Toward a General Dehydrogenase Enzymatic Scaffold for Industrial Biocatalysis

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Enzymes catalyze a wide range of reactions with high efficiency and exquisite specificity. As such, they lend themselves well for use in a myriad of applications from the production of fine chemicals to use in biofuel cells. Demand for enzymes with novel specificities has risen in recent years, as they are “green” catalysts and may find use as environmentally friendly replacements for conventional catalysts in a variety of chemical processes. However, their widespread use has been hindered by a number of challenges, including high cost, low stability, and the requirement of expensive cofactors for catalysis. A significant amount of research has been done to address these limitations, but the approaches taken are rarely general, and thus it remains difficult to engineer industrially compatible enzymes.

The ideal enzyme for use in these systems would be inexpensive to express and purify, extremely stable, easy to immobilize without loss of activity, able to use cheap, non-natural cofactors with improved stabilities and redox properties, and be rapidly evolvable for desired substrate specificities and reactions. Here, we
present a novel approach to satisfy these requirements. We begin with a designed enzyme scaffold with beneficial properties for use in these systems, and then engineer in cofactor and substrate specificity as required for the application.

A thermostable alcohol dehydrogenase, AdhD, from the hyperthermophilic archaea *Pyrococcus furiosus* was selected as the scaffold for this work, as it possesses several features which make it an attractive candidate for protein engineering and downstream industrial applications. It can be expressed recombinantly in *Escherichia coli* in high yield, and is readily purified due to its extreme thermostability (half-life of 130 min at 100°C). Additionally, a thermostable scaffold will increase enzyme lifetimes in industrial applications, and provide resistance to chemical and thermal inactivation. AdhD belongs to the aldo-keto reductase superfamily, a large and diverse family of oxidoreductase enzymes, and shares the canonical (α/β)₈-barrel fold and nicotinamide cofactor binding pocket. AdhD has a strong preference for NAD(H) over NADP(H), and is active with a broad range of substrates. Lastly, the enzyme is monomeric, with no metal centers or disulfides, further simplifying engineering efforts.

We began by examining cofactor binding in the AdhD enzyme through several rational mutations to the cofactor binding pocket. Guided by previous work examining cofactor specificity in the aldo-keto reductase superfamily, we
identified two mutations, K249G and H255R, which had a significant impact on cofactor binding and activity.

While characterizing the cofactor specificity double mutant, we discovered that the mutations also enabled the enzyme to utilize a truncated nicotinamide cofactor for catalysis. The benefit of improved cofactor diffusion was demonstrated through the creation of an enzymatic biofuel cell for the oxidation of D-arabinose.

Next we examined the substrate specificity of the enzyme, utilizing a rational loop-swapping approach. AdhD was readily imparted with aldose reductase activity through the grafting of substrate binding loops from another AKR, human aldose reductase. The chimeric loop mutants also retained activity with the model substrate for AdhD, but exhibited a complete reversal of cofactor specificity.

Finally, we discuss the design and preliminary results of a novel selection step for the directed evolution of substrate specificity and catalytic activity. Taken together, this work describes the development of a general dehydrogenase enzymatic platform that can be adapted for use in a wide range of applications.
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Chapter 1

INTRODUCTION

The field of enzyme engineering has evolved rapidly over the past 30 years, due in part to the growing demand for enzymes to replace conventional catalysts in industrial applications (Table 1.1). The worldwide market for industrial enzymes was estimated at $3.3 billion in 2010, with strong growth projected over the next decade (1). Enzymes are attractive alternatives to conventional catalysts, as they are derived from natural sources, work under mild conditions, and exhibit extremely high catalytic efficiency and selectivity (2). However, the use of natural enzymes in industrial applications is often hindered by their high cost, limited stability, strict cofactor requirements, and the limited number of reactions catalyzed by known enzymes. To address these limitations, researchers have developed a range of techniques to study enzyme function and mechanism, and to enable the engineering of enzymes with desirable properties.

Cofactor Specificity

Many industrially important enzymes require a cofactor for catalysis. The most common cofactors are nicotinamide adenine dinucleotide (phosphate) (NAD(P)(H)) and flavin adenine mononucleotide/dinucleotide (FMN(H)/FAD(H)). These cofactors
perform the function of electron donor or acceptor in dehydrogenase or reductase enzymatic reactions, and thus are required for activity. The nicotinamide cofactors NAD(H) and NADP(H) differ only by the presence of a 2'-phosphate group on the adenine ribose. Enzymes have evolved the ability to discriminate between these two cofactors so that both reduction and oxidation reactions can take place simultaneously within the cell. The physiological ratios of cofactors are $[\text{NADP(H)}] > [\text{NADP}^+]$ and $[\text{NAD}^+] > [\text{NADH}]$ (3). Thus enzymes that prefer NADP(H) generally act as reductases, while those that prefer NAD(H) act as oxidases.

The first reported alteration of cofactor specificity was by Scrutton et al. who utilized site-directed mutagenesis to alter the cofactor preference of glutathione reductase from NADP$^+$ to NAD$^+$ (4). Seven mutations were identified in the “fingerprint” region of the cofactor binding $\beta\alpha\beta$-fold motif to reverse cofactor specificity and result in a marked preference for NAD$^+$ as a cofactor. Since this pioneering work, there have been numerous reports of the alteration of cofactor specificity in a wide range of enzymes utilizing several different cofactor binding motifs (5-11). A review of cofactor engineering in the aldo-keto reductase superfamily appears later in this chapter. Traditionally, cofactor engineering is undertaken to address a cofactor imbalance in a process, or to increase activity with the less expensive cofactor NAD(H). There have also been a few attempts at engineering specificity for non-natural cofactors, with mixed results. Our efforts to broaden the nicotinamide cofactor specificity in a dehydrogenase
are discussed in Chapter 3, and we examine specificity for non-natural cofactors in Chapter 4.

### Substrate Specificity

Altering the substrate specificity of enzymes was one of the primary goals of early enzyme engineering efforts. While an immense number of enzymes have been identified, very few natively exhibit high levels of activity with industrially relevant or desirable substrates. As such, a significant amount of research has been devoted to elucidating the determinants of substrate specificity and in engineering enzymes for improved activity with various substrates. A wide range of enzymes have been successfully engineered in this regard, including proteases (12, 13), nucleases (14), hydrolases (15), and many others (16, 17). While much has been learned through rational engineering approaches to altering substrate specificity, more recent efforts have taken advantage of directed evolution techniques to identify beneficial mutations. A major limitation to these approaches is that, except in some specialized cases, mutants must be screened individually for the desired substrate specificity and activity. New techniques are constantly being developed, but a general and rapid method for creating enzymes with desired specificities remains elusive. A review of the approaches used to engineer novel substrate specificities appears in Chapter 6.
Enzyme Stability

Proteins have evolved to be only marginally stable in their native environment. It was once thought this was due to positive selection for increased activities of more flexible, and therefore less stable, proteins but it has since been suggested that the marginal stability is an artifact of neutral genetic drift and lack of selective pressure for increased stability (18). Regardless, wild-type enzymes are generally only slightly more stable than their parent organisms, and this low stability can lead to reduced lifetimes in industrial applications. For example an important class of industrial enzymes, lipases, are generally isolated from cold-adapted species and are therefore extremely thermolabile (19, 20). Various rational design and directed evolution approaches have been used to stabilize these enzymes against thermal or chemical denaturation, with moderate success (21, 22). Interestingly, it has been observed that directed evolution approaches generally yield superior results, as stabilizing mutations are not always easy to rationalize. This is well illustrated in the aldo-keto reductase superfamily, as *Pyrococcus furiosus* AdhD is extremely thermostable yet shares the same canonical tertiary structure as other mesostable AKRs. The difference is that AdhD contains numerous stabilizing mutations throughout the enzyme, and exhibits as low as 30% sequence homology with less stable members of the superfamily.
Enzyme Immobilization

Due to their high cost, the use of soluble proteins and enzymes in industrial applications is generally avoided. Instead, it is preferable to immobilize enzymes on some sort of solid support to facilitate separation and reuse. This immobilization can be as simple as absorption onto a surface, or may involve covalent linkages such as glutaraldehyde cross-linking, attachment through cysteines, or genetic fusion to a binding protein (23). The use of various polymers as immobilization matrices, such as chitosan or Nafion®, has also been investigated with good success (24, 25). Lastly, proteins and enzymes have been engineered themselves for self-assembly through the genetic fusion of various proteinaceous cross-linking domains.

Each of these immobilization strategies has advantages and disadvantages, but the primary concern is the impact of immobilization on enzymatic activity. Adsorption of the enzyme onto a surface or chemically-induced cross-linking may disrupt the native tertiary fold of the enzyme and lead to inactivity. Similarly, attachment through cysteines or fusion to another protein domain may block the active site. To address this problem, our lab has developed a general method for the immobilization of enzymes through the fusion of α-helical leucine zipper domains (26). These domains form non-covalent cross-links under mild conditions through the formation of tetrameric coiled-coil bundles. Furthermore, mixed macrohomogenous hydrogels are readily constructed
due to the general nature of this immobilization approach, and this could find utility in the creation of synthetic metabolic pathways. We have shown that this immobilization strategy can be extended to the AdhD enzyme, and the result is a thermostable enzymatically active hydrogel that retains its materials properties at temperatures in excess of 60°C (27). The design and construction of this bioactive hydrogel is discussed in Chapter 2.

The Aldo-Keto Reductase Superfamily

The aldo-keto reductase (AKR) superfamily consists of monomeric oxidoreductase enzymes approximately 300 residues in size. They share a common (α/β)_8-barrel tertiary structure and bind a nicotinamide cofactor without a Rossman-fold motif (28). AKRs appear in every living system, and catalyze an array of redox reactions involving a broad range of substrates (29). As such, these enzymes are of significant physiological importance and much work has been done to characterize their structures, substrate specificities, and reaction mechanism.

Sequence alignments of AKRs have revealed that the residues that make up the cofactor binding pocket and active site are highly conserved amongst members, while the substrate binding loops vary significantly. Thus it seems all AKRs share the same catalytic mechanism while tailoring substrate specificity through modification of the
loops near the active site. The highly conserved nature of the cofactor binding pocket and active site is useful in identifying putative members of the AKR superfamily, even though some members may exhibit less than 30% sequence homology overall (28, 30).

The catalytic mechanism of the AKRs has been elucidated through numerous site-directed mutations within the active site. The enzyme family has been shown to follow an ordered bi-bi reaction mechanism, with the cofactor binding first and leaving last (31). Catalysis involves direct hydride ion transfer between the 4-pro-R position of the nicotinamide ring C-4 directly to the re face of the substrate (31). There is some debate as to which active site residue serves as a general acid/base to accomplish proton transfer, however. Most evidence points to Tyr-55, as salt-bridges and hydrogen bonds formed by Asp-50 and Lys-84 serve to lower the pK\textsubscript{a} of this residue and facilitate proton donation (Rat 5α-HSD numbering) (8, 28). An early study made systematic conservative mutations to the conserved active site residues and discovered that any mutations of Tyr-55 were catastrophic to enzyme function (32). Later, a sensitive assay utilizing radiolabeled substrates was able to accurately measure the rate enhancement due to the conserved catalytic residues and their dependence on pH. Analysis of the rate enhancement with the Tyr-55 mutant combined with the observed dependence of k\textsubscript{cat} on pH convincingly demonstrated that Tyr-55 is the catalytic general acid/base. Furthermore, mutations of the adjacent residues Lys-84 and His-117 led to pH dependent shifts in activity, indicating that these residues play an important role in
modifying the pKₐ of Tyr-55. This was hypothesized to be due to a “push-pull” mechanism for proton transfer where His-117 and Lys-84 facilitate proton donation and removal, respectively (33). Structural studies with another AKR support the importance of Tyr-55 as the general acid/base, but question whether the “push-pull” mechanism is a hallmark feature of AKRs. Crystal structures indicate that the longer relative distances between Tyr-55, Lys-84, and His-117 in some AKRs are not conducive to proton transfer. Instead, it is hypothesized that the ε-NH₃⁺ group of Lys-84 is important for electrostatic stabilization while His-117 determines the orientation of the substrate in the active site (34) (Figure 1.1).

Further kinetic analysis with NADP(H) has suggested the rate limiting step for the reaction is the isomerization of the enzyme upon binding of the oxidized cofactor and before binding of the reduced cofactor (35). Pre-steady state kinetics were performed using stopped-flow fluorescence spectroscopy to analyze the change in intrinsic fluorescence of the enzyme as it bound the cofactor. The results were consistent with a two-step kinetic mechanism: A fast formation of a loose complex (E•NADP(H)), followed by a conformation change leading to a tightly bound complex (E*•NADP(H)) (36). The associated rates were in good agreement with the kinetic parameters calculated from steady state measurements.
The cofactor binding pocket is highly conserved amongst AKRs, with most having a preference for NADP(H) over NAD(H). As would be expected, the specificity seems to be highly dependent on the residues in the cofactor binding pocket that would interact with the 2’-phosphate of NADP(H). Attempts to relax the cofactor specificity of 2,5-diketo-D-gluconic acid (2,5-DKG) reductase from *Corynbacterium* by making several site-directed mutations in this area have identified a number of mutants having increased activity with NAD(H) (37). The best single mutant, R238H, exhibited a 7-fold improvement in activity with NAD(H), due mostly to an improvement in $k_{\text{cat}}$ (38). The arginine in the wild type enzyme interacts with the 2’-phosphate of NADP(H) whereas the histidine residue in the mutant can form a pi-pi stacking interaction with the adenine ring in either cofactor. Kinetic measurements indicated this mutation increased the ground state binding affinity for NADH by 0.13 kcal/mol and the binding affinity for the transition state by a significant 1.1 kcal/mol (38).

Other work has indicated that the residues in the nicotinamide pocket also play an important role in cofactor specificity (39). Site-directed mutations of residues interacting with the nicotinamide ring of NAD(P)(H) suggest different modes of binding of NAD(H) compared to NADP(H) in AKRs. The evidence for this is threefold: First, mutations in the nicotinamide pocket that affect the affinity for NADP(H) show no significant effect on NAD(H) affinity. Second, changes in the $K_m$ for NADP$^+$ are much greater than those for NAD$^+$, while the catalytic efficiency ($k_{\text{cat}}/K_m$) for NAD$^+$ is much
more severely impacted. Third, a stopped-flow fluorescence spectroscopy experiment showed the isomerization of the enzyme/NADP(H) complex fits a two-state model, while the kinetic transient was not observed with NAD(H) (36). An important caveat, however, is that all of these experiments were performed with rat liver 3α-HSD, which differs from most AKRs at a few highly conserved residues. Thus further investigation is required before this altered binding observation can be extended to other members of the superfamily.

For this work, we have selected a thermostable AKR from the hyperthermophilic archaea Pyrococcus furiosus, alcohol dehydrogenase D (AdhD). This enzyme has several features which make it an attractive candidate for protein engineering. The enzyme is small (32 kDa), monomeric, and requires no disulfide bonds or metal ions for activity. It has been expressed recombinantly in E. coli in high yield, and its extreme thermostability (half-life of 130 min at 100°C) allows it to be easily purified in a single step (40). Thermostable enzymes have also been shown to have long lifetimes in immobilized systems, and are more amenable to mutagenesis than their mesostable counterparts. The native preference of AdhD for NAD$^+$ and broad substrate specificity provide an ideal starting point for our engineering efforts, and much is known about altering cofactor and substrate specificity in this enzyme family.
Our first goal was to engineer a simple and general method of immobilizing the scaffold enzyme without impacting activity. Previously, we have shown that the fusion of α-helical leucine zipper domains to the termini of a protein of interest enables self-assembly into a hydrogel structure. In Chapter 2, we apply this methodology to the AdhD scaffold to create a bioactive enzymatic hydrogel. As the enzyme is thermostable, we characterize the activity and materials properties of the hydrogel over a range of temperatures. Surprisingly, the use of a thermostable protein in this construct was found to stabilize the hydrogel structure, and a robust, enzymatically-active hydrogel was formed at temperatures exceeding 60°C.

In Chapter 3, we explore the cofactor specificity of *Pyrococcus furiosus* AdhD through several rational mutations to the cofactor binding pocket. Guided by previous efforts to alter cofactor specificity in this family, we were able to identify a double mutant enzyme with broadened specificity and significantly improved activity compared to the wild-type AdhD. The impact of these mutations on cofactor binding and kinetics is characterized using several steady-state and pre-steady state kinetic techniques, and we identify a novel cofactor binding mechanism in the engineered double mutant enzyme.

In Chapter 4, we expand upon the cofactor engineering efforts from Chapter 3 with the goal of improving the performance of an immobilized enzyme system. As cofactor diffusion is often rate limiting in these architectures, the use of truncated nicotinamide
cofactors with improved diffusion rates is investigated. Fortuitously, the previously engineered double mutant enzyme is able to utilize the minimal cofactor nicotinamide mononucleotide for catalysis. The effect of the minimal cofactor on the turnover rate and diffusion are investigated, and an enzymatic biofuel cell for the oxidation of D-arabinose is constructed with the double mutant enzyme.

In Chapter 5, we take a rational approach to engineering the substrate specificity of AdhD through the modular exchange of AKR substrate binding loops. In order to improve the activity of the enzyme with sugars, we grafted in the substrate binding loops from human aldose reductase (hAR), which is active with glucose. Replacement of at least two substrate binding loops, Loops A and B, was required to impart hAR activity into the AdhD scaffold. We also discovered that these mutations were not additive with the previously discussed cofactor specificity mutant of AdhD. Grafting the same substrate binding loops into the double mutant scaffold, which itself displays hAR activity due to its broadened specificity, results in an inactive enzyme.

The final chapter provides a summary of our efforts toward engineering a general dehydrogenase enzymatic scaffold, and discusses preliminary results of a novel selection scheme to enable the directed evolution of catalytic activity.
Figure 1.1 The active site of 2,5-DKG reductase (1M9H) with bound NADH. It is believed that Tyr50 acts as the catalytic acid/base, His108 determines the orientation of the substrate in the active site, and Lys75 forms a salt bridge with Asp45 and is responsible for electrostatic stabilization.

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<tr>
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<td>Detergents, food industry (starch liquefaction/saccharification), textiles</td>
</tr>
<tr>
<td>Cellulases</td>
<td>Biomass degradation, textiles, detergents</td>
</tr>
<tr>
<td>Lipases</td>
<td>Detergents, chemical production, food industry, degreasing, organic synthesis (pharmaceutical intermediates)</td>
</tr>
<tr>
<td>Oxidoreductases</td>
<td>Chemical production, textiles, pulp and paper bleaching, organic synthesis (chiral compounds, pharmaceutical intermediates)</td>
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Chapter 2

A CHIMERIC FUSION PROTEIN ENGINEERED WITH DISPARATE FUNCTIONALITIES — ENZYMATIC ACTIVITY AND SELF ASSEMBLY†

Abstract: The fusion of protein domains is an important mechanism in molecular evolution, and a valuable strategy for protein engineering. We are interested in creating fusion proteins containing both globular and structural domains so that the final chimeric protein can be utilized to create novel bioactive biomaterials. Interactions between fused domains can be desirable in some fusion protein applications, but in this case the optimal configuration will enable the bioactivity to be unaffected by the structural cross-linking. To explore this concept, we have created a fusion consisting of a thermostable aldo-keto reductase, two α-helical leucine zipper domains, and a randomly coiled domain. The resulting protein is bifunctional in that: (1) it can self-assemble into a hydrogel material as the terminal leucine zipper domains form interprotein coiled coil cross-links; and (2) it expresses alcohol dehydrogenase and aldo-keto reductase activity native to AdhD from *Pyrococcus furiosus*. The kinetic parameters of the enzyme are minimally affected by the addition of the helical appendages, and rheological studies demonstrate that a supramolecular assembly of the bifunctional protein building blocks forms a hydrogel. An active hydrogel is produced at

† A version of this chapter is published in *The Journal of Molecular Biology* (2009), 392, 129-142, with co-authors Ian Wheeldon and Scott Banta. EC performed experiments and analyzed data (enzyme kinetics and CD).
temperatures up to 60 °C, and we demonstrate the functionality of the biomaterial by monitoring the oxidation and reduction of the native substrates by the gel. The design of chimeric fusion proteins with both globular and structural domains is an important advancement for the creation of bioactive biomaterials for biotechnology applications such as tissue engineering, bioelectrocatalysis, and biosensing and for the study of native assembled enzyme structures and clustered enzyme systems such as metabolons.

**Introduction**

Molecular evolution relies on diversity in protein structures, and one way this is accomplished is through the fusion of native protein domains. There has also been much success in the design and engineering of novel chimeric fusion proteins for new applications. Examples of globular protein fusions include enzymatic switches (1), light activated DNA binders (2), drug and gene delivery systems (3-5) single chain antibodies (6), tethered enzymes (7, 8), and many others. There has also been interest in creating chimeric structural fusion proteins, such as silk-elastin fusions (9, 10), and leucine zipper-elastin fusions (11). We are interested in creating chimeric fusions with both globular and structural functionalities such that both domains, and the functions of those domains, contribute independently to the final protein construct.
Examples of fusion proteins that combine structural functionality and chemical or bioactive functionality are relatively uncommon. The literature describes fusions of organophosphate hydrolase (12) (OPH) and the protein G Fc binding domain (13) to elastin-like peptides for immobilization to hydrophobic surfaces. Also described are OPH (14) and horseradish peroxidase (15) fusions to a cellulose binding domain, for immobilization on the cellulose surfaces and calmodulin fusions to OPH and β-lactamase for reversible immobilization on to appropriately modified surfaces (16).

Perhaps the most common use of protein fusions is in biotechnologies for heterologous expression of recombinant proteins and in the purification of such products (17-19). In expression and purification technologies, the fusion is often temporary as cleavage of the fusion protein generally occurs en route to the final product.

We have previously created bifunctional fusion proteins that self-assemble into bioactive and enzymatic biomaterials. So far, we have demonstrated the addition of structural self-assembly domains to green fluorescent protein (GFP), the tetrameric Discosoma red fluorescent protein (DSRed) and a polyphenol oxidase (SLAC) from Streptomyces coelicolor (20, 21). The bifunctional proteins are fusions of the globular mesostable protein-of-interest (GFP, DSRED and SLAC) and the hydrogel forming triblock polypeptide (22), AC10A (here termed HSH). The triblock polypeptide has a helix-random coil-helix structure; the terminal α-helical leucine zipper domains form tetrameric coiled coils leading to the formation of a supramolecular hydrogel structure.
Our bifunctional proteins are fusions of the domains of the triblock polypeptide to the termini of the protein-of-interest (or insertions of the protein-of-interest in triblock polypeptide). The addition of self-assembly functionality to globular proteins is highly useful in that with compatible assembly domains one can produce mixed supramolecular structures from more than one type of bifunctional fusion protein (20, 21).

In this study, we describe a new bifunctional enzyme that self-assembles to form a thermostable, 3-dimensional supramolecular hydrogel that has aldo-keto reductase (AKR) activity. This is again accomplished through N- and C-terminal fusions of α-helical leucine zipper cross-linking domains (22) to the α/β barrel structure of an alcohol dehydrogenase with AKR activity, AdhD from *Pyrococcus furiosus* (23). The monomers are able to self-assemble into a bioactive enzymatic hydrogel that is stable to temperatures in excess of 60 °C.

AdhD is a member of the AKR superfamily that catalyzes the oxidation of secondary alcohols under basic conditions (optimum pH 8.8) and reduction of ketones under slightly acidic conditions (optimum pH 6.1) with a strong preference towards NAD(H) as a cofactor. Activity increases up to 100 °C and AdhD exhibits latent activity towards primary alcohols, xylose, glucose, arabinose and glyceraldehydes, among others (23). A thermostable bifunctional AdhD is a platform from which one, with additional protein
engineering work to modify substrate specificity, could develop mimics to cellular metabolic pathways that require co-localization or multi-enzyme complexes (7). The complete oxidation of an alcohol to carbon dioxide for electrical power production (24, 25), and biosensing of transient intermediates within a metabolic pathway are applications that would benefit from such a hydrogel system.

The primary concern is that the addition of a second functionality by genetic fusion will eliminate or drastically inhibit the first functionality (21). We show that the fused α–helical domains maintain helical secondary structure and that the α/β barrel remains highly thermostable. Additionally, we demonstrate that the kinetic parameters measured in dilute solution for diol oxidation and ketone reduction are minimally affected by the fusions to AdhD. With rheological characterization and erosion studies we show supramolecular assemblies of the bifunctional enzyme to be robust and thermostable hydrogels. Finally, we demonstrate the functionality of the system as a hydrogel made from bifunctional AdhD building blocks can catalyze the oxidation of a secondary alcohol and the reduction of a ketone.

Materials and Methods

Chemicals and Reagents: Mono and dibasic sodium phosphate, glycine, sodium chloride, sodium hydroxide, 3-hydroxy-2-butanone, 2,3-butanediol, guanidine HCl,
Trizma HCl and Base (TRIS), hydrochloric acid, β-Nicotinamide adenine dinucleotide reduced disodium salt (NADH) and β-Nicotinamide adenine dinucleotide (NAD+) were purchased from Sigma-Aldrich and used without modification. Isopropyl β-D-1-thiogalactopyranoside (IPTG; Promega) was also used without modification. Ampicillin, kanamycin, spectinomycin, and Terrific Broth media were also purchased from Sigma-Aldrich. Premade sodium dodecyl sulfate polyacrylamide gels for electrophoresis (SDS-PAGE) were purchased from Invitrogen. All protein concentrations were determined by Bradford assay with bovine serum albumin standards (Pierce).

**Construction of pQE9HSadhH and pQE9HSadh:** The plasmid, pWUR85, with adhD from *Pyrococcus furiosus* was a kind gift from John van der Oost (Wageningen University, The Netherlands). The expression plasmid and the tRNA helper plasmid, pSJS1244, are described in ref(23). The adhD gene was extracted from pWUR85 by polymerase chain reaction with forward and reverse primers that include the addition of a SphI site both upstream and downstream of the gene (adhD(SphI)-F ATATAAGCATGCATGGAATGCAAAAAGGGTAAATG, the forward primer with unique SphI site (underlined) and adhD(SphI)-R, AATATAGCATGCCCCACACAACACTCCTTGCCAT, the reverse primer with unique SphI site (underlined)). The resulting fragment was ligated into pQE9AC10Acys (26) (a kind gift from David Tirrell, California Institute of Technology) at the unique SphI site
between the C10 and Acys domain encoding regions. Successful transformants were propagated in 5α *Escherichia coli* cell line (NEB). The resulting expression plasmid, pQE9HSadhH, was also transformed into SG13009 *Escherichia coli* (Qiagen) harboring the repressor plasmid pREP4 and pSJS1244. Successful expression of HS-Adh-H from SG13009 cell line requires ampicillin (pQE9HSadhH), kanamycin (pREP4) and spectinomycin (pSJS1244). The plasmid encoding HS-Adh was constructed in an identical manner as described above with one exception, the adhD gene was extracted from pWUR85 with a downstream primer adding a unique SpeI site (adhD(SpeI)-R, CGTATAACTAGTTCACACACACCTCCT-TGC with unique SpeI site (underlined)).

**Expression and purification of AdhD, HS-Adh-H and HS-Adh:** Expression of AdhD followed a previously described protocol (23). Expression of HS-Adh-H and HS-Adh was done in 750 mL batches of Terrific Broth media supplemented with 200 µg mL\(^{-1}\) ampicillin, 50 µg mL\(^{-1}\) kanamycin and 50 µg mL\(^{-1}\) spectinomycin inoculated with 10 mL of mature SG13009 *E. coli* harboring pQE9HSadhH (or pQE9HSadh), pREP4 and pSJS1244. Expression was induced with 0.5 mM of Isopropyl β-D-1-thiogalactopyranoside upon reaching an OD\(_{600}\) of 0.8-0.9. Expression was allowed to continue for 15-16 hours at 27 ºC prior to harvesting. Growth prior to induction occurred at 37 ºC. Cells were harvested by centrifugation at 10,000g for 10 minutes and resuspended in 100 mL of 20 mM TRIS pH 7.5 per 750 mL culture. Cells were lysed by heating to 80 ºC for 1 hour and clarified by centrifugation for 30 minutes at 10,000g. HS-
Adh-H (or HS-Adh) was purified from the heat stable lysate by Fast Protein Liquid Chromatography (ÄKTA FPLC, GE HealthCare) using a strong anion exchange column (Q-FF, GE HealthCare). After injection of the lysate, the column was washed with 20 mM TRIS pH 7.8 with 200 mM NaCl. The protein of interest was eluted from the column with a linear gradient of NaCl in 20 mM TRIS pH 7.8 from 200 mM NaCl to 500 mM NaCl. Ninety to ninety-five percent pure HS-Adh-H (as judged by SDS-PAGE) elutes in a broad peak from 300 mM to 450 mM NaCl. Fractions containing HS-Adh-H (or HS-Adh) were pooled and concentrated over a 30 kDa cellulose filter (Millipore) while exchanging the buffer to 10 mM dibasic sodium phosphate. The resulting concentrated samples of HS-Adh-H and HS-Adh were approximately 95% pure. Samples used in kinetic assays and circular dichroism experiments were further purified by size exclusion chromatography (SEC; HiLoad 16/20, Superdex 200, GE HealthCare) with 20 mM TRIS pH 7.8, 500 mM NaCl. Excess salt was removed from the size exclusion eluate by buffer exchange over 30 kDa cellulose filters (Amicon, Millipore). The SEC results were compared to low molecular weight calibrations standards for size estimation (Gel Filtration LMW Calibration Kit, GE HealthCare).

**Hydrogel formation:** Hydrogel samples ranging from 10 to 18 wt% (100-180 mg mL⁻¹) were prepared from lyophilized HS-Adh-H (or HS-Adh). Protein was lyophilized from anion exchange purified samples after buffer exchange to 10 mM dibasic sodium phosphate adjusted with 1 M NaOH to pH 9 and after concentration to 15-25 mg mL⁻¹.
(approximately 1/10 final hydrogel concentration). Aliquots of the samples were frozen to -80 °C and lyophilized to dryness. Hydrogels were formed by rehydrating the dried samples to the desired weight percent and buffer concentration while accounting for the initial sample buffer. Hydrogel pH was adjusted by adding 1 M NaOH or 1 M HCl in place of equal volumes of water required for re-hydration. Final hydrogel pH was measured by fine range pH paper (Whatman and pHydrion).

**Hydrogel Rheology:** Small amplitude oscillatory shear experiments were performed with a TA Instruments AR 1200 constant stress rheometer equipped with an 8 or 20 mm steel parallel plate with a gap of 500 μm, and a constant strain of 1% at 22 – 75 °C (Peltier plate temperature control). A bead of mineral oil around the edge of the sample was used to prevent dehydration of the hydrogels during testing.

**Hydrogel Erosion:** Hydrogels re-hydrated with 10 μl of aqueous solution were prepared in 96 well microtiter plates and covered with 250 μL of 100 mM sodium phosphate, pH 7.5. Percent erosion was determined by monitoring the absorbance at 280 nm of a sample of open buffer solution over time.

**Circular Dichroism:** Experiments were conducted with a Jasco J-815 CD spectrometer with Peltier junction temperature control. Five-μM samples of HS-Adh-H (purified by SEC) and AdhD in 10 mM sodium phosphate buffer were analyzed in a 1 mm quartz cuvette. Solution pH was adjusted with 1 M NaOH and 1 M HCl as required. Spectral
deconvolution was accomplished with the CDPro software (27). Each spectrum was
deconvoluted with each of SELCON3, CONTILL, and CDSSTR, in each case with 4
protein reference sets. The secondary structure composition is given as an average of
the 12 deconvolutions with the associated standard deviation. Alpha-helical and β-sheet
contents are stated as the sum of the ordered and disordered helical and sheet
deconvolution results.

**Protein denaturation studies:** The extent of folding was determined by monitoring the
circular dichroic absorbance at 222 nm while increasing temperature at a rate of 1 °C per
minute. Samples were prepared as described for all circular dichroism analysis. Six-
molar guanidine hydrochloride was used in place of phosphate buffer when required.
Melting temperature, T\text{M}, taken as the midpoint parameter of sigmoidal fits to
temperature scan data at 222 nm.

**Activity assays and determining the steady state kinetic parameters:** Oxidative
activity of AdhD and HS-Adh-H (purified by SEC) was measured with 2,3-butanediol
and NAD\textsuperscript{+} cofactor in 50 mM glycine buffer, pH 8.8. Reductive activity was measured
with 3-hydroxy-2-butanone and NADH cofactor in 100 mM sodium phosphate buffer,
pH 6.1. The steady state kinetic parameters of the ordered bi bi reaction mechanism, \( k\text{cat}, \)
\( K_{M,S} \) and \( K_{M,NAD(H)} \) were determined by fitting initial rate data to equation 1.
where $S$ is 2,3-butanediol in the oxidative reaction and 3-hydroxy-2-butanone in the reductive reaction. The constant $k_{iNAD(H)}$ was determined by fluorescence titration as described below. The initial rates were determined by following the absorbance of produced, or consumed, NADH at 340 nm ($\varepsilon = 6.22 \text{ mM}^{-1}\text{cm}^{-1}$) with a SpectraMax M2 microplate reader (Molecular Devices). Assays were performed in 96-well assay plates with an enzyme concentration of 1 – 5 $\mu$g mL$^{-1}$ at 45 °C. Reaction conditions for the oxidative reaction were combinations of 5, 10, 25, 50, 100, 250, 500, and 1000 $\mu$M NAD$^+$ cofactor and 5, 10, 20, 40, 60, 80, and 100 mM 2,3-butanediol. Reaction conditions for the reductive reaction were combinations of 1, 5, 10, 25, 50, 100, 175, and 250 $\mu$M NADH and 0.1, 0.5, 1, 2.5, 5, 10, 20, and 40 mM 3-hydroxy-2-butanone. Each data set was repeated in at least quadruplicate.

The steady state kinetic parameter $k_{iNAD(H)}$ is equivalent to the equilibrium binding constant, $K_D$, of the cofactor in the active site of the enzyme (28). A conveniently located tryptophan residue at position 92 in the wild type and position 272 in HS-Adh-H allows for accurate determination of the binding constant as tryptophan fluorescence is quenched upon binding (Ex. 280 nm, Em. 330-340 nm) (29). For both the wild type and the bifunctional enzyme, the NAD$^+$ and NADH equilibrium binding constants were determined by equation 2.
\[ \Phi = \frac{k_{D,NAD(H)}^{-1}[NAD(H)\right]}{1 + k_{D,NAD(H)}^{-1}[NAD(H)]} \]  \hspace{1cm} (2) 

where \( \Phi \) is the fraction of NAD(H) bound as determined by fluorescent titration.

Experiments were done in a 1 cm quartz cuvette with 2 mL of 2 \( \mu \)M enzyme, to which 2 \( \mu \)L aliquots of concentrated cofactor was added. At each point the fluorescence emission at 330 nm for \( \text{NAD}^+ \) titration or at 450 nm for NADH titrations, with excitation at 280 nm, was recorded. No more than 10 aliquots of concentrated NAD(H) (3, 5 or 10 mM) were added during each titration to ensure a negligible change in enzyme concentration. At least three titration were performed to determine each of \( K_{D,NAD^+} \) and \( K_{D,NADH} \) for both the wild-type and bifunctional enzyme. Titrations were performed at the same conditions as described above for the reduction and oxidation reactions.

The effect of temperature on enzyme activity was determined by initiating a buffered enzyme-substrate solution, equilibrated to the desired temperature, with cofactor. Assays were performed in a 1 cm quartz cuvette with a 1.5 mL reaction volume. The temperature dependent rate of degradation of NADH was determined from control assays without enzyme and subtracted when appropriate.

All data fits were done using IGOR Pro software with a 95 % confidence interval. Statistical significance is reached with \( p \leq 0.05 \) with Student’s t-test.
Hydrogel enzymatic activity: Enzymatic activity of hydrogel samples was determined by monitoring NADH fluorescence (Ex. 340 nm, Em. 450 nm). Hydrogel samples were re-hydrated with buffer containing NAD$^+$ cofactor and reaction was initiated by substrate addition. Assays were performed in 384 well black assay plates. Conversion of ‘in-gel’ NAD$^+$ cofactor to NADH and again to NAD$^+$ was accomplished in 20 µL hydrogel of 10 wt% HS-Adh-H rehydrated with 2 mM NAD$^+$ (final volume, wt% and concentration after addition of 2,3-butanediol), heated to 45 or 60 °C, and buffered to pH 7 with 100 mM sodium phosphate. A 3.8 µL aliquot of 100 mM 2,3-butanediol was added at t=0 to initiate the reduction of NAD$^+$ to NADH. Twenty-mM 3-hydroxy-2-butanoine was then added at t=10 minutes to initiate oxidation of ‘in-gel’ NADH. Sample pH and concentrations of substrates and cofactor were selected so that the equilibriums would favor near complete conversion of the limiting concentration of NAD$^+$ to NADH upon addition of diol, and the oxidation of NADH to NAD$^+$ upon ketone addition.

Results

Design, expression and purification of a bifunctional AKR

Of primary concern in the design of the bifunctional AdhD protein is that the desired functions, self-assembly and enzymatic activity, are retained in the final construct and
that neither is significantly altered. To aid in the design, a homology model of AdhD from *Pyrococcus furiosus* (accession no. Q8TZM9) was produced in order to evaluate the potential impact the bifunctional construct may have on catalytic activity. The homology model (Figure 2.1a.) was generated using ESyPred3D and MODELLER (30) with primary template prostaglandin F synthase from *Trypanosoma brucei* (1VBJ, 31.1% identities). Structures were analyzed using MolProbity (31) and verified against other members of the aldo-keto reductase superfamily. The conserved residues in the catalytic active site of the AKR superfamily (D58, Y63, K89, H121, AdhD numbering) lie at the top of the α/β barrel, at the same end but on the opposite side, as the C-terminal helix (H2, as per AKR nomenclature (32)). The N-terminus lacks a defined structure, is not buried in the α/β barrel structure, and is spatially located at the opposite end of the barrel.

Naïvely, inspection of the homology model suggests that fusions to the N-terminus should be innocuous, but that substantial modification to the C-terminus could impair catalytic activity. Concern about the latter modification is supported by evidence from other authors that have shown that single amino acid mutation to the C-terminus of different AKRs can significantly alter catalytic activity (33). We have previously shown that the fusions of an α-helical domain (H) and randomly coiled (S) domain in series to the N-terminus and a single H-domain to the C-terminus of GFP results in robust hydrogel with beneficial properties (20). We hypothesized that the asymmetric order of
the domain fusions leads to a reduction in the rate of hydrogel erosion in open buffer solution due to the suppression of closed loop formation. Therefore we employ a similar fusion strategy in this case. A schematic representation of AdhD with both N- and C-terminal modifications is shown in Figure 2.1b.

AdhD is active to temperatures in excess of 90 °C and has a half-life greater than 2 hours at 100 °C (23). An enzymatically active, thermostable hydrogel made from self-assembling HS-Adh-H building blocks requires that physical cross-linking through α-helical coiled coil formation also occur at elevated temperatures. The structure of the appended H-domains, if correctly folded, should confer thermostability to the coiled coil bundles to temperatures upwards of 80 °C at pH 6 and to 50 °C at pH 9 (22). Optimization of the coiled coil melting temperature at different pH values is possible (34); however, the helices used here are amenable to our design goals as they will allow for the investigation of both the reductive and oxidation reaction at high temperatures.

A new bifunctional protein that exhibits AKR activity and self-assembles to form a supramolecular hydrogel was produced. A gene encoding the protein, HS-Adh-H, was constructed by genetically fusing an H- and S-domain to the N-terminus of AdhD and a single H-domain to the C-terminus. The appended H-domains are identical in sequence. A control construct, HS-Adh, with only the N-terminal modification was also constructed. The full amino acid and genetic sequences of HS-Adh-H, HS-Adh, and
AdhD are presented in the supporting information. The H-and S-domains are blocks of an engineered tri-block polypeptide, HSH (22, 26).

The protein constructs were expressed in *Escherichia coli* and purified by cell lysis at 80 °C followed by strong anion exchange and size exclusion chromatography (SEC). The large amounts of protein required for hydrogel formation prevented extensive use of SEC, therefore hydrogel samples were purified by anion exchange purification only. The two-step purification resulted in samples of approximately 95% purity and three-step purification resulted in a slight increase in purity as judged by SDS-PAGE. Yields, prior to size exclusion chromatography, of 15 to 25 mg per liter of culture were achieved. Protein yields were not substantially reduced after SEC. HS-Adh-H elutes from SEC (20 mM TRIS, 500 mM NaCl, pH 7.8) in three broad peaks between protein standards of 44,000 and 75,000 kD; however, samples of each fraction appear identical with SDS-PAGE analysis.

*Structure and stability of a thermostable α/β barrel with cross-linking appendages*

Circular dichroism (CD) spectroscopy confirms that the H-domains of the bifunctional fusion protein HS-Adh-H do form α-helices. The CD spectra of HS-Adh-H and AdhD were recorded at intervals of one-half pH units from pH 6 to 9 each at a temperature of 22, 45, 60 and 90 °C. Spectra of HS-Adh-H and AdhD at pH 6 and 9, at 22 and 90 °C, are shown in Figures 2.2a. and b. No aggregation was observed upon heating and cooling.
of dilute solutions. The spectra suggest that the wild-type AdhD has no perceptible structural change with an increase in pH from 6 to 9 and only a small change with an increase in temperature from 22 to 90 °C. Conversely, both pH and temperature have an effect on the structure of HS-Adh-H.

Deconvolutions of the spectra support these claims. The α-helical and β-sheet content of HS-Adh-H and AdhD at pH 6 (top) and 9 (bottom) at temperature intervals of 22, 45, 60 and 90 °C are shown in Figure 2.2c. Wild-type AdhD shows a small exchange of α-helical to β-sheet secondary structure with increasing temperature. The random, or unstructured, content remains constant. In comparison to AdhD, the appended HS- and H-domains add to the α-helical content of HS-Adh-H at low temperatures, and the total β-sheet content is reduced by a similar amount. At 90 °C and pH 6, α-helical content of HS-Adh-H drops below that of AdhD (the β-sheet content increases concomitantly). The decrease in HS-Adh-H α-helical content occurs at a lower temperature when buffered to pH 9, with the percentage helices reaching a value slightly lower than that of AdhD at 45 °C. The decrease in α-helical content with increasing temperature at pH 9 is not as large as the decrease observed at pH 6, as at 22 °C and pH 9 the α-helical content of HS-Adh-H is only slightly higher than AdhD.

The deconvolution and spectral data strongly suggests that the HS- and H-domain fusions to the N- and C-terminus of AdhD, respectively, result in the addition of α-
helical secondary structure and that the α-helical content of HS-Adh-H decreases with increasing temperature and increasing pH. The effect that the fusions have on the ability of the H-domains to form physical cross-links through coiled coil formation is demonstrated in the rheological characterization of concentrated samples presented below.

A concern is that the appended domains will dramatically limit the stability of the highly thermostable α/β barrel structure of AdhD. The data presented in Figure 2.3a (and Figure 2.2c) shows that HS-Adh-H undergoes some thermal denaturation at temperatures below 90 °C, while wild type AdhD does not. We hypothesize that the initial change of HS-Adh-H from the native state is due to the loss of α-helical structure of the appended domains and not unfolding of the core of the α/β barrel. This partially unfolded structure (denatured helical appendages with an intact, or nearly intact, α/β barrel core) forms a stable intermediate prior to complete denaturation. The folded state of the α/β barrel core in HS-Adh-H at elevated temperature is supported by evidence of enzymatic activity at 90 °C presented below. AdhD in 6 M guanidine hydrochloride (GdHCl) buffered to pH 8 denatures with a single unfolding transition with a T_m of 76 °C (Figure 2.3b). Without denaturant, HS-Adh-H undergoes a structural change with a T_m of 40 °C at pH 8 (Figure 2.3a). With denaturant, HS-Adh-H undergoes a second unfolding transition with a T_m of 72 °C (Figure 2.3b). The observed unfolding transitions in HS-Adh-H are thermodynamically separated to such a degree that we
were unable to identify a denaturant concentration that allows for both transitions to occur within a single temperature scan. The stable α/β barrel core of HS-Adh-H in denaturant differ in molar ellipticity per residue in comparison to AdhD (Figure 2.3b) as the HS-Adh-H construct contains an additional 261 residues in random or unstructured conformation in 6 M GdHCl.

The data presented in Figure 2.3 supports the existence of the hypothesized intermediate of HS-Adh-H as unfolding proceeds through two thermally induced transitions: the first transition is reversible, and the second is not. Thermodynamic analysis of the folded-unfolded transition is not possible as complete unfolding of both AdhD and HS-Adh-H in denaturant are irreversible upon cooling. Aggregation of both AdhD and HS-Adh-H was observed upon cooling of denaturant solutions but not in solutions without denaturant. Comparison of the melting temperatures of AdhD and HS-Adh-H in 6 M GdHCl does suggest that the α/β barrel structure of HS-Adh-H is slightly destabilized by the appended domains.

The melting temperatures of the first transient of HS-Adh-H decrease with increasing pH (T_M > 85 °C at pH 6, 59 °C at pH 7, 40 °C at pH 8, 38 °C at pH 9, see Supplementary material Figure 2.8). The T_M of the triblock polypeptide HSH is similar to the T_M of the first unfolding transition of HS-Adh-H at neutral pH and under slightly acidic conditions (T_M of HSH at pH 6 > 80 °C, and at pH 7 ~55 °C) (22). Under basic conditions,
pH 9.5, $T_m$ of HSH is approximately 15 °C greater than the $T_m$ of HS-Adh-H at pH 9.0. Decrease in the stability of the coiled coil domains with increasing pH is expected as the negative charge of deprotonated glutamic acid side chains at positions $e$ and $g$ of the leucine zipper heptad repeat $abcdefg$ destabilize the structure (35).

The first functionality: alcohol dehydrogenase and aldo-keto reductase activities

The N- and C-terminal fusion of HS- and H-domains to AdhD, respectively, do not eliminate catalytic activity. In dilute solution assays (specifically, 90 nM or $5 \times 10^{-4}$ wt% enzyme) and under near saturating concentrations of substrate and cofactor there is no significant difference between the oxidative activity of HS-Adh-H and AdhD with 2,3-butanediol and NAD$^+$ at pH 8.8. Turnover number with 2 mM NAD$^+$ and 100 mM 2,3-butanediol increases from less than 0.1 s$^{-1}$ at 25 °C to nearly 40 s$^{-1}$ at 90 °C (Figure 2.4). Under conditions favoring diol oxidation, the CD analysis (presented above) provides evidence of thermal denaturation at temperatures above 38 °C. Catalytic activity at temperature greater than 45 °C confirms the existence of a stable, partially unfolded intermediate.

A similar trend of increasing activity with increasing temperature is observed in dilute solution kinetic assays with ketone reduction. There is no significant difference between HS-Adh-H and wild type AdhD turnover number with 250 μM NADH and 100 mM 3-hydroxy-2-butanone measured at 25, 45 and 60 °C (Figure 2.4). Activity increases from
0.1 s\(^{-1}\) at 25 °C to 1.5 s\(^{-1}\) at 60 °C. At 90 °C, reductive activity of HS-Adh-H increases to 12 ± 2 s\(^{-1}\), and to 7 ± 2 s\(^{-1}\) for the unmodified AdhD. The significant difference in reductive activities at 90 °C is unexpected as there is no significant difference in activity at all other temperatures for both reactions.

Characteristic to the AKR superfamily is an ordered bi bi reaction mechanism requiring the sequential binding of NAD(P)(H) cofactor followed by substrate binding (33, 36, 37). Consistent with this mechanism are the trends observed in double reciprocal plots (1/activity vs 1/[substrate]) of both AdhD and HS-Adh-H (38). The intersection of linear fits to a set of data series of inverse activity as a function of inverse cofactor concentration occurs below the x-axis (1/[NAD\(^+\)]) for the substrate oxidation reaction and above the x-axis (1/[NADH]) for the substrate reduction reaction (Figure 2.9).

The steady state kinetic parameter \(k_{i,NAD(H)}\) in the ordered bi bi mechanism (Equation 1, Materials and Methods) is equivalent to the equilibrium dissociation constant for the cofactors, \(K_{D,NAD(H)}\) (28). A conveniently located tryptophan residue in the cofactor binding pocket (W92, AdhD numbering) allows for accurate measurement of cofactor binding, as tryptophan fluorescence is quenched upon cofactor binding (29). Fluorescence titrations reveal a significant difference in dissociation constants, \(K_{D,NAD^+}\) and \(K_{D,NADH}\), of wild type AdhD and HS-Adh-H. The terminal fusions slightly increase affinity for NAD\(^+\) (HS-Adh-H, \(K_{D,NAD^+} = 106 \, \mu\text{M}\); AdhD, \(K_{D,NAD^+} = 110 \, \mu\text{M}\), while
decreasing the affinity towards NADH (HS-Adh-H, $K_{D,NADH} = 47 \, \mu M$; AdhD, $K_{D,NADH} = 38 \, \mu M$) (Table 2.1). The Michaelis constants $K_{M,NAD^+}$ and $K_{M,NADH}$ also reflect the change in cofactor binding, as a statistically significant reduction in $K_{M,NAD^+}$ (HS-Adh-H, 40 $\mu M$, AdhD, 57 $\mu M$) and a statistically significant increase in $K_{M,NADH}$ (HS-Adh-H, 225 $\mu M$, AdhD, 145 $\mu M$) are observed. A significant change in Michaelis constants for the substrate ($K_{M,S}$) is not observed in either the oxidation reaction (HS-Adh-H, 22 mM, AdhD, 21 mM) or the reduction reaction (HS-Adh-H 0.24 mM, AdhD, 0.67 mM).

The change in cofactor binding does not result in a change in maximum rate of kinetic turnover, as there is no statistical difference in $k_{cat}$ for either the oxidation reaction (HS-Adh-H, 3.0 s$^{-1}$, AdhD, 2.7 s$^{-1}$) or reduction reaction (HS-Adh-H, 0.9 s$^{-1}$, AdhD, 0.8 s$^{-1}$). It is possible that a difference in $k_{cat}$ arises at higher temperatures, as is seen in Figure 2.4. Additionally, the steady state kinetic parameters of the ordered bi bi mechanism presented in Table 2.1 capture, within experimental error, the turnover number measured at near saturating conditions of both reactions for AdhD and HS-Adh-H measured at 45 °C (Figure 2.4) (e.g. the predicted turnover number for HS-Adh-H with 2000 $\mu M$ NAD$^+$ and 100 mM 2,3-butandiol at pH 8.8 and 45 °C is 2.4 ±1.0, and the experimental value was measured to be 1.4 ±0.2 [p = 0.11, n = 5,4]). The AdhD results are consistent with previously published reports (23).
The second functionality: supramolecular assembly and hydrogel formation

At 10 wt%, HS-Adh-H forms a supramolecular hydrogel via physical cross-linking of monomers through coiled-coil formation of two or more H-domains (it has previously been shown that the H-domains used in this work tend to form tetrameric coiled-coils (39)). It is also possible that cross-linking between monomers occurs due to dimer formation between the α/β barrel cores as is seen in some AKR family members (40). Three separate negative controls, 8 wt% HS-Adh-H, 10 wt% HS-Adh and 20 wt% AdhD confirm that two H-domains per monomer are required to form a sufficiently cross-linked structure, and that a minimum of 10 wt% HS-Adh-H is required to form a stable hydrogel structure. Evidence of hydrogel formation, i.e. a shear storage modulus (G’) that is greater than the shear loss modulus (G”) over a range of oscillation frequencies (41), along with the G’ values of 10 and 14 wt% samples of HS-Adh-H and negative controls of 10 wt% HS-Adh and 8 wt% HS-Adh-H are shown in Figure 2.5a.

A minimum concentration of 10 wt% HS-Adh-H is common to hydrogels at pH 6.3, 7.0, 8.0 and 9.0 (all hydrogel pH values ±0.2). The shear storage modulus of 10 wt% samples of HS-Adh-H at all studied pH values is between 100 and 200 Pa with a loss modulus no greater than 50 Pa (Figure 2.5b and Table 2.2). As expected, at low pH (pH < 4) we observed protein precipitation and at high pH (pH >12) hydrogel structure is lost due to a loss in secondary structure of the appended H-domains (20, 22).
Previous works have demonstrated that $G'$ increases with wt% protein used to make the hydrogel material (20, 21, 42). As applications such as enzymatic surface modifications and bioelectrocatalysis generally require more rigid hydrogels we investigated the temperature dependence of 14 and 18 wt% hydrogels at pH 7 and 9, respectively. At 14 wt%, 22 °C and pH 7, HS-Adh-H forms a hydrogel with a $G'$ of 960 ±140 Pa, a value similar to a previously reported monomeric fluorescence bifunctional protein HS-ECFP-H, which attains a $G'$ value of 1000 Pa at 15 wt%. With additional monomers within the structure (18 wt%), $G'$ increases to 3000 ±540 Pa, 22 °C and pH 9. An 18 wt% hydrogel sample at pH 9 demonstrates that the increase in hydrogel strength is not limited to neutral pH and that $G'$ can be increased to at least 3000 kPa. In both cases (14 wt%, pH 7 and 18 wt%, pH 9) there is no meaningful change in $G'$ or $G''$ up to 60 °C (Figure 2.5c.). At temperatures above 60 °C, $G'$ decreases and $G''$ increases, but a hydrogel persists (as $G' > G''$) to the end of the temperature ramp at 75 °C. Ten-wt% samples of HS-Adh-H also persist at high temperatures. At pH 6.3, 7.0, 8.0, and 9.0, 10 wt% samples of HS-Adh-H maintain hydrogel characteristics at temperatures up to 65 °C (Figure 2.5c and Figure 2.10).

The coiled coil tertiary structure is transient in that strand exchange occurs between coiled coils (26, 43). That is to say that an individual H-domain within a coiled coil can exchange places with another H-domain of a different coiled coil. Strand exchange results in a small number of monomers forming a separate unit (closed loop) that is not
attached to the larger hydrogel structure. Surface erosion, the loss of protein multimers (or monomers) from the surface of the hydrogel, occurs when closed loops at the surface of the hydrogel diffuse away into open buffer solution. We have previously shown that the asymmetrical structures of HS-GFP-H and HS-DSRED result in suppression of closed loop formation leading to an increase in hydrogel longevity (20). Erosion rate can also be suppressed by creating a mismatch of aggregation number between N- and C-terminal H-domains (43). We have also shown that covalent cross-linking after hydrogel formation results in a near complete suppression of erosion (21).

As expected, HS-Adh-H does erode in quiescent buffer solution (Figure 2.6). At 25 °C and pH 7, a 12 wt% (2.2 mM) HS-Adh-H hydrogel erodes at a rate of 120 ±10 pmol min⁻¹cm⁻² (a 10 µL gel is nearly 30% eroded after 2.5 hrs), a value comparable to that of HS-GFP-H (18 wt% or 3.4 mM, 93 pmol min⁻¹cm⁻²) (20). Under similar conditions a 7.5 wt% (3.4 mM) sample of triblock polypeptide, HSH, readily forms closed loops and completely erodes within 150 minutes (43). At 45 °C, the erosion rate of HS-Adh-H increases to 390 ±30 pmol min⁻¹cm⁻², likely due to an increase in the rate of strand exchange at the elevated temperature.

Hydrogel formation does not prevent enzymatic activity. In Figure 2.7 we demonstrate the bifunctionality of HS-Adh-H: enzymatic reaction within a self-assembled hydrogel of HS-Adh-H building blocks. In hydrogel samples re-hydrated with buffer containing
NAD⁺ cofactor we monitor the production and consumption of NADH by fluorescence detection upon initiating the oxidative and reductive reactions at 45 °C with 2,3-butanediol and 3-hydroxy-2-butanoine, respectively. After the addition of the substrate solutions the final HS-Adh-H concentration was 10 wt%, a concentration sufficient to form a hydrogel structure. Importantly, we observe that liquid solutions added to HS-Adh-H hydrogel samples are rapidly absorbed into the hydrogel. Reaction and hydrogel conditions were optimized so that we could observe a second change in the redox state of ‘in-gel’ cofactor while moving towards a new equilibrium point after addition of 3-hydroxy-2-butanoine. An optimized set of conditions were found at pH 7 were the reaction rate of the oxidation reaction is sub-optimial (23).

Upon addition of 3.8 µL of 100 mM 2,3-butanediol to a 14.2 µL sample of HS-Adh-H containing NAD⁺, the reaction proceeds towards an equilibrium state favoring NADH due to the limiting concentration of cofactor and high enzyme loading (Figure 2.7a). Upon addition of 2 µL of 20 mM 3-hydroxy-2-butanoine, the reduction reaction proceeds towards an equilibrium that favors NAD⁺ and diol. Enzymatic activity is due to HS-Adh-H monomers that are incorporated within the hydrogel as there is no open buffer solution at the surface of the hydrogel for erosion to occur. The reaction rate during the first minute after addition of oxidation of 2,3-butanediol in figure 2.7a is greater than the reaction rate due to eroded monomers alone, if erosion had been able to occur during that time (Figure 2.11).
Similar reaction profiles with the initiation of the oxidation and reduction reactions are observed at 60 °C (Figure 2.12). Additionally, control of the redox state of ‘in-gel’ cofactor is possible through changes in hydrogel pH (Figure 2.7b). A basic shift from slightly acidic to basic conditions induces the concomitant oxidation of 2,3-butanediol and reduction of NAD$^+$ while the system moves towards a new equilibrium that favors reduced cofactor.

**Discussion**

Here we demonstrate that a fusion protein of α-helical leucine zipper domains to the termini of the thermostable AdhD from *Pyrococcus furiosus* results in a bifunctional protein building block that self-assembles into a thermostable enzymatic hydrogel. The bifunctional protein building block, HS-Adh-H, expresses the disparate functions of its constituent parts. The α/β core of AdhD is catalytically active and the α-helical leucine zipper domains form coiled coil cross-links in a supramolecular hydrogel structure.

In contrast to some members of the AKR superfamily (33, 36), mutation to the C-terminus of AdhD, specifically addition to the C-terminus, does not dramatically affect substrate binding or catalytic functionality. The N- and C-terminal fusions to AdhD do alter cofactor binding, but in such a way as to not inhibit turnover under saturating conditions. AdhD lacks substrate binding loops common among some members of the
AKR superfamily, and one or more of these loops is often at the C-terminus (36) (Figure 2.13). The relatively benign nature of the C-terminal fusion to AdhD in HS-Adh-H is evidence for the lack of importance of the C-terminal domain in substrate binding and enzymatic activity of AdhD. The terminal fusions to AdhD from *Pyrococcus furiosus* do not eliminate enzymatic activity. In fact, catalytic turnover at saturating conditions is unaffected. Cofactor binding is affected, but modification to the C-terminus results in a less than one fold difference in $k_{cat}/K_{M,NAD^{+}}$ for the oxidation of 2,3-butanediol (AdhD, 50 s$^{-1}$mM$^{-1}$, HS-Adh-H, 75 s$^{-1}$mM$^{-1}$ ) and $k_{cat}/K_{M,NADH}$ for the reduction of 3-hydroxy-2-butanone is remains relatively unchanged (AdhD, 6 s$^{-1}$mM$^{-1}$, HS-Adh-H, 4 s$^{-1}$mM$^{-1}$).

The minimal disruption of AdhD enzymatic activity in the HS-Adh-H fusion is noteworthy as it is not always the case that fusions are benign. A bifunctional protein of similar design to HS-Adh-H, HS-SLAC (SLAC is a dimeric polyphenol oxidase) results in more than two orders of magnitude decrease in $k_{cat}/K_{M}$ (21). Conversely, fusion of an elastin-like peptide domain to organophosphate hydrolase (OPH) results in only a 16% decrease in $k_{cat}/K_{M}$ (12). OPH activity is minimally affect in a cellulose binding domain fusion (CBD) (14), but enzymatic activity is decreased by one order of magnitude in a calmodulin-OPH fusion (16). Additionally, there is no measurable difference in horseradish peroxidase (HRP) activity between the wild-type and a CBD-HRP fusion (15). Cross-comparison of the different fusions does not provide specific insight into the
different protein engineering problems, but does highlight the success of the HS-Adh-H fusion and leads to the simple observation that each case is unique.

The demonstration of enzymatic activity within a hydrogel sample of HS-Adh-H presented in Figure 2.7 does not provide data for evaluation of the specific activity of active sites within the hydrogel construct. It is possible that immobilization AdhD within the supramolecular structure results in a decrease in enzymatic capacity either through structural change to the active site or due to substrate and cofactor diffusion limitations within the hydrogel. The enzymatic activity shown in Figures 2.7 and 2.12 is due to monomers that are crosslinked within the hydrogel as there is no open buffer solution for erosion to occur as added substrate solution is quickly absorbed into the hydrogel and a hydrogel structure is maintain throughout the course of the experiment. It should also be noted that in the experiments where reversibility is demonstrated (Figure 2.7a, Figure 2.12), the reactions were performed at pH 7.0 which is not optimal for either the oxidative or reductive reactions, and therefore the kinetics of the enzymes will be slower than what is reported in Table 2.1 at the more optimal pH values.

Circular dichroism analysis suggests that HS-Adh-H passes through an enzymatically active but partially unfolded intermediate in which the appended helical domains change conformation while the AdhD α/β barrel core remains intact. The pH dependent \( T_m \) of the first unfolding transition is greater than 85 °C at pH 6 and decreases to 38 °C.
at pH 9. The destabilization of the coiled-coil structure with increasing pH is due to the increase in negative charge with progressive deprotonation of glutamic acid residues at positions e and g (35). The apparent decrease in stability of the H-domains of HS-Adh-H with respect to HSH could possibly be a result of some interference of helical coil formation by the AdhD protein core. Since there is no linker region on the C-terminal side, it is possible that this H-domain is sterically inhibited from complete formation of coiled coils.

A second thermal unfolding transition in HS-Adh-H is observed with 6 M GdHCl at a T_m 4 °C less than the single thermal unfolding transition of AdhD in 6 M GdHCl (Figure 2.3b). The two state unfolding of HS-Adh-H is confirmed by the catalytic activity of the HS-Adh-H fusion at pH 6.1 and 8.8 at 90 °C (Figure 2.4). The minimal difference in kinetic parameters of HS-Adh-H with respect to the wild type enzyme and the measurement of enzymatic activity at temperatures above the first unfolding transition imply that the α/β barrel core of HS-Adh-H remains folded and the observed unfolding transition at low temperature is the loss or change of structure of the appended helical domains. Combined the CD analysis and dilute solution kinetic assays demonstrate two aspects of the structure of HS-Adh-H: 1) the terminal fusions add α-helical structure to the protein independent of the α/β barrel core; and 2) the fusions do not substantially reduce the highly thermostable nature of the AdhD core.
Secondary structure analysis of the CD measurements suggests that at pH > 7 α-helical formation, and consequently hydrogel formation, is limited to temperatures of approximately 40 °C. At pH 8 and 9, 10 wt% samples of HS-Adh-H at pH from 6.3 to 9.0 are stable to temperatures up to 65 °C (Figure 2.5c, Figure 2.12 and Table 2.2). The T_m of the H-domains at pH 8 and 9, as determined under dilute solution conditions required for the CD analysis, are 40 °C and 38 °C respectively. It is likely that α-helical secondary structure is concentration dependent and is stabilized by the formation of coiled coil bundles; consequently, hydrogel stability (through physical cross-linking between monomers by coiled-coil formation and potentially by protein-protein interactions) is limited not to the temperature dependence observed in the dilute solution experiments. The T_m data does not predict the temperature-dependent behavior of the secondary structure of HS-Adh-H samples at hydrogel forming concentrations (i.e. concentrations that are more than three orders of magnitude higher than the CD analysis). The discrepancy in thermal stability of the α-helical secondary structure in dilute solution and HS-Adh-H hydrogels is also observed at neutral pH.

Five-wt% samples of HSH (22) and 10 wt% samples of a triblock polypeptide with HSH structure but with different H-domain sequences (44) show an increase in liquid-like character as temperature is increased from 23 to 55 °C; however, direct comparison of an HS-Adh-H supramolecular structure to one of HSH is inappropriate as the structures are distinctly different. In the case presented here, the C-terminal cross-links are
immobilized by a thermostable $\alpha/\beta$ barrel potentially adding to the stability of the motif. Additionally, an increase in cross-linking density could result from specific and non-specific protein-protein interactions between $\alpha/\beta$ cores and suppression of closed loop formation increases connectivity of the network. While the differences in systems are substantial, a comparison of the two systems reveals that the inclusion of the AdhD protein with the supramolecular structure has significant implications on the mechanisms of connectivity within the hydrogel.

The N-terminal fusion of a randomly coiled domain is included in the design as it is highly soluble (22) and it allows for physical separation between protein cores within the hydrogel. It has been shown that it is not essential to hydrogel formation (45) provided that the construct is sufficiently soluble. Also, we envision control over hydrogel porosity by controlling the length and placement of the S-domain.

**Conclusions**

The chimeric fusion protein, HS-Adh-H, self-assembles to form a thermostable enzymatic hydrogel. The protein is bifunctional in that it forms the physical structure of a hydrogel while retaining the enzymatic activity of the enzyme. The appended $\alpha$-helical leucine zipper domains are responsible for the formation of a physically cross-linked hydrogel at a minimum concentration of 10 wt% protein. The N- and C-terminal
fusions to AdhD minimally affect native enzymatic activity. Enzymatic activity of the bifunctional protein increases with temperature, and hydrogel formation is lost at high temperatures; we produce a rigid hydrogel with enzymatic activity at 60 °C. Our design will have use in a broad range of biotechnology applications such as enzymatic hydrogels for heterogeneous catalysis, electrode modifications for bioelectrocatalysis, enzymatically active surface coatings for biosensors, tissue engineering scaffolds, and the development of artificial metabolons. We also present it as an example of a general design for functional and multifunctional hydrogels.
## Tables and Figures

### Table 2.1 Kinetic constants for HS-Adh-H and wild type AdhD for the oxidation of 2,3-butanediol at pH 8.8 and 45 °C and the reduction of 3-hydroxy-2-butanone at pH 6.1 and 45 °C. Statistically significant difference indicated by * (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>k$_{cat}$ (s$^{-1}$)</th>
<th>K$_{M,S}$ (mM)</th>
<th>K$_{M,NAD(H)}$ (µM)</th>
<th>K$_{D,NAD(H)}$ (µM)</th>
</tr>
</thead>
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<tr>
<td><strong>oxidation reaction, pH 8.8</strong></td>
<td></td>
<td></td>
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<tr>
<td>HS-Adh-H</td>
<td>3.0 ±0.7</td>
<td>22 ±2.4</td>
<td>40 ±6.2$^*$</td>
<td>106 ±1.8$^*$</td>
</tr>
<tr>
<td>AdhD</td>
<td>2.7 ±0.6</td>
<td>21 ±1.7</td>
<td>57 ±5.7$^*$</td>
<td>110 ±1.5$^*$</td>
</tr>
<tr>
<td><strong>reduction reaction, pH 6.1</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>HS-Adh-H</td>
<td>0.9 ±0.2</td>
<td>0.24 ±0.04</td>
<td>225 ±35$^*$</td>
<td>47 ±2$^*$</td>
</tr>
<tr>
<td>AdhD</td>
<td>0.8 ±0.2</td>
<td>0.67 ±0.13</td>
<td>145 ±26$^*$</td>
<td>38 ±1$^*$</td>
</tr>
</tbody>
</table>
**Figure 2.1** Structure of AdhD and bifunctional HS-Adh-H.  

**a.** Top view of the homology model of AdhD from *Pyrococcus furiosus* with N- and C-termini indicated and active site residue side chains shown.  

- **N-Terminus:** at the top of the α/β barrel, out of the page.  
- **C-Terminus:** at the bottom of the α/β barrel, into the page.  

**b.** Schematic of AdhD with α-helical (H) and randomly coiled (S) domains fused to the N-terminus and an α-helical (H) domain fused to the C-terminus.  

Partial protein sequences are stated below, full sequence is provided in the Supplemental Information.
Figure 2.2 Circular dichroism (CD) analysis of HS-Adh-H and wild type AdhD.

a. CD spectra in molar ellipticity per residue of 5 μM HS-Adh-H, 10 mM phosphate buffer.  
b. CD spectra of AdhD, conditions same as a.  
c. α-helix and β-sheet content, determined by spectral deconvolution, of spectra of HS-Adh-H and AdhD at pH 6 and 9 at temperatures of 22, 45, 60 and 90 °C.
Figure 2.3 Thermal denaturing of HS-Adh-H and AdhD. Molar ellipticity per residue at 222 nm, $[\phi]_{222}$, of HS-Adh-H (solid lines) and AdhD (dots) from 25 to 90 °C. Samples prepared in (a) 10 mM sodium phosphate buffer, pH 8 and (b) 6 M guanidine hydrochloride.
Figure 2.4 Turnover number of AdhD (grey) and HS-Adh-H (black) with saturating substrate concentrations at 25, 45, 60 and 90 °C. Oxidation of 100 mM 2,3-butanediol (top) with 2000 μM NAD⁺, buffered to pH 8.8 with 100 mM sodium phosphate. Reduction of 100 mM 3-hydroxy-2-butanone (bottom) with 250 μM NADH, buffered to pH 6.1 with 100 mM sodium phosphate. Statistically significant difference indicated by * (p<0.05). Error bars are standard deviations.
Figure 2.5 HS-Adh-H hydrogel rheology.

(a) Storage modulus, $G'$, of 8 wt% HS-Adh-H and 10 wt% HS-Adh negative controls and 10 and 14 wt% HS-Adh-H hydrogels at 22 °C, pH 7. Small amplitude oscillatory shear frequency sweeps depicting $G'$ (filled) and $G''$ (open) of each sample is depicted as an inset above each data bar.

(b) $G'$ and $G''$ of 10 wt% samples of HS-Adh-H at pH 6, 7, 8 and 9. All pH values ±0.2.

(c) Temperature dependence of 10, 14 and 18 wt% HS-Adh-H hydrogels: 10 and 14 wt% data presented for hydrogels at pH 7, 18 wt% data taken a pH 9. $G'$ of each wt% sample at 22, 45, 60 and 65 °C are bars and temperature scans of $G'$ (filled) and $G''$ (open) for 14 and 18 wt% samples provided as insets.
Figure 2.6 Fraction eroded of a 12 wt% (2.2 mM) hydrogel of HS-Adh-H, pH 7, 25 °C (closed) and 45 °C (open circles). Ten-μL hydrogel samples in 25X quiescent buffer solution, 100 mM sodium phosphate, pH 7. Error bars are standard deviations, n≥5.
Figure 2.7 Enzymatic hydrogel activity.

(a) Conversion of in-gel NAD$^+$ to NADH by initiating the oxidation of 2,3-butanediol and the conversion of produced NADH to NAD$^+$ by initiating the reduction of 3-hydroxy-2-butanone at pH 7, 45 °C in a 10 wt% hydrogel of HS-Adh-H (final wt% after the additions of 2,3-butanediol at t=0 and 3-hydroxy-2-butanone at t=8 minutes). Hydrogel rehydrated with 2 mM NAD$^+$ (final concentration). Reduction of NAD$^+$ to NADH initiated with 21 mM 2,3-butanediol (diol). Perturbation to a new equilibrium initiated with 20 mM 3-hydroxy-2-butanone (ketone).

(b.) Initiation of the oxidation of 2,3-butanediol and the conversion of in-gel NAD$^+$ to NADH by a basic shift in pH. Times of substrate additions indicated by arrows.
Supplemental Information

AdhD, HS-Adh and HS-Adh-H amino acid sequences.

**AdhD**

MAKRVNAFND LKRIGDDKVTL AIGMGTWGI GRETDPYSRD KESIEAIRYG LELGMNLIDT 60
AEFYGAGHA EIVGEAIKEF EREDIFIVSK VWPTHFGYEE AKKAARASAK RLGTYIDLYL 120
LHPWDDFKK IEETLHALED LVDEGVIRYI GVSFNLEELL QRSQEMRKY EIVANQVKYS 180
VKDRWPEATG LLDYMKREGI ALMAYTPLEK GTLANECLA KIGEKYGKTA AQVALNYLIW 240
EENVVAIPKA SNKEHLKENF GAMWRLSEE DREMARRCV 279

**HS-Adh**

MRGSHHHHHH GSDDDKWAS GDLENEVAQL EREVRSLEDE AAELEQKVSR LKNIEIDLKA 60
EIGDHVAPRD TSYRDMGAG AGAGPEGAGA GAGPEGAGAG AGPEGAGAGAG AGPEGAGAGAG 120
PEGAGAGAGP EGAGAGAGPE GAGAGAGPEG AGAGAGGPE AGAGAGGPEGH MGHMAKRVNA 180
FNDLKRIIGDD KVTAIGMTGW GIGGRETPDY SRDKESIEAI RYGELGMNL IDTAEFYGAG 240
HAEEOVGEAI KEFEDIFIV SVDWTHFEG YEEAKKAARA SAKRLGTYID LYLLHPVD 300
FKKIEETLHA LEDLVEGVRI RYIGVSNFL ELLQDSQEM RKYEIVANQV KYSVKDRWPE 360
TTGLLLDMKR EGIALMAEYTP LEKTLARNE CLAKIGEYG KTAQVALNY LIWEENV 420
PKASNKEHLK ENFGAMGWRL SEEDREMARR CV 452

**HS-Adh-H**

MRGSHHHHHH GSDDDKWAS GDLENEVAQL EREVRSLEDE AAELEQKVSR LKNIEIDLKA 60
EIGDHVAPRD TSYRDMGAG AGAGPEGAGA GAGPEGAGAG AGPEGAGAGAG AGPEGAGAGAG 120
PEGAGAGAGP EGAGAGAGPE GAGAGAGPEG AGAGAGGPE AGAGAGGPEGH MGHMAKRVNA 180
AFNDLKRIIGDD DKVTAIMGT WGIGGRETDP YSRDSIESIA ERYGELGMN LIDTAEFYGA 240
GHAEEIVGEAI KEFEDIFIV SVDWTHFEG YEEAKKAARA SAKRLGTYID LYLLHPVD 300
DNA sequences of AdhD, HS-Adh and HS-Adh-H

**AdhD**

ATGGCAAAAA GGTTAAATGC ATTCAACGAC CTTAAGCGTA TAGGAGATGA TAAGGTAACG
GCAATTGGAA TGGGAACATG GGGAATAGGA GGGAGAGAGA CCCCAGACTA TTCTAGGGAT
AAGGAAAGCA TAGAAGCAAT AAGATATGGA CTTGAATTAG GAATGAATTT AATCGACACA
GCGGAATTCT ATGGAGCTGG TCATGCTGAG GAAATAGTTG GAGAGGCCAT TAAAGAATTC
GAACGTGAGG ACATCTT CAT AGTGAAGCAAG CTCTGGCCAA CTCACTTTGG GTATGAGGAA
GCAAAGAAGG CTGCTAGAGC AAGTGCTAAA AGGTTAGGAA CTTATATTGA CCTTTATTTG
TTGCACTGCC CCGTTGATGA CTTCAAGAAG ATAGAGGAGA CACTTCACGC TTTGGAAGAC
CTCGTAGATG AGGGAGTGAT AAGTACATT GGAAGTACAT TTACTTACCT GGAACCTTCTC
CAGCGCTCCC AGGGTATCAG GAGAATGTG TAGATTGTAG CAAATCAAGT TAAATACTCA
GTGAAAGACC GCTGGCCCGA AACTACAGGA CTTCTCGACT ACATGAAGCG TGAAGGAATA
GCATTAATGG CGTACACACC TCTAGAAAAG GGAACTCTTG CAAGGAATGA ATGTCTAGCT
AAAATTGGAG AAAAATACCC AAAAACACTG GCCTAAGTGG CTTTAAACTA CCTGATTTGG
GAGGAAAATG TTGTAGCAA TCCAAAAAGA AGCAACAAGG AACCACCTCAA AGAAAACTTT
GGAGCTATGG GATGGAGGCT TTCAGAGGAG GATAGAGAGA TGCAAGGAG GTGTGTGTGA

**HS-Adh**

ATGACGATGA CAAATGGGCT AGCGGTACC TGGAAAAACGA AGTGGCCTAG CTGGAAAGGA
AAGTTGGCTC TCTGGAAGAT GAAGCGGCTG AACTGGAACA AAAAGTCTGG AGACTGAAA
ATGAAATCGA AGACCTGAAA GCCGAAATTG GTGACCATGT GGCGCCTCGA GACACTAGCT
ATCGCGATCC GATACTGGCC GGCGCTGGTG CGGGCCCGGA AGGTGCAGGC GCTGGTGCGG

61
GCCCGGAAGG TGCCGGCGCT GGTGCGGGCC CGGAAGGTGC AGGCGCTGGT GCGGGCCCGG 300
AAGGTGCCGG CGCTGGTGCG GGCCCGGGCG GAAGGTGCCG CCGCTGGTGC GGGCCCGGAA 420
CGGCGCTGG TGCCGGCCCG GAAGGTGCCG CGCTGGTGC GGGCCCGGAA GGTGCCGGCG 540
CTGTTGCGGG CCCGGAGGTT GCAGGGCGCT GTGCGGGCCC GGAAGGATGC CGCATGCATG 660
GAAAAAAAA TAAATGCATT CAACGACCTT AAGCGTATAG GAGATGATAA GGTAACCGCA 780
ATTGGAATGG GAACATGGGG AATAGGAGGG AGAGAGACCC CAGACTATTC TAGGGATAG 900
GCCCGGAAGG TGCCGGCGCT GGTGCGGGCC CGGAAGGTGC AGGCGCTGGT GCGGGCCCGG 1020
AAGGTGCCGG CGCTGGTGCG GGCCCGGGCG GAAGGTGCCG CCGCTGGTGC GGGCCCGGAA 1140
GAAGATGCAGG TACATTGGAAG GTACATTGGACA CTGGTGGTCA TGCTGAGGAA ATAGGGTAGA 1260
GAAAGCATAG AAGCAATAAG ATATGGACTT GAATTAGGAA TGAATTTAAT CGACACACGC 1380
GAATTGTATG GAGCTGGTCA TGCTGAGGAA ATAGGGTAGA 1500

HS-Adh-H

ATGAGAGGAT CGCATCACCA TCACCACCA GGATCCATAG ACGATGACAA ATGGGCTAGC 60
GGTGACCTGG AAAACGAAGT GGCCCAGCTG GAAAGGGAAG TTAGATCTCT GGAAGATGAA 120
GGCGCTGGTGG AAAACGAAGT GGCCCAGCTG GAAAGGGAAG TTAGATCTCT GGAAGATGAA 180
AAAATGTCAGG TACATGATGC CATTTGCTCT GCTCGAGACA GGGTGCCGGC 240
GGCGCTGGTGG AAAACGAAGT GGCCCAGCTG GAAAGGGAAG TTAGATCTCT GGAAGATGAA 300
GGCGCTGGTGG AAAACGAAGT GGCCCAGCTG GAAAGGGAAG TTAGATCTCT GGAAGATGAA 360
GGCGCTGGTGG AAAACGAAGT GGCCCAGCTG GAAAGGGAAG TTAGATCTCT GGAAGATGAA 420
GGCGCTGGTGG AAAACGAAGT GGCCCAGCTG GAAAGGGAAG TTAGATCTCT GGAAGATGAA 480
GGCGCTGGTGG AAAACGAAGT GGCCCAGCTG GAAAGGGAAG TTAGATCTCT GGAAGATGAA 540
GGCGCTGGTGG AAAACGAAGT GGCCCAGCTG GAAAGGGAAG TTAGATCTCT GGAAGATGAA 600
TGGGGAATAG GAGGGAGAGA GACCCCAGAC TATTCTAGGG ATAAGGAAAG CATAGAAGCA 660
ATAAGATATG GACTTGAATT AGGAATGAAT TTAATCGACA CAGCGGAATT CTATGGAGCT 720
GGTCATGCTG AGGAAATAGT TGGAGAGGCC ATTAAAGAAT TCGAACGTGA GGACATCTTC 780
ATAAGTGAGCA AGGTCTGCCC AACTCACCTT GGGTATGAGG AAGCAAAGAA GGCTGCTAGA 840
GCAAGTGCTA AAAGGTTAGG AACTTATATT GACCTTTATT TGTTGACTG GCCCCGTGAT 900
GACTTCAAGA AGATAGAGGA GACACTTCAC GCTTTGGAAG ACCTCGTAGA TGAGGGAGTG 960
ATAAGGTACA TTGGAGTTAG CAACTTCAAT CTGGAACTTC TCCAGCGCTC CCAGGAGGTC 1020
ATGAGGAAGT ATGAGATTGT AGCAAATCAA GTTAAATACT CAGTGAAGA AGGCTGGCCC 1080
GAAAATACAG GACTTTCGGA CTACATGAAG CGTGAAGGAA TAGCATTAAT GGCGTACACA 1140
CCTCTAGAAA AGGGAACTCT TGCAAGGGAT GAATGTCTAG CTAAAATGG AGAAAAATAC 1200
GGAAAAACAG CTGCTCAAGT GGCTTTAAC TACCTGATTT GGGAGGAAA TGTTGTAGCA 1260
ATTCCAAAAG CAAGCAACAA GGAACACCTC AAAGAAAACT TTGGAGCTAT GGGATGGAGG 1320
CTTTCAAGAG AGGATAGAGA GATGGCAAGG AGGTGTGTGG GCATGCCGAC TAGCGGTGAC 1380
CTGGAAAACG AAGTGGCCCA GCTGGAAAGG GAAGTTAGAT CTCTGGAAGA TGAAGCGGCT 1440
GAACTGGAAC AAAAGTCTC CAGACTGAAA AATGAAATCG AAGACCTGAA AGCCGAAATT 1500
GGTGACCATG TGGCGCCTCG AGACACTAGT ATGGGTGGCT GCTAGGATCC GTCGACCTGC 1560
AGCCAAGCTT AATTAGCTGA GCTGGAGCTC CTGGTAGATG ATCCAGTAAT GACCTCAGAA 1620
CTCCATCTGG ATTTGTTCAG AAGCGCTCGGT TGCCGCAGGG CGTTTTTTAT TGGTGA
Figure 2.8 Molar ellipticity per residue, $\theta$, of HS-Adh-H at 222 nm, temperature ramp of 1 °C min$^{-1}$ for samples buffered to pH 6(black), 6.5(light blue), 7(yellow), 7.5(red), 8(green), 8.5(purple) and 9(dark blue). Scans of 5 μM samples in 10 mM sodium phosphate buffer, pH adjusted with 1 M NaOH or 1 M HCl as required. Melting temperature, $T_M$, taken as midpoint of the sigmoidal fits to the temperature scan data at 222 nm (solid lines).
Figure 2.9 Lineweaver-Burk plots. Inverse specific activity towards the oxidation of 2,3-butanediol with NAD$^+$ cofactor, buffered to pH 8.8 with 50 mM glycine at 45 °C for HS-Adh-H (a) and AdhD (b). Inverse specific activity towards the reduction of 3-hydroxy-2-butanone with NADH cofactor, buffered to pH 6.1 with 100 mM NaP at 45 °C for HS-Adh-H(c) and AdhD(d). Error bars are standard deviations of quadruplicate data sets.
**Figure 2.10** Small amplitude oscillatory shear of 10 wt% samples of HS-Adh-H at pH 7, 45 °C (left), and 60 °C (right). Data taken with a 20 mm steel parallel plate, 500 µm gap, 1% strain with frequencies from 1 to 50 to 1 rad s\(^{-1}\).

<table>
<thead>
<tr>
<th>HS-Adh-H wt%</th>
<th>pH (±0.2)</th>
<th>(G'); (G'') (Pa) at 65 °C</th>
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<tr>
<td>10</td>
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<td>30 ; 10</td>
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<tr>
<td>10</td>
<td>7.0</td>
<td>110 ; 25</td>
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<tr>
<td>10</td>
<td>8.0</td>
<td>150 ; 30</td>
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<tr>
<td>10</td>
<td>9.0</td>
<td>200 ; 30</td>
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**Table 2.2** Storage and loss modulus of HS-Adh-H at high temperature. Experimental conditions: 20 mm steel parallel plate, 500 µm gap, 1% strain, 10 rad s\(^{-1}\), temperature ramp from 25 to 75 °C, 0.5 °C per minute.
Figure 2.11 Experimental and model ‘in gel’ activity with HS-Adh-H hydrogels. The first two minutes of the data presented in Figure 2.7a is presented here as open circles. The reaction profile predicted from the kinetic parameters of the oxidation reaction at pH 8.8 and 45 °C for a solution of HS-Adh-H equal to the hydrogel forming concentration of HS-Adh-H and substrate and cofactor concentrations as presented in Figure 2.7a is plotted as black squares. The apparent decrease in activity from the predicted reaction profile to the experimental reaction profile can be explained by several reasons. The kinetic parameters were measured at pH 8.8 while the hydrogel
experiments were performed at pH 7.0 where the enzyme does not exhibit maximal activity. Also, in the gel state there will be significant effects due to diffusion of substrates and cofactors within the hydrogel structure. And it is also possible that a decrease in activity of HS-Adh-H occurs as a result of structural changes due to cross-linking that were not observed in dilute solution. Also presented is the predicted activity from HS-Adh-H monomers that would potentially be eroded from the bulk hydrogel during a 1 minute period at a rate of 390 pmole per minute per cm² (black line). The predicted reaction profile of the eroded sample assumes: 1) the amount eroded in 1 minute occurs instantly at the initial time point; 2) the concentration of NAD⁺ is constant at 2000 μM; 3) only eroded monomers are active; 4) the amount of open buffer solution on top of the hydrogel sample is equal to 3.8 μL (the amount of 100 mM 2,3-butanediol solution added to initiate the reaction); and 5) the reaction in the eroded volume occurs at the optimal reaction pH, pH 8.8.
Figure 2.12 Conversion of ‘in-gel’ NAD$^+$ cofactor to NADH and again to NAD$^+$. Twenty-µL hydrogel of 10 wt% HS-Adh-H rehydrated with 2 mM NAD$^+$ (final volume, wt% and concentration), heated to 60 °C, and buffered to pH 7 with 100 mM sodium phosphate. Twenty one-mM (in-gel concentration) of 2,3 butanediol added at $t=0$ to initiate the reduction of NAD$^+$ to NADH. Twenty-mM 3-hydroxy-2-butanone added at $t=10$ mins. to initiate oxidation of in-gel NADH. Sample pH and concentrations of substrates and cofactor were selected so that the equilibriums would favor near complete conversion of the limiting concentration of NAD$^+$ to NADH upon addition of diol, and the oxidation of NADH to NAD$^+$ upon ketone addition.
Figure 2.13 Amino acid sequence alignment of AdhD from *Pyrococcus furiosus* (Q8TZM9) and a Chicken AKR (Q90W83) demonstrating the lack of substrate binding loops A, B and C commonly found in the AKR superfamily. The primary accession number is given in parentheses.
References


Chapter 3

BROADENING THE COFACTOR SPECIFICITY OF A THERMOSTABLE ALCOHOL DEHYDROGENASE USING RATIONAL PROTEIN DESIGN INTRODUCES NOVEL KINETIC TRANSIENT BEHAVIOR

Abstract: Cofactor specificity in the aldo-keto reductase (AKR) superfamily has been well-studied, and several groups have reported the rational alteration of cofactor specificity in these enzymes. Although most efforts have focused on mesostable AKRs, several putative AKRs have recently been identified from hyperthermophiles. The few that have been characterized exhibit a strong preference for NAD(H) as a cofactor, in contrast to the NADP(H) preference of the mesophilic AKRs. Using the design rules elucidated from mesostable AKRs, we introduced two site-directed mutations in the cofactor binding pocket to investigate cofactor specificity in a thermostable AKR, AdhD, which is an alcohol dehydrogenase from Pyrococcus furiosus. The resulting double mutant exhibited significantly improved activity and broadened cofactor specificity as compared to the wild-type. Results of previous pre-steady state kinetic experiments suggest that the high affinity of the mesostable AKRs for NADP(H) stems from a conformational change upon cofactor binding which is mediated by interactions

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between a canonical arginine and the 2’-phosphate of the cofactor. Pre-steady state kinetics with AdhD and the new mutants show a rich conformational behavior that is independent of the canonical arginine or the 2’-phosphate. Additionally, experiments with the highly active double mutant using NADPH as a cofactor demonstrate an unprecedented transient behavior where the binding mechanism appears to be dependent on cofactor concentration. These results suggest that the structural features involved in cofactor specificity in the AKRs are conserved within the superfamily, but the dynamic interactions of the enzyme with cofactors are unexpectedly complex.

**Introduction**

The aldo-keto reductases (AKRs) are a family of oxidoreductases with a common (α/β)₈-barrel structure. They are found in almost every living system and catalyze a wide range of redox reactions (1, 2). Characteristic to this superfamily is a highly conserved cofactor binding pocket that binds a nicotinamide cofactor in the extended conformation without a Rossmann fold motif (3). Most members of the superfamily that have been studied exhibit a strong preference for NADP(H), suggesting a physiological role as reductases (4).

Understanding the determinants of cofactor specificity of dehydrogenases has significant importance from an engineering perspective, as the native cofactor
specificity of these enzymes is often not ideal for use in synthetic metabolic pathways and other industrial applications. Altering cofactor specificity of an enzyme in an artificial metabolic pathway can potentially correct a redox imbalance in a process or improve overall product yield, and therefore cofactor engineering is important in applications ranging from cofactor regeneration to bioelectrocatalysis (5-11). We are particularly interested in engineering these dehydrogenase enzymes for use in enzymatic biofuel cells, where the choice of the cofactor (acting as the electron mediator between the enzyme and the electrode) is of critical importance (12-14). 

Several groups have used site-directed mutagenesis to study the structural determinants of cofactor specificity in the AKRs (15-24) and there have been a few reports of the broadening of the cofactor specificity to increase the activity of these enzymes with NAD(H) (6, 25-28). Through these efforts, several hot spots for mutagenesis have been identified. The first is a lysine residue that appears partially buried under the bound cofactor and interacts with the pyrophosphate backbone, adenine ribose, and 2'-phosphate of NADP(H). This residue has been conservatively mutated in human aldose reductase (15, 24) and these studies suggest that interactions with the lysine are important for properly orienting the cofactor within the binding pocket and for positioning the nicotinamide head group for hydride transfer. Later, in an effort to improve the activity of an AKR with NADH, a lysine → glycine mutant was identified with improved kinetic properties (26).
A highly conserved arginine residue has also been shown to form important interactions with the adenosine 2'-phosphate. Studies mutating this canonical arginine have demonstrated a significant impact on activity with NADP(H), while changes with NAD(H) were minor (18, 22). The mechanism of cofactor binding in a model AKR, rat 3α-hydroxysteroid dehydrogenase (3α-HSD), has been extensively studied and demonstrates a multi-step binding mechanism for the NADP(H) cofactor (22). A comparison of the crystal structures for the apo enzyme and the enzyme-NADPH binary complex suggests a conformational change takes place upon cofactor binding, similar to that observed in other AKRs (3, 29, 30). Using an arginine→methionine mutant, it was demonstrated that the conformational change was due to the formation of a salt bridge between the arginine and 2'-phosphate of NADP(H), which could be observed as a fluorescence kinetic transient. No transient was observed in the arginine→methionine mutant or when NAD(H) was used as a cofactor, suggesting this transient and corresponding conformational change were dependent upon interactions between the arginine and adenosine 2'-phosphate group (22). The stopped-flow fluorescence data was consistent with a two-step binding mechanism, where an initial rapid bi-molecular association is followed by a slow isomerization to a tightly bound complex. This serves to greatly increase the affinity of the enzyme for the cofactor, and locks the enzyme in a primed state ready to immediately act upon a substrate (19). In mutagenesis work performed with Cornybacaterium 2,5-diketo-D-gluconic acid reductase
(2,5-DKGR), it was demonstrated that an arginine→histidine mutant at this position increased activity with NADH while retaining activity with NADP(H) (26). The solved crystal structure of this mutant shows the histidine side-chain forms a π-stacking interaction with the indole ring of the cofactor, and a kinetic analysis demonstrated an improvement in the free energy of cofactor binding, consistent with the introduction of this stabilizing interaction.

Newly available genome sequences from a variety of hyperthermophiles has led to the identification of several putative thermostable AKRs. Although few have been characterized, sequence alignments indicate that these thermostable AKRs contain a histidine residue in the cofactor binding pocket in place of the highly conserved arginine residue found at this position in mesophilic AKRs (Table 3.1). One such AKR, an alcohol dehydrogenase identified from the hyperthermophilic archaeon *Pyrococcus furiosus* (AdhD), exhibits a strong preference towards NAD(H) as a cofactor (31). The hyperthermophile sequence data and experimental evidence of the preference of AdhD towards NAD(H) combined with the arginine to histidine mutation identified in NADP(H)-biased AKRs seems to suggest that hyperthermophilic AKRs may preferentially utilize NAD(H).

In the present work, we have rationally mutated the cofactor binding pocket in the thermostable AKR, AdhD, from *Pyrococcus furiosus* guided by the design rules
elucidated in the mesostable AKRs. A K249G/H255R double mutant exhibited the greatest improvement in activity with NADP(H), and also had superior activity with NAD(H) compared to the wild-type and the other enzyme variants tested. All enzyme forms also exhibited varying degrees of kinetic transients upon cofactor binding, in contrast to the previous results obtained with a mesostable AKR (22). Most interestingly, the highly active double mutant exhibited bi-exponential kinetic transients with NADPH where the direction of the fast transient was concentration dependent. Taken together, these results suggest that amino acids identified in the mesostable AKRs can be used to modify the cofactor specificity of AdhD, and the observed kinetic transients are independent of the formation of a guanido-phosphate salt bridge.

Materials and Methods

Chemicals and plasmids: Oligonucleotides were from Integrated DNA Technologies. The QuikChange Site Directed Mutagenesis kit was from Stratagene. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was from Promega. E. coli BL21(DE3) competent cells were from New England Biolabs. Precast sodium dodecyl sulfate-polyacrylamide gels, NuPAGE MOPS running buffer, and broad-range molecular weight marker were from Invitrogen. All other chemicals were from Sigma-Aldrich and used without
modification. The *Pyrococcus furiosus* AdhD expression plasmid pWUR85 and tRNA helper plasmid pSJS1244 were a kind gift from Dr. John van der Oost (Wageningen University, The Netherlands) and are described in (31).

**Mutant Construction:** Single mutants K249G and H255R and double mutant K249G/H255R were created using the QuikChange Site-directed Mutagenesis Kit (see SI). All mutations were verified by DNA sequencing.

**AdhD Expression and Purification:** Expression and purification of AdhD followed a previously established protocol with minor modifications (31). After expression, cells were harvested by centrifugation, and pellets were resuspended in 1/10th volume 20 mM Tris-HCl (pH 7.5) before being incubated at 80°C for 1 h. Endogenous proteins and cell debris were then removed by centrifugation for 20 min at 10,000 x g. The supernatant was retained as the heat-stable cell-free extract (HSCFE). Samples were concentrated over a centrifugal filter (30 kDa MWCO) before being applied to a gel filtration column (Superdex 16/200, GE Healthcare) equilibrated in 20 mM Tris HCl (pH 7.8), 100 mM NaCl. Fractions containing active enzyme were pooled and concentrated. Enzyme stocks were diluted to working concentration in 20 mM Tris HCl (pH 7.8) before use. Expression and purification of AdhD mutants followed the same protocol. All enzyme concentrations were determined from A$_{280}$ measurements with a calculated molar extinction coefficient of ε$_{280}$ = 52495 M$^{-1}$ cm$^{-1}$.
SDS-PAGE: Protein composition was analyzed using NuPAGE 4-12% Bis-Tris Gels with a Novex Mini-Cell system. Samples were prepared as described previously (31). A broad-range protein marker was used for molecular weight estimation.

Homology Modeling: A homology model of AdhD was generated using ESyPred3D (32) and MODELLER with primary template prostaglandin F synthase from Trypanosoma brucei (1VBJ, 31.1% identities). Structures were analyzed using MolProbity (33) and verified against other members of the aldo-keto reductase superfamily. Cofactors were inserted into the binding pocket by aligning the backbone of the homology model with 2,5-DKGR from Corynebacterium (1A80 with bound NADPH, 1M9H with bound NADH) (27). Figures were generated using YASARA.

Activity Assays: The activity of each mutant was first examined at fixed substrate concentrations above the previously reported Michaelis constants for AdhD. Reaction mixtures containing 50 mM glycine (pH 8.8), 100 mM 2,3-butanediol (oxidation reaction) or 100 mM sodium phosphate (pH 6.1), 80 mM 3-hydroxy-2-butanone (reduction reaction) and enzyme were incubated in a 96-well UV-transparent microplate at 45°C in a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). Reactions were initiated by the addition of 1-1000 µM cofactor. Initial rates were determined by following the production or depletion of NAD(P)H at 340 nm (ε = 6.22 mM$^{-1}$ cm$^{-1}$). Data were collected in triplicate, and experiments were repeated three times with fresh solutions. All points were fit simultaneously to (Eq. 1) using non-linear least-squares regression (Igor Pro, Wavemetrics, Inc.) to obtain estimates for the apparent $k_{cat}$ and Michaelis constant for each cofactor (34). Reported errors are standard deviations.
Statistically significant differences from wild-type AdhD were determined by Student’s t-test.

\[ V = \frac{E_k^{\text{cat}} A}{K_A^{\text{app}} + A} \quad \text{Eq. 1} \]

**Fluorescence Titrations:** Dissociation constants for the enzyme-cofactor complexes were determined by fluorescence titration (22, 35, 36). Briefly, 2 μM enzyme in 50 mM glycine (pH 8.8) (for NAD(P)^+) or 10 mM potassium phosphate (pH 7.0) (for NAD(P)H) was stirred in a 1 cm quartz cuvette placed in a J-815 spectrometer (Jasco Inc., Easton, MD) equipped with a Peltier junction temperature control. Samples were excited at 280 nm, and the fluorescence change upon cofactor binding was monitored at 330 nm (NAD(P)^+) or 450 nm (NAD(P)H). The total volume of cofactor added was less than 1% of the reaction volume to limit dilution effects. Experiments were repeated in at least triplicate, and data were fit to a saturation adsorption isotherm.

**Steady-state Kinetics:** The full kinetic parameters for the wild-type and double mutant AdhD were determined for both the oxidation and reduction reactions with NAD(P)(H). Initial rates at 45°C were measured using a SpectraMax M2 plate reader by following the production or depletion of NAD(P)H at 340 nm (ε = 6.22 mM⁻¹ cm⁻¹). Oxidation reactions contained 50 mM glycine (pH 8.8), 1-100 mM 2,3-butanediol, and the appropriate amount of enzyme and were initiated with 1-1000 μM NAD(P)^+. Reduction reactions contained 100 mM sodium phosphate (pH 6.1), 1-100 mM 3-
hydroxy-2-butanone, and enzyme, and were initiated by the addition of 1-500 µM NAD(P)H. Some cofactor inhibition was observed at concentrations in excess of 1 mM (data not shown). Reactions were initiated with cofactor to limit cofactor degradation during incubation at elevated temperatures, however control experiments indicated that cofactor degradation was not significant over the time the reaction was monitored. Data were collected in at least triplicate, and were fit simultaneously to the ordered bi-bi rate equation ((34), Eq. 2) using non-linear least-squares regression. This reaction mechanism was previously verified (37). Reported errors are standard deviations.

\[ V = \frac{E_k a_{cat} AB}{K_{ia} K_p + K_{iB} B + K_{iA} A + AB} \]  

Eq. 2

**Determination of Protein Stability:** Unfolding was assessed by following the CD signal at 222 nm in a J-815 CD Spectrophotometer equipped with a Peltier junction temperature control. Scans were made over a range of guanidine hydrochloride (GdnHCl) concentrations with a 1°C min\(^{-1}\) temperature ramp from 25°C to 90°C. Prior to analysis, enzyme samples were allowed to equilibrate overnight at room temperature in the appropriate concentration of GdnHCl. The midpoint of a sigmoidal fit to the data at 80 °C was taken as the denaturation midpoint.

**Kinetics of Cofactor Binding:** The kinetics of cofactor binding were investigated using a SFM-20 stopped flow system (BioLogic Inc., Knoxville, TN) equipped with a 20 µl
fluorescence cuvette (dead time ≈ 13 ms) attached to a J-815 CD Spectrophotometer. All experiments were performed at 25°C. Samples of enzyme (0.75 µM) and cofactor (0.5 – 30 µM) were mixed, and the quenching of intrinsic protein fluorescence (for NAD(P)⁺, 320 nm cutoff) or the energy transfer between the protein and cofactor (for NAD(P)H, 430 nm cutoff) was monitored upon exciting at 280 nm. Each fluorescence trace is the average of 3-5 shots, and each experiment was repeated three times with fresh solutions. Traces were fit to a mono-exponential or bi-exponential function where applicable, and the resulting rate constants were plotted versus cofactor concentration. These plots were used to obtain estimates of the rate constants for cofactor binding as described previously (38). All concentrations are given as the final concentration in the cuvette.

Results

Expression and Purification of AdhD: Site-directed mutagenesis was used to create three new mutant AdhD enzymes, K249G, H255R, and K249G/H255R. The wild-type and new mutant AdhDs expressed in high yields in E. coli and were readily purified due to their extreme thermostabilities. A simple and rapid purification scheme consisting of heating the re-suspended cell pellets to both lyse the cells and denature
endogenous proteins, followed by centrifugation, concentration, and size exclusion chromatography yielded homogenous samples as judged by SDS-PAGE (Figure 3.6).

**Homology Modeling:** Previous work and structural insights guided the decision to create the two single mutants and the double mutant of the thermostable AKR AdhD. In order to visualize the potential impact of these mutations on the cofactor binding pocket, a homology model was created. The highly conserved structure of the AKR superfamily enabled the addition of cofactors into the homology model by alignment with crystal structures of a similar AKR (2,5-DKGR) containing bound cofactor. The structural alignment had a RMSD of 1.0 Å over 232 aligned residues, and allowed us to identify amino acids that could potentially interact with the cofactor (Figure 3.1). It seems likely that His255 is in position to form a stacking interaction with the adenine ring of the cofactor, and potentially an ionic interaction with the 2’-phosphate of NADP(H) as well. Additionally, replacement of Lys249 with glycine should increase the volume of the cofactor binding pocket and allow for increased conformational flexibility.

**Fluorescence Titrations:** The new mutations were made to impact cofactor binding and thereby cofactor specificity. Conveniently located tryptophan residues in the cofactor binding pocket allow for the determination of cofactor dissociation constants for the different mutants by fluorescence titration (Table 3.5). Comparison of dissociation
constants between the wild-type enzyme and mutants can then be used to calculate the changes in ground state cofactor binding energies. At 25°C, the H255R mutant lost 0.2 kcal/mol of binding energy with NAD⁺ but gained 2.6 kcal/mol with NADP⁺. The change in binding energy with NADP⁺ is less than was observed in an arginine→methionine mutant of 3α-HSD (22), but is comparable in magnitude to the gain of an electrostatic interaction. The K249G single mutant gained 2.2 and 2.4 kcal/mol of binding energy with NAD⁺ and NADP⁺, respectively, while the K249G/H255R double mutant exhibited a slight gain of 0.5 kcal/mol for NAD⁺ and a larger gain of 2.1 kcal/mol with NADP⁺. Small gains in binding energies were observed with the reduced cofactors in every case, ranging from 0.14 kcal/mol with NADPH for the K249G mutant to 0.57 kcal/mol with NADPH for the H255R mutant. The double mutant gained 0.31 kcal/mol with NADH and 0.25 kcal/mol with NADPH.

Fluorescence titrations were also performed at 45°C with wild-type AdhD and the K249G/H255R mutant to allow for comparison with the $K_{ia}$ term of the ordered bi-bi rate equation obtained from steady-state kinetic experiments as described below (Table 3.2).

**Steady-State Kinetic Analysis:** A simplified kinetic analysis of the oxidation and reduction reactions for the wild-type enzyme and the three mutants was performed at a fixed substrate concentration (Table 3.6). In order to estimate the effect of the mutations
on cofactor specificity, the apparent catalytic efficiency ($k_{cat}/K_A$) was compared (Figure 3.2). In the oxidation reaction, the wild-type enzyme exhibits a similar preference for both NAD$^+$ and NADP$^+$. No significant difference in catalytic efficiency between the mutants and the wild-type with either cofactor was observed. However, the H255R mutant demonstrated a 2-fold preference for NADP$^+$ over NAD$^+$, which agrees with the proposed role of Arg255 in NADP(H) binding. For the double mutant in the two oxidation reactions, the Michaelis constants for the substrate ($K_B$) were later calculated to be greater than the substrate concentration utilized, and therefore the assumption of saturating substrate in these cases is invalid which would lead to an underestimation of the $k_{cat}^{app}$.

In the reduction reaction, larger changes in the apparent kinetic parameters were observed. The wild-type enzyme exhibited significant specificity for NADH over NADPH as evidenced by an order of magnitude difference in the catalytic efficiency. For the H255R mutant, the catalytic efficiency doubled with NADH as compared to the wild-type and it increased by an order of magnitude with NADPH so that the mutant had no significant specificity between the cofactors. For the K249G mutant, the catalytic efficiency increased 5-fold for NADH while the efficiency with NADPH increased more than 30-fold in comparison to the wild-type. For the double mutant, the catalytic efficiency with NADH increased 4-fold while the efficiency with NADPH increased more than 16-fold.
Since the double mutant exhibited the largest increase in the apparent $k_{cat}$ with both cofactors, the full steady-state kinetic experiments were performed for this mutant and compared to the values for the wild-type enzyme (Table 3.2, Figure 3.7). In the oxidation reaction, the $k_{cat}$ with NAD$^+$ improved by 15-fold for the double mutant over the wild-type enzyme. The impact on activity with NADP$^+$ was even greater, as the double mutant had a $k_{cat}$ nearly two orders of magnitude larger than the wild-type. However, the Michaelis constant for the cofactor also increased significantly in both cases, from 63 µM to 460 µM for NAD$^+$ and from 5.1 µM to 78 µM for NADP$^+$ in the wild-type and double mutant, respectively. The Michaelis constant for the substrate also increased significantly, from 29 mM for the wild-type to 690 mM for the double mutant with NAD$^+$, and from 1.3 mM to 200 mM with NADP$^+$. In the reduction reaction, the double mutant has a $k_{cat}$ 3-fold greater with NADH and 6-fold greater with NADPH compared to the wild-type. While the Michaelis constants for the cofactor and substrate increased for the double mutant in the oxidation reaction, they mostly decreased in the reduction reaction. For the cofactor, $K_A$ with NADH decreased from 190 µM to 50 µM, and with NADPH the value decreased from 280 µM to 33 µM. The $K_B$ value increased from 0.9 mM to 13 mM when NADH was the cofactor, but decreased from 6.7 mM to 5.0 mM when NADPH was the cofactor. In the case of the reduction reaction with NADPH, the $K_{ia}$ values were unable to be fit by the model and so the $K_D$ values obtained by fluorescence titration at 45°C were used instead.
In order to simplify the comparison of the impact of the mutations on the steady-state kinetics, the parameters were used to estimate the microscopic rate constants for the simplified reaction mechanism described in Equation 3 (Table 3.3). Generally, the on-rate of the cofactor ($k_{1ss}$) increased by 2 to 10-fold in the double mutant compared to the wild-type. In most cases the off-rate ($k_{2ss}$) was found to decrease, except in the case of the double mutant with NADPH where the off-rate increased. The net on-rate of the substrate ($k_{3ss}$) in the oxidation reaction was only slightly impacted by the mutations, whereas a much stronger effect was observed in the reduction reaction. The on-rate of the substrate with NADH decreased almost 5-fold in the double mutant versus the wild-type, but increased 8-fold with NADPH. The ratio of $k_{1ss}k_{3ss}/k_{2ss}$ is a convenient single parameter for examining the catalytic performance of the mutants (9). When judged by this composite rate constant, the double mutant enzyme is shown to be substantially improved with NADP$^+$ in the oxidative direction and with both NADH and NADPH in the reductive reaction compared to the wild-type (Figure 3.3).

\[
E + A \xrightleftharpoons[k_{2ss}]{k_{1ss}} EA \quad EA + B \xrightarrow{k_{3ss}} EAB \quad \text{Eq. 3}
\]

**Determination of Enzyme Stability:** Guanidine denaturation curves were generated for wild-type AdhD and the K249G/H255R double mutant in order to assess the effect of the mutations on enzyme stability. Both enzymes appeared stable in up to 6M GdnHCl.
at room temperature, and temperatures greater than 70°C were required to observe an unfolding transition. At 80°C, the denaturation midpoints of both enzymes were comparable (4.8M for the wild-type, K249G and H255R mutants and 4.9M for the K249G/H255R mutant), suggesting that the mutations had little effect on stability (Figure 3.8). The unfolding did not appear to be reversible, however, as little CD signal was regained upon cooling. Thus this data could not be used to calculate ΔG values.

**Kinetics of Cofactor Binding:** Stopped-flow fluorescence spectroscopy was used to further investigate the mode of cofactor binding in the wild-type and mutant enzymes (16, 35, 39). AdhD contains six tryptophan residues, two of which are located near the active site. These residues act as distal reporters of cofactor binding, as the quenching of intrinsic protein fluorescence or energy transfer with the reduced cofactor can be followed (Figure 3.4). The signal voltage is inversely proportional to the fluorescence intensity, such that negative amplitude corresponds to an increase in fluorescence and vice versa. In this study, all four enzymes tested displayed observable fluorescence transients with NADP(H), and all except H255R displayed observable transients with NAD(H) (Figure 3.9). In some cases, transients may have occurred mostly within the dead-time of the stopped-flow (such as H255R with NADH), and in these cases no rate data were obtained. The existence of transients in these cases was confirmed by control stopped-flow experiments diluting the enzyme into buffer and by comparison with the steady-state fluorescence titration data. Plots of the observed rate constant versus
cofactor concentration displayed saturation kinetics and were well fit by a hyperbolic function (Table 3.4, Figure 3.10). These are consistent with a two-step binding mechanism (Eq. 4), in which a rapid bimolecular association step is followed by a slow isomerization step (35, 38). Note that the cofactor binding constants $k_{1}^{ss}$ and $k_{2}^{ss}$ obtained from analysis of the steady-state kinetics data include the isomerization step and thus are different than $k_{1}$ and $k_{2}$ obtained from the transient kinetic data.

Unprecedented Transients with the Double Mutant and NADPH: Fluorescence traces of NADPH binding with the double mutant exhibited cofactor concentration dependent amplitudes and were best fit with a bi-exponential function. Three regimes were identified based on the amplitude and direction of the fast transient, as the rate of the slow transient remained relatively constant (Figure 3.5). At low NADPH concentrations (< 2 μM), the fast transient had a negative amplitude, consistent with an increase in FRET efficiency as the nicotinamide head group binds near the active site. At slightly higher concentrations, the amplitude of this fast transient was too small to be reliably fit with a rate constant. Above 5 μM NADPH the amplitude of the fast transient became positive, indicating an initial rapid decrease in fluorescence. Interestingly, a hyperbolic
fit to the rates of the fast transient in the first regime extrapolates roughly to the measured rates of the fast transients in the third regime (Figure 3.5).

**Discussion**

Knowledge of the cofactor binding mechanism and determinants of cofactor specificity obtained with mesostable AKRs allowed us to readily broaden the cofactor specificity in a thermostable AKR, AdhD. As an arginine→histidine mutation has been previously demonstrated to increase activity with NAD(H), we reasoned the reverse would hold and that a histidine→arginine mutation would increase activity with NADP(H). Additionally, a lysine→glycine mutation was investigated as it was previously found to improve overall activity (9, 26). Combining both mutations in 2,5-DKGR yielded a double mutant with significantly improved kinetic properties (9).

In the present work with AdhD, the H255R single mutant exhibited an increased binding affinity toward NADP⁺ and a concomitant reduction in affinity for NAD⁺. A similar trend was observed using a simplified kinetic analysis, as the apparent kcat for H255R was only about 60% of that of the wild-type with NAD⁺, but was six-fold higher than the wild-type with NADP⁺. These results support the idea that an arginine at position 255 is important for recognizing NADP(H), but is not the sole determinant of cofactor specificity.
Kinetics with the K249G single mutant demonstrated a significant increase in $k_{cat}$ compared to both the wild-type and the H255R single mutant with NAD(P)$^+$ and NADH. Previous kinetic and structural studies suggest this residue is important in properly orienting the cofactor in the active site (9, 15, 24, 26). As the natural substrates for 2,5-DKGR and AdhD are not known, it is possible this mutation better positions the cofactor for turnover with the non-natural substrates and would impair wild-type functionality. These mutations do not seem to have an additive effect on cofactor binding energy in AdhD, however, as the double mutant only exhibits a slight increase in affinity for NAD$^+$ and a moderate increase in affinity for NADP$^+$, which is less than would be expected given the changes in binding affinities observed in the single mutants. Regardless, the K249G/H255R double mutant was significantly more active than the wild-type and single mutant enzymes with both NAD(H) and NADP(H) both at moderate (Table 3.6) and high temperatures (Table 3.7), and these mutations slightly improved the thermostability of the enzyme (Figure 3.8). This impressive result confirmed the design rules established for relaxing cofactor specificity in AKRs and prompted us to further investigate the basis for this change and whether mesostable and thermostable AKRs share a conserved cofactor binding mechanism.

Given the increase in $k_{cat}$ observed in the K249G/H255R double mutant, it is useful to compare individual rate constants rather than overall catalytic efficiencies (Table 3.3, Figure 3.3). The composite parameter $(k_3/k_2)^{1/2}$ highlights the significant improvement in
activity and broadened specificity observed with the K249G/H255R double mutant over the wild-type enzyme. Further, the double mutant exhibits an order of magnitude improvement in this parameter when NADP⁺ is used as the cofactor in place of NAD⁺. This is largely a result of a decrease in the off-rate of NADP⁺, likely due to anchoring of the 2′-phosphate by Arg255 as has been previously proposed.

Stopped-flow fluorescence spectroscopy has been used to probe the difference in binding mechanism between NADP(H) and NAD(H) in rat liver 3α-HSD and suggests the canonical arginine residue forms an electrostatic linkage with the 2′-phosphate of NADP(H), which is observed as a fluorescence kinetic transient (22). The interaction is accompanied by a conformational change in the cofactor binding pocket which increases the affinity of the enzyme for the cofactor. A kinetic transient was not observed in an arginine→methionine mutant or when NAD(H) was used as a cofactor, suggesting that the transient (and associated conformational change) was both arginine and 2′-phosphate dependent.

Introduction of the canonical arginine residue into AdhD allowed us to examine whether the cofactor binding mechanism established in the 3α-HSD enzyme applies to the thermostable AdhD. Unexpectedly, the reported arginine and 2′-phosphate dependent fluorescence transient observed upon cofactor binding in the mesostable AKRs does not seem to hold for AdhD. In the present work, we demonstrate that a
kinetic transient exists in wild-type AdhD which contains a histidine at this position, and also when NAD(H) is used as a cofactor. Similar behavior was observed in the three cofactor binding pocket mutants used to further investigate cofactor specificity, suggesting this conformational behavior is less sensitive to the presence of the arginine residue and 2′-phosphate of NADP(H) than previously suggested. Additionally, when protonated, the histidine mutation is relatively conservative compared to the previously described methionine mutants. This could explain the existence of fluorescence kinetic transients with both the wild-type and H255R enzymes upon NADP(H) binding, as the histidine may be able to form an electrostatic linkage with the negatively charged 2′-phosphate of the cofactor similarly to the canonical arginine. Further exploration of these mutations in mesophilic AKRs, especially the transient behavior of His255 and Gly249 mutants, would lead to a better understanding of these differences. Also, it will be interesting to see whether other thermostable AKRs demonstrate a similar cofactor binding mechanism.

Unprecedented transient behavior was observed in the K249G/H255R double mutant when NADPH was used as a cofactor. Fluorescence traces appeared to be at least bi-exponential, and the amplitude of the fast transient was surprisingly dependent on the concentration of the cofactor. At low NADPH concentrations (< 2 µM), a fast increase in fluorescence intensity was followed by a slow decay to the steady-state value. As the fluorescence signal is due to energy transfer between the enzyme and cofactor, this
suggests an initial rapid binding step that brings the nicotinamide head group close to the active site, followed by a slow isomerization moving the head group away to an equilibrium position. As the cofactor concentration increased, the amplitude of the fast transient decreased to the point where the signal was dominated by the slow transient. Above 5 µM NADPH, a fast transient was again observed, but with an amplitude opposite of that at lower cofactor concentrations. A plot of the fast transient observed in regime I versus NADPH concentration is best fit by a hyperbola, suggesting at least a three step reaction mechanism (36, 38). To the best of our knowledge, this behavior has not been previously reported in the literature.

The dynamics of NADPH binding suggest the cofactor samples several configurations before reaching an equilibrium position. The increased volume of the cofactor binding pocket afforded by the lysine→glycine mutation seems likely to contribute to the increased conformational flexibility of the cofactor. Multiple cofactor molecules competing for the same binding site could also explain the inverse amplitude observed above 5 µM NADPH, but this does not agree with the steady-state kinetics where cofactor inhibition was only observed at cofactor concentrations several orders of magnitude higher (data not shown).

Estimates of cofactor dissociation constants were obtained through three orthogonal methods: fluorescence titrations, steady-state kinetics, and transient-state kinetics. Fits
to the ordered bi-bi rate equation (Eq. 2) were used to determine the full steady-state kinetic parameters for the wild-type and K249G/H255R double mutant. The fit parameter $K_{av}$ is equivalent to the dissociation constant of the enzyme-cofactor complex (34), and was generally in good agreement with the dissociation constant as measured by fluorescence titrations (Table 3.2). The transient-state kinetics investigated by stopped flow fluorescence spectroscopy can also be used to calculate the microscopic rate constants corresponding to each step in the cofactor binding mechanism (38). The overall dissociation constant can then be calculated from the microscopic rate constants, and compared to that obtained by fluorescence titrations (35). These results are summarized in Table 3.4. Almost universally, the dissociation constants calculated from the microscopic rate constants significantly underestimate those obtained by fluorescence titration and steady-state kinetics. Although the source of this disparity is unknown, some difficulty in reconciling stopped-flow fluorescence data with that measured at steady-state has been reported by others (21, 22, 29, 40). Control experiments were performed to rule out artifacts due to mixing effects, non-specific binding, or photobleaching with the fluorescence methods, and the introduction of additional steps in the cofactor binding mechanism could only further decrease the calculated dissociation constants. Further experiments using T-jump spectroscopy or ITC may be necessary to reconcile these observations and fully elucidate the cofactor binding mechanism.
Broadening cofactor specificity in the AKR superfamily has become almost formulaic, although the mechanism of cofactor binding does not yet seem to be fully elucidated. The ability to change or relax the cofactor specificity of AKRs will be useful in industrial applications, as NAD(H) is more stable and less expensive than NADP(H) (8), and the use of AKRs in specialized applications will benefit from knowledge obtained during cofactor specificity engineering exercises, as it may be advantageous to increase activity with non-natural cofactors that are optimized for the final application (41).
### Table 3.1: Multiple sequence alignment of cofactor binding pocket residues of selected mesostable and thermostable AKRs.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Origin</th>
<th>Residue…</th>
<th>24</th>
<th>50</th>
<th>166</th>
<th>167</th>
<th>190</th>
<th>216</th>
<th>219</th>
<th>221</th>
<th>270</th>
<th>271</th>
<th>272</th>
<th>276</th>
<th>279</th>
<th>280</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 aldehyde reductase</td>
<td>Human</td>
<td></td>
<td>W</td>
<td>D</td>
<td>S</td>
<td>N</td>
<td>Q</td>
<td>Y</td>
<td>L</td>
<td>S</td>
<td>K</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>Q</td>
<td>N</td>
</tr>
<tr>
<td>2 3α-HSD</td>
<td>Rat</td>
<td></td>
<td>T</td>
<td>D</td>
<td>S</td>
<td>N</td>
<td>Q</td>
<td>Y</td>
<td>L</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>F</td>
<td>R</td>
<td>E</td>
<td>L</td>
</tr>
<tr>
<td>3 2,5-DKGR</td>
<td>Mesostable bacterium</td>
<td></td>
<td>F</td>
<td>D</td>
<td>S</td>
<td>N</td>
<td>Q</td>
<td>W</td>
<td>L</td>
<td>Q</td>
<td>K</td>
<td>S</td>
<td>V</td>
<td>R</td>
<td>E</td>
<td>N</td>
</tr>
<tr>
<td>4 AdhD</td>
<td>Thermostable archaeon</td>
<td></td>
<td>W</td>
<td>D</td>
<td>S</td>
<td>N</td>
<td>Q</td>
<td>Y</td>
<td>L</td>
<td>K</td>
<td>K</td>
<td>A</td>
<td>S</td>
<td>H</td>
<td>E</td>
<td>N</td>
</tr>
<tr>
<td>5 Putative AKR</td>
<td>Thermostable archaeon</td>
<td></td>
<td>W</td>
<td>D</td>
<td>S</td>
<td>N</td>
<td>Q</td>
<td>Y</td>
<td>L</td>
<td>K</td>
<td>K</td>
<td>A</td>
<td>I</td>
<td>H</td>
<td>E</td>
<td>N</td>
</tr>
<tr>
<td>6 Putative AKR</td>
<td>Thermostable archaeon</td>
<td></td>
<td>Y</td>
<td>D</td>
<td>S</td>
<td>N</td>
<td>Q</td>
<td>W</td>
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<td>H</td>
<td>R</td>
<td>A</td>
<td>S</td>
<td>H</td>
<td>E</td>
<td>N</td>
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<tr>
<td>7 Putative AKR</td>
<td>Thermostable bacterium</td>
<td></td>
<td>Y</td>
<td>D</td>
<td>A</td>
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<td>L</td>
<td>V</td>
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<td>M</td>
<td>S</td>
<td>H</td>
<td>E</td>
<td>N</td>
</tr>
</tbody>
</table>

Rat liver 3α-HSD numbering, the shaded positions correspond to AdhD residues 249 and 255 as mutated in this study. 1. Human aldehyde reductase (Accession #P14550), 2. Rat liver 3α-hydroxysteroid dehydrogenase (Accession #P23457), 3. Corynebacterium 2,5-diketo-D-gluconic acid reductase A (Accession #P06632), 4. Pyrococcus furiosus alcohol dehydrogenase D (Accession #NP_579689), 5. Putative AKR from Thermococcus barophilus (Accession #EDY40262), 6. Putative AKR from Thermococcus volcanium (Accession #NP_111671), 7. Putative AKR from Aquifex aeolicus (Accession #NP_213220).
Table 3.2: Full steady state kinetic parameters for wild type AdhD and the K249G/H255R double mutant

<table>
<thead>
<tr>
<th></th>
<th>Oxidation</th>
<th>Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_D (µM)</td>
<td>K_La (µM)</td>
</tr>
<tr>
<td>wt AdhD^+ NAD</td>
<td>59 ±1</td>
<td>37 ±2</td>
</tr>
<tr>
<td>K249G/H255R</td>
<td>45 ±2</td>
<td>11 ±1</td>
</tr>
<tr>
<td>wt AdhD^- NADP</td>
<td>5.7 ±1.0</td>
<td>20 ±8</td>
</tr>
<tr>
<td>K249G/H255R</td>
<td>0.66 ±0.10</td>
<td>12 ±0.2</td>
</tr>
</tbody>
</table>

Oxidation reactions were performed at 45°C in 50 mM glycine (pH 8.8) with 2,3-butanediol substrate. Reduction reactions were performed at 45°C in 100 mM sodium phosphate (pH 6.1) with 3-hydroxy-2-butanone substrate. K_D is the cofactor dissociation constant as determined by fluorescence titration under the same conditions. K_A and K_B are the Michaelis constants for the cofactor and substrate, respectively (Equation 2). Reactions were performed in at least triplicate, and errors are standard deviations. ND: The K_La term was unable to be fit by the model, and was instead set equal to the measured K_D (34).
Table 3.3: Microscopic rate constants calculated from steady-state kinetic parameters.

<table>
<thead>
<tr>
<th></th>
<th>Oxidation</th>
<th>Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_1^{ss}$</td>
<td>$k_2^{ss}$</td>
</tr>
<tr>
<td>wt AdhD</td>
<td>0.016 s^{-1}</td>
<td>0.59 s^{-1}</td>
</tr>
<tr>
<td>K249G/ H255R</td>
<td>0.033 s^{-1}</td>
<td>0.36 s^{-1}</td>
</tr>
<tr>
<td>wt AdhD</td>
<td>0.0059 s^{-1}</td>
<td>0.12 s^{-1}</td>
</tr>
<tr>
<td>K249G/ H255R</td>
<td>0.060 s^{-1}</td>
<td>0.07 s^{-1}</td>
</tr>
</tbody>
</table>

Rate constants calculated from the relationships: $k_1^{ss} = k_{cat}/K_A$, $k_2^{ss} = k_{cat}K_{ia}/K_A$, and $k_3^{ss} = k_{cat}/K_b$, for the mechanism described in Equation 3.
Table 3.4: Comparison of cofactor dissociation constants measured by fluorescence titrations and stopped-flow fluorescence spectroscopy

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cofactor</th>
<th>$K_D$ (µM) Measured</th>
<th>$K_D$ (µM) Calculated</th>
<th>Microscopic Rate Constants</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$K_1$ (µM)</td>
<td>$k_2$ ($s^{-1}$)</td>
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<td></td>
<td></td>
<td></td>
<td>$K_1$ (µM)</td>
<td>$k_2$ ($s^{-1}$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$K_1$ (µM)</td>
<td>$k_2$ ($s^{-1}$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$K_1$ (µM)</td>
<td>$k_2$ ($s^{-1}$)</td>
</tr>
<tr>
<td>wt AdhD</td>
<td>NAD$^+$</td>
<td>65 ±2</td>
<td>0.65</td>
<td>5.5</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>NADH</td>
<td>42 ±1</td>
<td>ND</td>
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<td>-</td>
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<tr>
<td></td>
<td>NADP$^+$</td>
<td>25 ±1</td>
<td>0.01</td>
<td>3.2</td>
<td>9.6</td>
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<tr>
<td></td>
<td>NADPH</td>
<td>29 ±1</td>
<td>0.11</td>
<td>0.70</td>
<td>4.6</td>
</tr>
<tr>
<td>K249G</td>
<td>NAD$^+$</td>
<td>1.7 ±0.1</td>
<td>NA</td>
<td>1.5</td>
<td>7.9</td>
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<td></td>
<td>NADH</td>
<td>20 ±1</td>
<td>0.66</td>
<td>3.3</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>NADP$^+$</td>
<td>0.4 ±0.1</td>
<td>NA</td>
<td>3.2</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>23 ±1</td>
<td>ND</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H255R</td>
<td>NAD$^+$</td>
<td>91 ±2</td>
<td>ND</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NADH</td>
<td>29 ±1</td>
<td>ND</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NADP$^+$</td>
<td>0.3 ±0.1</td>
<td>0.13</td>
<td>14</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>11 ±1</td>
<td>0.10</td>
<td>3.0</td>
<td>15</td>
</tr>
<tr>
<td>K249G/H255R</td>
<td>NAD$^+$</td>
<td>29 ±1</td>
<td>11</td>
<td>78</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>NADH</td>
<td>25 ±1</td>
<td>ND</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NADP$^+$</td>
<td>0.7 ±0.1</td>
<td>1.5</td>
<td>18</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>19 ±1</td>
<td>0.31</td>
<td>1.7</td>
<td>38</td>
</tr>
</tbody>
</table>

Dissociation constants were calculated as previously described from hyperbolic fits to the observed rate constants of the kinetic transients versus the cofactor concentrations (Figure 3.5, Figure 3.10). Measured dissociation constants were obtained by fluorescence titrations performed under the same conditions as the stopped flow experiments. NA: Not applicable as no estimate was able to be obtained for $k_{-2}$.  

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Figure 3.1: Homology model of the AdhD cofactor binding pocket with bound cofactors

Homology model of AdhD with bound NAD(H) (left) and NADP(H) (right). Side chains of Lys249 and His255 of the wild-type AdhD are shown in purple, and Arg255 of the double mutant is shown in yellow. His255 is in position to form a stacking interaction with the adenine moiety of the cofactor, while Arg255 can form an electrostatic interaction with the 2’-phosphate in NADP(H). Lys249 extends beneath the pyrophosphate group and also forms an electrostatic interaction with the 2’-phosphate in NADP(H).
Figure 3.2: Apparent catalytic efficiencies ($k_{cat}/K_M$)$_{app}$ of wild-type AdhD and mutants in the oxidation and reduction reactions.

Apparent catalytic efficiencies determined under fixed substrate conditions were calculated using Equation 1. Reaction mixtures contained 50 mM glycine (pH 8.8), 100 mM 2,3-butanediol, 1-1000 μM NAD(P)$^+$, and enzyme (oxidation reaction, A) or 100 mM sodium phosphate (pH 6.1), 80 mM 3-hydroxy-2-butanone, 1-500 μM NAD(P)H, and enzyme (reduction reaction, B) at 45°C. Measurements were performed in triplicate, and experiments were repeated three times with fresh solutions. Error bars are standard deviations. Asterisks indicate statistically significant difference from wild-type AdhD at $p < 0.05$. +: fits to Equation 3 suggest substrate concentration is not saturating, thus the actual $k_{cat}$ is likely higher.
Figure 3.3: Activity of wt AdhD and K249G/H255R with each cofactor

Microscopic rate constants calculated from the steady-state kinetic parameters (Table 3.3). Comparing the value of \( \frac{k_1 k_3}{k_2} \) demonstrates the significant improvement in activity and broadened specificity of the double mutant enzyme.
Figure 3.4: Fluorescence kinetic transients observed upon cofactor binding for the K249G AdhD mutant

Representative fluorescence traces of K249G AdhD mutant (0.75 µM) with (A) 7.5 µM NADH and (B) 7.5 µM NAD$^+$ fit to a mono-exponential function. (C) Plot of the observed rate constant as a function of cofactor concentration for K249G AdhD with NAD$^+$. Error bars are standard deviations of at least three independent measurements. The data was fit with a hyperbola to obtain estimates of the microscopic rate constants (Table 3.4).
Figure 3.5: Identification of three regimes of kinetic transients observed upon NADPH binding to the K249G/H255R double mutant

The observed rate constants for the fast and slow fluorescence transients observed upon mixing 1.5μM K249G/H255R with NADPH. Samples were excited at 280 nm, and fluorescence of the cofactor due to energy transfer from tryptophan residues was detected through a 430 nm cutoff filter. A hyperbola was fit to the fast transient in the first regime (≤ 2 μM NADPH), and was extrapolated to the third regime. Inset plots contain representative fluorescence traces from each regime. In regime I, a fast increase in fluorescence is followed by a slow decay to the steady-state value. In regime II, the amplitude of the fast transient is too small to obtain an estimate, and the slow transient dominates the signal. In regime III, a fast initial decay is followed by a slow decay to the steady-state value. These results are consistent with a concentration-dependent reversal in the direction of the reorientation that occurs in the cofactor binding pocket during the transient conformational change.
Supplemental Information

Experimental Procedures

Oligos used for site-directed mutagenesis (mutations in italics):

K249Gs:
5'- GGA AAA TGT TGT AGC AAT TCC AGC AAG CAA CAA GGA ACA CC -3'
K249Gas:
5'- GGT GTT CCT TGT TGC TTG CTG GAA TTG CTA CAA CAT TTT CC -3'
H255Rs:
5'- CCA AAA GCA AGC AAC AAG GAA CTC AAA GAA AAC TTT GG -3'
H255Ras:
5'- CCA AAG TTT TCT TTG AGG CGT TCC TTG TTG CTT GCT TTT GG -3'
Tables

Table 3.5: Changes in free energy of cofactor binding calculated from equilibrium fluorescence titrations.

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_D$ (µM) @ 25°C</td>
<td>$K_D$ (µM) @ 45°C</td>
<td>$\Delta \Delta G_b$ (kcal/mol) @ 25°C</td>
<td>$\Delta \Delta G_b$ (kcal/mol) @ 45°C</td>
<td></td>
</tr>
<tr>
<td>wt AdhD</td>
<td>NAD$^+$ 65 ± 2</td>
<td>59 ± 1</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NADP$^+$ 25 ± 1</td>
<td>5.7 ± 1.0</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>K249G</td>
<td>NAD$^+$ 1.7 ± 0.1</td>
<td>N/A</td>
<td>-2.2</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NADP$^+$ 0.40 ± 0.10</td>
<td>N/A</td>
<td>-2.4</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>H255R</td>
<td>NAD$^+$ 91 ± 2</td>
<td>N/A</td>
<td>0.20</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NADP$^+$ 0.30 ± 0.10</td>
<td>N/A</td>
<td>-2.6</td>
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<td>K249G/ H255R</td>
<td>NAD$^+$ 29 ± 1</td>
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<td>-0.48</td>
<td>-0.16</td>
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<tr>
<td></td>
<td>NADP$^+$ 0.70 ± 0.10</td>
<td>0.70 ± 0.10</td>
<td>-2.1</td>
<td>-1.7</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.6: Apparent Kinetic Parameters in the Oxidation and Reduction Reactions.

Oxidation reaction performed at 45°C in 50 mM glycine (pH 8.8) with 100 mM 2,3-butanediol as the substrate. Reduction reaction performed at 45°C in 100 mM sodium phosphate (pH 6.1) with 80 mM 3-hydroxy-2-butanone as the substrate. Statistically significant difference between values obtained with NAD(H) and NADP(H) for a particular mutant are indicated by italics, statistically significant difference from the values obtained with the wild-type enzyme are indicated by asterisks (*). Statistical significance was reached at a $p$ value < 0.05.
Table 3.7: Apparent Kinetic Parameters in the Oxidation Reactions at 70 °C.

Oxidation reaction performed at 70°C in 50 mM glycine (pH 8.8) with 100 mM 2,3-butanediol as the substrate. The change in absorbance at 340nm was monitored using a Jasco J-815 spectrometer in a 1cm stirred quartz cuvette, and data were fit simultaneously to Eq. 1. Reported errors are standard deviations. NA: The observed activity of the wt AdhD with NADP⁺ at the above conditions was <0.5 s⁻¹, and a value for $K_A^{app}$ could not be reliably obtained.
Figures

Figure 3.6: SDS-PAGE analysis of heterologously expressed AdhD and mutants after gel filtration.

MW: molecular weight marker, lane 1: wt AdhD, lane 2: K249G, lane 3: H255R, lane 4: K249G/H255R. Samples were prepared by heating for 1 h at 100°C in the presence of sample buffer (see text). A single band is observed at ~32 kDa, consistent with the calculated molecular mass of AdhD.
Figure 3.7: Ordered bi-bi Kinetics Fits

Plots of ordered bi-bi rate equation fits to steady-state kinetics data for the wild type AdhD enzyme and the K249G/H255R double mutant.
Figure 3.8: GdnHCl Denaturation Curves

CD signal at 222 nm for the wild type AdhD (blue, open circles), K249G AdhD (green, open squares), H255R AdhD (black, open triangles) and the K249G/H255R mutant (red, closed circles) at 80°C as a function of GdnHCl concentration. Lines are least-squares fits using a sigmoidal function to obtain the denaturation midpoint. Fits are only shown for the wild-type and K249G/H255R mutant curves for clarity. Wild-type AdhD, K249G AdhD, and H255R AdhD have an apparent denaturation midpoint of 4.8M GdnHCl while the K249G/H255R mutant has a similar apparent denaturation midpoint of 4.9M GdnHCl.
Figure 3.9: Representative stopped-flow fluorescence traces for each enzyme/cofactor pair.

Fluorescence signal upon mixing of 0.75 μM enzyme and 5 μM cofactor. Each trace is the average of 3-5 shots. Fits are to a mono-exponential function (except K249G/H255R with NADPH, which is fit to a bi-exponential function) and residuals are shown above the trace. Data was collected from 90 ms before the stop in all experiments to establish a baseline.
Figure 3.10: Plots of observed rate constant versus cofactor concentration for each enzyme/cofactor pair used in the determination of microscopic rate constants.

Lines are hyperbolic fits to the data as described in the main text. Error bars are standard deviations.
References:


Chapter 4

AN ENZYMATIC BIOFUEL CELL UTILIZING A BIOMIMETIC COFACTOR

Abstract: The performance of enzyme-based biofuel cells, biosensors, and bioelectrocatalytic systems is often limited by poor mass transport within immobilized architectures and poor cofactor regeneration kinetics. As the dehydrogenase enzymes commonly used in these applications require a nicotinamide cofactor as an electron mediator, we have explored the use of biomimetic cofactors with higher diffusion coefficients to address these limitations. Here, we demonstrate a biofuel cell anode constructed with an engineered dehydrogenase enzyme capable of utilizing nicotinamide mononucleotide (NMN\(^+\)) to oxidize D-arabinose. While the enzymatic activity with NMN\(^+\) is significantly reduced as compared to NAD\(^+\), the maximum power density of the biofuel cell is comparable for both cofactors. Additionally, an increase in the limiting current is observed with NMN(H), suggesting increased cofactor diffusion. While protein engineering efforts have often focused on improving the kinetics of wild-type enzymes, this work suggests that significant performance gains can be obtained by engineering enzymes for activity with biomimetic cofactors with

\[^{\S}\] A version of this chapter has been submitted to Nature Communications with co-authors Matthew Meredith, Shelley Minteer, and Scott Banta. EC and MM contributed equally to this work and are co-first authors.
desirable properties, such as improved diffusion and faster cofactor regeneration kinetics at electrode surfaces.

**Introduction**

Immobilization of enzymes in polymer films has been used extensively in bioelectrode and biosensor applications (1-3). Compared to soluble enzyme systems, the use of immobilized enzymes reduces the amount of protein required and greatly increases the stability and lifetime of the system (4). Nafion®, a perfluorosulfonated ion-exchange polymer, has been widely used in these systems, as it provides a mechanically and chemically stable layer that can be easily cast onto an electrode surface. Further, modifying the Nafion® membrane by casting in the presence of quaternary ammonium salts has been shown to increase mass transport through the film (1, 5, 6). This also provides a more favorable pH environment for the immobilized enzymes, as the superacid character of the polymer is reduced through the exchange of protons on the sulfonic acid groups. While these modified Nafion® membranes provide improved mass transport compared to unmodified films, cofactor diffusion has still been suggested to be rate-limiting in these systems (7). In order to address this significant limitation, we have examined the use of minimal cofactor analogs to improve performance through an increased diffusion rate.
Typically, enzymes possess a high specificity for their natural cofactor, which allows them to perform a variety of chemistries in vivo. As such, wild-type enzymes generally exhibit very poor activity with non-native biomimetic cofactors, and there have been few reports of engineering enzymes for this novel activity. The most notable work in this area has been performed by Fish et al., who tested a series of N-benzylnicotinamide derivatives and β-nicotinamide-5′-ribose methyl phosphate for the stereospecific reduction of a variety of compounds by horse liver alcohol dehydrogenase, with concomitant cofactor regeneration catalyzed by [Cp*Rh(bpy)(H)]⁺ and formate (8). However, the observed activities were extremely low (≈28 d⁻¹), and the investigated cofactor analogues were sensitive to oxidation. A later set of experiments performed with bacterial Cytochrome P450s and 2-hydroxybiphenyl 3-monooxygenase (HbpA) yielded much higher activities, and a few previously reported cofactor specificity mutants were able to utilize the nicotinamide derivatives with better than wild-type activity (9, 10).

Previously, we engineered a thermostable NAD(H)-dependent alcohol dehydrogenase from Pyrococcus furiosus (AdhD) for broadened cofactor specificity and improved activity (11). The engineered enzyme contains two mutations to the cofactor binding pocket obtained by rational design. One, a histidine to arginine mutation (H255R), is positioned in a cleft distal to the active site where the adenine indol of the natural cofactor (and the 2′-phosphate of NADP(H)) binds, and has been shown to be important
in determining cofactor specificity (12). The other, a lysine to glycine mutation (K249G), is located in the bottom of the cofactor binding pocket along the pyrophosphate backbone of the cofactor (13, 14). Previous work suggests the elimination of this bulky lysine side chain increases the conformational flexibility of the cofactor in the binding pocket, and allows for an increased turnover rate and broadened specificity (12-15). Analysis of the two single AdhD mutants supports this hypothesis, with the H255R mutation increasing activity with NADP(H), while the K249G mutation improves activity with both cofactors (11).

We subsequently discovered that the engineered double mutant AdhD was able to utilize the minimal cofactor nicotinamide mononucleotide (NMN+) for catalysis. NMN+ represents the electroactive half of a natural nicotinamide cofactor, cleaved at the pyrophosphate backbone, and differs from the previously studied β-nicotinamide-5′-ribose methyl phosphate only by the lack of the methyl group (Figure 4.1a). Here we describe the effect of using this biomimetic cofactor in a biofuel cell as compared to the native cofactor for the enzyme, NAD+ (Figure 4.1b).
Materials and Methods

Chemicals: NAD$^+$, NMN$^+$, neutral red, Nafion® 1100EW suspension, and all salts were purchased from Aldrich and used as received. Nafion® modified with tetrabutylammonium bromide (modified Nafion®) was prepared according to a previous protocol (5). All other chemicals were from Sigma-Aldrich and used without modification.

Protein Expression and Purification: Expression and purification of wt AdhD, K249G AdhD, H255R AdhD, and the K249G/H255R AdhD double mutant followed a previously established protocol (11). Purity was assessed by SDS-PAGE and standardized activity assays, and purified enzyme was stored lyophilized. All enzyme concentrations were determined from $A_{280}$ measurements with a calculated molar extinction coefficient of $\varepsilon_{280} = 52495$ M$^{-1}$ cm$^{-1}$ and confirmed using the BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL).

Fluorescence Titrations: Dissociation constants for the enzyme-cofactor complexes were determined by fluorescence titration (16-18). Briefly, 2 μM enzyme in 50mM glycine (pH 8.8) was stirred in a 1 cm quartz cuvette placed in a J-815 spectrometer (Jasco Inc., Easton, MD) equipped with a Peltier junction temperature control. Samples were excited at 280 nm, and the fluorescence quenching upon cofactor binding was monitored at 330 nm. The total volume of cofactor added was less than 1% of the
reaction volume to limit dilution effects. Experiments were repeated in at least triplicate, and data were fit to a saturation adsorption isotherm.

**Steady-state Kinetics:** The full kinetic parameters for the K249G/H255R double mutant AdhD were determined with both NAD$^+$ and NMN$^+$ using 2,3-butanediol or D-arabinose as a substrate. Initial rates at 25°C were measured using a SpectraMax M2 plate reader by following the production of the reduced cofactor at 340 nm ($\varepsilon_{340} = 6.22$ mM$^{-1}$ cm$^{-1}$). Reactions contained 50 mM glycine (pH 8.8), 1-100 mM substrate, and the appropriate amount of enzyme and were initiated with 1-1000 µM NAD$^+$ or 50-2500 µM NMN$^+$. Data were collected in at least triplicate, and were fit simultaneously to the ordered bi-bi rate equation (Eq. 1) (19) using non-linear least-squares regression. This reaction mechanism was previously verified (20). Reported errors are standard deviations.

$$V = \frac{Eck_{cat}AB}{K_{ia}K_B + K_{BA} + K_{AB} + AB} \quad (1)$$

**Electrode Fabrication:** Glassy carbon rotating disc electrodes (5 mm diameter) were purchased from Pine Instruments and a Pine Rotator (Model AFM-SRX) was used for all rotating experiments. Electrochemical measurements were taken with a CH Instruments model 810 potentiostat interfaced with a PC. All voltammetric experiments were carried out using a platinum mesh counter electrode and a saturated calomel reference electrode (SCE). Neutral red was electropolymerized according to previously
published protocols (21, 22) as follows: Neutral red (11.5 mg) was dissolved into 100 mL of a pH 6 buffer solution consisting of 0.25 M phosphate and 0.1 M NaNO₃. The potential was swept at a scan rate of 50 mV/s between 0.8 V and -0.8 V (vs. SCE) for 6 complete cycles (12 scans). After electropolymerization, the electrodes were carefully washed with 18MΩ water to remove any residual monomer and dried under a gentle stream of nitrogen. Modified Nafion® (10 μL in of a 5% by wt suspension in 100% ethanol) was drop-cast on top of the poly(neutral red) (PNR)-modified GC electrodes and allowed to dry for 4-6 hours. These modified electrodes were then soaked in Tris buffer solutions (10 mM Tris-HCl, 10 mM KCl, pH 7) containing 10 mM of either NAD⁺ or NMN⁺ for 18 hours before use.

Biofuel cell anodes utilizing poly(methylene green) (PMG) as an electrocatalyst were prepared similarly to previously published procedures (1) as follows: Methylene green was polymerized onto 1 cm² pieces of Toray paper (TGP-060, E-Tek) by performing cyclic voltammetry (6 complete cycles, -0.3 V to 1.3 V) at a scan rate of 50 mV/s in a degassed solution containing 0.4 mM methylene green, 0.1 M sodium nitrate, and 10 mM sodium borate. The electrode was rinsed and then allowed to dry overnight. Enzyme/Nafion® casting solutions (50 μL of 5 wt% by wt. modified Nafion® in 100% ethanol combined with 150 μL of pH 7.4 phosphate buffer containing 1 mg/mL enzyme and 1 mg/mL NAD⁺ or 0.5 mg/mL NMN⁺) were pipetted in 50 μL aliquots onto each PMG-modified electrode and allowed to dry for 4-6 hours. The bioanodes were soaked
in a fuel solution (pH 8.0) consisting of 50 mM phosphate, 100 mM sodium nitrate, 50 mM arabinose, and 1 mM NAD$^+$ or NMN$^+$ overnight before use in the biofuel cell.

**Biofuel Cell Testing:** Bioanodes were tested in a biofuel cell apparatus that has been previously described (1). The cell consisted of two vertical glass chambers, separated by the cathode, which was coated with a Nafion® polymer electrolyte membrane. The upper glass chamber contained the fuel solution, and the bottom chamber was open to the air to allow O$_2$ to flow to the cathode. The cathode material was an ELAT electrode with 20% Pt on Vulcan XC-72 (E-Tek). The cathode was hot pressed to the backside of a Nafion® NRE 212 PEM with the catalyst side facing the membrane for 1 minute. The fuel solution was identical to the anode soaking solution, consisting of 50 mM sodium phosphate, 100 mM sodium nitrate, 50 mM arabinose, and 1 mM NAD$^+$ or NMN$^+$. Data was collected using a CH Instruments model 810 potentiostat. The reference and counter electrodes were connected to the bioanode, and the working electrode was connected to the cathode. The biofuel cell was allowed to reach a steady open circuit potential, after which a polarization curve was taken at a scan rate of 1 mV/s.

**Cyclic Voltammetry and Rotating Disc Voltammetry:** Cyclic voltammetry was carried out at various scan rates on each electrode in the pH 7 Tris buffer, using a potential window of 0.2 V to -0.8 V. Rotating disc voltammetry was carried out at various
rotation rates on each electrode at a scan rate of 10 mV/s. Electrodes were rotated for 10 minutes at each rotation rate before the voltammetry was performed.

**Results**

We hypothesize that the increased volume of the cofactor binding pocket of AdhD afforded by the K249G mutation allows the truncated cofactor to adopt a conformation relative to the substrate that favors catalysis. This is supported by the observation that both the wild-type enzyme and the H255R single mutant possess very low activity with NMN⁺, while the K249G single mutant exhibits over an order of magnitude increase in activity (Table 4.4). Surprisingly, the K249G/H255R double mutant exhibits a further 2-fold increase in activity with NMN⁺, and a higher affinity for the truncated cofactor. However, the activity of the double mutant with NMN⁺ is still one to two orders of magnitude lower than the wild-type enzyme with its natural cofactor.

The affinity of the various AdhD enzymes for the minimal cofactor NMN⁺ is clearly lower than for the natural cofactors (Table 4.4). Nicotinamide-dependent enzymes typically have a high specificity for either NAD(H) or NADP(H), with NAD(H)-dependent enzymes typically acting as oxidases while NADP(H)-dependent enzymes act as reductases (23). Thus the high specificity allows different enzymes to perform both reduction and oxidation reactions simultaneously. As NMN⁺ lacks the specificity
determining half of the molecule, cofactor affinity is significantly decreased. This is observed both in the cofactor dissociation constants measured by fluorescence titrations and the kinetic parameters (K_{Sa} and K_{A}, Table 4.1) from steady-state kinetics. The reduced affinity is unlikely to have a large impact in immobilized applications, however, due to relatively high enzyme loadings and an increased local concentration of cofactor and substrate in the polymeric films.

As the model substrate for AdhD (2,3-butanediol) was not compatible with the Nafion/PMG anode, an alternate substrate (D-arabinose) that displays rapid kinetics with the enzyme was used in the biofuel cell. A full kinetic analysis of the engineered double mutant AdhD enzyme was performed using both substrates and both the native (NAD\(^+\)) and truncated (NMN\(^+\)) cofactors (Table 4.1). In dilute solution, the enzyme exhibits a k_{cat} two to three orders of magnitude greater with NAD\(^+\) than NMN\(^+\) (15 s\(^{-1}\) with NAD\(^+\) vs. 0.018 s\(^{-1}\) with NMN\(^+\) for 2,3-butanediol; 65 s\(^{-1}\) with NAD\(^+\) vs. 0.55 s\(^{-1}\) with NMN\(^+\) for D-arabinose). The impact of the cofactor on the Michaelis constants varies unexpectedly with the substrate, however. When 2,3-butanediol is the substrate, the Michaelis constants for both the cofactor and substrate decrease (K_{A} from 460\(\mu\)M with NAD\(^+\) to 140\(\mu\)M with NMN\(^+\), and K_{B} from 690mM with NAD\(^+\) to 17mM with NMN\(^+\)), whereas the Michaelis constants increase when D-arabinose is the substrate (K_{A} from 480\(\mu\)M with NAD\(^+\) to 1100\(\mu\)M with NMN\(^+\), and K_{B} from 72mM with NAD\(^+\) to 130mM with NMN\(^+\)). Thus, enzyme performance is expected to be much higher with NAD\(^+\) as a
cofactor, owing both to the two order of magnitude increase in $k_{\text{cat}}$ and the significant decreases in the Michaelis constants.

The diffusion coefficients of $\text{NAD}^+$ and $\text{NMN}^+$ through modified Nafion® and their extraction coefficients into the polymer films were determined by cyclic voltammetry (CV) and rotating disc voltammetry (RDV). The biofuel cells described later in this study utilize poly(methyelene green) (MG) as an electrocatalyst to oxidize the NADH or NMNH produced by the enzymes during operation. However, MG cannot catalyze the reverse reaction to reduce $\text{NAD}^+$, so a different electrocatalyst was needed to measure the transport properties of $\text{NAD}^+$ and $\text{NMN}^+$ through the films. Poly(neutral red) (NR) has been shown to be an effective electrocatalyst for the two-electron reduction of $\text{NAD}^+$ (21, 22), and was used in this study to determine the rate at which $\text{NAD}^+$ and $\text{NMN}^+$ diffused through the film to the electrode surface. The diffusion coefficients and extraction coefficients of $\text{NAD}^+$ and $\text{NMN}^+$ are shown in Table 4.2, as determined by Saveant (24) analysis of the RDE data (Figure 4.4) as well as analysis (25) of the variable scan rate CV experiments (Figure 4.2). As seen in the table, $\text{NMN}^+$ diffuses through modified Nafion® faster than $\text{NAD}^+$ by an order of magnitude. This is likely due to the smaller size of $\text{NMN}^+$, relative to $\text{NAD}^+$ (Figure 4.1a,b). $\text{NAD}^+$ was shown to have a higher extraction coefficient into the modified Nafion® films. This may be due to the more hydrophobic nature of $\text{NAD}^+$ relative to $\text{NMN}^+$, which results from the aromatic
adenine group attached to NAD⁺. Overall, the flux of NMN⁺ through modified Nafion® is higher than NAD⁺, as expected for a smaller molecule.

A schematic of the bioanode is shown in Figure 4.1c. Methylene green (MG) is polymerized onto the carbon paper electrode to act as a mediator for cofactor oxidation. The MG lowers the overpotential required to oxidize the reduced cofactor by ~500 mV and produces a greater anodic current as compared to an unmodified electrode (26, 27).

Even though the double mutant AdhD enzyme had a much lower turnover rate with NMN⁺ in dilute solution, biofuel cells using NMN(H) as a cofactor performed similarly to ones using NAD(H) (Figure 4.3, Table 4.3). The open circuit potential for NAD(H) biofuel cells was higher than for NMN(H) fuel cells (0.642 vs 0.593 V), while the maximum power densities of the biofuel cells using each cofactor were not statistically different (1.52 ± 0.27 vs 1.37 ± 0.24 µW/cm², respectively). Interestingly, the use of NMN(H) resulted in a 40% increase in maximum current density, which suggests an improvement in mass transfer for the truncated cofactor.

Discussion

Although non-natural biomimetic cofactors may possess superior properties for some applications, their use is hindered by the fact that they are generally poor substrates for wild type enzymes. There currently exists no framework or general rules for the
engineering of enzymes to use non-natural cofactors, but the changing of cofactor specificity between the two natural cofactors NAD(H) and NADP(H) has been extensively researched over the past two decades (28, 29). Interestingly, the same mutations that have been identified in some enzymes to broaden or reverse cofactor specificity seem to improve activity with non-natural cofactors. This effect has been observed both in the Cytochrome P450 studied by Ryan et al. and the alcohol dehydrogenase (AdhD) examined in this study. These wild-type enzymes show little to no activity with the non-natural cofactors, while some of the cofactor specificity mutants are able to use these truncated cofactors with nearly wild-type levels of activity. The high specificity of enzymes for their cofactors has evolved so that enzymes catalyzing oxidation reactions do not need to be separated from enzymes catalyzing reduction reactions. It is therefore not unexpected that relaxing this specificity allows the enzymes to become more permissive in accepting non-natural biomimetic cofactors, which depending on the rate-limiting step of the reaction, can lead to high levels of activity.

Analysis of the polarization curves obtained with each cofactor provides insight into the processes affecting biofuel cell performance. The open circuit potential (OCP) of the fuel cell (the y-intercept of the polarization curve), is the cell potential at infinite resistance (open circuit), and is dependent on the formal potential of the cofactor at the poly(methylene green) electrocatalyst as well as the rate of accumulation of the reduced
cofactor at the electrode surface. The formal potential differences combined with the increased turnover rate of the enzyme with the natural cofactor leads to a higher OCP in the NAD$^+$ fuel cell. Conversely the limiting current, measured under “short circuit” conditions, is predominantly a function of mass transfer within the system. Here, the order of magnitude increase in the diffusion coefficient of NMN$^+$ compared to NAD$^+$ results in a greater than 40% increase in the limiting current. As current is proportional to the number of electrons transferred to the electrode, it follows that the faster diffusing NMN(H) can shuttle more electrons between the enzyme active site and electrode surface per time than NAD(H). Lastly, the maximum power density is dependent on both the kinetic rates of cofactor reduction by the enzyme and cofactor oxidation by MG on the electrode surface, as well as mass transfer effects and ohmic losses within the system. Surprisingly, the maximum power densities observed with NAD(H) and NMN(H) were not statistically different. This suggests that the rate of turnover by the enzyme is not limiting in this system; rather performance is dominated by mass transfer effects or by the rate of oxidation of the reduced cofactors by MG.

The use of poly(neutral red) as an electroreduction catalyst to measure the diffusion coefficients of the oxidized cofactors through modified-Nafion® may also provide insight into the relative rates of oxidation of the reduced cofactors at MG. The rate of NMN$^+$ reduction at the PNR modified electrode was found to be much faster than for NAD$^+$ (Figure 4.2), as shown by the decreased ΔEp from 354 mV for NAD$^+$ to 229 mV.
for NMN⁺. Given the structural similarities between MG and PNR, it is possible the rate of NMNH oxidation by MG may be comparatively higher than that of NADH. While the reasons for this are not clear, the presence of the adenine moiety in NAD(H) may sterically hinder the nicotinamide group from reaching the surface, and may also cause the cofactor to adsorb onto the electrode surface through interactions with MG. As this half of the cofactor is absent in NMN(H), the nicotinamide group may be able to more freely interact with the MG, promoting charge transfer.

The present work opens new avenues of research involving electron relay systems, and will have important applications in many biocatalysis applications. We have demonstrated that the use of the truncated cofactor NMN(H) resulted in similar power densities and increased current densities compared to NAD(H), and that the rates of cofactor diffusion and cofactor oxidation at the electrode surface are much more important than the rate of enzyme turnover. The critical bottleneck in using alternative cofactors is the lack of enzymes engineered for altered or broadened cofactor specificity. In addition, further improvements to the biomimetic cofactors are also likely to improve system performance including stability, redox potential, turnover at the electrode surface, and cost.
### Table 4.1 – K249G/H255R AdhD Kinetic Parameters

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>( k_{cat} ) (s(^{-1}))</th>
<th>( K_i ) (µM)</th>
<th>( K_A ) (µM)</th>
<th>( K_B ) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>wt AdhD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3-butanediol</td>
<td>NAD(^+)</td>
<td>1.0 ± 0.1</td>
<td>37 ±2</td>
<td>63 ±2</td>
</tr>
<tr>
<td></td>
<td>NMN(^+)</td>
<td>&lt;0.0005</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>K249G/H255R AdhD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3-butanediol</td>
<td>NAD(^+)</td>
<td>15 ± 2</td>
<td>11 ± 1</td>
<td>460 ± 60</td>
</tr>
<tr>
<td></td>
<td>NMN(^+)</td>
<td>0.018 ± 0.002</td>
<td>880 ± 10</td>
<td>140 ± 20</td>
</tr>
<tr>
<td>D-arabinose</td>
<td>NAD(^+)</td>
<td>65 ± 1</td>
<td>78 ± 3</td>
<td>480 ± 10</td>
</tr>
<tr>
<td></td>
<td>NMN(^+)</td>
<td>0.55 ± 0.03</td>
<td>1700 ± 100</td>
<td>1100 ± 100</td>
</tr>
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</table>
### Table 4.2 – Properties of Nicotinamide Cofactors in Nafion® Films

<table>
<thead>
<tr>
<th></th>
<th>Diffusion Coefficient (cm²/s)</th>
<th>Extraction coefficient</th>
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</thead>
<tbody>
<tr>
<td>NAD⁺</td>
<td>5.45 ± 0.37 x 10⁻⁹</td>
<td>1.15 ± 0.09</td>
</tr>
<tr>
<td>NMN⁺</td>
<td>4.32 ± 0.43 x 10⁻⁸</td>
<td>0.44 ± 0.05</td>
</tr>
</tbody>
</table>

### Table 4.3 – Summary of Biofuel Cell Performance

<table>
<thead>
<tr>
<th></th>
<th>Open Circuit Potential (V)</th>
<th>Max Power Density (Watt/cm²)</th>
<th>Max Current Density (A/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD⁺</td>
<td>0.642 ± 0.011</td>
<td>1.52 ± 0.27 x 10⁻⁶</td>
<td>1.61 ± 0.36 x 10⁻³</td>
</tr>
<tr>
<td>NMN⁺</td>
<td>0.593 ± 0.069</td>
<td>1.37 ± 0.24 x 10⁻⁶</td>
<td>2.28 ± 0.26 x 10⁻³</td>
</tr>
</tbody>
</table>
Figure 4.1 (a) Structure of the biomimetic cofactor NMN$^+$ and (b) natural cofactor NAD$^+$. (c) Schematic of the bioanode. Methylene green mediator is polymerized on carbon paper electrode. Enzyme and cofactor are immobilized in TBAB-modified Nafion® on electrode surface. A commercially available air-breathing platinum cathode completes the fuel cell.
Figure 4.2 Representative cyclic voltammograms of NAD$^+$ (A) and NMN$^+$ (B) at a PNR-modified GC electrode coated with modified Nafion$^\circledR$ at a variety of scan rates. Conditions: Quiescent solution, room temperature, 10 mM Tris-HCl, 10 mM KCl, 10 mM NAD$^+$/NMN$^+$, pH 7.0. Insets: Plot showing linear relationship between current and the square root of the scan rate.
Figure 4.3  Representative polarization curves of biofuel cells using either NAD$^+$ or NMN$^+$ as cofactors. Conditions: Quiescent solution, room temperature, 100 mM sodium phosphate, 100 mM NaNO$_3$, 50 mM arabinose, pH 8.0.
Supplemental Information

Table 4.4 – Apparent Kinetic Parameters of wt AdhD and Mutants

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_M$ (µM)</th>
<th>$K_D$ (µM)</th>
<th>$k_{cat}/K_M$ ($\times 10^5$ µM$^{-1}$ min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt AdhD</td>
<td>0.03</td>
<td>2500</td>
<td>875</td>
<td>1.2</td>
</tr>
<tr>
<td>H255R</td>
<td>0.07</td>
<td>2100</td>
<td>ND</td>
<td>3.3</td>
</tr>
<tr>
<td>K249G</td>
<td>0.8</td>
<td>3800</td>
<td>ND</td>
<td>21</td>
</tr>
<tr>
<td>K249G/H255R</td>
<td>1.6</td>
<td>2600</td>
<td>826</td>
<td>62</td>
</tr>
<tr>
<td>wt AdhD</td>
<td>85</td>
<td>60</td>
<td>59</td>
<td>140000</td>
</tr>
<tr>
<td>K249G/H255R</td>
<td>180</td>
<td>460</td>
<td>45</td>
<td>40000</td>
</tr>
</tbody>
</table>

Reaction conditions: 50mM glycine, 100mM 2,3-butanediol (pH 8.8), 45°C. Reactions were initiated by the addition of NAD$^+$ (1 - 1000 µM) or NMN$^+$ (5 – 3000 µM), and monitored at 340nm. Each experiment was run in triplicate, and the data was fit to a simplified rate equation (Eq. S1). $K_D$ values were obtained by fluorescence titration, as previously described.

$\nu = \frac{k_{cat}^{app} E_t A}{k_{cat}^{app} A}$  \hspace{1cm} Eq. S1
Figure 4.4 Representative rotating disc voltammograms of NAD$^+$ (A) and NMN$^+$ (B) at a PNR-modified GC electrode coated with modified Nafion® at a variety of rotation rates. Conditions: Quiescent solution, room temperature, 10 mM Tris-HCl, 10 mM KCl, 10 mM NAD$^+$/NMN$^+$, pH 7.0. Insets: Koutechy-Levich plots showing a linear relationship between inverse current and inverse square root of rotation rate.
References:


Chapter 5

MODULAR EXCHANGE OF SUBSTRATE BINDING LOOPS ALTERS BOTH SUBSTRATE AND COFACTOR SPECIFICITY IN A MEMBER OF THE ALDO-KETO REDUCTASE SUPERFAMILY

Abstract: Substrate specificity in the aldo-keto reductase (AKR) superfamily is determined by three mobile loops positioned at the top of the canonical (α/β)_8-barrel structure. These loops have previously been demonstrated to be modular in a well studied class of AKRs, in that exchanging loops between two similar hydroxysteroid dehydrogenases resulted in a complete alteration of substrate specificity (1). Here, we further examine the modularity of these loops by grafting those from human aldose reductase (hAR) into the hyperthermostable AKR, AdhD, from *Pyrococcus furiosus*. Replacement of Loops A and B were sufficient to impart hAR activity into AdhD, and the resulting chimera retained the thermostability of the parent enzyme. However, no active chimeras were observed when the hAR loops were grafted into a previously engineered cofactor specificity mutant of AdhD, which displayed similar kinetics to hAR with the model substrate DL-glyceraldehyde. The non-additivity of these mutations suggests that efficient turnover is more dependent on the relative positioning.

A version of this chapter has been submitted to *Biochemistry* with co-authors Sara Chuang, and Scott Banta. EC designed the experiments, performed the experiments, analyzed data, and wrote the manuscript.
of the cofactor and substrate in the active site than on binding of the individual species. The ability to impart the substrate specificities of a variety of mesostable AKRs into a thermostable scaffold will be useful in a variety of applications including immobilized enzyme systems for biofuel cells and fine chemical synthesis.

**Introduction**

Aldo-keto reductases (AKRs) comprise a large, diverse family of oxidoreductase enzymes and are found in nearly every species (2, 3). They share a common (α/β)8-barrel structure and catalytic mechanism, but some members of the superfamily share less than 30% sequence homology. These enzymes bind a nicotinamide cofactor in an extended conformation along a cleft that runs through the C-terminal face of the barrel, in contrast to the Rossman-fold motif common in other dehydrogenases. Three mobile loops on the same face form the substrate binding pocket. The physiological role of many of these enzymes is unknown, but they are generally thought to fall into one of three classes. The most studied members of this family are mammalian AKRs involved in steroid and prostaglandin metabolism (4-6). These enzymes often have long substrate binding loops and are highly specific for their substrates. Another well studied class of AKRs, the aldose reductases, are involved in the interconversion of glucose to sorbitol and have been investigated as drug targets to prevent complications from diabetes (7,
A third class of AKRs, which have been identified in a wide range of species, have no known function (9). The substrate binding loops in these enzymes are often truncated, imparting them with fairly broad substrate specificity. Additionally, they have been shown to be upregulated in response to stress, leading to the hypothesis that their physiological role is of general detoxification, metabolizing various aldehydes and ketones to less toxic species (10).

The advent of high-throughput sequencing has allowed the complete genomes of several species to be elucidated, and the sequence data has yielded several putative members of the aldo-keto reductase superfamily (11, 12). One of these enzymes, AdhD, was identified in the hyperthermophilic archaea *Pyrococcus furiosus*, and has been characterized by our group and others. The enzyme has a strong preference for NAD(H) as a cofactor, and oxidizes a range of sugars and alcohols (12, 13). The substrate binding loops in this enzyme are significantly truncated compared to AKRs identified from other organisms, with the C-terminal loop (Loop C), completely absent. This likely contributes to the broad substrate specificity and extreme thermostability of the enzyme.

The importance of the mobile loops in substrate binding and specificity was elegantly demonstrated through the creation of several chimeric hydroxysteroid dehydrogenases (HSD) where the substrate binding loops from a 20α-HSD were grafted into a 3α-HSD
enzyme scaffold (1). Replacement of only Loop A resulted in an enzyme with novel 17β-HSD activity, while swapping all three substrate binding loops resulted in a complete alteration of substrate specificity, with an increase in catalytic efficiency for the 20α-HSD reaction of $10^{11}$ compared to the wild-type 3α-HSD enzyme.

Based on this impressive work, we decided to investigate a similar strategy to rationally alter the substrate specificity of AdhD. In an attempt to improve the activity of AdhD with sugars, we created several loop chimeras inserting the substrate binding loops from human aldose reductase, which has activity with glucose (7, 14-17). These loop chimeras are also compared to and combined with a cofactor specificity double mutant of AdhD (K249G/H255R) that exhibits broadened cofactor specificity and improved activity compared to the wild-type enzyme (13). A summary of loop chimera constructs appears in Table 5.1.

Whereas the previous work exchanged substrate binding loops between similar hydroxysteroid dehydrogenases, the present work investigates exchanging loops between two distinct AKRs which share less than 30% sequence homology. While AdhD is an extremely thermostable archaeal enzyme with broad substrate specificity, human aldose reductase (hAR) is a mesostable mammalian AKR with a specialized function. Also, AdhD has a strong preference for NAD(H) as a cofactor, while hAR has a strong preference for NADP(H). Thus, in addition to the change in substrate
specificity expected due to changing the substrate binding loops, it will also be interesting to observe the effects on cofactor specificity and thermostability.

**Materials and Methods**

In order to design the AdhD/hAR loop chimeras, sequence and structural alignments were performed. Sequence alignments of AdhD (GenBank 1469842) and hAR (GenBank AAA51713) were performed using the CLUSTALW tool, and structural alignments of AdhD and hAR (PDB 2ACQ) were performed with Yasara. DNA oligos corresponding to the hAR substrate binding loops were obtained from IDT DNA, Inc. (Coralville, IA) and assembled into the AdhD gene using overlap extension PCR (see SI). PCR fragments were doubly digested with NcoI and HindIII and cloned into a similarly digested pET-24d vector. All constructs were verified by DNA sequencing.

AdhD/hAR Loop chimeras were initially expressed in 50ml cultures and purified by heating of the cell extracts, as described previously (13). Two constructs, A (wt AdhD with Loop A) and D (DM AdhD with Loops A + B), were not well expressed despite numerous attempts to optimize the expression and purification of these samples. Thus they were not investigated further. Relatively pure protein (estimated > 90% pure by SDS-PAGE) was obtained in the heat-stable extract, and was used without further purification for initial studies.
Large scale expression and purification of wild-type AdhD and mutants followed a previously described protocol (13). Typical yields were on the order of 300 - 1200 mg L\(^{-1}\) of culture, and samples were estimated to be >98% pure by SDS-PAGE.

The hAR gene was amplified from human placenta QUICK-clone cDNA (Clontech, Mountain View, CA) using forward primer 5’-GGTCTGGGGAGCGCAGCAGC-3’ and reverse primer 5’-TTCGAAGCTTTCAAAACTCTTCATGGAAGGGGTAAATCCTT-3’. The reverse primer inserted a unique HindIII restriction site (underlined). The purified PCR fragment was doubly digested with NcoI and HindIII and ligated into a similarly digested pET-24d vector containing an N-terminal RGSHis tag for purification. Ligated plasmids were transformed into electrocompetent BLR E. coli (Novagen, Gibbstown, NJ) and plated on LB-Kan selection plates. Individual colonies were picked and grown overnight in LB medium supplemented with 50 μg ml\(^{-1}\) kanamycin and stored as glycerol stocks. Proper insertion of the hAR gene was verified by DNA sequencing.

Expression and purification of hAR followed a different protocol, as the enzyme is not highly thermostable. One liter expression cultures of Terrific Broth containing 50 μg ml\(^{-1}\) kanamycin were inoculated from an overnight culture, and expression was induced at \(\text{OD}_{600} \approx 0.6\) by the addition of IPTG to 0.2mM. Expression continued for 16h at 37°C with agitation. Cells were harvested by centrifugation, and resuspended in 1/10\(^{th}\) volume Binding Buffer (20mM Tris-HCl, 150mM NaCl, 40mM imidazole, pH 7.5) supplemented
with 1x HALT Protease Inhibitor (Fisher Scientific). Cells were lysed by sonication on ice for a total of 8 minutes, following cycles of 5 seconds on and 5 seconds off. Cell debris was removed by centrifugation for 20 mins at 10000g. Samples were then loaded onto a HisTrap column (GE Healthcare, Piscataway, NJ) equilibrated in Binding Buffer. After rinsing with 10 column volumes of Binding Buffer, His-tagged hAR was eluted with a gradient of 0-100% Elution Buffer (20mM Tris-HCl, 150mM NaCl, 500mM imidazole, pH 7.5) over 20 column volumes. hAR eluted in a single peak at an imidazole concentration of ~150mM. Fractions containing hAR were pooled and concentrated over a 30kDa centrifugal filter and applied to a Superdex 16/200 gel filtration column (GE Healthcare, Piscataway, NJ) equilibrated in 20mM Tris-HCl (pH 7.5) containing 150mM NaCl. Fractions containing enzyme were pooled and concentrated over a 30kDa filter, before being diluted to the desired working concentration in 20mM Tris-HCl (pH 7.5). Typical yields were on the order of 30 mg L⁻¹ of culture, and samples were estimated to be >98% pure by SDS-PAGE.

All loop mutants were initially screened in a 96-well plate assay. To test for activity in the oxidation reaction, 10μl of partially purified enzyme was added to 290μl 50mM glycine (pH 8.8) containing 1mM NAD⁺ or NADP⁺ and 10mM of the indicated substrate in a 96-well UV-transparent microplate. For the reduction reaction, 10μl of partially purified enzyme was added to 290μl 100mM sodium phosphate (pH 6.1) containing 500μM NADH or NADPH and 10mM of the indicated substrate. Plates were incubated
at 37°C and imaged under UV light at various time points to monitor the production or depletion of reduced cofactor.

Full kinetic assays were performed on the active enzymes identified by the plate assay. For the oxidation reaction, 10μl of the appropriate concentration of purified enzyme was added to 290μl 50mM glycine (pH 8.8) containing 5-2000μM NAD+ or NADP+ and 1-100mM of the indicated substrate. For the reduction reaction, 10μl of the appropriate concentration of purified enzyme was added to 290μl 100mM sodium phosphate (pH 6.1) containing 1-500μM NADH or NADPH and 1-100mM of the indicated substrate. Plates were incubated (at 25°C for the reduction reaction, 37°C for the oxidation reaction) in a SpectraMax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA) and the absorbance at 340nm was followed to monitor the production or depletion of NAD(P)H (ε340nm = 6220 M⁻¹ cm⁻¹). Experiments were performed in at least triplicate. Kinetic data were fit to the ordered bi-bi rate equation using a non-linear regression program (Igor Pro, Wavemetrics, Inc.).

Cofactor dissociation constants were measured by fluorescence titration, following a previously described protocol (18-20). Proper folding of the loop chimeras and determination of thermal stability were investigated by CD spectroscopy as described previously (21).
Results

A sequence and structural alignment of hAR (PDB ID 2ACQ) with a previously generated homology model of AdhD (13) guided the insertion of the hAR binding loops into AdhD (Figure 5.1). The structural alignment also identified a short loop in AdhD (corresponding to residues 182-184) not present in hAR, which could potentially sterically interfere with Loop C and prevent it from properly folding over the top of the barrel. Thus additional mutants were generated with this short loop removed (denoted Δ182-184) to increase the likelihood of Loop C adopting its native conformation.

Loops were inserted at the genetic level through a series of oligonucleotide primers, which were used to PCR fragments of the gene containing the desired loops (see SI). These fragments were then reassembled into a full length gene using overlap-extension PCR, and cloned into a vector for expression.

A concern when grafting in the large substrate binding loops from hAR was a decrease in the thermostability of the AdhD scaffold. Thus mutants were characterized by CD spectroscopy and thermal denaturation experiments. Surprisingly, the impact on enzyme stability was minimal, as no change in CD signal was observed from 25°C to 90°C (data not shown).

Loop mutants were initially screened in a plate assay. Mutants were tested for their ability to reduce DL-glyceraldehyde with NADPH, the model substrates for hAR, and
in the oxidation and reduction of 2,3-butanediol and 3-hydroxy-2-butanone respectively, the model substrates for AdhD, using both NAD(H) and NADP(H) cofactors. Plates were illuminated by UV to visualize the reduced cofactor and photographed at regular intervals. A representative image of the plate after 45 minutes of incubation is shown in Figure 5.2. At this time point, only hAR showed appreciable activity with DL-glyceraldehyde, and clearly had a preference for NADPH over NADH. The AdhD double mutant showed the highest activity with 2,3-butanediol and 3-hydroxy-2-butanone, with little difference apparent between the NAD(H) and NADP(H) cofactors. The activity of wt AdhD was lower with these substrates, and a marked preference for NAD$^+$ was observed with 2,3-butanediol. hAR also demonstrated activity with both 2,3-butanediol and 3-hydroxy-2-butanone, and had a slight preference for its preferred cofactor NADP(H) with these model AdhD substrates. Interestingly, wt Loops A+B and wt Loops A-C also retained significant activity with these substrates, but only when NADP(H) was the cofactor. At longer time points, NADPH/DL-glyceraldehyde activity was also observed in these loop chimeras.

Enzymes that were identified as active in the plate assay were grown in large scale expression cultures and purified to homogeneity as described. A full kinetic analysis was performed with these samples to allow for fitting to the ordered bi-bi rate equation. Parameters are summarized in Table 5.2. While the wt AdhD exhibited very little activity with NADPH and DL-glyceraldehyde, the cofactor specificity double mutant
had a turnover rate faster than hAR. This was offset by significant increases in the
dissociation constant and Michaelis constant for NADPH, however, leading to a lower
catalytic efficiency. The two loop chimeras identified as active in the plate assay
demonstrated reasonable kinetics with NADPH and DL-glyceraldehyde, but again the
Michaelis constants were significantly larger than those for hAR or the DM AdhD. With
the model AdhD substrate 2,3-butanediol, an interesting effect is observed with the loop
chimeras. Both constructs C and E retained activity with this substrate, but had a strict
requirement for NADP$^+$ as a cofactor, in contrast to the NAD$^+$ preference exhibited by
wt AdhD. hAR was also found to have high catalytic rates with this substrate with both
NAD$^+$ and NADP$^+$, however the catalytic efficiency was much higher with its preferred
cofactor NADP$^+$.

The combination of relatively low turnover numbers and high Michaelis constants
impeded the accurate determination of the full kinetic parameters for the two loop
chimeras. In order to enable fitting to the ordered bi-bi rate equation, the $K_{ia}$ term was
set equal to the dissociation constant measured by fluorescence titration (22). As proper
saturating conditions were not achieved with these mutants, the resulting kinetic
parameters are given as apparent parameters.
Discussion

The modular nature of the aldo-keto reductase substrate binding loops has been confirmed in this work, as the AdhD scaffold was successfully imparted with hAR activity through a loop grafting approach. Whereas a complete reversal of substrate specificity was previously shown to require the exchange of all three substrate binding loops, here it appears that only two loops are necessary for activity. Additionally, the chimeric mutants studied here maintained the high thermostability of the parent enzyme, suggesting that this technique can be used to rapidly stabilize other mesophilic AKRs.

It is difficult to directly compare the catalytic efficiencies of the various enzyme constructs due to the large difference in Michaelis constants between enzymes. Thus, activities were examined under saturating conditions by looking at the turnover rate, $k_{\text{cat}}$. The catalytic rate of wt AdhD is much lower than that of hAR with NADP(H), both in the oxidation of the model AdhD substrate 2,3-butanediol and the reduction of the model hAR substrate DL-glycerladehyde. The active loop chimeras fall in between, with the wt Loops A+B construct having similar activities with both substrates while the wt Loops A-C construct has a much faster turnover rate with 2,3-butanediol. Interestingly, the DM AdhD has a much higher turnover rate with DL-glyceraldehyde than even
hAR, but is comparable to the wt Loops A+B construct with 2,3-butanediol (Figure 5.3A).

The steady-state kinetic parameters can also be used to calculate changes in the cofactor binding energies relative to the wt AdhD enzyme (23, 24). In the ground state, the loop chimeras destabilized the binding of NADP⁺, while hAR and the previously engineered DM AdhD had more favorable binding energies. All constructs except wt Loops A-C also demonstrated a decreased free energy of binding with NADPH relative to the wt AdhD, which partially explains the improvement in activity observed in these constructs (Figure 5.3B). The effect of the loop chimeras is most apparent when comparing the transition-state binding energies. Here, both wt Loops A+B and wt Loops A-C have a significantly lower transition-state binding energy with DL-glyceraldehyde/NADPH compared to the wt AdhD, while those with 2,3-butanediol/NADP⁺ remain relatively unaffected. (Figure 5.3C)

In contrast to the previous work, both active loop chimeras (constructs C and E) retained activity with their native substrate, but with a strict requirement for the less-preferred cofactor NADP⁺. As hAR has been shown to prefer NADP(H), this suggests the substrate binding loops can also impact cofactor specificity. In fact, Loop B of some AKRs has been demonstrated to take part in cofactor binding through electrostatic interactions. The crystal structure of hAR indicates that residue Asp216 on Loop B
forms a salt-bridge with Lys262 to form the canonical AKR “seat-belt” over the pyrophosphate backbone of the cofactor, thereby locking it into the binding pocket. This motif is likely absent in the wt AdhD, as Loop B is significantly truncated and lacks the charged residue required to form an electrostatic interaction. Grafting Loop B from hAR into AdhD may therefore reconstitute the “seat-belt”, and promote binding and proper orientation of the cofactor in the binding pocket. Interestingly, the wt Loops A+B construct has the second lowest dissociation constant for NADPH, behind only hAR.

Given the success in grafting the hAR loops into the AdhD scaffold, it is interesting that the same loops grafted into the double mutant AdhD scaffold, which itself possesses hAR-like activity, results in an inactive enzyme. As the wt AdhD requires at least Loop B of hAR for activity with DL-glyceraldehyde, this supports the hypothesis that cofactor binding and orientation provided by the “seat-belt” mechanism is important for catalysis. One of the mutations in the double mutant, K249G, removes the lysine residue that interacts with the arginine of Loop B. Thus the formation of a “seat-belt” in the DM AdhD loop constructs is unlikely, and if this has a detrimental effect on cofactor binding, could explain the lack of activity observed in these chimeras.
### Tables and Figures

**Table 5.1 – AdhD / hAR Loop Chimera Constructs**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Enzyme / Loops</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>wt Loop A</td>
</tr>
<tr>
<td>B</td>
<td>DM Loop A</td>
</tr>
<tr>
<td>C</td>
<td>wt Loops A+B</td>
</tr>
<tr>
<td>D</td>
<td>DM Loops A+B</td>
</tr>
<tr>
<td>E</td>
<td>wt Loops A–C</td>
</tr>
<tr>
<td>F</td>
<td>DM Loops A–C</td>
</tr>
<tr>
<td>G</td>
<td>wt Loops A–C + Δ182–184</td>
</tr>
<tr>
<td>H</td>
<td>DM Loops A–C + Δ182–184</td>
</tr>
<tr>
<td>Enzyme</td>
<td>Cofactor</td>
</tr>
<tr>
<td>------------</td>
<td>----------</td>
</tr>
<tr>
<td>wt AdhD</td>
<td>NADPH</td>
</tr>
<tr>
<td>DM AdhD</td>
<td>NADPH</td>
</tr>
<tr>
<td>wt Loops A+B</td>
<td>NADPH</td>
</tr>
<tr>
<td>wt Loops A-C</td>
<td>NADPH</td>
</tr>
<tr>
<td>hAR</td>
<td>NADPH</td>
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<tr>
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<td>NAD$^+$</td>
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<tr>
<td>wt AdhD</td>
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<td>DM AdhD</td>
<td>NAD$^+$</td>
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<tr>
<td>DM AdhD</td>
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<td>wt Loops A+B</td>
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<tr>
<td>hAR</td>
<td>NADP$^+$</td>
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</table>

*Apparent parameters, $^a$Measured by fluorescence titration.
Figure 5.1 (A) Homology model of AdhD and (B) crystal structure of hAR (PDB ID 2ACQ) with substrate binding loops indicated. (C) Sequence alignment of hAR and AdhD showing the location of the substrate binding loops and their absence in AdhD.
Figure 5.2 Plate Assay of hAR Loop Chimeras

Partially purified enzyme samples were incubated with 10mM of the indicated substrate, and 500μM cofactor (NAD(P)H) in 100mM sodium phosphate, pH 6.1 (reduction reactions) or 500μM cofactor (NAD(P)⁺) in 50mM glycine, pH 8.8 (oxidation reaction) for 45 minutes at 37°C. The reduced cofactor (NAD(P)H) fluoresces under UV light.
Figure 5.3 Effect of Loop Insertions on Activity and Binding Energy.

(A) Comparison of turnover rate for AdhD, hAR, and active loop chimeras with DL-glyceraldehyde/NADPH and 2,3-butanediol/NADP⁺ as substrates. The value of $k_{cat}$ was obtained by fits of kinetic data to the ordered bi-bi rate equation. (B) Change in the ground-state cofactor binding energy$^a$ of constructs with NADPH and NADP⁺ relative to wt AdhD. (C) Change in the transition-state binding energies$^b$ of constructs with DL-glyceraldehyde/NADPH and 2,3-butanediol/NADP⁺ relative to wt AdhD.

\[ \Delta \Delta G_a = -RT \ln \left( \frac{K_{ia_{construct}}}{K_{ia_{wt AdhD}}} \right) \]
\[ \Delta \Delta G_b = RT \ln \left( \frac{k_{cat/K_A_{construct}}}{k_{cat/K_A_{wt AdhD}}} \right) \]
Supplemental Information

Materials & Methods

Overlap Extension PCR Primers
NcoI forward
5’- CGACTCACTATAGGGGAATTGTGAGC -3’

HindIII reverse
5’- ATCTCAGTGTTGGGTGGTGGTG -3’

Loop A forward
5’-
GAGTTCTTTCCGCTGGATGAAAGCGGTAACGTGCCGTCGGACAAGAAGATAGAGGAGACACTTCACG -3’

Loop A reverse
5’-
GCTTTCATCCAGCGAAAGGAACCTCTTTGCTTTGAAGCCGGTGCGCCAGTGCAACAAATAAAGG -3’

Loop B forward
5’-
CCGGATCGCCCGTGGGCGAAACCGGAAGACCCGTCTCTTGCAAGGAATGATCTAGCTAAAATTGG -3’

Loop B reverse
5’- GTTTCGCCACGGGCGATCCGGCCTTTCTAGAGGTGTGTACGCCATTAATGC -3’

Loop C forward
5’-
TGCACATCCCATAAAGATTATCCGTTTCACGAAGGTTCTGAGGATCCGAATTCGAGCTCCGTCGACAAGC -3’
Loop C reverse

5’- TAATCTTTATGGGATGTGCAAGAAAGCAGCGGCACACGCAGCAGTTACGCACACACCTCCTTGCATCTC -3’

Δ182-184 forward

5’- ATCAAGTTAAATACTCAGTGCGCCGAAACTACAGGACTTCTCG -3’

Δ182-184 reverse

5’- AAGTCCTGTAGTTTCCGGCCACACTGAGTTATTTAACCTGATTTCG -3’
References:


Chapter 6

SUMMARY

In this work we describe our efforts to engineer a general dehydrogenase enzymatic scaffold for immobilized systems and industrial applications. The ideal scaffold would be inexpensive to make and purify, stable, readily immobilized, able to use inexpensive cofactors, and easily engineered for activity with a variety of substrates. To meet these goals, we have selected a thermostable alcohol dehydrogenase, AdhD, from Pyrococcus furiosus as the basis for our engineering efforts. The hyperthermophilic nature of this organism requires that all of its constituent proteins, including AdhD, remain well-folded and active under extreme conditions. Thus the inherent thermostability of our scaffold protein provides many benefits in addition to long active lifetimes in immobilized systems. First, it allows the enzyme to be easily purified from a mesophilic host. Combined with the high expression levels achievable in E. coli, large amounts of purified protein can be quickly and inexpensively obtained by heating the cultures and removing the denatured endogenous proteins and cell debris by centrifugation. Next, it has been shown that thermostable proteins are more tolerant to mutations and thus more amenable to protein engineering and mutagenesis (1). As we envision extensively engineering this scaffold for a range of applications, a high intrinsic stability will allow
a more thorough exploration of sequence space while maintaining a well-folded structure.

In Chapter 2, we addressed the problem of enzyme immobilization through the fusion of protein cross-linking domains, thereby functionalizing the enzyme for self-assembly. Importantly, we have demonstrated that these fusions minimally affect the kinetic properties and thermostability of the enzyme, and that a robust hydrogel structure persists over a wide temperature range. The general nature of these cross-linking domains allows for the creation of mixed macrohomogenous hydrogels, and may find utility in the design of clustered enzyme systems, such as synthetic metabolic pathways.

In Chapter 3, we studied the cofactor specificity of *Pyrococcus furiosus* AdhD and engineered a highly active double mutant with broadened specificity. A detailed analysis of the cofactor binding mechanism using steady-state and pre-steady state kinetic techniques revealed a novel transient upon cofactor binding which has not been previously observed in the aldo-keto reductase superfamily. This kinetic analysis also allowed us to calculate the microscopic rate constants corresponding to each step in the cofactor binding mechanism and assess the impact of the mutations on binding and catalysis.

The relaxed specificity of the highly active double mutant engineered in Chapter 3 allowed the enzyme to utilize a truncated nicotinamide cofactor for catalysis. In Chapter
4, we demonstrated the benefits of utilizing a minimal cofactor in immobilized systems through the creation of an enzymatic bioanode for the oxidation of D-arabinose. Fuel cells constructed with the double mutant AdhD using either NAD(H) or NMN(H) as a cofactor exhibited similar maximum power densities, despite a two order of magnitude decrease in the activity of the enzyme with NMN\(^+\). Additionally, the fuel cell constructed with the minimal cofactor exhibited a 40% increase in the maximum current density, suggesting that significant gains in performance can be obtained through increases in the rate of cofactor diffusion.

Finally, in Chapter 5, we explore strategies to engineer novel substrate specificities into our designed scaffold. One approach to altering the substrate specificity of enzymes is through the exchange of substrate binding loops. As this has previously shown to be effective in two closely related members of the AKR superfamily, we decided to apply this method to AdhD. In an attempt to improve the activity of the enzyme with sugars, we grafted in the substrate binding loops from another AKR, human aldose reductase. Even though the two enzymes share less than 30% sequence homology, replacement of two of the substrate binding loops was sufficient to impart hAR activity on AdhD. It is interesting to note that these mutations were not additive with the previously engineered cofactor specificity mutant, as grafting the same substrate binding loops onto the double mutant AdhD (which itself exhibited hAR activity) resulted in an inactive enzyme. This suggests that cofactor and substrate binding cannot be altered in
a completely independent fashion, and this will be important to keep in mind for future engineering efforts. We hypothesize that this lack of activity is due to improper positioning of the substrate relative to the cofactor in the active site, and not due to lack of binding of either molecule. As the K249G mutation increases the volume of the cofactor binding pocket (and presumably the conformational flexibility of the bound cofactor), the catalytic rate enhancement observed in these mutants can be explained by a more favorable positioning of the nicotinamide head group within the active site.

The wide range of substrate specificities exhibited by members of the AKR superfamily could potentially be accessed through this modular loop swapping approach. However, it is likely that substrate specificities will be desired beyond those of known AKRs. Thus, a more general approach to identifying and optimizing scaffolds with novel activities is required.

Directed Evolution of Catalytic Activity

Directed evolution of peptides or proteins with high affinity for a ligand is relatively straightforward, and along with recent developments in selection strategies, binders with picomolar affinities are readily attainable (2, 3). Generally, these selection

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1 Note that directed evolution experiments also require a genotype-phenotype linkage. As a wide range of techniques for accomplishing this have been described, it will not be further discussed here.
strategies involve immobilizing the target ligand and passing a library of mutants over it. Thus proteins with affinity to the target are immobilized and retained while non-functional proteins are washed away. Successive rounds of mutagenesis and selection can be combined to identify high affinity binders, and further improvements in affinity may be obtained by selecting for a slow off-rate (2). Currently, protein engineering techniques have yielded binders with femtomolar ligand affinities, far exceeding those of the best natural binders (4).

Extending these techniques to obtain novel or improved enzymes has proved challenging, however, as it is much more difficult to identify mutants for improved catalytic activity. These reactions generally involve multiple molecules, and require precise positioning and a specific environment in the active site. Numerous approaches have been developed to identify mutants with improved activity, with varying levels of success.

The simplest and lowest-throughput method is to screen all mutants individually for catalytic activity. The efficiency of this process is highly dependent on the sensitivity of the assay, and is generally limited to examining a few thousand mutants. Thus, this technique is most commonly used to select improved mutants after site-directed mutagenesis of a few key amino acids. Since an efficient directed evolution experiment
requires exploration of a large region of sequence space (libraries of $10^{11} - 10^{14}$ are not uncommon), a high-throughput selection step is preferred.

Any enzyme that can be directly linked to cell survival provides a useful selection scheme. Usually, these *in vivo* selections complement a function that has been knocked out (an auxotroph), or provide resistance against a toxin or synthesize a substance essential for growth. Unfortunately, the *in vivo* nature of the selection severely limits the scope of reactions that can be selected for, and often the cells find alternate ways of generating the desired phenotype. For example, a common result of increased selection pressure is increased expression levels of the enzyme rather than increased catalytic efficiency (5).

Several indirect selection protocols have been developed on the basis of binding to substrate, product, or transition state analogues (TSA). The latter takes advantage of Pauling’s theory that an enzyme stabilizes a transition state, and thus has much higher affinity for the transition state than for the substrates or products (6). Drawing on previous successes, researchers attempted to synthesize stable transition state analogues that would mimic the geometry and charge distribution of the transition state and evolve high affinity binders towards them. This technique was successfully used to evolve an antibody with catalytic activity, but the observed reaction rates were quite low (7).
A similar approach using a specially designed suicide substrate has also been developed. Here, a relatively non-reactive substrate analogue is transformed by the enzyme into a reactive inhibitor, thereby irreversibly blocking the enzyme. Thus active enzymes become covalently bound to the inhibitor and can be readily selected if the inhibitor is labeled with an affinity tag. Fast kinetics can be selected for using a limiting concentration of inhibitor and reacting for a short time. This technique has been successfully used to select proteins with β-lactamase activity from a mock library (8).

Both of these indirect selection techniques (TSAs and suicide substrates) are severely limited by the availability of the appropriate molecule. Their design is not trivial, and the efficiency of each technique hinges on the molecule accurately representing the desired reaction mechanism. Furthermore, catalytic activity is usually limited by mutations that favor the selection step. For example, a suicide substrate may select for nucleophiles in the active site that are more reactive with the inhibitor, but do not necessarily lead to increased catalytic activity (5).

These limitations have motivated the development of novel methods to directly select for catalytic activity. This type of selection is most easily performed for enzymes that catalyze bond formation or breakage. A compelling example is the recent evolution of novel RNA ligases from a partially randomized zinc-finger scaffold. Active ligases created a covalent linkage between a linked mRNA tail and an immobilized RNA
target, enabling efficient recovery. The resulting ligases exhibited a rate enhancement for the ligation reaction of greater than $2 \times 10^6$ (9). Some metallo-enzymes can also lend themselves well to direct selection. Enzymes can first be inactivated by extraction of the catalytic metal ion with EDTA and selected based on binding to a substrate. Active enzyme can then be selectively eluted upon addition of the metal ion and the conversion of the substrate to product, to which the enzyme has a lower affinity (10).

Another approach has been described in which in vitro compartmentalization is used to label microbeads with a single gene and multiple copies of its protein product. The microbeads are then re-emulsified with a tagged substrate, and after incubation the substrate and product are coupled to the beads. Microbeads with active enzyme are then fluorescently labeled with an anti-product antibody and selected using flow cytometry (11). Again, there are a number of drawbacks to these approaches and their application is limited to specific cases.

Thus no “best” way of evolving catalytic activity has been identified, but the periodic successes indicate the selection step should be based on the properties of the enzyme you are trying to optimize. As such, we envision taking advantage of the reaction mechanism and unique structure of the AKR superfamily to evolve enzymes with novel specificities.
Kinetic Based Enzyme Capture

Immobilized cofactors have been used extensively for affinity chromatography in an attempt to purify cofactor-dependent enzymes. The addition of a non-reactive substrate analogue to the enzyme solution prior to affinity chromatography has been shown to greatly increase the specificity of this purification step, and enzymes can be selectively purified based on their substrate specificity (12). This kinetic based enzyme capture (KBEC) technique takes advantage of the ordered bi-bi kinetic mechanism shared by a large number of enzymes, including dehydrogenases, wherein the cofactor binds first and leaves last. In these enzymes, the substrate binding site is usually spatially located above the cofactor, preventing the cofactor from dissociating while the substrate is bound. Thus the presence of a saturating concentration of substrate analogue in solution “locks-on” the enzyme to the cofactor such that enzymes not specific to the cofactor/substrate combination are washed away. Enzymes non-specifically bound to the immobilized cofactor can also be competitively eluted using soluble fragments of NAD(P)(H) (i.e. 5′-AMP). This technique has been extensively characterized and optimized for several dehydrogenase enzymes with regards to immobilization chemistry, reaction conditions, and substrate analogues (13-15). Additionally, KBEC has been demonstrated to be powerful enough to purify yeast alcohol dehydrogenase from crude cell extracts in a single bioaffinity chromatographic step (16).
To achieve the final goal of being able to rapidly evolve novel substrate specificities into our designed scaffold, we have explored the use of KBEC as a selection step for directed evolution. In this case, a library of enzyme mutants would be passed over an immobilized cofactor column in the presence of a substrate or substrate analogue. Mutants that bound could then be released upon a (pH, temperature, etc) shift to conditions that promote catalysis, or through competition with the desired substrate. The duration and stringency of the wash and elution steps could be modulated over successive rounds to increase the selectivity and activity of the recovered mutants.

As a proof of concept, we have attempted several mock enrichments using various fluorescently labeled enzyme constructs. While we have been able to repeat the KBEC results of O'Flaherty et al. with yeast alcohol dehydrogenase (YADH) (16), identifying conditions for the specific binding and elution of AdhD has proven difficult.

While immobilized cofactor matrices of various chemistries are commercially available, we have obtained superior results with homemade preparations. The nicotinamide cofactors can be readily linked through the adenine amine to epoxy-activated Sepharose beads under slightly basic conditions. Immobilized cofactor columns prepared in this fashion have a higher binding capacity for YADH than commercial preparations, and linking at this position ensures enzyme accessibility. As AKRs bind the cofactor in an extended conformation, the adenine indol sits in a cleft at the edge of the α/β barrel and
a linker attached at this position would likely have a minimal impact on binding affinity (Figure 3.1). Indeed, cofactors attached at this position seem to be accessible to both YADH and AdhD, as both enzymes are able to use the immobilized cofactors for catalysis. Additionally, preparations of immobilized reduced cofactor can be prepared in this manner to examine the effect of charge and cofactor oxidation state on KBEC efficiency.

We first examined the salt concentration and ionic strength of the binding and elution buffers to minimize non-specific interactions. Under low salt conditions, we observed a significant amount of non-specific binding to the cofactor resin, which could be reversed upon the addition of a high salt buffer. Several methods were attempted to titrate the ionic strength for selectivity, but no optimum was found. We were able to identify conditions where wt AdhD could be enriched from a mixture with the double mutant enzyme, but as this was shown to be independent of the “locking-on” ligand and only on cofactor binding, it would not be a useful selection scheme (Figure 6.1).

We have also examined a variety of “locking-on” ligands for binding and selectivity. As we have been unable to identify an inhibitor for AdhD, a range of molecules similar to its preferred substrate were tested. Some success was seen using a combination of immobilized oxidized cofactor (NAD(P)^+) and the preferred substrate for the reduction reaction, 2,3-butanedione. However, the elution peaks were extremely broad, and use of
the diketone presented some experimental difficulties due to its reactivity and intrinsic fluorescence.

Despite our limited success with mock selection experiments, this technique may still prove valuable as a directed evolution selection step. The broad substrate specificity and poor affinities demonstrated by the wild-type and double mutant constructs may preclude their selection using this technique, but it is possible that enzymes with high specificities to desired substrates could still be obtained from a randomized library. Thus this technique is worthy of continued research, as few alternatives yet exist for the directed evolution of novel enzyme activities.
Figures

**Figure 6.1** Chromatogram of fluorescently labeled wt AdhD and K249G/H255R AdhD

Conditions: 1 ml NADP⁺-Sepharose column at 25°C, equilibrated in 20mM sodium phosphate (pH 7.0) containing 20-1000mM NaCl (blue dashed line) and indicated ligands. Double mutant AdhD elutes in the protein breakthrough peak, while wt AdhD is retained. A salt gradient is used to elute bound enzyme. Additional double mutant AdhD elutes at ca. 190 mM NaCl, while a significant wt AdhD peak is observed ca. 240 mM NaCl. The observed elution behavior was later shown to be independent of the locking-on ligand, and thus would not be useful from a selection perspective.
References


