs, the pathway genes including Ald6, Acs1, Atf1, and the scaffold were assembled on a high copy number plasmid (Supplementary

Table 7.2). Given that expression of genes and the scaffold may change kinetic background of whole cell lysate, a similar genetic background expressing Atf1 and the scaffold was used as the vector control. Expression of the vector control showed an activity of 0.16 ± 0.01 $\mu\text{mol}/\text{min}/\text{mg}$ for Acs reaction. The activity was increased to 0.19 ± 0.00 $\mu\text{mol}/\text{min}/\text{mg}$ when the pathway genes were expressed. A similar result was observed for Ald reaction. Expression of the vector control exhibited an activity of 0.62 ± 0.06 $\mu\text{mol}/\text{min}/\text{mg}$. The activity was elevated to 0.82 ± 0.05 $\mu\text{mol}/\text{min}/\text{mg}$ when the pathway genes were expressed (**Fig. 7.3c**). These results suggested that, with the cushion partitioning enzymes and the LD surface, Ald6 and Acs1 were functionally localized to LDs.

Evaluation of pathway efficiency in vitro and in vivo

The strength of promoters driving gene expression was taken into account when expressing pathway genes. Based on the kinetic results of Ald6 and Acs1 in this study along with the result of Atf1 from our previous work [17], we estimated the order of activity was Ald6 > Atf1 > Acs1. According to the results characterized by Sun [40] and Lee [41] et al., the promoter strength was found $P_{\text{TDH3}} > P_{\text{TEF1}} > P_{\text{TDH2}} > P_{\text{ADH1}}$. Therefore, P_{TDH3} , P_{TEF1} , P_{TDH2} , and P_{ADH1} were used to drive gene expression of Atf1, Acs1, Ald6, and the scaffold, respectively. Atf1 is the last metabolic enzyme of ethyl acetate production in *S. cerevisiae*. Maintaining similar expression level of the enzyme among the controls and the assembled pathway on LDs is essential for comparison of pathway efficiency. Given that a significant reduction of expression of Atf1 was observed when more genes of the pathway were expressed on a high copy plasmid, we

hypothesized that overexpression of pathway proteins may have titrated out given cellular transcriptional and/or translational machinery in cells (**Supplementary Fig. 7.4**). Thus, we integrated the gene cassette of Atf1 at the site of *yprcΔ15* on Chr XVI, a previously characterized integration site with high expression of heterologous gene [42] while gene cassettes of Ald6, Acs1 and the scaffold were assembled on a single copy plasmid (**Fig. 7.4a**).

Enzyme co-localization of metabolic or synthetic pathways on scaffolds have been shown improving yields of mevalonate [5], hydrogen [6], and resveratrol [7] production. The proximity of enzymes along the pathway provides channeling effect for reaction intermediates. Nevertheless, it is unclear to us that organizing the cytoplasmic enzyme to a membrane-bound enzyme would improve pathway efficiency since the co-localized enzymes on the membrane may not be adjacent to each other. To address this question, we focused on the catalytic step from Acs1 to Atf1 and evaluated performance of the assembled pathway beginning with synthesis of ethyl acetate *in vitro*, which overcomes the limit of substrate and cofactor supply *in vivo*. Whole cell lysates prepared from aerobic cultures at stationary phase were first equilibrated with the substrate and cofactors, including ethanol, ATP, and CoA (**Fig. 7.4b**). The pathway was initiated by the addition of acetate and production of ethyl acetate was measured by GC. To confirm that LDs remained intact under the experimental procedure, whole cell lysate containing Oleosin-CFP was made parallel and observed for LD morphology with fluorescence microscopy (**Fig. 7.4c**) Because of suppression of Atf1 expression under aerobic condition, the strain with an additional copy of Atf1 on chromosome was used as the

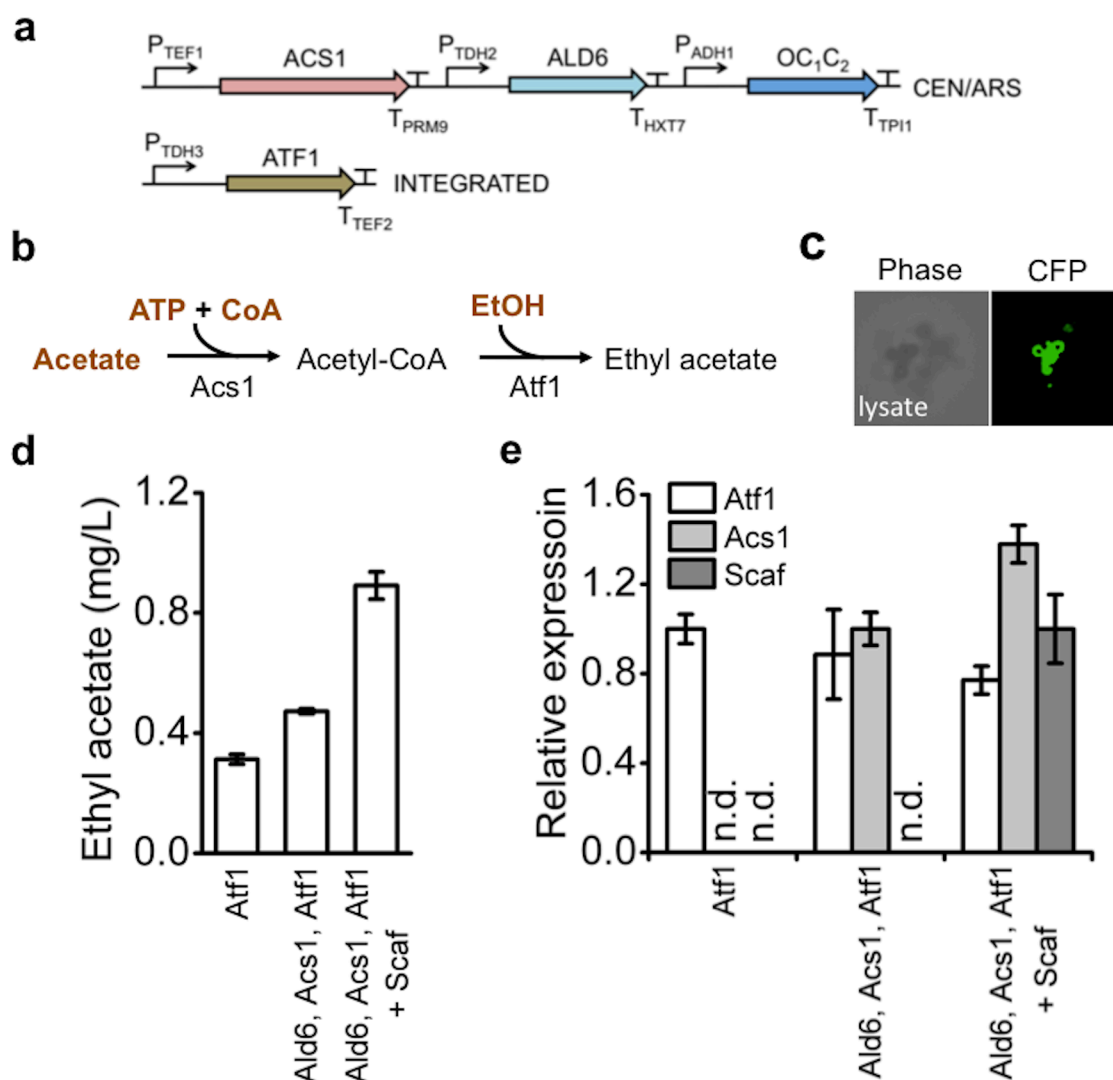


Figure 7.4. Evaluation of pathway efficiency from Acs1 to Atf1 in vitro. (a) The strain and construct used to overexpress pathway genes. To maintain similar expression of Atf1, the gene was integrated to chromosome. The rest genes including Acs1, Ald6, and the scaffold were expressed on a low copy number vector (CEN/ARS). Promoters and terminators driving gene expression and maintaining mRNA stability are shown in the scheme. (b) Evaluation of the channeling effect from Acs1 to Atf1. Substrates and cofactors fed to the pathway were shown in brown. (c) Demonstration of integrated LDs after cell lysis. Cells were cultured to stationary phase, and whole cell lysates were prepared with glass bead lysis method. (d) Ethyl acetate produced with the constructed strains *in vitro*. Gas chromatography (GC) was used to quantify ester production. (e) Quantification of protein expression for Acs1, Atf1, and the scaffold with western blot analysis. See Supplementary Fig. 7.5 for a detailed analysis.

single enzyme control. The strain constitutively expressing Atf1 produced 0.31 ± 0.02 mg/L ethyl acetate. The titer was increased to 0.47 ± 0.01 mg/L when Ald6 and Acs1 were expressed. A maximum production of 0.89 ± 0.05 mg/L was observed when organizing enzymes on LDs. 1.9- and 2.9-fold enhancement were gained when comparing the assembled pathway to the unassembled pathway and single enzyme pathway (**Fig. 7.4d**) To rule out the possibility that a dramatic change of protein expression results in the kinetic enhancement, quantification of proteins was performed by western blot. The expression of Atf1 was found similar in Atf1 integrated strain and the unassembled pathway, and a reduction of Atf1 was observed in the assembled pathway. Acs1 was expressed in the unassembled and assembled pathway, and an increase of expression was observed in the assembled pathway. The synthetic scaffold was only expressed in the assembled pathway (**Fig. 7.4e** and **Supplementary Fig. 7.5**).

Development of a colorimetric assay for ethyl acetate determination

Screening a combinatorial library tuning gene expression and enzyme stoichiometry on the scaffold for pathway optimization requires a facile and high throughput detection method. Previous detection method for measurement of esters heavily relies on GC analysis, which has become a hurdle for screening pathway variants. As such, based on a previously reported assay for ester detection [28], we developed a colorimetric assay that can quantify ethyl acetate from cultures. In general, ester dissolved in solvent reacts with alkaline hydroxylamine solution to form hydroxamic acid, which subsequently reacts with ferric ions to form ferric chelate complex (**Fig. 7.5a**). To test the feasibility of this assay, we first dissolved ethyl acetate in ethanol for the reaction. 0 to 5000 mg/L ethyl

acetate was tested for the assay (**Fig. 7.5b**), and the quantified result was shown in **Supplementary Fig. 7.6a**). Clearly, a linear range from 0 to 100 mg/L was observed, which had covered the highest reported titer from previous metabolic engineering efforts. To extend the assay for direct quantification of ethyl acetate from cultures and expand the throughput, we applied solvent-solvent extraction and performed the reactions in microplates. With a positive control of cells overexpressing Atf1 and a negative control with cells harboring an empty vector, a Z' factor of 0.66 was achieved from the microplate based processing, indicating the quality of the assay (**Supplementary Fig. 7.6b**).

Optimization of the pathway to improve titer

To optimize metabolic flux of the pathway, we evaluated relative expression pathway enzymes. Using GFP as the standard, the western blot result suggested a relatively low expression of Acs1 from the assembled pathway, comparing to those of Ald6 and Atf1 (**Supplementary Fig. 7.7**). In combination with the kinetic results, we reasoned that metabolic flux from Acs1 was limited. Therefore, an optimization strategy increasing Acs1 expression, and tuning expression of the scaffold and Acs1 stoichiometry on the scaffold was applied to balance metabolic flux of the pathway. Here, gene cassettes expressing Acs1 and Ald6 from the prototype construct were fixed (**Fig. 7.4a**), but an additional copy of Acs1 and altered scaffold architectures with 2- and 3- copies of Cohesin (Ct) were incorporated to the constructs. To tune gene expression, promoter with different strengths driving transcription and terminator conferring different mRNA stabilities were applied to the optimization. As a result, a combinatorial library containing

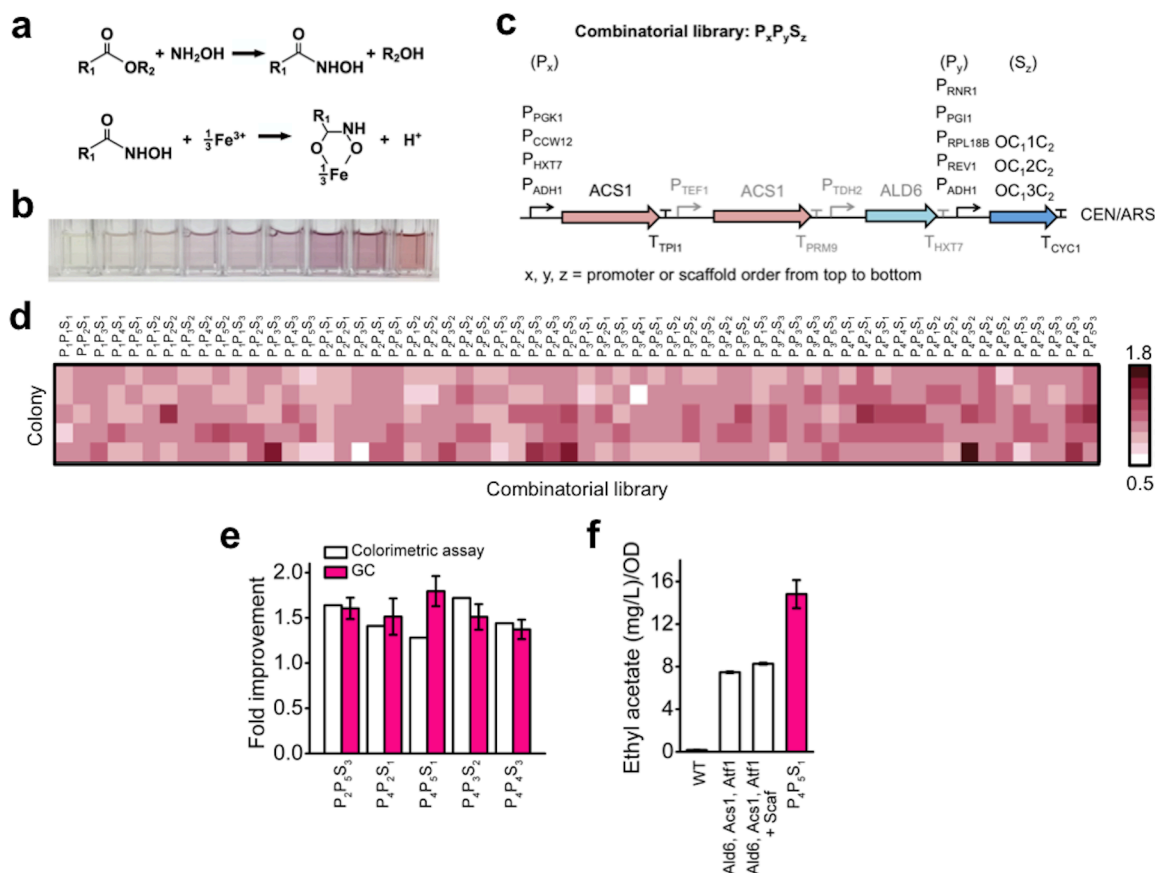


Figure 7.5. Optimization of metabolic flux for improvement of ethyl acetate yield. (a) Chemical reactions used in the colorimetric assay. Ester reacts with hydroxylamine to form hydroxamic acid, which subsequently reacts with ferric ion to form a red ferric hydroxamate complex. Ethyl acetate can be readily quantified by measuring absorbance at 520 nm derived from the complex. (b) Reaction solutions after assaying hexane-dissolved ethyl acetate with a range of concentration from 0, 20, 40, 60, 80, 100, 500, 1000, to 5000 mg/L. (c) A combinatorial library of the pathway tuning stoichiometry of C_2 , and expression of Acs1 and the scaffolds was created. (d) Heat map of fold improvement retrieved from strains of the combinatorial library. The fold improvement was calculated from a comparison of the library strain with the prototype shown in Figure 4 (a). 10% glucose was fed to yeast for fermentation of 24 h. (e) A comparison of results of top five producers from the screening assay and GC measurement. 2% glucose was used for fermentation of 24 h in GC analysis. (f) Specific titers of ethyl acetate production of wild type and the evolved pathways.

60 pathway variants was created to maximize the titer (**Fig. 7.5c**). For the convenience describing the construct architectures, a naming rule P_x , P_y , and S_z was incorporated where PPS representing the **p**romoter of Acs1, and the **p**romoter of the scaffold, and the

scaffold architecture, and x, y, z stand for numbers of promoters or scaffolds counting from top to bottom in the library (**Fig. 7.5c**). We transformed the library to the Atf1 integrated strain, and five colonies of each pathway variant were screened, resulting in screening a total number of 300 samples. To enable facile quantification of ethyl acetate fermented from glucose with the colorimetric assay, 10% glucose was fed to the cultures. The results were presented in fold enhancement, a normalization of the screening result to that of the prototype assembled pathway (**Fig. 7.5d and Supplementary Table 7.4**). We randomly selected 5 colonies with a fold enhancement over 1.2-fold. To rule out the possibility that the retrieved phenotype was resulted from random integration on chromosomes, constructs assembled from DNA assembler were isolated, amplified, and re-transformed to the Atf1 integrated strain. Expression of proteins on constructs was analyzed with western blot. Ethyl acetate production was further confirmed by fermenting 2% glucose and analyzed with GC. The results from the colorimetric screening and GC measurement were compared. P₂P₅P₃, P₄P₂P₁, P₄P₄P₃ showed no significant difference between the two methods, whereas P₄P₅P₁ and P₄P₃P₂ slightly deviated the screening. Interestingly, the construct architecture of the highest producer was with P_{ADH1} for the additional copy of Acs1 and the scaffold, and one copy of C₂ on the scaffold. Finally, we compared the optimized pathway to wild type, the unassembled pathway and assembled pathway, 83-, 2.0-, and 1.8-fold improvement of ethyl acetate production were obtained, respectively, with a final titer of 51.6 mg/L achieved in this work.

7.5 DISCUSSION

Engineering microbial production of desirable chemicals often require an array of enzymes that may be naturally organized in distinct subcellular compartments. Interruption of metabolic reactions given by unorganized subcellular localization of enzymes may compromise the performance of an overall pathway resulted from a challenge of metabolite transport and utilization of competing pathways. The concept of enzyme spatial organization overcoming the challenge has been taken for syntheses of natural products in plant and fungi with a long evolutionary history. However, studies of recapitulation of such strategy in engineered microorganisms for metabolic engineering was limited. Part of the reasons is a lack of synthetic biology tools for enzyme spatial organization. Development of synthetic biology tools for localization of enzymes on the organellar membranes (e.g. LDs and the ER) and in organellar matrices (e.g. peroxisomes and mitochondria) will accelerate our capabilities to reorganize modularized metabolic reactions along a synthetic pathway for the sake of metabolic channeling. We believe that this work suggests a niche for the strategy organizing metabolic pathways for membrane-bound enzymes to metabolic engineering.

Despite synthetic biology tools organizing metabolic enzymes in proximity in cytoplasm or organellar compartments have been developed and shown improvement of product yields, a demonstration exploring the channeling effects of enzyme co-localization on the intracellular membranes has yet to be shown. The Oleosin-Cohesin-Dockerin based synthetic scaffolds developed here redirect cytoplasmic enzymes to LDs and, meanwhile, maintain enzyme activities. Without further optimization for enzyme

activity and stoichiometry of the scaffold, the cascade reaction co-localizing Ald6 and Acs1 to Atf1 on LDs has clearly demonstrated a kinetic advantage with 1.9-fold fold enhancement over a freely diffused pathway in buffered solution. In contrast to the efficacy of assembled pathway *in vitro*, it appears that the channeling effect was compromised in intracellular conditions (**Fig. 7.4d and 7.5f**). Part of the reactions may be due to limitation of cofactors availability, which requires supplies of NAPD^+ , ATP, and CoA. In addition, the early onset of oleosin targeting to LDs may reduce time of co-localization of the scaffold and Atf1 on the intracellular membrane. The targeting of oleosin to LDs was observed at 10 h (**Fig. 7.2a**); however, LD localization of Atf1 was observed at 18 h in our previous study [9], leading to a mismatch of co-localization in time. A dynamic control system that can regulate molecular targeting of proteins from the ER to organelles needs to be developed for elaboration of enzyme co-localization strategy.

Maturation of cloning techniques has enabled us to rapidly assemble metabolic pathway or gene clusters. Yet, technologies that enable us to rapidly dissect phenotypes fall far behind. Quantification of esters relies heavily on GC or GC-MS, which have lowered the throughput of screening. In this work, we developed a colorimetric assay that can be used to quantify ethyl acetate directly from cultures, which has increased the throughput of screening. An optimization strategy coupling this assay was used to obtain high producers. Phenotypic characterization of pathway variants was first performed with the colorimetric assay, which had guaranteed improvements of ethyl acetate production. The constructs were then isolated and re-transformed into yeast for confirmation product

synthesis. This processes has decreased the number for sampling and shortened the time required for GC analysis.

The synthetic biology tools and colorimetric assay presented here provide new approaches for strain optimization. The modularity of the synthetic scaffolds can be generalizable to other intracellular membranes if cohesin is fused to membrane anchors. Moreover, the colorimetric assay quantifying esters can be expanded to detect other esters if solvents and reaction conditions are optimized. Most importantly, this is the first effort of spatial organization of cytoplasmic enzymes on the intracellular membrane for pathway engineering, which demonstrates the advancement of our ability to manipulate biology in the context of cellular organization.

7.6 ACKNOWLEDGEMENTS

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7.8 SUPPLEMENTARY INFORMATION

Supplementary Table 7.1. *E. coli* and yeast strains used in this study.

Strains	Genotype	Source
<i>E. coli</i> DH5 α	<i>F</i> – Φ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA</i> – <i>argF</i>) <i>U169 recA1 endA1 hsdR17 (rK–, mK+)</i>	Life technologies
<i>E. coli</i> BL21 (DE3)	<i>phoA supE44 λ– thi-1 gyrA96 relA1</i> <i>fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS</i> λ DE3 = λ <i>sBamHI</i> o Δ EcoRI–B int::(<i>lacI</i> :: <i>PlacUV5</i> :: <i>T7 gene1</i>) <i>i21 Δnin5</i>	NEB
<i>S. cerevisiae</i> BY4742	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	GE Healthcare
YS218	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ERG6::DsRed</i>	Lin and Wheeldon ¹
YS219	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 SEC61::DsRed</i>	Lin and Wheeldon ¹
YS419	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 yprcΔ15::P_{TDH3}–ATF1–myc–T_{TEF2}</i>	this study

Supplementary Table 7.2. Plasmids used in this study.

Name	Description	Cloning technique	Source
pIW7	pRS426		GE Healthcare
pIW14	pRS426-PPGK1-TPGK1	R. E.	Lin and Wheeldon
pIW119	pRS426-PPGK1-AAM-B (1-38)-CFP-TPGK1	OE-PCR and R. E.	this study
pIW120	pRS426-PPGK1-Viperin (1-42)-CFP-TPGK1	OE-PCR and R. E.	this study
pIW121	pRS426-PPGK1-HCV cp (118-164)-CFP-TPGK1	OE-PCR and R. E.	this study
pIW122	pRS426-PPGK1-17 β HSD11 (1-28)-CFP-TPGK1	OE-PCR and R. E.	this study
pIW123	pRS426-PPGK1-GBF1 (1004-1048)-CFP-TPGK1	OE-PCR and R. E.	this study
pIW124	pRS426-PPGK1-CFP-TPGK1	R. E.	this study
pIW147	pRS426-PPGK1-Oleosisin (1-186)-CFP-TPGK1	R. E.	this study
pIW131	pRS426-PPGK1-ACSI-CFP-TPGK1	R. E.	this study
pIW149	pRS426-PPGK1-ACSI-Oleosisin-CFP-TPGK1	R. E.	this study
pIW215	pRS426-PPGK1-ACSI-HA-Doc (C2)-TPGK1	OE-PCR and R. E.	this study
pIW134	pRS426-PPGK1-ALD6-CFP-TPGK1	R. E.	this study
pIW151	pRS426-PPGK1-ALD6-Oleosisin-CFP-TPGK1	R. E.	this study
pIW242	pRS426-PPGK1-ALD6-His tag-Doc (C1)-TPGK1	OE-PCR and R. E.	this study
pIW107	pRS426-PPGK1-ATF1-myc-TPGK1	R. E.	Lin and Wheeldon
pIW251	pRS426-PTEF1-Oleosisin-X82 (C1)-Coh (C2)-V5-TTP11-PPGK1p-ATF1-myc-TTEF2	DNA assembler	this study
pIW254	pRS426-PTEF1-Oleosisin-X82 (C1)-Coh (C2)-V5-TTP11-PPGK1p-ATF1-myc-TTEF2-PTP11-ACSI-HA-Doc (C2)-TCYC1-PTDH2-ALD6-His tag-Doc (C1)-THXT7	DNA assembler	this study
pIW189	pRS426-PPGK1-Oleosisin-Cohesin (C1)-His tag-YFP-TPGK1	R. E.	this study
pIW152	p413-PGPD-TCYC1		Mumberg
pIW192	p413-PGPD-ACSI-CFP-Dockerin (C2)-TCYC1	OE-PCR and R. E.	this study
pIW193	p413-PGPD-ALD6-CFP-Dockerin (C2)-TCYC1	OE-PCR and R. E.	this study
pIW234	pRS426-PPGK1-ATF1-DsRed-PGK1t	R. E.	this study
pIW239	p413-PGPD-ALD6-YFP-Doc (C1)-TCYC1	OE-PCR and R. E.	this study
pIW240	pRS426-PPGK1-Oleosisin-X82 (C1)-Coh (C2)-V5-TPGK1	OE-PCR and R. E.	this study
pIW247	pRS316-PTEF1-Oleosisin-X82 (C1)-Coh (C2)-V5-TTP11-PPGK1-ATF1-DsRed-TTEF2-PTP11-ACSI-CFP-Doc (C2)-TCYC1-PTDH2-ALD6-YFP-Doc (C1)-THXT7	DNA assembler	this study
pIW266	pRS316-PPGK1-ATF1-DsRed-TTEF2-PTP11-ACSI-CFP-Doc (C2)-TCYC1-PTDH2-ALD6-YFP-Doc (C1)-THXT7	DNA assembler	this study
pIW310	pRS316-PTEF1-ACSI-HA-Doc (C2)-TPRM9-PTDH2-ALD6-His tag-Doc (C1)-THXT7-PADH1-Oleosisin-X82 (C1)-Coh (C2)-V5-TTP11	DNA assembler	this study
pIW313	pRS316-PTEF1-ACSI-HA-Doc (C2)-TPRM9-PTDH2-ALD6-His tag-Doc (C1)-THXT7	DNA assembler	this study
pIW323	pRS426-PPGK1-X82 (C1)-Coh (C2)-Coh (C2)-V5-TPGK1	Gibson assembly	this study
pIW324	pRS426-PPGK1-X82 (C1)-Coh (C2)-Coh (C2)-Coh (C2)-V5-TPGK1	Gibson assembly	this study
pIW334	pRS426-PPGK1-ACSI-TTP11	Gibson assembly	this study
pIW335	pRS426-PCCW12-ACSI-TTP11	Gibson assembly	this study
pIW336	pRS426-PHXT7-ACSI-TTP11	Gibson assembly	this study
pIW337	pRS426-PADH1-ACSI-TTP11	Gibson assembly	this study
pIW338	pRS426-PRNR1-Oleosisin-X82 (C1)-Coh (C2)-TTP11	Gibson assembly	this study
pIW339	pRS426-PPG11-Oleosisin-X82 (C1)-Coh (C2)-TTP11	Gibson assembly	this study
pIW340	pRS426-PRPL18B-Oleosisin-X82 (C1)-Coh (C2)-TTP11	Gibson assembly	this study
pIW341	pRS426-PREV1-Oleosisin-X82 (C1)-Coh (C2)-TTP11	Gibson assembly	this study
pIW342	pRS426-PADH1-Oleosisin-X82 (C1)-Coh (C2)-TTP11	Gibson assembly	this study
pIW343	pRS426-PRNR1-Oleosisin-X82 (C1)-Coh (C2)-Coh (C2)-TTP11	Gibson assembly	this study
pIW344	pRS426-PPG11-Oleosisin-X82 (C1)-Coh (C2)-Coh (C2)-TTP11	Gibson assembly	this study
pIW345	pRS426-PRPL18B-Oleosisin-X82 (C1)-Coh (C2)-Coh (C2)-TTP11	Gibson assembly	this study
pIW346	pRS426-PREV1-Oleosisin-X82 (C1)-Coh (C2)-Coh (C2)-TTP11	Gibson assembly	this study
pIW347	pRS426-PADH1-Oleosisin-X82 (C1)-Coh (C2)-Coh (C2)-TTP11	Gibson assembly	this study
pIW348	pRS426-PRNR1-Oleosisin-X82 (C1)-Coh (C2)-Coh (C2)-Coh (C2)-TTP11	Gibson assembly	this study
pIW349	pRS426-PPG11-Oleosisin-X82 (C1)-Coh (C2)-Coh (C2)-Coh (C2)-TTP11	Gibson assembly	this study
pIW350	pRS426-PRPL18B-Oleosisin-X82 (C1)-Coh (C2)-Coh (C2)-Coh (C2)-TTP11	Gibson assembly	this study
pIW351	pRS426-PREV1-Oleosisin-X82 (C1)-Coh (C2)-Coh (C2)-Coh (C2)-TTP11	Gibson assembly	this study
pIW352	pRS426-PADH1-Oleosisin-X82 (C1)-Coh (C2)-Coh (C2)-Coh (C2)-TTP11	Gibson assembly	this study
pIW306	pRS316-LEU2-PTDH3-ATF1-myc-TTEF2	Gibson assembly	this study
pIW185	pET29-HA-myc-GFP-(His)6	R. E.	this study

Supplementary Table 7.3. DNA sequences of protein parts including LD tags, cohesin, and dockerin used in this study.

AAM-B (114 bp)

```
ATGGAGTTGA CAATTTTAT TTTAAGGTTA GCCATTTATA TCCTAACGTT
TCCACTATAT CTATTAACT TTTTAGGACT ATGGTCTTGG ATATGCAAGA
AGTGGTTTCC ATAT
```

Viperin (126 bp)

```
ATGGGTATGT TAGTGCCTAC GGCACCTAGCT GCTAGACTGT TAAGTTTGT
CCAACAACAA TTAGGAAGCC TGTGGTCAGG TTTAGCTATT TTATTCTGCT
GGTTACGTAT CGCCCTAGGT TGGTTG
```

HCV core protein (144 bp)

```
ATGAATTTAG GCAAAGTGAT TGACACCCTG ACTTGTGGTT TCGCCGATTT
GATGGGGTAC ATTCCTCTTG TAGGGGCTCC CTTGGGAGGT GCCGCTAGAG
CTCTAGCACA TGGTGTTCGT GTATTAGAAG ACGGTGTTAA TTAT
```

17b-HSD11 (84)

```
ATGAAGTTTC TACTGGATAT CTTATTGTTG CTTCCACTTT TAATAGTCTG
CAGCTTGGA TCTTTCGTAA AACTATTTAT ACCC
```

GBF1 (138 bp)

```
ATGAAAGCCC ATATCGCAGC TAAGACGGTC TTTCATTTAG CACACAGACA
TGGTGACATT CTACGTGAAG GCTGGAAGAA TATTATGGAA GCCATGTTAC
AATTATTTAG GGCACAGTTA CTGCCTAAGG CAATGATT
```

Oleosin (558 bp)

```
ATGGCTGATA GAGATAGGTC TGGTATTTAT GGGGGTGCTC ACGCGACTTA
TGGTCAACAA CAGCAACAAG GTGGAGGTGG TAGGCCAATG GGTGAACAAG
TTAAAGGTAT GTTGCATGAT AAAGGTCCAA CTGCTTCTCA AGCTTTGACT
GTGGCTACTT TGTTTCCATT GGGTGGGCTT TTGTTGGTTT TGTCTGGTTT
GGCCTTGACT GCGTCTGTGG TCGGTTTtagC TGTTGCTACA CCTGTTTTCT
TGATTTTTTC TCCGGTTTTG GTTCCAGCTG CACTATTGAT TGGTACTGCT
GTTATGGGTT TTTTGACTAG TGGTGCTTTG GGTTTGGGCG GTTTGTCTAG
TTTGACATGT TTGGCTAATA CTGCTCGTCA AGCCTTTCAA AGAACTCCAG
ATTATGTTGA AGAAGCACAT AGAAGAATGG CCGAGGCTGC TGCTCATGCT
GGGCATAAAA CTGCCCAAGC TGGTCAAGCT ATTCAAGGTA GAGCTCAAGA
AGCTGGGGCA GGGGGTGGTG CAGGTGCGGG TGCTGGTGGC GGTGGAAGAG
CCTCTAGC
```

Cohesin (*C. thermocellum*) 486 bp

```
ATGGCCTCTG ATGGGGTGGT TGTGGAAATT GGAAAAGTTA CTGGGAGCGT
GGGTACCACT GTTGAAATCC CGGTCTATTT CAGGGGAGTT CCATCTAAAG
```

GTATCGCAAA	CTGTGACTTT	GTCTTTAGAT	ACGACCCTAA	CGTTCTTGAG
ATAATCGGTA	TAGATCCAGG	CGATATCATT	GTGGATCCTA	ATCCTACTAA
GAGCTTCGAC	ACGGCTATAT	ATCCAGATAG	AAAAATTATT	GTATTTTTGT
TTGCAGAAGA	CTCAGGGACT	GGGGCATATG	CTATCACTAA	GGATGGTGTG
TTTGCTAAAA	TACGTGCTAC	CGTTAAGTCC	TCAGCACCTG	GTTATATCAC
TTTCGACGAA	GTTGGTGGCT	TTGCCGACAA	TGATCTAGTG	GAACAAAAGG
TATCATTCAT	TGATGGTGGT	GTAAATGTCT	GTAATGCAAC	ACCGACCAAA
GGAGCCACTC	CCACTAACAC	AGCAACTCCA	ACCAAA	

Dockerin (*C. thermocellum*)195 bp

GGTGGTGGTG	GTTCTATGGG	TGATGTTAAT	GGTGATGGCA	CGATAAACTC
CACGGATCTT	ACTATGTAA	AAAGATCTGT	TTTACGTGCG	ATCACATTAA
CAGACGATGC	TAAGGCAAGG	GCTGATGTAG	ATAAAAATGG	TTCAATCAAT
AGCACAGATG	TCTTACTATT	ATCCAGATAC	CTTTTGAGAG	TGATT

X82 (*C. perfringens*)468 bp

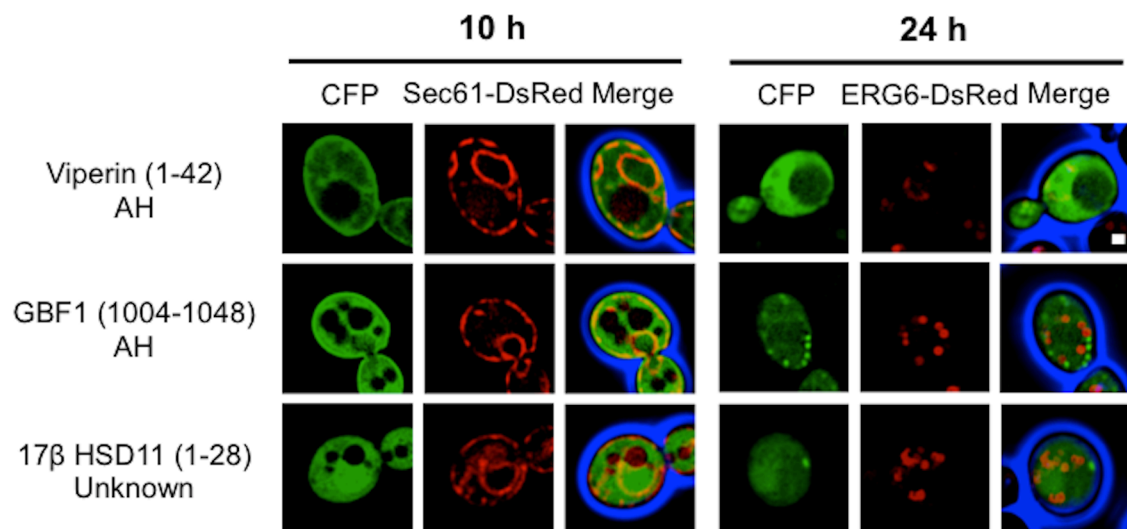
ATGTCCAGCG	GACTTGTCCC	AAGAGGAAGT	CATATGGCCA	GTAAATTAAA
AGAAAATGCA	GAGGTGACTG	GATCTGTCAG	TCTAGAAGCT	TTGGAAGAAG
TACAAGTTGG	AGAAAATCTA	GAGGTGGGTG	TAGGAATAGA	TGAATTGGTC
AACGCGGAAG	CATTTGCTTA	TGACTTTACT	TTGAATTATG	ATGAAAATGC
TTTCGAGTAT	GTGGAAGCTA	TCTCTGATGA	TGGTGTGTTC	GTTAATGCGA
AAAAGATTGA	GGATGGCAAG	GTGAGAGTCT	TAGTTTCTTC	ACTTACTGGT
GAACCCTTAC	CTGCTAAGGA	GGTACTGGCC	AAAGTCGTCT	TAAGAGCTGA
AGCCAAAGCA	GAAGGATCCA	ACCTTTCAGT	GACTAATTCT	TCCGTCGGTG
ACGGCGAAGG	GTTGGTTCAC	GAGATCGCTG	GGACTGAGAA	AACCGTCAAC
ATCATCGAAG	GAACATCT			

Dockerin (*C. perfringens*)159 bp

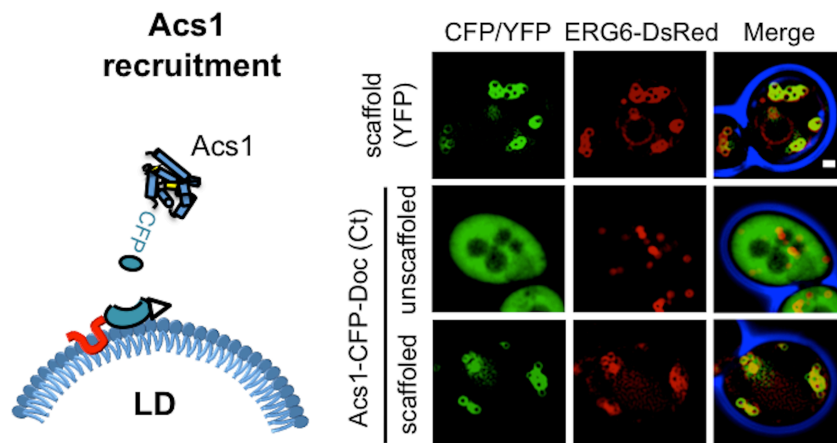
GGAGATTTCA	ATGGTAATGG	AAAAATAGAT	ATCGGCGATT	TGGCTATGGT
TTCTAAGAAT	ATTGGGAGCA	CCACAAACAC	TTCATTAGAT	TTGAATAAGG
ACGGGTCTAT	TGATGAATAT	GAAATTTTCAT	TCATAAACCA	CCGTATTTTA
AATTTAGAA				

Supplementary Table 7.4. Fold enhancements shown in the heat map. Colony 1 to 5 in the table were shown in the heat map with the order from bottom to top.

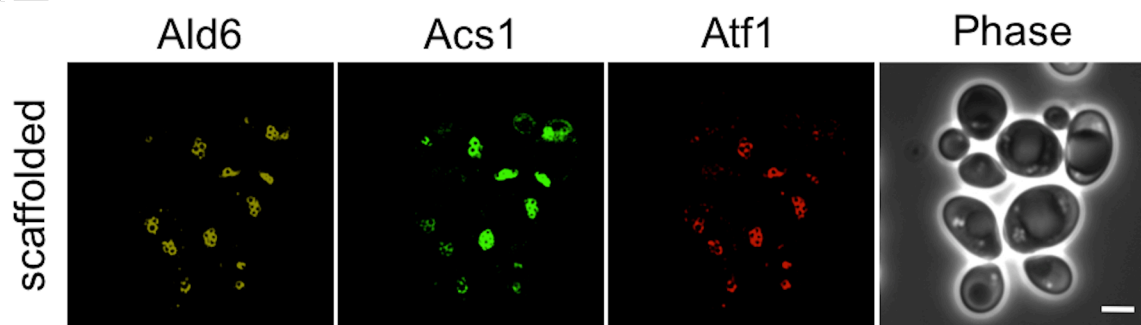
Construct	Colony				
	1	2	3	4	5
P ₁ P ₁ S ₁	1.1	0.7	1.0	0.8	0.8
P ₁ P ₂ S ₁	1.1	1.1	0.8	1.0	1.0
P ₁ P ₃ S ₁	1.1	1.0	1.0	1.0	1.0
P ₁ P ₄ S ₁	0.8	1.2	0.9	0.9	1.0
P ₁ P ₅ S ₁	0.9	1.1	0.9	1.0	0.8
P ₁ P ₁ S ₂	1.0	1.1	1.1	0.9	1.0
P ₁ P ₂ S ₂	0.9	1.1	1.4	0.8	1.0
P ₁ P ₃ S ₂	1.0	1.0	1.0	1.0	0.9
P ₁ P ₄ S ₂	1.0	1.2	1.1	1.0	0.9
P ₁ P ₅ S ₂	1.0	1.3	1.1	0.9	1.0
P ₁ P ₁ S ₃	0.9	1.1	1.0	0.9	0.9
P ₁ P ₂ S ₃	1.2	1.2	0.9	1.1	1.1
P ₁ P ₃ S ₃	1.6	1.0	0.9	0.9	1.0
P ₁ P ₄ S ₃	0.9	1.0	1.2	1.1	0.9
P ₁ P ₅ S ₃	0.8	1.1	1.0	0.8	0.7
P ₂ P ₁ S ₁	0.7	0.9	0.9	0.9	0.8
P ₂ P ₂ S ₁	1.0	1.0	1.0	0.9	0.9
P ₂ P ₃ S ₁	0.5	1.0	1.1	1.0	1.0
P ₂ P ₄ S ₁	1.1	1.0	1.0	1.0	1.0
P ₂ P ₅ S ₁	1.2	1.2	1.1	1.0	1.0
P ₂ P ₁ S ₂	0.8	1.1	0.9	0.8	0.8
P ₂ P ₂ S ₂	1.0	1.1	1.1	0.8	0.9
P ₂ P ₃ S ₂	1.3	1.0	1.0	1.0	0.9
P ₂ P ₄ S ₂	1.0	1.2	1.0	1.1	1.0
P ₂ P ₅ S ₂	1.1	1.1	0.8	1.0	0.9
P ₂ P ₁ S ₃	0.8	0.9	0.9	1.0	0.9
P ₂ P ₂ S ₃	0.9	0.8	1.2	0.9	1.0
P ₂ P ₃ S ₃	1.4	1.1	1.2	0.9	1.0
P ₂ P ₄ S ₃	1.3	1.2	1.2	1.0	1.1
P ₂ P ₅ S ₃	1.6	1.1	1.3	1.0	1.1
P ₃ P ₁ S ₁	1.0	1.1	1.0	0.8	0.8
P ₃ P ₂ S ₁	0.8	1.0	1.2	0.9	1.0
P ₃ P ₃ S ₁	1.0	1.2	0.9	1.0	0.9
P ₃ P ₄ S ₁	0.9	1.1	1.0	0.5	1.0
P ₃ P ₅ S ₁	0.8	1.0	1.1	1.0	0.9
P ₃ P ₁ S ₂	0.9	1.1	1.0	1.0	1.0
P ₃ P ₂ S ₂	0.8	1.1	1.1	1.0	0.9
P ₃ P ₃ S ₂	1.0	1.0	1.0	1.0	0.9
P ₃ P ₄ S ₂	1.0	1.2	1.1	1.0	1.0
P ₃ P ₅ S ₂	1.0	1.2	1.3	1.0	1.0
P ₃ P ₁ S ₃	1.0	1.0	1.1	0.9	0.8
P ₃ P ₂ S ₃	0.9	1.1	1.1	1.1	0.9
P ₃ P ₃ S ₃	1.0	1.0	1.1	1.0	1.0
P ₃ P ₄ S ₃	1.1	1.2	1.0	1.2	0.9
P ₃ P ₅ S ₃	1.1	1.0	0.9	1.0	1.0
P ₄ P ₁ S ₁	1.1	1.1	1.0	1.1	0.9
P ₄ P ₂ S ₁	1.1	1.2	1.4	1.2	1.1
P ₄ P ₃ S ₁	1.0	1.2	1.3	1.2	0.9
P ₄ P ₄ S ₁	1.0	1.2	1.4	1.0	1.1
P ₄ P ₅ S ₁	1.1	1.2	1.3	1.0	1.0
P ₄ P ₁ S ₂	1.0	1.2	1.0	1.1	1.1
P ₄ P ₂ S ₂	0.9	1.1	1.0	1.2	1.1
P ₄ P ₃ S ₂	1.7	1.0	1.3	1.1	1.0
P ₄ P ₄ S ₂	1.0	1.1	1.2	1.2	1.2
P ₄ P ₅ S ₂	1.2	1.0	1.1	1.0	0.8
P ₄ P ₁ S ₃	0.7	1.2	1.2	0.9	1.0
P ₄ P ₂ S ₃	1.0	1.2	1.0	1.0	1.0
P ₄ P ₃ S ₃	1.0	1.0	1.1	0.9	1.1
P ₄ P ₄ S ₃	1.4	1.1	1.3	1.0	1.1
P ₄ P ₅ S ₃	1.0	1.1	1.5	1.2	1.1



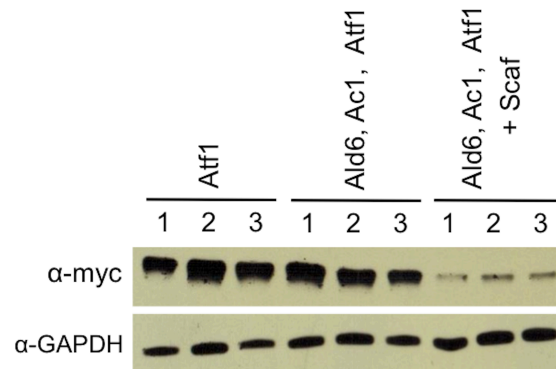
Supplementary Figure 7.1. Fluorescence microscopy analysis of subcellular localization of three potential LD tags including N-terminal 1-42 of Viperin, internal 1004-1048 of GBF1, and N-terminal 1-28 of 17 β HSD11 at different time course of aerobic growth. Scale bar: 1 μ m.



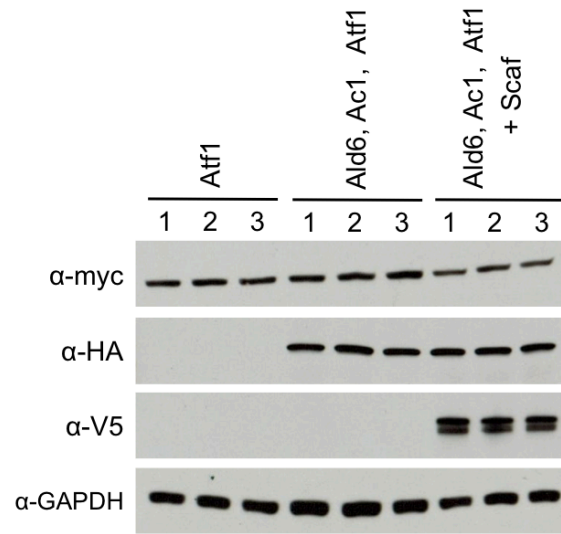
Supplementary Figure 7.2. Fluorescence microscopy analysis of subcellular localization of the scaffold: Oleosin-Coh (Ct)-YFP, and Acs1-CFP-Doc (Ct) in the absence or presence of the scaffold. Scale bar: 1 μ m.



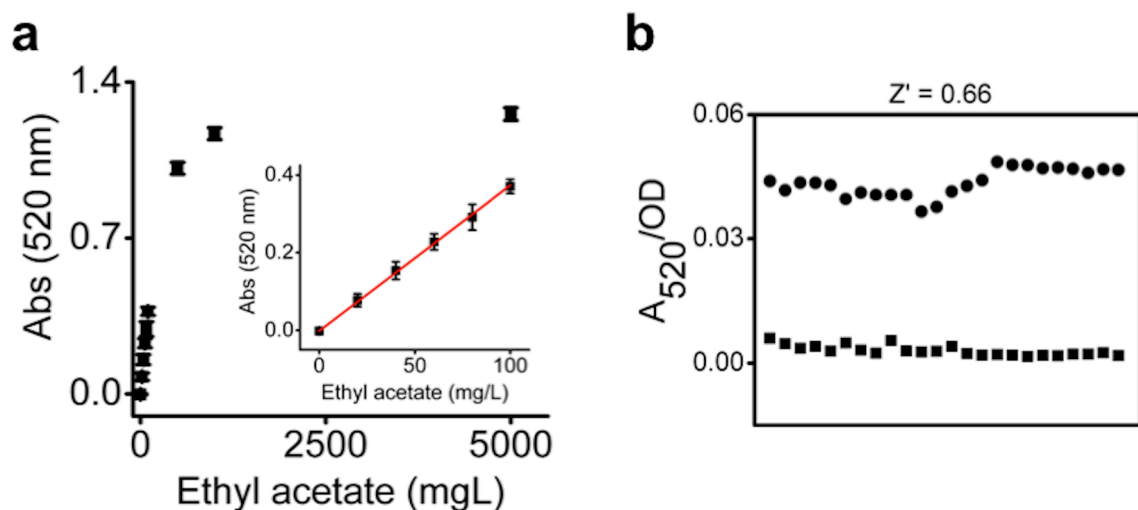
Supplementary Figure 7.3. Fluorescence microscopy analysis of subcellular localization of Ald6, Acs1, and Atf1 in presence of the scaffold (Oleosin-X82 (Cp)-Coh (Ct)). Cells were cultured to stationary phase in fermented condition. Scale bar: 3 μ m.



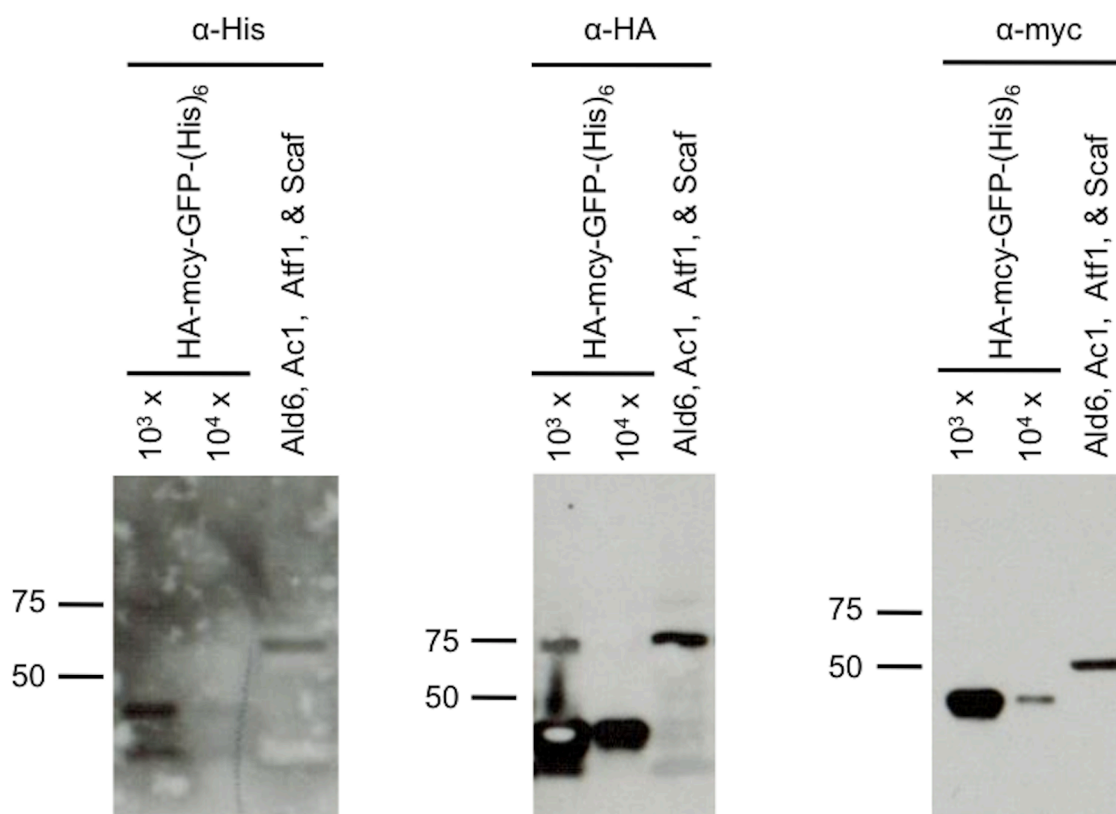
Supplementary Figure 7.4. Western blot analysis of protein expression of Atf1 in three different strains. Genes were overexpressed with high copy number plasmids (pRS426). anti-myc antibody was used to detect Atf1; GAPDH was used as the loading control. Three independent samples were tested for expression.



Supplementary Figure 7.5. Western blot analysis of protein expression of Acs1, Atf1, and the scaffold in three different strains. Atf1 was overexpressed by chromosomal integration of an additional copy while Acs1 and the scaffold (Oleosin-X82 (Cp)-Coh (Ct)) were expressed with low copy number plasmids (pRS316). anti-myc, anti-HA, and anti-V5 antibody were used to against Atf1, Acs1, and the scaffold, respectively. GAPDH was used as the loading control. Three independent samples were tested for expression.



Supplementary Figure 7.6. Development of the colorimetric assay for ethyl acetate quantification. (a) Demonstration of the assay responding to different ethyl acetate concentrations. 0 to 5000 mg/L ethyl acetate dissolved in ethanol was tested with the assay. A linear relationship between absorbance and ethyl acetate concentration is shown in the inset. (b) Z' factor analysis for the assay. Yeast strains BY4742 *yprcA15::PTDH3-ATF1-myc-TTEF2* and BY4742 transformed with pRS316 were used as the positive and negative control, respectively. Ethyl acetate extracted from the fermented cultures was quantified with the assay. A detailed experimental protocol was described in Screening of the combinatorial library of Materials and Methods. Five independent colonies were analyzed and each of them was performed at least in quadruplicate.



Supplementary Figure 7.7. Western blot analysis for estimation of relative expression of Ald6, Acs1, and Atf1. Atf1 was overexpressed by chromosomal integration of an additional copy while Acs1 and the scaffold (Oleosin-X82 (Cp)-Coh (Ct)) were expressed with low copy number plasmids (pRS316). A GFP containing HA, myc, and His tags was used as the standard for comparison. Basically, 2.5 OD cells expressing pET28-HA-myc-GFP-(His)₆ in BL21 (DE3) *E. coli* strain was resuspended in 250 μ L SDS-PAGE sample buffer and boiled for lysate preparation. 10 μ L of *E. coli* lysate with 10^3 - or 10^4 -fold dilution was loaded for analysis. anti-His, anti-HA, and anti-myc antibody were used to against Ald6, Acs1, and Atf1, respectively.

Chapter 8. Conclusions

Industrial biosynthesis of high value and commodity chemicals requires bioprocesses that operate in high yield and productivity. To achieve this, enzymes with high activity and specificity, and metabolic pathways with balanced flux must be engineered. The overall goal of this thesis is to engineer efficient biocatalysts and metabolic pathways. The advancement of new technologies and fundamental understanding of underlying mechanisms will tremendously speed up the progress of industrial production. In this regard, various scientific questions and engineering challenges have been addressed.

Engineering substrate or metabolic channeling effect for biocatalysis and metabolic engineering is one of the objectives that researchers strive to achieve. In Chapter 2, I introduced three methodologies to characterize the channeling effect—transient time analysis, kinetic challenge with a competing reaction to the intermediate, and overall catalysis of initial reaction rate. This theoretical presentation has been applied to evaluate channeling of Acs1 to Atf1 on LDs (Chapter 7). With co-localizing Acs1 to Atf1, the production of ethyl acetate was enhanced 1.9-fold when required substrates and cofactors were fed to the pathway *in vitro*. Noticeably, it has yet to be determined whether there is a physical interaction between Acs1 and Atf1.

DNA is a prevailing biomaterial that bioengineers use to scaffold cascade enzymes in close proximity *in vitro*. Though the efficiency of the consecutive reactions is increased due to enzyme co-localization, the kinetic effect of DNA scaffolds on the catalysis of enzyme-DNA nanostructures remains under explored. In Chapter 3, I have shown that kinetic enhancements can be obtained through conjugation of HRP to DNA scaffolds,

and such enhancements are substrate binding energy dependent. The best enhancement occurred when the binding between substrate and DNA was intermediate, whereas weak and strong binding showed minimal effects. The concept abstracted from the volcano shape result mimicking the Sabatier Principle can be extended to rational designing of DNA sequences for specific substrates to controlling kinetics of biocatalysts.

High throughput screening has always been a critical step for protein engineering and enzyme discoveries. In Chapter 4, with an extension of α -ketoglutarate dehydrogenase reaction to AATase, the rate of ester synthesis can be monitored by measuring absorbance of NADH at 340 nm. This coupled enzyme assay was further advanced for high throughput screening with microtiter plates. Aided with this facile assay, we screened a panel of AATases toward a library of acyl-CoAs and revealed that Atf-SI from tomato exhibited high activity toward C4 to C10 acyl-CoAs and 1-pentanol. Though only AATase synthesizing short- and medium- chain esters were tested and demonstrated in this work, this assay can be possibly applied to long chain waxy ester synthases. Furthermore, the poor activity and substrate specificity of AATases used in metabolic engineering has limited the titer and product purity for ester synthesis. This high throughput assay can be readily used to screening of enzyme variants with rational design.

With the advent of next-generation DNA sequencing, numerous microorganisms with unique characteristic have been isolated and genotyped. The diversity of identified microbes has provided metabolic engineers with a variety of host chassis to produce target molecules. Despite the availability of new microbial hosts, heterologous expression of

enzymes in a new host may sabotage activity due to altered intracellular conditions. In Chapter 5, heterologous expression of AATases in *E. coli* and *S. cerevisiae* offers a cue for microbial host selection. For example, when the membrane localized Atf1-S.c is expressed in *E. coli*, a lack of organellar membrane structure may result in forming cytoplasmic aggregates. A loss of enzyme activity may be, in part, resulted from blocking the active site from accessing substrates from the cytoplasm. As a result, a more suitable microbial host for heterologous expression of membrane-bound enzymes from plants and other eukaryotes may go to organisms with similar cellular structures, unless a cytosolic counterpart of the membrane-bound one is found.

Cellular localization of enzymes can be dynamic. The destination of enzymes in cells is regulated by cellular physiology. The elusive membrane localization on the ER and LDs of Atf1 in *S. cerevisiae* has hindered engineering of enzyme co-localization. As such, in Chapter 6, I have investigated the potential molecular targeting mechanism and monitored dynamic membrane targeting of Atf1. The study reveals a growth phase dependent subcellular localization of Atf1 from the ER at lag and log phase to LDs at stationary phase. This membrane association is N- and C-terminal amphipathic helices (AHs) dependent. According to this finding, I have developed synthetic protein scaffolds capable of organizing cytoplasmic enzymes to LDs for co-localization effects (Chapter 7). The synthetic scaffolds consisting of oleosin-cohesin-dockerin protein parts can functionally localize Ald6 and Acs1 to Atf1 on LDs. Because of the modularity of cohesin-dockerin, it is applicable to other organellar membranes if a membrane anchor is defined. In addition, by extracting ethyl acetate from fermented cultures with hexane, this

ester could be quantified with spectrophotometer at 520 nm via absorbance of ferric chelate complex. The high throughput manner of the colorimetric assay has greatly shortened time scale for pathway library screening. The assay is generally applicable to other esters if the extraction solvent is miscible to the assay reactions.

Metabolic engineering promises green and renewable syntheses of various molecules that petrochemical refineries and organic syntheses can not accomplish, there remains myriad challenges that we need to overcome. For examples, current high throughput screening methods amenable to fluorescence activated cell sorting (FACS) or microfluidics are not generalizable to every molecule; computational algorithms predicting enzyme structures and functions and metabolic flux need to be advanced; and dynamic expression systems responding to environmental or physiological cues are only limited to certain chemicals. As more analytical and computational technologies, and synthetic biology tools being developed and matured, I envision a revolution of industrial chemical syntheses.