The Hydricity and Reactivity Relationship in [FeFe]-hydrogenases

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Article

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Abstract

Reactivity of transition metal catalysts is controlled by covalent and non-covalent interactions that tune thermodynamic properties including hydricity. Hydricity is critical to catalytic activity and for modulating the reduction or oxidation of chemical compounds. Likewise, enzymes can employ transition metal cofactors and use metal-hydride intermediates tuned by protein frameworks to selectively control reactivity. One example, the [FeFe]-hydrogenases, catalyze reversible H\textsubscript{2} activation with H\textsubscript{2} oxidation to H\textsuperscript{+} reduction ratios spanning $\sim 10^7$ in rate, offering a model to determine the extent that hydricity controls reactivity. To address this question, the hydricity of the catalytic H cluster of two [FeFe]-hydrogenases, CpI and CpII, were compared. We show that for CpI, the higher rates of H\textsuperscript{+} reduction correspond to a more hydridic H cluster, whereas CpII, which strongly favors H\textsubscript{2} oxidation, has a less hydridic H cluster. The results demonstrate that enzymes manipulate metal cofactor hydricity to enable an extraordinary range of chemical reactivity.

Introduction

Metal-ligand complexes have fundamental roles in reduction-oxidation reactions, where covalent and non-covalent interactions strongly influence the metal-ligand reactivity.\textsuperscript{1} One class of metal-ligand complexes, the transition metal hydrides, functions as intermediates in a broad number of chemical transformations.\textsuperscript{2,3} For example, in small molecule activation reactions, hydricity of a catalytic metal site strongly influences the control of turnover rates and end-product specificities.\textsuperscript{4} This effect of metal hydricity on a reduction-oxidation reaction is exemplified by control of H\textsubscript{2} reactivity in Ni-diphosphine complexes.\textsuperscript{5} In these series of compounds, a more hydridic Ni-site strongly favors reduction of protons to form H\textsubscript{2}, whereas weakly hydridic Ni sites favor H\textsubscript{2} binding and activation.\textsuperscript{6} A caveat is that proton donor-acceptor p\textsubscript{Ka} influences the reactivity trends, and matching of hydricity to p\textsubscript{Ka} provides a means to further tune the end-product preferences of metal hydride based catalysts and their chemical reactions.\textsuperscript{7,8}

Transition metals are critical to biological energy transformation reactions and are found in many enzyme active sites, including the FeMo cofactor in nitrogenase, leading to reduction of dinitrogen to ammonia,\textsuperscript{9} the tungsten- or molybdenum-containing formate dehydrogenases, which reversibly reduce CO\textsubscript{2} to formate,\textsuperscript{10} methyl coenzyme reductase,\textsuperscript{11} which catalyzes the reduction of CH\textsubscript{3}-S-CoM to methane, and hydrogenases, which catalyze reversible H\textsubscript{2} activation chemistry.\textsuperscript{12,13} For the latter, striking differences in preferences for H\textsubscript{2} oxidation or H\textsubscript{2} production reactivity for a series of three unique [FeFe]-hydrogenases from Clostridium pasteurianum has been shown.\textsuperscript{12,14} Each [FeFe]-hydrogenase incorporates a organometallic, iron-sulfur rich H cluster (Fig. 1), with additional iron-sulfur clusters, or F clusters, that can provide electron transfer functions.\textsuperscript{15} The H cluster is composed of a [4Fe-4S] cubane linked by a protein cysteine thiolate ligand to a organometallic, diiron subsite ([2Fe]). The [2Fe] subsite has pairs of terminal CO and CN (t-CO and t-CN) ligands, a bridging CO (µ-CO) of the Fe atom pair, and a bridging azadithiolate ligand (Fig. 1).\textsuperscript{16–19} The CO and CN ligands are critical for tuning the H cluster
electronic structure for heterolytic bond cleavage of H₂, where the µ-CO contributes a significant trans-effect for chemical H₂ activation at the distal Fe site (FeD) of [2Fe] subsite.²⁰,²¹ The residues surrounding the H cluster further modulate electronic structure,²²−²⁴ electrostatics,¹² and redox potentials,²⁵ and observed differences in residue compositions may explain the reactivity variations among [FeFe]-hydrogenases. In support of this hypothesis, differences in the local H cluster electrostatics have been proposed to account for the variations in reactivity among the different C. pasteurianum [FeFe]-hydrogenases.¹²

Among the C. pasteurianum [FeFe]-hydrogenases, [FeFe]-hydrogenase II, or CplII, has a unique preference for H₂ oxidation, with a 10⁴-fold higher reaction rate than proton reduction whereas C. pasteurianum [FeFe]-hydrogenase I, or Cpl, has high rates of both proton reduction and H₂ oxidation, or more neutral reactivity.¹²,²⁶ The catalytic site of CplII also differs from Cpl due to several changes in nearby non-covalent amino-acid interactions that are proposed to create a more hydrophobic environment around the catalytic site H cluster.¹² In this study, the biophysical properties of the CplII catalytic intermediates were compared to Cpl in order to determine if there are underlying electronic properties that change H cluster thermodynamics to account for differences in reactivity. The outcomes clearly demonstrate that the CplII H cluster electronic structure is unique among [FeFe]-hydrogenases, and that the reactivity differences result from substantial changes in the H cluster hydricity that tune reactivity to favor H₂ oxidation.

Results And Discussion

CplII Resting State Electronic Structure. To identify possible differences in the electronic structure of the H cluster in CplII compared to other [FeFe]-hydrogenases, EPR and FTIR measurements were initially carried out on the well-characterized resting state of the enzyme, H₀x. For CplII, H₀x has a unique S=1/2 EPR signal, with g-values of 2.08, 2.03, and 2.00 (Figure S1). However, the Topt value was 40 K,²⁷ and higher than the Topt average of 15 K for other [FeFe]-hydrogenases with differing reactivity profiles (Table S1). The difference in the relaxation property of the signal suggests that the H cluster in CplII has more [2Fe-2S] cluster character²⁸ compared to [FeFe]-hydrogenases with more neutral reactivity such as Cpl, suggesting that CplII may have subtle differences in its electronic structure or distribution of spin on [2Fe-2S]. The corresponding FTIR spectra (Fig. 2) of the resting state CplII (H₀x) has νCN bands at 2082 and 2069 cm⁻¹, and terminal νCO bands at 1969 and 1944 cm⁻¹, however the νCO band of the µ-CO that bridges the diiron sub-site Fe atoms was at 1752 cm⁻¹, or ~50 cm⁻¹ downshifted compared to the FTIR spectra of H₀x for Cpl (Table 1) and other [FeFe]-hydrogenases (Table S2). This signifies an increase of π back-bonding from FeD → µ-CO in CplII, which is further illustrated by the differences in the FTIR spectrum of H₀x sample treated with CO (H₀x-CO). CO is a π-acceptor ligand that terminally binds at the ligand exchangeable site of the FeD atom (Fig. 1).²¹ For CplII, the H₀x-CO form has an exogenous νCO band at 2023 cm⁻¹, which is upshifted by 6 cm⁻¹ relative to same band at 2017 cm⁻¹ in Cpl H₀x-CO (Fig. 2, Table 1). Thus, the downshift of µ-CO frequency in CplII (greater π back-bonding from FeD into µ-CO)
compared to CpI is also matched by an upshift in \( t\)-CO frequency (less \( \pi \) back-bonding from Fe\(_D\) into \( t\)-CO) in CpII relative to CpI (Table 1), owing to differences in the underlying H cluster electronic structures between the two enzymes. Collectively, the EPR and FTIR properties of resting state CpII indicate differences in electronic structure compared to CpI, which is likely to affect the properties of catalytic intermediates.

| Enzyme | State  | Terminal \( v\)CO | \( \Delta v\)CO |
|--------|--------|------------------|----------------|
| CpI\(^a\) | \( H_{ox} \) | 1971, 1948 | 23 |
| CpII | \( H_{ox} \) | 1969, 1944 | 25 |
| CpII\(^{c\rightarrow s}\) | \( H_{ox} \) | 1973, 1950 | 23 |
| CpI\(^a\) | \( H_{ox}^{\cdots}CO \) | 2017, 1974, 1971 | 43, 3 |
| CpII | \( H_{ox}^{\cdots}CO \) | 2023, 1975, 1960 | 48, 15 |

\(^a\) values for CpI.\(^{56}\)

Properties of the Reduced Intermediates of CpII. Redox poising of [FeFe]-hydrogenases at defined potentials in combination with EPR and FTIR spectroscopy can identify the spectral signatures of catalytic intermediates that arise from changes in the H cluster oxidation state and electronic structure.\(^{29-32}\) Each of the H cluster intermediates in Fig. 1 has a distinctive EPR and FTIR signal, for example, the reduction of \( H_{ox}\) by 1-electron leads to formation of \( H_{\text{red}}\), and a second reduction step leads to formation of either \( H_{\text{sred}}\), or \( H_{\text{hyd}}\) that has a terminally bound hydride. Poising samples can be used to identify and determine the relative populations of the 2-electron reduced intermediates, \( H_{\text{sred}}\) and \( H_{\text{hyd}}\), and thus inform on the hydricity of the H cluster.\(^{33}\)

When poised under reducing conditions, the EPR spectrum of CpII is composed of two signals, a fast-relaxing signal (\( g = 2.070, 1.936, 1.867 \)) with a \( T_{\text{opt}} \) of 5 K, which can be assigned to \( H_{\text{sred}}\);\(^{34,35}\) and an additional rhombic signal (\( g = 2.058, 1.92, 1.89 \)) with a \( T_{\text{opt}} \) of 15 K, which can be assigned to the reduced, paramagnetic F clusters (Figure S2, Table S3).\(^{26}\) As potentials became more negative, CpII formed only \( H_{\text{sred}}\), and there was no equivalent \( H_{\text{hyd}}\) signal\(^{29,36}\) even at potentials as low as -625 mV (Figure S2). The addition of the natural substrate, \( H_2\) and poising at -490 mV, led to higher enrichments of \( H_{\text{sred}}\) (Table S3), which is a result of \( H_2\) oxidation. Nonetheless, the addition of \( H_2\) also failed to lead to a detectable formation of the hydride intermediate, \( H_{\text{hyd}}\), EPR signal originating from a reduced [4Fe-4S] subsite (Fig. 1).\(^{29}\)
The corresponding FTIR spectra of CplII were measured as it is possible to observe a more complete profile of the reduced state populations due to all the reduced states having unique IR spectral signatures (Table S2). This is evident as collective downshifts of t-CO bands for both the H$_{\text{red}}$ and H$_{\text{sred}}$ states compared to H$_{\text{ox}}$ (Fig. 2) owing to the formal reduction of the H cluster subsites. Under the reducing potentials used here, the H cluster of CplII predominantly equilibrates into the 1-electron reduced H$_{\text{red}}$ state, with a smaller population of the 2-electron reduced H$_{\text{sred}}$ state being observed at -625 mV (Fig. 3A), consistent with the EPR results (Table S3). A spectral feature of the H cluster hydride intermediate, H$_{\text{hyd}}$, in [FeFe]-hydrogenases is the presence of a μ-CO band at ~ 1850–1870 cm$^{-1}$, which arises from a terminally bound hydride at the open coordination site of Fe$_D$ of the [2Fe] subsite. This feature was not detected in the FTIR spectra of CplII poised at reducing potentials (Fig. 3A), consistent with the EPR results from Figure S2, Table S3.

**CplII C→S Traps the 2-electron Reduced Intermediate of CplII.** A strictly conserved cysteine residue in [FeFe]-hydrogenase that is proximal to the H cluster, forms a part of the proton-transfer relay to the active-site. When the cysteine is changed to a serine (C→S) this exchanges a -SH for a -OH, which disrupts proton-transfer and traps the enrichment of the 2-electron reduced state in [FeFe]-hydrogenases. For neutral bias enzymes this results in a pronounced enrichment of the H$_{\text{hyd}}$ state. Thus, a C→S variation of the proton-relay can reveal the preferred 2-electron reduced state, and directly inform on whether the hydricity of the H cluster changes in enzymes that have different catalytic site microenvironments.

The effect of the C→S variation on CplII was determined under redox poising (Fig. 3) and compared to CplII. At the less reducing potential of -375 mV, CplII C→S is primarily poised in the 1-electron reduced state H$_{\text{red}}$ with t-CO bands at 1901 and 1882 cm$^{-1}$ consistent with the addition of electron density to the [2Fe$_H$] compared to the resting state spectrum (Fig. 3). Further reduction of CplII C→S to more reducing potentials led to a more enriched formation of H$_{\text{sred}}$ compared to CplII (Fig. 3A). Likewise, the corresponding EPR spectra recorded at 5 K showed an overall weak signal that increased in intensity at lower reduction potentials due to the increased presence of both H$_{\text{sred}}$ and reduced F clusters (Fig. 3B, Figure S3, Table S3). Overall, the 1906 and 1872 cm$^{-1}$ t-CO bands in CplII C→S are consistent with assignment to the 2-electron reduced H$_{\text{sred}}$ intermediate, which matches to a weaker H$_{\text{sred}}$ t-CO band at 1879 cm$^{-1}$ in CplII poised at -625 mV. Thus, in contrast to what has been observed for neutral bias [FeFe]-hydrogenases, the C→S variation leads to enrichment of H$_{\text{sred}}$ in CplII, supported by a lack of H$_{\text{hyd}}$ in any of the reduced or H$_2$ treated samples.

**H/D Isotope Editing of Reduced CplII.** In order to further corroborate that the preferred 2-electron reduced intermediate of CplII is H$_{\text{sred}}$ rather than H$_{\text{hyd}}$, H/D isotope experiments were carried out under H$_2$ or D$_2$ gas. H/D isotope-editing combined with FTIR spectroscopy of [FeFe]-hydrogenases has been used to identify the H$_{\text{hyd}}$ intermediate, which is observed as a H→D isotope induced shift of the μ-CO IR band due to the trans-effect of the terminally bound hydride on the adjacent Fe$_D$ atom (Fig. 1). Treatment of
CpII with either H₂ (H₂O) or D₂ (D₂O) (Fig. 4) led to a downshift of the νCO bands owing to binding and activation of H₂ or D₂ accompanied by reduction of the resting state H cluster (Fig. 1). The FTIR spectra indicate CpII is mainly poised in the H_{red} state with terminal νCO at 1918 and 1889 cm⁻¹, and a µ-νCO band at 1730 cm⁻¹, with a smaller population of H_{sred} (Fig. 4, top panel). A clear lack of an H/D isotope sensitive µ-νCO band, the defining feature of the H_{hyd} spectrum (Table S2), strongly supports that the H cluster of CpII is tuned to favor H_{sred} in the 2-electron reduced state over H_{hyd}, and again consistent with enrichment of H_{sred} in CpII→S. Due to the lack of an observable “H_{hyd}” state, the H/D exchange activity of CpII was measured in order to determine whether the catalytic mechanism involves heterolytic H₂ activation. In reactions under H₂ in D₂O, purified CpII co-evolved both HD and D₂ (Figure S4), confirming the catalytic mechanism of CpII involves formation of a H_{hyd} state, which due to low hydricity favors H₂ oxidation (Figure S5).

**CpII→S Reactivity has Increased Bias Towards H₂ oxidation.** The spectroscopic properties of CpII→S demonstrate a change in the proton relay that leads to a greater stabilization of H_{sred}, compared to CpII. Based on the observed scaling relationship between H cluster hydricity and enzymatic reactivity in CpII versus Cpl, where H₂ oxidation rates are favored by a less hydridic H cluster, the reactivity of CpII→S is predicted to further shift towards H₂ oxidation compared to CpII. The reactivity ratio of CpII→S for H₂ oxidation-to-proton reduction is 10⁴ versus 10³ for CpII (Table S4), a difference of 10-fold in favor of H₂ oxidation. Thus, a change in the hydricity of the H cluster leads to more favorable formation of H_{sred} over H_{hyd} and favors H₂ oxidation over proton reduction. This effect is accentuated for CpII by a shift in the pKa landscape of proton transfer, where the electronic structure leads to a more acidic FeD relative to the proton relay Cys residue (see Fig. 1). This difference is further magnified in CpII→S where -SH to -OH creates an even larger difference in the pKa between the proton donor and hydride binding sites.

**Conclusions**

Redox enzymes exhibit broad preferences for catalyzing the reduction or oxidation of a chemical substrate. For hydrogenases, several mechanisms have been proposed to account for differences reversibility of H₂ activation as a preference for H⁺ reduction or H₂ oxidation. Due to the fact that hydrogenases share a common catalytic site cofactor and use similar redox intermediates, reactivity differences have been mainly attributed to differences in accessory clusters and extended proton transfer pathways.

A less obvious property of enzymes that is known to profoundly affect reactivity of transition metal complexes is the thermodynamic hydricity. The understanding of hydricity and reactivity is embodied in scaling relationships that directly account for observed rate differences of reduction-oxidation reactions. Model compounds control broad reactivity ranges using primary and secondary sphere chemistry. This relationship is exemplified for Cpl and CpII in Fig. 5 where the dramatic difference in reactivity is a
direct outcome of the thermodynamic hydricity. This result now explains why manipulation of [FeFe]-
hydrogenase catalytic site microenvironments\cite{29,36} can be modeled as changes in hydricity by
computational analysis.\cite{33}

What these studies reveal is that tuning of the H cluster hydricity and pK\textsubscript{a} regimes provides for kinetic
control over reactive intermediates in the heterolytic exchange of H\textsubscript{2}, H\textsuperscript{+}, and electrons (Figure S5).\cite{50} The
H cluster microenvironment in CpI, and likewise for other neutral bias [FeFe]-hydrogenases, favors more
reversible equilibrium of reduced states like H\textsubscript{hyd}\cite{29,38} and H\textsubscript{sred}. In contrast, this work reveals that the
modulation of the H cluster electronic structure in CpII leads to changes in the pK\textsubscript{a} landscape for
protonation-deprotonation of Fe\textsubscript{D}. This effect manifests as differences in the acidity of the Fe\textsubscript{D} site on the
H cluster (i.e., a lower \(\mu\)-CO frequency (50–70 cm\textsuperscript{-1}, Table S2) for all oxidation states due to greater back-
bonding from Fe\textsubscript{D}.\cite{21,51,52} As a result, the Fe\textsubscript{D} site is more acidic, and protonation of the reduced H cluster
is strongly disfavored in CpII, an effect which is strengthened in CpII\textsuperscript{C→S}. In CpII, the outcome is a
significantly less stable H\textsubscript{hyd} state and kinetic equilibrium favoring H\textsubscript{red} and H\textsubscript{sred} and H\textsubscript{2} oxidation.

The organic framework of Ni-phosphines, diiron-organometallic complexes have been extensively
modified to understand how hydricity, pK\textsubscript{a}, and redox potential of metal sites determine catalytic
properties.\cite{53–55} Metal site steric, solvation networks, electronic structure, hydrogen bonding and
electrostatics contribute to the hydricity, by scaling relationships. It is possible that the subtle changes in
the H cluster microenvironment of CpII versus CpI exacts a similar range of control. Model complexes
have an additional layer of control through changing the solvent system or using rare-earth metals.
However, the broad range of reactivities in [FeFe]-hydrogenases demonstrates that reactivity may be
sufficiently tuned using earth-abundant materials and aqueous solvent.

Summary

We have conducted a thorough analysis of the catalytic site electronic structure and relationship to the H\textsubscript{2}
activation mechanism of [FeFe]-hydrogenase CpII. These changes underlie and determine the hydricity to
favor the formal 2-electron reduced catalytic site (lower hydricity) over a hydride bound intermediate, and
reactivity towards H\textsubscript{2} oxidation. Changing the proton-donor amino acid from acidic to basic, further shifts
reactivity towards H\textsubscript{2} oxidation by introducing a barrier to protonation of the hydride bound H cluster.
These changes are likely elicited by subtle differences in the amino acids that comprise the
microenvironment of catalytic site H cluster, establishing a template for how the natural chemistry of
redox enzymes can be utilized to optimize organometallic site reactivity. It may also make possible the
implementation of rational design to reengineer redox enzymes and their reactivities for optimized
production of desired compounds.

Declarations
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**Competing Interests.** The authors declare no competing interests.

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