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Current Opinion in Chemical Biology

Emerging artificial metalloenzymes for asymmetric hydrogenation reactions

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Abstract

Artificial metalloenzymes (ArMs) utilize the best properties of homogenous transition metal catalysts and naturally occurring proteins. While synthetic metal complexes offer high tunability and broad-scope reactivity with a variety of substrates, enzymes further endow these complexes with enhanced aqueous stability and stereoselectivity. For these reasons, dozens of ArMs have been designed to perform catalytic asymmetric hydrogenation reactions, and hydrogenase ArMs are, in fact, the oldest class of ArMs. Herein, we report recent advances in the design of hydrogenase ArMs, including (i) the modification of natural [Fe]-hydrogenase by insertion of artificial metallocofactors, (ii) design of a novel ArM system from the tractable and inexpensive protein β-lactoglobulin to afford a high-performing transfer hydrogenase, and (iii) the design of chimeric streptavidin scaffolds that drastically alter the secondary coordination sphere of previously reported streptavidin/biotin transfer hydrogenase ArMs.

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Current Opinion in Chemical Biology 2022, **66**:102096

This review comes from a themed issue on **Bioinorganic Chemistry**

Edited by **Emily Que**

For a complete overview see the Issue and the Editorial

https://doi.org/10.1016/j.cbpa.2021.102096

Keywords

Hydrogenase, Asymmetric hydrogenation, Catalysis, Metalloenzymes, Artificial metalloenzymes.

Introduction

Artificial metalloenzymes (ArMs) are a class of hybrid catalysts wherein a protein (natural, mutant, or *de novo*) is given a non-natural, metal-containing cofactor. These systems leverage the most attractive properties of both homogenous transition metal catalysts (highly tunable primary coordination sphere, facile incorporation of precious metals, and reactivity with a wide range of substrates) and natural enzymes (high stereoselectivity, diverse well-defined secondary coordination spheres, and high turnover numbers). Many ArMs have been designed to perform asymmetric hydrogenation of unsaturated functional groups, such as nitriles [1,2], carbonyls [3], and imines [3], with H_2 or H_2-donors to yield a chiral product. Asymmetric hydrogenation is a crucial step in the synthesis of many pharmaceuticals, agrochemicals, fragrances, and other small organic molecules [4,5], and biocatalysis is predicted to play a crucial role in the future of industrial small molecule synthesis [6]. The design of the chiral ligands and metal complexes needed for this catalysis is often significantly more difficult, time-consuming, and costly than analogous achiral catalysts. Proteins exist as one stereoisomer, which can be used to design hybrid systems containing an achiral metal catalyst embedded in a rigidly defined chiral environment. Here, we will examine three different ArM systems using different design approaches that provided chemically robust, high-turnover, stereoselective hydrogenation catalysts (summarized in Table 1).

Insertion of synthetic Mn-cofactors into apo-[Fe]-hydrogenase

Tetrahydromethanopterin dehydrogenase (Hmd or [Fe]-hydrogenase) is a homodimeric metalloenzyme expressed in several species of methanogenic archaea. The iron-guanosylylpyridinol (FeGP) cofactor in the active site of Hmd performs heterolytic cleavage of H_2 to generate a proton and a hydride, and the latter is stereoselectively transferred to the *methenyl*-tetrahydromethanopterin (methenyl-H_4MPT^+) substrate, thus providing *methylene*-tetrahydromethanopterin (methylene-H_4MPT) [7] (Figure 1). This transformation is the third of four reductions in the metabolism of carbon dioxide to methane but is the only one that directly uses H_2 as a reducing equivalent [8]. The FeGP cofactor has been the target of synthetic modeling by organometallic and bio-inorganic chemists since the unambiguous structural determination of its active site in 2009 [9]. While a vast array of Hmd synthetic models have been reported — many reconstructing the active site primary coordination sphere with atom-for-atom accuracy — only two models (reported by our group) showed enzyme-like reactivity (in the absence of a protein environment) [10,11]. One potential explanation for this lack of reactivity is the

Table 1

Comparison of design principles employed for recently reported artificial hydrogenases.

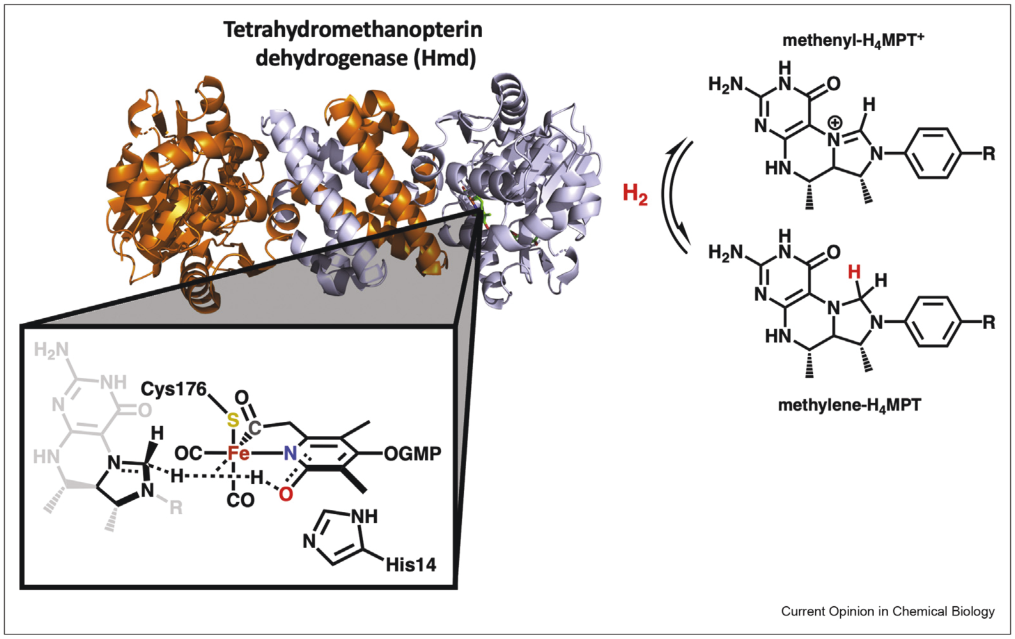
	Hmd	βLG	Sav
Hydrogenation mechanism	Fe-mediated heterolytic H_2 cleavage	Transfer hydrogenation	Transfer hydrogenation
Metallocofactor binding mechanism	Coordination of Cys to metal center	Hydrophobic interaction of nonpolar ligand with calyx	H-bonding to biotin moiety on cofactor
Design innovation	Non-native [Mn] cofactor	Novel use of a commercially available protein scaffold	Alteration of protein scaffold's tertiary structure
Transition metals	Fe(II), Mn(I)	Rh(III), Ru(II)	Ir(III)

secondary interactions between the protein scaffold and the FeGP cofactor that exist in the natural enzyme but are difficult to reproduce in a small molecule system. First, a hydrogen bond interaction between the amide backbone of Cys176 and the acyl oxygen of the FeGP cofactor may cause contraction of the Fe—C_{acyl} bond to a remarkably short 1.88 Å [12]. This more Fischer carbene-type structure (Fe=C—O) of the Fe-acyl (Fe—C=O) unit likely has a great effect on the interaction of the Fe center with H_2. Second, QM/MM computational studies have invoked His14 as a crucial general base, allowing thermoneutral deprotonation of the FeGP pyridinol moiety that precedes H_2 activation [13]. Hu et al. — noting that even the natural FeGP cofactor extracted from Hmd exhibits hydrogenase reactivity only when bound to the Hmd protein — reasoned that incorporation of Hmd synthetic models into a protein environment may result in catalytic enhancement. In their first report [14], reconstitution of apo-Hmd from *Methanocaldococcus jannaschii* (jHmd) with the iron pyridinol complex **1** (Figure 2) resulted in a reactive bioconjugate **1**⊂jHmd with ∼1% of the specific activity of the natural enzyme. Even so, **1**⊂jHmd exhibits a higher turnover frequency (2 s^{-1} for the forward reaction and 1 s^{-1} for the reverse) than a number of known Fe-based hydrogenation catalysts (10^{-3}–10^{-1} s^{-1}) [15,16]. Importantly, **1** exhibited no hydrogenation reactivity in the absence of jHmd.

Because the [Fe]-hydrogenase model **1**⊂jHmd exhibited low activity, the design was repurposed to develop a family of [Mn]-hydrogenase ArMs [17,18]. Isoelectronic Mn(I) analogs of the ferrous carbonyls are of interest as Mn(I) complexes often display both greater hydrogenation and transfer hydrogenation reactivity and enhanced stability [19] causing one to question the reason Nature never evolved a hydrogenation catalyst using a Mn active site. Reconstitution of jHmd with **2** (Figure 2) afforded their first [Mn]-hydrogenase ArM [17]. The calculated active site occupancy was only about 20%, and FTIR indicated that a significant proportion of **2** binds nonspecifically, possibly attributable to the unfavorable CO ligand displacement needed to bond **2** to Cys176. When adjusted for active site occupancy, the [Mn]-hydrogenase displayed greatly enhanced activity for hydrogenation of H_4MPT^+ and turnover frequency, outperforming semi-synthetic [Fe]-hydrogenase and, therefore, most iron-based hydrogenation catalysts. Remarkably, the [Mn]-hydrogenase was strongly biased toward the forward hydrogenation reaction, whereas all the [Fe]-hydrogenases (natural and artificial) display only a slight bias toward the forward reaction. This bias toward the forward reaction was attributed to the higher pK_a of the 2-hydroxy group of **2** compared with its Fe(II) analogs, hindering the ability of **2** to protonate a putative Fe—H intermediate to form H_2 in the dehydrogenation reaction.

In a following report [18], Hu et al. developed a series of [Mn]-hydrogenase ArMs with the aim of demonstrating the necessary presence of an internal base on the metallocofactor for efficient hydrogenation/dehydrogenation catalysis to occur. Apo-jHmd was reconstituted with a number of derivatives of complex **2**, each featuring different functionalities at the acyl methylene and 2-pyridine positions (Figure 2). As it has been hypothesized that either of these sites could act as a pendant base during H_2 activation, each of these models was designed to exclude one or both sites to probe the effect on catalysis. Interestingly, only [Mn]-hydrogenases reconstituted with metal complexes containing a pendant base functionality (**2**, **3**, **4**, and **5**) showed hydrogenase reactivity. In addition, mutation of crucial amino acid residues (Cys176 or His14) rendered the resulting ArMs negligibly active or completely inactive. The development of Hmd-derived ArMs has proven impactful in the elucidation of the natural enzyme's H_2 heterolysis mechanism, as well as highlighting the importance of the enzyme scaffold for catalytic activity. Designing such ArMs affords researchers the opportunity to wield precise synthetic control over the metallocofactor catalyst, thus discerning the effects of single functional group substitutions or amino acid mutations on catalysis.

Figure 1



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X-ray crystal structure of Hmd in the open confirmation (PDB 6HAV) and catalytic hydride transfer/abstraction of methenyl-H_4MPT^+/methylene-H_4MPT. The proposed transition state of the H_2 heterolysis step is shown in the outset. H_4MPT^+, *methenyl*-tetrahydromethanopterin.

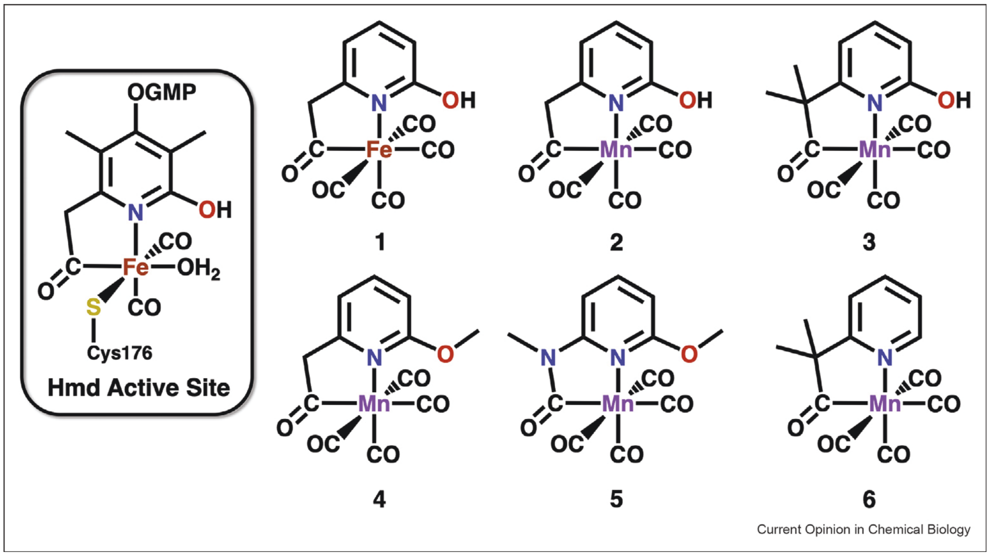
Bovine β-lactoglobulin as a novel scaffold for asymmetric transfer hydrogenation

Bovine β-lactoglobulin (βLG) is the most abundant whey protein found in bovine milk, comprising some 50–60% of total whey composition. While the complete physiological function of βLG is still unknown, it is believed to be involved in the transport of small hydrophobic molecules, such as fatty acids, phospholipids and steroids [20]. Among the benefits of the βLG system for designing ArMs is the protein's high affinity for fatty acids, with K_D values ranging from 0.49 μM for palmitic acid (C_{16}) to 5.9 μM for lauric acid (C_{12}) [21]. The binding of fatty acids is influenced by three factors, (i) hydrophobic interactions of the long aliphatic chain with residues in the interior of the calyx, (ii) polar interactions with charged amino acids at the aperture of the calyx, and (iii) pH-induced conformational changes which restrict access to the calyx below pH 7 [22,23]. It is commercially available in high purity and low cost (∼$2/mg), exhibits high stability and has well-characterized behavior across a fairly wide range of conditions [24,25]. Metal complexes can be easily modified to include a high-affinity binding moiety through either condensation with a fatty acid derivative or substitution with an *n*-halogenated linear hydrocarbon. Thus, βLG represents an attractive ArM design template.

The first ArMs to use βLG were reported by Chevalley and Salmain [26]. Their designs featured Rh^{3+} or Ru^{2+} half-sandwich complexes ligated by 2,2′-dipyridylamine, which was functionalized with either lauric or palmitic acid. The binding of this family of complexes to βLG was qualitatively determined by circular dichroism wherein the association of an achiral guest molecule to the chiral protein resulted in a significant spectral intensity shift. The crystal structure of one of these ArMs, **7**⊂βLG (Figure 2B) was later determined [27], showing that the Rh cofactor binds to βLG in the expected fashion; however, while the aliphatic 'tail' appeared well-ordered in their solved structure, the metal-containing 'head' was poorly defined in the observed electron density. Nonetheless, **7**⊂βLG was shown to catalyze the enantioselective transfer hydrogenation of 2,2,2-trifluoroacetophenone to the corresponding alcohol at 72% yield and 24% *ee* (versus 50% yield and 0% *ee* for **7** alone).

In a later report [28], this group reported another series of βLG-derived ArMs, again based on transition metal half-sandwich complexes. In this case, however, Ru^{2+}, Rh^{3+} and Ir^{3+} precursors of the form $[M(Ar)\ (\mu\text{-}Cl)Cl]_2$ were first hydrolyzed to give a mixture of monomer and dimer species and then incubated with βLG. Their

Figure 2



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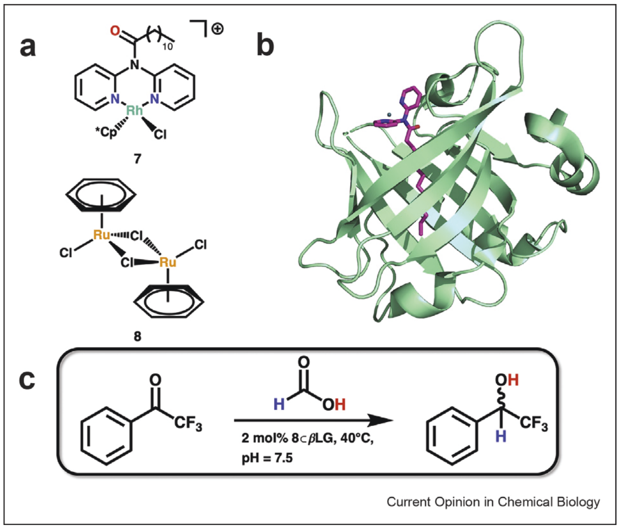
The FeGP cofactor of Hmd and its synthetic models used to generate artificial [Fe]- and [Mn]-hydrogenases. FeGP, iron-guanosylylpyridinol; Hmd, tetrahydromethanopterin dehydrogenase.

report focuses on the **8**⊂βLG system, which demonstrated the most impressive catalytic activity. Although no aliphatic moiety was appended to this metallocofactor, inductively coupled plasma optical emission spectroscopy (ICP-OES) of metal-incubated βLG (purified by gel desalting) showed Ru/βLG ratios of 1:1. Native mass spectrometry corroborated this Ru/βLG ratio, furthermore showing that a detectable population of βLG with two Ru complexes bound; indeed, a putative second binding site has long been postulated for βLG [29]. The hybrid **8**⊂βLG showed remarkable catalytic enhancement in asymmetric transfer hydrogenation versus the mixture resulting from the hydrolysis of [Ru(η^6-benz)(μ-Cl)Cl]$_2$. Using sodium formate as the sacrificial hydrogen donor, **8**⊂βLG converted trifluoroacetophenone to its corresponding alcohol with 87% yield and an impressive 82% *ee* (versus 28% yield and 0% *ee* for **8** alone) (Figure 3c). This finding is quite remarkable as it suggests that the well-defined, highly specific binding of a synthetic metallocofactor to βLG is possible without the inclusion of a long aliphatic binding moiety. In fact, designing a metallocofactor that binds to βLG in the same manner as natural fatty acids may serve to erode *ee*, as the cofactor may rotate freely in the calyx and not exhibit one preferred binding mode.

Designing chimeric streptavidin scaffolds to enhance transfer hydrogenation reactivity

The streptavidin (Sav)/biotin bioconjugate system represents perhaps the best-developed family of ArMs. As reported by Whitesides in 1978 [30], avidin (a close structural relative of Sav) was in fact the first example of a well-defined ArM. This system featured a biotinylated Rh(I) pincer complex bound to Sav, catalyzing asymmetric olefin hydrogenation with 41% *ee* and an impressive turnover frequency of 500 s^{-1}. Since then, dozens of Sav/biotin-based ArMs have been designed and studied. This ArM system is particularly robust as biotin binds essentially irreversibly to Sav ($K_D = 10^{-14}$ M) [31]. Since

Figure 3



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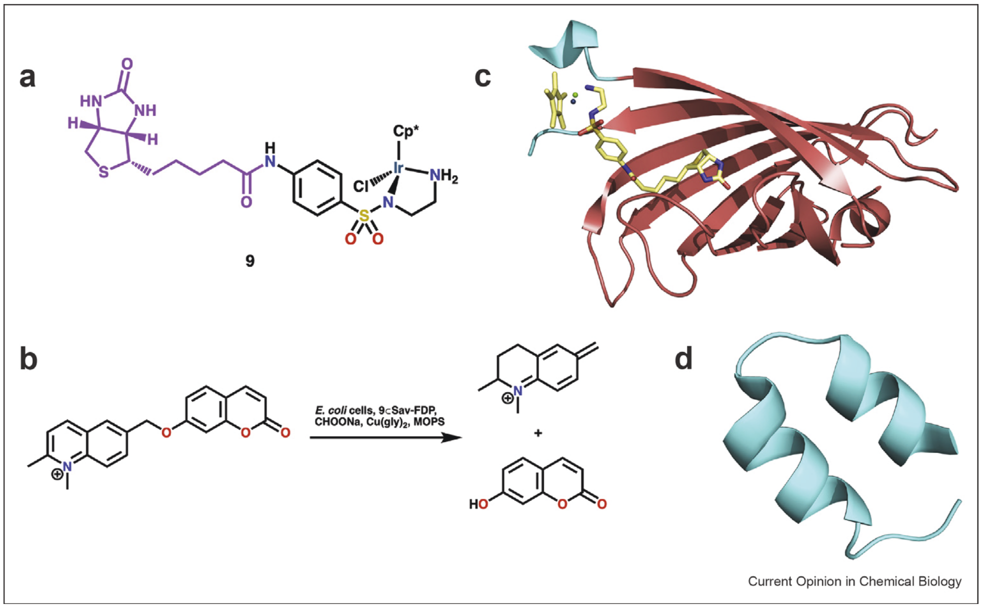
Highlighted features of b-lactoglobulin-based transfer hydrogenases. (**a**) Metallocofactors **7** and **8** used to make reactive βLG-derived ArMs. (**b**) X-ray crystal structure of **7**⊂ βLG (PDB 4KII). Metallocofactor **7** is shown in magenta. (**c**) Transfer hydrogenation of 2,2,2-trifluoroacetophenone to 2,2,2-trifluoro-1-phenylethanol catalyzed by **8**⊂βLG.

2003, Chatterjee et al. [32] have vastly expanded the scope of reactions catalyzed by Sav/biotin-derived ArMs to include such exotic non-natural transformations as Suzuki cross-couplings, olefin metathesis [33,34], and transfer hydrogenation [35–37]. Through almost 20 years of work with Sav/biotin hydrogenase ArMs, this group has developed an impressive biochemical toolbox whereby the Sav protein scaffold can be modified to tune the reactivity and selectivity of a metallocofactor. Their streamlined procedure for expressing and screening Sav mutants allows for rapid identification of promising new protein scaffolds that enhance the yield and *ee* of their previously developed metallocofactors [38]. This is quite the converse approach to the systems previously discussed in this review, although it must be noted that Hmd and βLG ArMs systems are in a far earlier stage of development.

While the optimization of Sav/biotin hydrogenase ArMs focused on the identification of new Sav mutants that enhanced catalysis compared with wild-type Sav or previously characterized mutants, they recently noted that there may be an optimization ceiling for this method. Point mutations often do not result in a significant change of the secondary, tertiary, or quaternary structure of a protein, thus limiting the possible scaffolds for hydrogenation catalyst to structures that closely resemble wild-type Sav. Chimeric Savs allow for entire novel protein subunits to be appended to or inserted into Sav, allowing for a far greater variety of protein scaffold structural motifs. In the first report of chimeric Sav hydrogenase ArMs [39], a number of Sav variants containing loops expanded with exogenous peptide fragments with well-defined secondary structures were overexpressed in *Escherichia coli*. These fragments featuring α-helices and β-sheets were inserted into one of several unstructured loops that surround the 'vestibule' or metallocofactor-binding active site of Sav. The chimeric Savs were then treated with the Ir(III) half-sandwich complex **9** (Figure 4a), which has been used in previously reported Sav transfer hydrogenase ArMs [40,41]. Insertion of two of the α-helix loop fragments into Sav loop 3/4 resulted in ArMs with enhanced TON and *ee* for transfer hydrogenation of cyclic imine substrates, improving TON by up to 700% relative to previously reported non-chimeric Sav variants. It was hypothesized that insertion into loop 3/4 projects the appended peptide sequence into the closest proximity of **9** as compared to insertion into other loops, thus affording the best catalytic enhancement; however, all crystal structures of these chimeric Sav ArMs were highly disordered at the expanded loops, so a definitive structural argument for this hypothesis is at this time not possible.

In a subsequent report [42], Ward et al. applied a chimeric Sav/biotin hydrogenase ArM in an *in vivo* system. Here, Sav was modified by insertion of an FPD peptide between residues 115 and 117 of the previously mentioned unorganized loop. This chimeric Sav was

Figure 4



Highlighted features of chimeric streptavidin ArMs. (**a**) Metallocofactor **9** used in both chimeric Sav ArMs [31,34]. The protein-binding biotin moiety is highlighted in purple. (**b**) Transfer hydrogenation of self-immolative substrate catalyzed by **9**⊂Sav-FDP S112V K121A. (**c**) Partial X-ray crystal structure of chimeric **9**⊂Sav (PDB 3PK2). The termini of the partially-solved FDP loop are shown in cyan. (**d**) FDP loop fragment (PDB 3U0S) appended to loop 3/4 of Sav. ArMs, artificial metalloenzymes.

loaded with **9** and catalyzed the transfer hydrogenation of a self-immolative substrate. In this transformation (Figure 4b), hydrogenation of the quinolinium substrate causes a C–O bond cleavage, which releases a fluorophore fragment. This not only allows for facile reaction progress monitoring by fluorescence spectroscopy but also has applications in prodrugs [43] and biomolecular imaging [44,45]. *E. coli* engineered to express Sav-FDP (Figure 4c and d) in their periplasm were incubated with **9** and then washed with Cu(gly)$_2$ to remove glutathione present in the periplasm, which poisons the Ir catalyst. In the presence of formate and substrate, the self-immolative release of fluorophore in 33% yield was observed (versus 1–8% for **9** in the absence of Sav-FDP under various conditions). To optimize this system, a library of Sav-FDP mutants was screened. The highest-performing mutant, Sav-FDP S112V K121A, was characterized by X-ray crystallography; however, the structure of the appended FDP peptide could not be solved (as before), most likely due to its conformational flexibility. Interestingly, metallocofactor **9** was found to have a significantly higher occupancy in this structure than in WT-Sav (80% versus 50%), indicating that the FDP loop serves to shield the active site. The development of chimeric Sav variants represent a new chapter in ArM design. The decades of study of Sav in ArM systems have shown that alteration of secondary structure about a bound synthetic metallocofactor can only improve catalytic efficiency up to a point. Through the addition of entirely new structural domains to Sav, researchers may now modify the tertiary structure of Sav, affording the opportunity to access improved or entirely novel reactivity for this system.

Conclusion and outlook

The development of hydrogenase ArMs began with the very first ArM reported by Whitesides over 40 years ago [30]. In the intervening decades, great strides have been taken to develop robust ArMs that perform catalytic hydrogenation reactions on a variety of functional groups and with high yield and stereoselectivity. With both yield and *ee* approaching 100% for ArMs reported in the 2010s [46], bioinorganic chemists sought to develop systems that combine these efficiencies with loftier goals, such as using inexpensive durable protein scaffolds such as βLG, taking advantage of modern synthetic biology to custom design structurally diverse chimeric Savs or screening a library of synthetic metal cofactors in Hmd to elucidate the still unverified mechanism of H_2 cleavage in natural [Fe]-hydrogenase. These lines of inquiry have proven promising in the last few years; however, these systems with more ambitious designs still fall short of the high conversions and stereoselectivity of previously developed models. Just as there exists no universally ideal, multifunctional hydrogenase enzyme in nature, there is similarly not likely to be a universally ideal hydrogenase ArM that is concomitantly broad in substrate scope, highly stereoselective and highly active. Therefore, the development of numerous hydrogenase ArMs based on a variety of design principles will be critical in the coming years to expand both the scope and efficacy of this utile class of catalysts.

Author contributions

Sean T. Goralski: Conceptualization, writing (original draft), writing (review and editing), visualization.

Michael J. Rose: Conceptualization, writing (reviewing and editing), supervision, funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank the National Science Foundation (CHE-1808311) and the Welch Foundation (F-1822) for funding this endeavor.

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