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Stereoselective Hydrogenation of Olefins Using Rhodium-Substituted Carbonic Anhydrase—A New Reductase

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Abstract: One useful synthetic reaction missing from nature's toolbox is the direct hydrogenation of substrates using hydrogen. Instead nature uses cofactors like NADH to reduce organic substrates, which adds complexity and cost to these reductions. To create an enzyme that can directly reduce organic substrates with hydrogen, researchers have combined metal hydrogenation catalysts with proteins. One approach is an indirect link where a ligand is linked to a protein and the metal binds to the ligand. Another approach is direct linking of the metal to protein, but nonspecific binding of the metal limits this approach. Herein, we report a direct hydrogenation of olefins catalyzed by rhodium(I) bound to carbonic anhydrase (CA-[Rh]). We minimized nonspecific binding of rhodium by replacing histidine residues on the protein surface using site-directed mutagenesis or by chemically modifying the histidine residues. Hydrogenation catalyzed by CA-[Rh] is slightly slower than for uncomplexed rhodium(I), but the protein environment induces stereoselectivity favoring *cis*- over *trans*-stilbene by about 20:1. This enzyme is the first cofactor-independent reductase that reduces organic molecules using hydrogen. This catalyst is a good starting point to create variants with tailored reactivity and selectivity. This strategy to insert transition metals in the active site of metalloenzymes opens opportunities to a wider range of enzyme-catalyzed reactions.

Keywords: carbonic anhydrase · enzyme catalysis · hydrogenation · metalloenzymes · protein modifications

Introduction

Although nature's enzymes efficiently catalyze a wide range of chemical reactions, their catalytic range is limited to reactions that offer a selective advantage to living organisms. One notable reaction missing from nature's toolbox is the direct hydrogenation of substrates using hydrogen gas. This is a common and useful reaction in organic synthesis, but nature does not use it. Hydrogenases are enzymes that cleave hydrogen to protons and electron, but they do not reduce organic molecules.[1]

To reduce organic molecules during metabolism nature uses cofactors such as NAD(P)H or $FADH_2$, which provides evolutionary advantages over the direct use of hydrogen. These cofactors are large to allow tight binding and precise positioning in an active site. This likely makes the enzymes more efficient than they would be with hydrogen. Cofactors differ in their redox balance in the cell. Cells contain approximately equal amounts of NADH and NAD^+, but they contain 200-fold more NADPH than $NADP^+$. This difference allows NADPH-utilizing enzymes to be more efficient at reducing substrates than they would be if the entire cell had a single redox balance. Finally, cofactors are charged and do not diffuse out of cells. These evolutionary advantages do not accrue to enzyme-catalyzed synthesis using isolated enzymes in vitro. In these cases, cofactors are a disadvantage since they are unstable and add complexity and cost to the syntheses.

Recognizing this need for enzymes that directly reduce substrates with hydrogen, researchers created hybrids of chemical hydrogenation catalysts and proteins.[2] One approach was an indirect link. Researchers bound the metal catalyst to a water-soluble ligand (often a phosphine) and then linked the ligand to the protein. Most researchers used a phosphorus ligand containing a biotin moiety, which binds tightly to the protein avidin.[3] In the best cases—hydrogenation of α-acetamidoacrylic acid and α-acetamidocinnamic acid—the catalysts were highly enantioselective ($\leq$96% *ee*) and gave high yields.[3c–d] Another group bound a rhodium–

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phosphine complex to a monoclonal antibody.[4] The catalyst showed high enantioselectivity for one substrate, but the yield was disappointingly low (23%). The disadvantage of this indirect linking is that optimization still involves multistep synthesis of the ligands and linkers or generation of monoclonal antibodies.

A second approach is directly linking hydrogenation catalysts such as rhodium(I) or rhodium(II) complexes to protein surfaces. Unfortunately, rhodium and similar metals bind at multiple sites so the resulting catalysts are nonselective. For example, a rhodium(II) complex—$[Rh_2(carboxylate)_4]$ binds weakly to serum albumin ($K \approx 10^3$) in a molar ratio of about 8 Rh per serum albumin.[5] The rhodium probably binds to the side chains of cysteine (one residue in human serum albumin) and histidine (sixteen residues). Circular dichroism shows a loss in helicity indicating that the binding distorts the protein's secondary structure. In some cases, the binding increased the stability of the albumin to unfolding, possibly by forming cross-links between several imidazoyl groups.[6] Marchetti and co-workers used a similar complex (serum albumin and a 30-fold molar excess of rhodium(I)) to catalyze hydroformylation. A preliminary report suggested some selectivity, but the full paper did not report any stereoselectivity.[7] A direct-linking approach that did yield a size-selective hydrogenation catalyst was the entrapment of 460 palladium atoms within the 12 nm-diameter cavity of apoferritin.[8] Presumably, the larger substrates could not enter the pores of the apoferritin and thus did not react.

A third approach—not yet realized—is to replace the active-site metal of a metalloenzyme with rhodium or a similar metal. Carbonic anhydrase is a good starting point for several reasons. First, both zinc (atomic number 30) and rhodium (atomic number 45) have similar ionic radii,[9] so the rhodium may not distort the active site. Second, the metal ligands are the imidazoyl groups of three histidines,[10] which are also good ligands for rhodium. Third, bovine carbonic anhydrase lacks cysteine residues that could interfere with binding of rhodium in the active site; human carbonic anhydrase has one deeply buried cysteine residue. Fourth, we, and others, previously replaced the active-site zinc in carbonic anhydrase (CA) with manganese to create a catalyst for the enantioselective epoxidation of styrene.[11]

Herein, we describe our replacement of the active site zinc in carbonic anhydrase with rhodium, our strategies to minimize binding of extra rhodium ions at the protein's surface and the *cis*-selective hydrogenation of stilbene with the resulting catalyst.

Results

Replacing zinc with rhodium in carbonic anhydrase: Previous researchers developed methods to replace the active site zinc in carbonic anhydrase with another metal ion.[12] Dialysis of carbonic anhydrase against zinc chelator—2,6-pyridinedicarboxylate—removes the zinc to form apo(carbonic anhydrase). A new metal ion added to the solution will bind forming metal-substituted carbonic anhydrase (Figure 1) We used bovine carbonic anhydrase isoenzyme II (bCAII, commercially available), human carbonic anhydrase isoenzyme II (hCAII, produced by a recombinant strain of *E. coli* according to Fierke's procedure[13]) as well as variants of hCAII where selected histidine residues were replaced by other amino acid residues, see below. We tested several rhodium(III) and rhodium(I) salts as metal sources. $[Rh(cod)_2]BF_4$ (cod = 1,5-cyclooctadiene) gave a rhodium-carbonic anhydrase complex with the highest activity in hydrogenation and the most stable to precipitation. We used $[Rh(cod)_2]BF_4$ for all subsequent experiments.

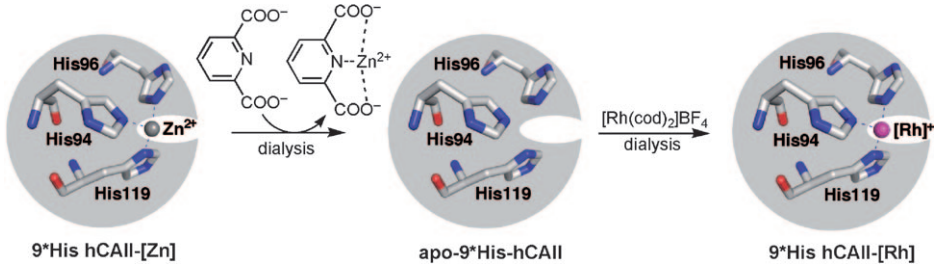


Figure 1. Dialysis of carbonic anhydrase against a zinc chelator—2,6-pyridinedicarboxylate—removed 90–95% of the active-site zinc. Subsequent dialysis against a solution of $[Rh(cod)_2]BF_4$ yielded rhodium(I)-substituted carbonic anhydrase. 9*His hCAII is a variant of human carbonic anhydrase isoenzyme II, in which site-directed mutatgenesis replaced nine of the histidine residues on the surface by arginine, alanine, or phenylalanine residues. cod = 1,5-cyclooctadiene.

Like previous researchers that prepared rhodium–albumin complexes,[5,6] we found that carbonic anhydrase forms stable, pale yellow complexes with the rhodium salts and that each carbonic anhydrase binds multiple rhodium ions (Table 1). Inductively coupled plasma mass spectrometry (ICP-MS) revealed the amount of rhodium, while a Bradford dye-binding assay[14] revealed the amount of carbonic anhydrase. Both bovine carbonic anhydrase (Table 1, entry 1) and human CA (Table 1, entry 3) complexed an average of 7.5 and 6.5 rhodium ions, respectively.

Minimizing the surface-bound rhodium: We hypothesized that the extra rhodium binds on the protein surface most likely to the side chains of histidine or lysine. Bovine CA II contains eight histidine and eighteen lysine residues outside the active site, while the human enzyme[15] contains nine and twenty-four, respectively.[16] We tested this hypothesis by selective chemical modification of the lysine and either site-directed mutagenesis or a combination of chemical modification and site-directed mutagenesis for the histidine residues.

Table 1. Rhodium to protein ratios of various rhodium–carbonic anhydrase complexes.[a]

Entry	Protein	Total Rh	Extra Rh
1	bCAII-[Rh]	7.5	6.5
2[b]	Acetyl-bCAII-[Rh]	10.0	9.0
3	hCAII-[Rh]	6.5	5.5
4	H4/10R + H17F-[Rh]	4.3	3.3
5	H3/4/10R + H17F-[Rh]	4.2	3.2
6[c]	9*His hCAII-[Rh]	1.8	0.8
7[d]	DEPC-H4/10R + H17F-[Rh]	2.3	1.3

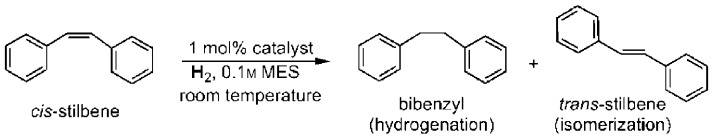
[a] Rhodium content was measured by inductively coupled plasma mass spectrometry, while the protein content was measured by Bradford dye assay at 595 nm. bCAII = bovine carbonic anhydrase II (entries 1 and 2); hCAII = human carbonic anhydrase II (entries 3–7). All variants refer to hCAII. [b] Treated with acetic anhydride to block the ε-amino group of lysine residues. [c] 9*His refers to the mutant H3/4/10/36R + H15/17/107/122F + H64A, in which the 9 histidine residues were replaced by arginine, phenylalanine, or alanine as indicated. [d] hCAII was treated with diethylpyrocarbonate to block the imidazole amino group of the histidine residues before replacement of zinc with rhodium.

Acetylation of the ε-amino group of lysine decreases their basicity and ability to bind metals. Acetylation of bovine carbonic anhydrase with acetic anhydride[17] added an average of fourteen acetyl groups (see mass spectra in the Supporting Information). Surprisingly, this acetylation *increased* the number of bound rhodium ions from 7.5 to 10.0 (Table 1, entries 1 and 2). The increase may be due to nonspecific binding caused by the increased negative charge on the acetylated carbonic anhydrase. Bovine carbonic anhydrase is slightly negatively charged at pH 7 (its isoelectric point is 6.4), and acetylation of fourteen lysine residues increases the overall negative charge by fourteen. This increase in the number of bound rhodium ions indicates that lysine residues probably do not bind the extra rhodium ions.

Diethylpyrocarbonate (DEPC), a histidine-selective reagent, adds an ethoxycarbonyl group to one of the imidazole nitrogens. This addition of an electron-withdrawing group makes the remaining imidazole nitrogen a poorer ligand. Unfortunately, treatment of hCAII with DEPC to modify all nine non-active-site histidines (see Figure S1 in the Supporting Information), yielded an unstable protein,[18] so we used either site-directed mutagenesis or a combination of site-directed mutagenesis and modification with DEPC to block the binding of rhodium to surface histidines. In the site-directed mutagenesis approach, we replaced these nine histidine residues in hCAII with arginine (maintains positive charge), phenylalanine (maintains shape and hydrophobic character), or alanine (creates more space nearby the active site). This variant yielded a rhodium–CA complex that had only 0.8 extra Rh. In the chemical modification approach, we used variant H4/10R + H17F because it was more stable than the wild-type hCAII. Treatment of H4/10R + H17F-hCAII with DEPC to modify the six remaining surface histidines followed by replacement of the active site zinc with rhodium yielded a protein that bound only 1.3 extra rhodium ions. These results indicate that the extra rhodium likely binds to the non-active-site histidine residues. Thus, two approaches—site-directed mutagenesis and combined site-directed mutagenesis with chemical modification—minimized the binding of rhodium to the surface of carbonic anhydrase while allowing a rhodium to bind to the active site. As a result, we could test whether this hybrid catalyzes hydrogenation and whether the protein controls the selectivity of the hydrogenation.

Hydrogenation: As expected, native carbonic anhydrase, which contains zinc in the active site, does not catalyze hydrogenation of *cis*-stilbene (Table 2, entry 1). Substituting

Table 2. Hydrogenation and isomerization of *cis*-stilbene catalyzed by rhodium-carbonic anhydrase complexes.[a]



Entry	(Enzymatic) catalysts	H_2 [atm]	H[b] [%]	I[c] [%]	H/I[d] ratio
1	bCAII-[Zn]	1	–	–	–
2	bCAII-[Rh]	1	15.8	16.4	0.9
3[e]	hCAII-[Rh]	1	4.2	5.8	0.7
4	hCAII-[Rh]	5	55.5	15.8	3.5
5	H4/10R + H17F-hCAII-[Rh]	1	21.3	21.2	1.0
6	H4/10R + H17F- hCAII-[Rh]	5	80.5	19.0	4.2
7	H3/4/10R + H17F- hCAII-[Rh]	1	11.6	9.8	1.2
8	H3/4/10R + H17F- hCAII-[Rh]	5	42.5	5.6	7.6
9	DEPC-H4/10R + H17F-hCAII-[Rh]	5	46.8	2.8	16.7
10[f]	9*His- hCAII-[Rh]	5	55.6	2.7	20.6
11	$[Rh(cod)_2]BF_4$	5	80.0	12.6	6.3

[a] Reaction time 12 h. MES = 2-(*N*-morpholino)ethanesulfonic acid. [b] Hydrogenation. [c] Isomerization. [d] Hydrogenation/isomerization ratio. [e] Entries 3–9 refer to human CA or variants. [f] 9*His-hCAII refers to variant H3/4/10/36R + H15/17/107/122F + H64A, in which nine histidine residues were replaced by the amino acids indicated.

the zinc by rhodium yields a hydrogenation catalyst for *cis*-stilbene (Table 2, entries 2–4; note the change in H_2 pressure for entries 4, 6, 8–11). Complexation to the protein slightly slows the hydrogenation as indicated by the lower hydrogenation conversion for hCAII-[Rh] as compared to uncomplexed rhodium, 55 versus 80% (Table 2, entries 4 vs. 11). In both cases, isomerization of *cis*-stilbene to the more stable *trans*-stilbene accompanied the hydrogenation—the ratio of hydrogenation to isomerization is 3.5 (Table 2, entry 4) and 6.3 (Table 2, entry 11).

We hypothesized that the rhodium outside the active site catalyzed most of the isomerization, while the rhodium in the active site catalyzed mostly hydrogenation. Consistent with this hypothesis, variants of carbonic anhydrase binding fewer extra rhodium atoms showed less isomerization and therefore elevated ratios of hydrogenation to isomerization. The DEPC-H4/10R + H17F-hCAII-[Rh] variant showed a hydrogenation to isomerization ratio of 16.7 (Table 2, entry 9) and 9*His-hCAII-[Rh] showed a ratio of 20.6 (Table 2, entry 10). These ratios are 2.7 and 3.3-fold higher than the ratio for $[Rh(cod)_2]BF_4$ (Table 1, entry 11). These

results demonstrate that the protein environment controls reactivity—it reduces the amount of isomerization to the *trans* isomer during hydrogenation.

To confirm that the rhodium at the active site catalyzes hydrogenation, we compared the hydrogenation of a rhodium–carbonic anhydrase hybrid to a variant where zinc remained in the active site, but rhodium bound to the surface. This variant catalyzed mainly isomerization (Table 3, entry 2), whereas the variant containing rhodium both in the active site and the surface catalyzed both hydrogenation and isomerization (Table 2, entry 1). If we assume that the surface-bound rhodium behaves similarly in the two cases, then 80% of the hydrogenation occurs in the active site for the variant containing rhodium in both the active site and on the surface. Similarly, about 25% of the isomerization occurs in the active site.

Table 3. Hydrogenation of *cis*-stilbene catalyzed by rhodium-substituted hCAII and a variant that retains zinc in the active site, but has rhodium on the surface.[a]

Entry	Enzymatic catalysts	H[b] [%]	I[c] [%]	H[b] at active site [%][d]
1	H4/10R+H17F-hCAII-[Rh]	21.3	21.2	80.3
2	H4/10R+H17F-hCAII-[Zn]-[Rh]	4.2	16.0	–

[a] Reaction conditions: 1 atm H_2, 12 h reaction time, at room temperature. The H4/10R+H17F variant contains 3.3 extra rhodium ions. We chose this variant for the experiment because it is more stable than other variants. [b] Hydrogenation. [c] Isomerization. [d] (21.3–4.2)/21.3 = 80.3%.

Stereoselective hydrogenation of *cis*-stilbene over *trans*-stilbene: The lower amount of isomerization of *cis*-stilbene to *trans*-stilbene during hydrogenation by the rhodium–carbonic anhydrase hybrids with the least extra rhodium ions suggests that these catalysts show stereoselective hydrogenation favoring *cis*-stilbene over *trans*-stilbene. $[Rh(cod)_2]BF_4$ catalyzed hydrogenation of both *cis*- and *trans*-stilbene with similar degrees of conversion under our conditions: 80% versus 57% conversion (Table 4, entries 1 vs. 2; stereoselectivity approximately 1.4). In contrast, rhodium–carbonic anhydrase hybrids favored the hydrogenation of *cis*-stilbene over *trans*-stilbene by more than 20-fold: 80.5% versus 3.5% conversion (Table 4, entries 3 vs. 4, for H4/10R+H17F-hCAII-[Rh]), 55.6% versus 2.1% (Table 4, entries 5 vs. 6, for 9*His-hCAII-[Rh]).

Table 4. Stereoselective hydrogenation of *cis*- over *trans*-stilbene.[a]

Entry	Catalysts	Substrate isomer	H[b] [%]	I[c] [%]	*cis*/*trans*[d]
1	$[Rh(cod)_2]BF_4$	*cis*	80.0	12.6	1.4
2	$[Rh(cod)_2]BF_4$	*trans*	57.5	5.7	
3	H4/10R+H17F-hCAII-[Rh]	*cis*	80.5	19.0	23.0
4	H4/10R+H17F-hCAII-[Rh]	*trans*	3.5	–[e]	
5	9*His- hCAII-[Rh]	*cis*	55.6	2.7	26.4
6	9*His- hCAII-[Rh]	*trans*	2.1	–[e]	

[a] Reactions at 5 atm H_2, room temperature, 12 h. [b] Hydrogenation. [c] Isomerization. [d] Hydrogenation conversion ratio of *cis*/*trans* by the same catalyst. [e] Conversion was too low to determine.

Computer modeling was consistent with the notion that *cis*-stilbene fits in the active site of human carbonic anhydrase II better than *trans*-stilbene. Reliable molecular mechanics parameters were not available for rhodium, so the model contained zinc in the active site. This modeling identifies the fit of the two stereoisomers in the active site, but does not model the hydrogenation reaction itself.[19] One phenyl group of the stilbene stereoisomers fits into a hydrophobic pocket in the active site largely defined by Val121, Val143, Leu198, and Trp209 (Figure 2).[20] The geometry-

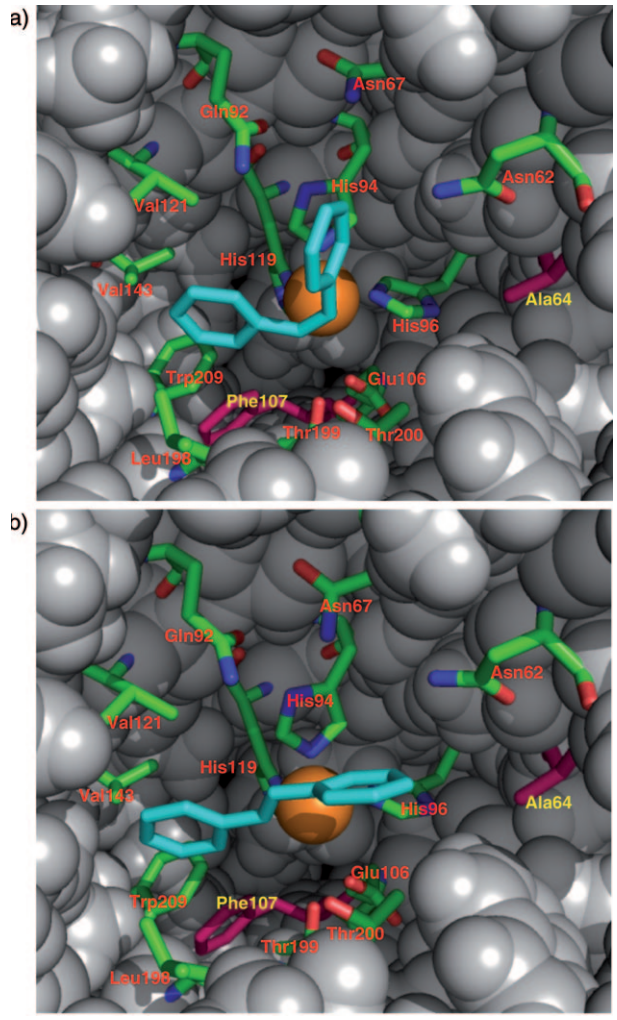


Figure 2. Modeling fits the double bond of *cis*-stilbene facing the metal ion in human carbonic anhydrase II (a), but the double bond of *trans*-stilbene does not face the metal ion (b). The zinc ion is the orange ball and amino acids nearest the active site are shown as sticks in CPK colors. Two histidines close the active site (His64, His107) are among those replaced by other amino acids. The residues are shown as the replaced amino acids (Ala64, Phe107) in pink. The surrounding protein atoms are in grey space-filling representation and the *cis*- or *trans*-stilbene are shown as sticks in cyan. Modeling included the water molecules defined in the X-ray crystal structure, but these are not shown in the figure for clarity. The hydrophobic pocket of the active site is largely defined by Val121, Val143, Leu198, and Trp209.[20]

optimized structures placed *cis*-stilbene close to the metal ion (3.8 and 4.3 Å between the olefinic carbons and the zinc[21]), but placed *trans*-stilbene farther away (4.7 and 5.7 Å). Hydrogenation would require that the π-electron cloud of the double bond face the metal center. This is the orientation seen for *cis*-stilbene, but *trans*-stilbene orients in a manner that is unlikely to be catalytically productive. Manually reorienting *trans*-stilbene closer to metal ion with its double bond facing the metal creates steric clashes with Leu 198, Thr 200, His 94/96, Asn 62, and Ala 64.

No stereoselectivity for *cis*- versus *trans*-3-hexen-1-ol: A smaller substrate, 3-hexen-1-ol, showed similar conversions in the hydrogenation of either *cis* or *trans* isomers. Further, *cis–trans* isomerization accompanied the hydrogenation (see the Supporting Information), suggesting that the rhodium–carbonic anhydrase is not stereoselective for this olefin. Computer modeling supported this suggestion since the model placed both *cis*- and *trans*-3-hexen-1-ol at similar distances above, rather than in, the active site, possibly due to only weak interactions with the hydrophobic region of the active site.

Preliminary hydrogenation experiments with prochiral substrates (e.g. methyl α-acetamidoacrylate or methyl α-acetamidocinnamate) yielded $<10\,\%$ *ee* in the products. This poor enantioselectivity may be due to the spaciousness around the active site.

Discussion

Removal of the active site zinc from bovine or human carbonic anhydrase isoenzyme II followed by addition of the rhodium(I) salt $[Rh(cod)_2]BF_4$ gave rhodium–carbonic anhydrase complexes each containing 6.5–7.5 rhodium ions. We hypothesized that one of these rhodium ions bound to the active site and the others on the surface of the carbonic anhydrase. Modification of the histidine residues on the surface either by site-directed mutagenesis or a combination of site-directed mutagenesis and chemical modification reduced the amount of rhodium bound to approximately two. Presumably one binds in the active site and the other on the protein surface. This ability to minimize nonspecific binding allowed the behavior of a metal hydrogenation catalyst to be tested for the first time at the active site of a metalloenzyme.

The rhodium–carbonic anhydrase complexes with minimal extra rhodium showed about 20:1 selectivity for *cis*-stilbene over *trans*-stilbene. Evidence for this stereoselectivity is the faster hydrogenation of *cis*-stilbene as compared to *trans*-stilbene and the lower amount of isomerization of *cis*-stilbene to *trans*-stilbene during hydrogenation. Modeling suggests that the shape of the active site (steric effects) favors binding *cis*-stilbene, but not *trans*-stilbene, in a catalytically productive orientation. This is the first biocatalyst that directly reduces a substrate with hydrogen gas without an added phosphine ligand. This biocatalyst would fit into enzyme classification 1.12.99: oxidoreductase acting on hydrogen as a donor with other acceptors. Current examples in this classification reduce cofactors or other enzymes; none reduce an organic substrate, so this hybrid catalyst is the first example.

Several issues still limit the application of this catalyst to synthesis. First, even with all the histidines blocked or replaced, one extra rhodium remains, which may contribute to catalysis and lower the selectivity. Reducing the overall negative charge of the carbonic anhydrases may remove this last extra rhodium. The modifications we made to remove or modify histidine (partially positively charged amino acid side chain) likely increased the overall negative charge of the protein. This increased negative charge may help bind the extra rhodium ion to the surface. Second, the protein does not accelerate the hydrogenation reaction. Hydrogenation by $[Rh(cod)_2]BF_4$ gives a slightly higher conversion than 9*His-hCAII-[Rh] (Table 2, entries 10 and 11) suggesting that all the catalytic power comes from the rhodium. Modification of the active site should be able to increase the rate of hydrogenation. Third, the substrate range of the catalyst has not been explored. If it proves to be limited, mutagenesis guided by either rational design or directed evolution may expand it.

Nature also used the metal replacement approach to create new catalytic activities. For example, the vicinal oxygen chelate superfamily consists of proteins with similar structure, but different metals bound at the active site to catalyze different reactions.[22] Glyoxylase I (zinc or nickel in active site) and methylmalonyl-CoA epimerase (cobalt in active site) catalyze rearrangements via acid–base reactions involving similar enediol intermediates. In contrast, extradiol dioxygenases (iron or manganese in active site) catalyze oxidative *meta* ring cleavage of catechol or similar substrates. Fosfomycin resistance protein (manganese at active site) catalyzes nucleophilic attack of glutathione on the epoxide ring of fosfomycin.

Previous work on changing the catalytic activity of metalloenzymes by replacing the active site metal ion involved only oxidation reactions. The first example was replacement of the active-site zinc(II) in carboxypeptidase with copper(II) to create a slow oxidase.[23] Recently, we and others replaced the active-site zinc in carbonic anhydrase with manganese to create an oxidase.[11] There are two additional borderline examples. Sheldon and co-workers removed the active site zinc from the protease thermolysin and added not a metal ion, but an oxoanion—tungstate, molybdate, or selenate.[24] The resulting complex catalyzed oxidations, but this experiment was unlikely a simple replacement. Zinc and the oxoanions have opposite charge and differ dramatically in size, so they likely bind in different places. Another borderline example is not a replacement because the starting enzyme did not contain a metal ion. Two groups started with a phosphatase that contains no metal ion and added vanadate, a transition state analog for phosphate hydrolysis.[25] The resulting complexes catalyzed the enantioselective oxidation of sulfides.

In the future this active-site-metal-replacement approach may greatly expand the catalytic range of enzymes. Natural enzymes do not contain precious metals because they are rare in nature. Examples of interesting transition-metal-catalyzed reactions that occur in water include hydroformylation, cycloaddition, olefin metathesis and Heck-type reactions.[26] An important advantage of protein-based catalysts is ease of optimization using directed evolution. Unlike tedious chemical synthesis of chemical ligands, molecular biology methods can create thousands or millions of protein variants and screening can identify those with improved properties. Carbonic anhydrases occur with three different protein folds,[27] so there are several starting points for creation of new catalysts; in addition, other metalloenzymes may also be good starting points.

Experimental Section

General: The Supporting Information contains details for preparation of human carbonic anhydrase isoenzyme II and its variants. Dialysis experiments used Spectra/Por 2 tubing with a molecular weight cut off of 12–14000 g mol^{-1}.

Replacement of zinc in carbonic anhydrase with rhodium: A dialysis tube containing carbonic anhydrase (75 mg, 2.4 μmol) in buffer (0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.0, 5.0 mL) was dialyzed overnight against 2,6-pyridinedicarboxylic acid (2,6-PDCA, 557 mg, 3.34 mmol) in sodium acetate buffer (500 mL, 0.1 M, pH 5.5). The tube was removed and dialyzed for 20 h against buffer (50 mM MES, pH 6.0, 500 mL) to remove the excess of 2,6-PDCA and yield apo(carbonic anhydrase). The rhodium ion was introduced by dialysis for 10 h against $[Rh(cod)_2]BF_4$ (203 mg, 0.5 mmol in 500 mL of 50 mM MES buffer, pH 6.0). To remove the traces of unbound rhodium the dialysis tubing was dialyzed twice for 2.5 h against buffer (50 mM MES, pH 6.0). The total volume of Rh-substituted CA solution increased by about 10% (to 5.5 mL) during the entire process. During the preliminary work, we tested $RhCl_3$, $[(Cp^*RhCl_2)_2]$ (Cp^* = pentamethylcyclopentadiene), and $[Rh(CO)_2(acac)]$ (acac = acetylacetonato), but found that these yielded either lower hydrogenation or less stable protein. A ruthenium metal precursor, $[\{Ru(p\text{-cymene})Cl_2\}_2]$, was also tested, but after dialysis against buffer, most of the Ru^{II} was lost suggesting that it did not bind the protein.

Modification of human carbonic anhydrase by diethylpyrocarbonate (DEPC): Human carbonic anhydrase was modified by DEPC according to a literature procedure for protein modification.[28] DEPC solution (69 μL, 27.6 μmol of 400 mM DEPC in 30% ethanol) was added to a solution of human carbonic anhydrase II (20 mg mL^{-1}, 0.685 μmol, in 2.0 mL of a mixture of 50 mM MES buffer and 0.10 M borate buffer, pH 7) and stirred for 20 min at room temperature. The mixture was dialyzed overnight against MES buffer (400 mL of 50 mM, pH 6.0). ESI-MS spectra (see Supporting Information, Figure S2) showed a mixture of proteins with increased molecular weight consistent with addition of 8–12 carboxyethoxyl moieties.

Typical hydrogenation procedure:[29] Metal-substituted carbonic anhydrase (200 μL of 0.225 μmol mL^{-1}, 0.045 μmol) followed by substrate (18 μL of 0.25 M in CH_3CN, 4.5 μmol) was added to a 5 mL vial containing a stirring bar (7 mm) and MES buffer (200 μL, 50 mM, pH 6.0). The vial was placed in an autoclave; the autoclave was sealed, filled with hydrogen to 7 atm and then released. The fill–release was repeated twice more and the hydrogen pressure was adjusted to 1 or 5 atm. The reaction was stirred for 12 h, then extracted with ethyl acetate and the extract analyzed by gas chromatography. This procedure was also used for hydrogenation of *trans*-stilbene, *cis*- or *trans*-3-hexene-1-ol and hydrogenation catalyzed by $[Rh(cod)_2]BF_4$ (in 50 mM MES buffer, pH 6.0). The hydrogenation of stilbene was analyzed on a chiral column Chirasil-Dex CB 25 M × 0.25 (oven temperature: 150 °C, injector temperature 250 °C, detector temperature 300 °C); flow rate: 1.2 mL min^{-1}; retention time of *cis*-stilbene, 5.2 min; retention time of bibenzyl, 5.5 min; retention time of *trans*-stilbene 13.6 min. The details for the hydrogenation of 3-hexene-1-ol are in the Supporting Information.

Molecular modeling: Molecular modeling was performed with Maestro (version 8.0, Schrödinger, New York, NY) using the Merck molecular force field (MMFF)[30] starting with the X-ray crystal structure of zinc-containing human carbonic anhydrase II at 2.0 Å resolution, PDB file ID: 4CAC.[15] Even though the zinc-containing enzyme does not catalyze hydrogenation, we did not replace it with rhodium because molecular mechanics parameters for rhodium are limited and because we were interested in the fit of the isomeric alkenes in the active site, not the metal-olefin interaction. The dielectric constant was set to 1 to mimic the solvation effects of water. Hydrogen atoms were added and His 119 was changed to its less common tautomer because the X-ray structure shows that this tautomer coordinates to zinc. The structure was geometry optimized stepwise. First, the geometry of the hydrogen atoms on water were optimized, next the hydrogen atoms on the protein, and then the whole protein with water molecules, finally the water, whole protein and the bound substrate (*cis*- or *trans*-stilbene). Each geometry optimization step used the PolaRibiere conjugated gradient (PRCG) method with a maximum of 500 interations and a convergence threshold of 0.05 kcal Å^{-1} mol^{-1}.

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