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A [RuRu] Analogue of an [FeFe]-Hydrogenase Traps the Key Hydride Intermediate of the Catalytic Cycle

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Abstract

The active site of the [FeFe]-hydrogenases features a binuclear $[2\text{Fe}]_{\text{H}}$ sub-cluster that contains a unique bridging amine moiety close to an exposed iron center. Heterolytic splitting of H_2 results in the formation of a transient terminal hydride at this iron site, which, however is difficult to stabilize. We show that the hydride intermediate forms immediately when $[2\text{Fe}]_{\text{H}}$ is replaced with $[2\text{Ru}]_{\text{H}}$ analogues through artificial maturation. Outside the protein, the $[2\text{Ru}]_{\text{H}}$ analogues form bridging hydrides, which rearrange to terminal hydrides after insertion into the apo-protein. H/D exchange of the hydride only occurs for $[2\text{Ru}]_{\text{H}}$ analogues containing the bridging amine moiety.

Abstract

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Conflict of interest

The authors declare no conflict of interest.

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Wechsel im Zentrum: Mit künstlicher Reifung und Ruthenium-Synthesechemie (wurde eine Ru₂-Einheit in das aktive) Zentrum einer [FeFe]-Hydrogenase eingebracht. Das Protein begünstigt die Bildung terminaler Hydride, der Amin-Kofaktor ermöglicht einen Hydrid-Isotopenaustausch, und die Dimetallhydride sind redoxaktiv in der Katalyse.

Keywords

hydrides; hydrogenases; iron; metalloenzymes; ruthenium

The [FeFe]-Hydrogenases catalyze the fundamental reaction $\text{H}_2 \rightleftharpoons 2\text{H}^+ + 2\text{e}^-$.^[1] The active site (H-cluster) contains the binuclear iron complex $[\text{Fe}_2(\text{ADT})(\text{CO})_3(\text{CN})_2]^{2-}$ (ADT = $\text{HN}(\text{CH}_2\text{S}^-)_2$), referred to as $[\text{2Fe}]_{\text{H}}$, linked to a generic $[\text{4Fe-4S}]_{\text{H}}$ cluster. The distal iron features an open coordination site close to the pendant amine base of the bridging azadithiolate (ADT) ligand, which shuttles protons in and out of the active site via the intermediacy of hydride intermediates (Figure 1).^[2] The detailed properties of these hydride states are of great interest.

Mechanistic analysis of [FeFe]-hydrogenases has greatly benefited from the discovery of “artificial maturation”^[3] whereby the apo-enzyme lacking the $[\text{2Fe}]_{\text{H}}$ sub-site but still containing the $[\text{4Fe-4S}]_{\text{H}}$ cluster is heterologously expressed in *E. coli* and subsequently reconstituted with $[\text{Fe}_2(\text{ADT})-(\text{CO})_4(\text{CN})_2]^{2-}$.^[4] While many variants of $[\text{2Fe}]_{\text{H}}$ focused on modified dithiolate cofactors^[3a, 5, 6] have been introduced, the question remains, could $[\text{2Fe}]_{\text{H}}$ be replaced with non-native metals? In this report, we address this question and describe the unexpected consequences. Specifically, we report the characterization of apo-HydA1 from *Chlamydomonas reinhardtii* matured with $[\text{2Ru}]_{\text{H}}$ to afford an artificial $[\text{RuRu}]$ -hydrogenase.

As precursor of $[\text{2Ru}]_{\text{H}}$, we prepared $[\text{Ru}_2(\text{ADT})(\text{CO})_4-(\text{CN})_2]^{2-}$ through cyanation of $[\text{Ru}_2(\text{ADT})(\text{CO})_6]$ by following the protocol for preparing $[\text{Ru}_2(\text{PDT})(\text{CO})_4(\text{CN})_2]^{2-}$ (PDT = $\text{CH}_2(\text{CH}_2\text{S}^-)_2$).^[7] The intermediate $[\text{Ru}_2(\text{ADT})-(\text{CO})_6]$ was obtained using carboxybenzyl-protected ADT $[\text{CbzN}(\text{CH}_2\text{SH})_2]$ according to Scheme 1.

Dissolution of $[\text{Ru}_2(\text{ADT})(\text{CO})_4(\text{CN})_2]^{2-}$ in water resulted in a color change from orange to pale yellow over the course of 15 min yielding $[(\mu\text{-H})\text{Ru}_2(\text{ADT})(\text{CO})_4(\text{CN})_2]^-$. The protonation reaction causes ν_{CO} bands to shift 63–82 cm^{-1} towards higher energy (Figure

2B and Figure S8). The ^1H NMR spectrum of the product exhibits a signal at $\delta = -13.9$ ppm, a region characteristic of bridging hydride ligands.^[8] Similar IR and ^1H NMR signals were observed when a DMSO solution of $(\text{Et}_4\text{N})_2[\text{Ru}_2(\text{ADT})(\text{CO})_4(\text{CN})_2]$ was treated with one equivalent of trichloroacetic acid (Figure S7). In contrast to the behavior of $[(\mu\text{-H})\text{Ru}_2(\text{ADT})(\text{CO})_4(\text{CN})_2]^-$, the analogous $[(\mu\text{-H})\text{Fe}_2(\text{ADT})(\text{CO})_4(\text{CN})_2]^-$ is unstable in solution at room temperature.^[9] We observed no evidence for the formation of terminal hydrides, in complete contrast to our observations of the ruthenated protein (see below).

Initial maturation experiments were conducted with $[\text{Ru}_2(\text{PDT})(\text{CO})_4(\text{CN})_2]^{2-}$. This precursor lacks the amine function of ADT. Figure 3A,B presents the FTIR spectrum of HydA1 matured with $[\text{Ru}_2(\text{PDT})(\text{CO})_4(\text{CN})_2]^{2-}$ dissolved in DMSO added 2:1 to the apo-enzyme in aqueous buffer at pH 8 and rebuffed to pH 6. Maturation proceeds very cleanly. Five FTIR bands are observed for the HydA1-[2Ru]_H-PDT variant, two at higher wavenumbers arising from CN^- ligands and three carbonyl stretches between 1990–1850 cm^{-1} . The narrow spectral linewidth of these bands is characteristic for insertion of the complex into the protein (compare spectra in Figure 2 and Figure 3). Interestingly, the enzyme can also be matured with the [2Ru]-PDT complex predissolved in aqueous buffer, in which it, similar to the ADT complex, forms a bridging hydride (Figure S8), in other words, $[(\mu\text{-H})\text{Ru}_2(\text{PDT})(\text{CO})_4(\text{CN})_2]^-$.^[7] However, the FTIR spectrum of the inserted [2Ru]-PDT complex is identical to that of the enzyme matured with the precursor predissolved in DMSO.

Just like the HydA1-[2Fe]_H-PDT H-cluster analogue,^[10] the HydA1-[2Ru]_H-PDT complex shows two redox states (Figure 3A,B). The oxidized and reduced states are distinguished by relative small (ca. 10 cm^{-1}) shifts in the corresponding FTIR spectra. The small shifts suggest that only the $[\text{4Fe-4S}]_{\text{H}}$ sub-cluster participated in this redox event.^[10] The HydA1-[2Ru]_H-PDT sub-site seems to be trapped in a single redox state, which is different from the mixed-valence state $[\text{Fe}^{\text{I}}\text{Fe}^{\text{II}}]$ found for the PDT variant of HydA1.^[10] The FTIR spectra of HydA1-[2Ru]_H-PDT strongly resemble those of the recently identified terminal hydride state (H_{hyd}).^[11] In particular, the blue-shifted bridging CO band at 1860 cm^{-1} is characteristic of an H-cluster state with an oxidized $[\text{Ru}^{\text{II}}\text{Ru}^{\text{II}}]$ core and an electron-donating ligand (i.e. H^-) in the open coordination site. Due to the coupling of the bridging CO and metal hydride stretch vibrations, the presence of a hydride state can be identified through H/D exchange (Figure S9 and S10).^[12] Figure 3C shows the FTIR of HydA1-[2Ru]_H-PDT matured in D_2O (pD=8.0). The $\nu(\mu\text{CO})$ feature shows an additional peak that is 10 cm^{-1} blue-shifted with respect to its position in H_2O buffer. The incomplete conversion of the $\nu(\mu\text{CO})$ band suggests that the hydride is partly formed through equilibration of the buffer with the 2.0% H_2 atmosphere present in the anaerobic chamber. Indeed, when the atmosphere is exchanged for 2.5% D_2 , the $\nu(\mu\text{CO})$ band of the hydride species at 1852 cm^{-1} decreases in intensity (Figure 3C). The occurrence of the characteristic bridging CO band (1853–1863 cm^{-1}) and its sensitivity to H/D exchange signifies that the hydride is required to be terminal. Insertion of the complex into the protein therefore induces a rearrangement of the bridging hydride into a terminal hydride.

These experiments also establish that the Ru-H/D species do not readily exchange with buffer. Indeed, rebuffing the in D_2O matured enzyme in H_2O did not alter the FTIR

spectrum of the $[2\text{Ru}]_{\text{H}}$ unit at all (Figure S11). This result leads us to conclude that the terminal hydride in $\text{HydA1-}[2\text{Ru}]_{\text{H}}\text{-PDT}$ is very stable and unreactive and does not exchange with the solvent. The two redox states observed in the $\text{HydA1-}[2\text{Ru}]_{\text{H}}\text{-PDT}$ variant of the enzyme can thus be represented as $[\text{4Fe-4S}]^{2+}[\text{Ru}^{\text{II}}\text{Ru}^{\text{II}}](\text{H}^-)$ and $[\text{4Fe-4S}]^{+}[\text{Ru}^{\text{II}}\text{Ru}^{\text{II}}](\text{H}^-)$. The latter state has a reduced $[\text{4Fe-4S}]_{\text{H}}$ sub-cluster as is also evidenced by its EPR spectrum (Figure S12). Interestingly, at pH 6 its FTIR bands show a blue shift of approximately 3 cm^{-1} (Figure 3B) with respect to the spectrum recorded at pH 8, thus suggesting a protonation event close to the H-cluster but not on the $\text{HydA1-}[2\text{Ru}]_{\text{H}}\text{-PDT}$ sub-site itself.

The same maturation experiments were subsequently conducted using $[\text{Ru}_2(\text{ADT})(\text{CO})_4(\text{CN})_2]^{2-}$ (pre-dissolved in DMSO) as well as $[(\mu\text{-H})\text{Ru}_2(\text{ADT})(\text{CO})_4(\text{CN})_2]^{-}$ (predissolved in H_2O). Both precursors matured the enzyme, which, however, turned out to be inactive in hydrogen conversion. With the ADT derivative, a mixture of two states is obtained, which is dominated by the terminal hydride state H_{hyd} (CO bands 1984, 1961, 1857 cm^{-1} at pH 8, oxidized) that was also observed for the PDT-containing precursor (compare Figures 3A,B and 4A,B). The new species, associated with FTIR bands 1971, 1931, 1769 cm^{-1} (pH 8, oxidized), is tentatively assigned to a state that lacks the hydride but is presumably protonated at the ADT amine moiety. This assignment is based on the strongly red-shifted FTIR signature (suggesting a $[\text{Ru}^{\text{I}}\text{Ru}^{\text{I}}]$ configuration) and the similarity to the recently reported H_{red}^* state observed in a sensory $[\text{FeFe}]$ -hydrogenase, which is characterized by a $[\text{Fe}^{\text{I}}\text{Fe}^{\text{I}}]$ core coordinated by a bridging CO ligand.^[13] Smallmodel DFT calculations predict that amine protonation of the reduced $\text{HydA1-}[2\text{Fe}]_{\text{H}}\text{-ADT}$ sub-site induces opening of the bridging CO,^[14] as is also observed experimentally.^[15] The same type of calculation also predicts that for the rutheniumsubstituted sub-site, the bridging CO is retained upon amine protonation (Figure S14). Analogous to the $[\text{Ru}^{\text{II}}\text{Ru}^{\text{II}}]$ hydride state, the $[\text{Ru}^{\text{I}}\text{Ru}^{\text{I}}]$ species shows two redox states characterized by small (10 cm^{-1}) shifts of the CO bands, again suggesting a redox event on the $[\text{4Fe-4S}]_{\text{H}}$ subcluster. Therefore, these redox states can be represented as $[\text{4Fe-4S}]^{2+}[\text{Ru}^{\text{I}}\text{Ru}^{\text{I}}]$ and $[\text{4Fe-4S}]^{+}[\text{Ru}^{\text{I}}\text{Ru}^{\text{I}}]$.

The H/D exchange experiment presented in Figure 4C indicates that for the $\text{HydA1-}[2\text{Ru}]_{\text{H}}\text{-ADT}$ variant, in contrast to $\text{HydA1-}[2\text{Ru}]_{\text{H}}\text{-PDT}$, the hydride fully exchanges with the solvent. Maturation in D_2O buffer leads to a blue-shifted $\nu(\mu\text{CO})$ peak (Figure 4C), which is restored to its original position upon rebuffering in H_2O (Figure S13). The $[\text{Ru}^{\text{I}}\text{Ru}^{\text{I}}]$ species is, however, unaffected by H/D exchange, thus confirming that no hydride is associated with this state. Given the simultaneous occurrence of both hydride and $[\text{Ru}^{\text{I}}\text{Ru}^{\text{I}}]$ states in the ADT enzyme, with a ratio largely independent of pH and redox potential, it is tempting to assume that both states are protonated, that is, either at the ADT amine or at Ru_{d} . The two states are in rapid exchange (Figure S15), thus explaining the fact that for the $\text{HydA1-}[2\text{Ru}]_{\text{H}}\text{-ADT}$ enzyme, H/D exchange of the hydride is possible through buffer exchange while exchange is not observed for the $\text{HydA1-}[2\text{Ru}]_{\text{H}}\text{-PDT}$ matured enzyme (see also Figure S16). Figure S17 shows the FTIR-spectroelectrochemistry of $\text{HydA1-}[2\text{Ru}]_{\text{H}}\text{-ADT}$ at pH 6. Both hydride and $[\text{Ru}^{\text{I}}\text{Ru}^{\text{I}}]$ states show a reduction event, assigned to the $[\text{4Fe-4S}]_{\text{H}}$ sub-cluster. The hydride state with its formally oxidized $[\text{Ru}^{\text{II}}\text{Ru}^{\text{II}}]$ core shows the highest redox potential (ca. -270 mV), while the $[\text{Ru}^{\text{I}}\text{Ru}^{\text{I}}]$ state is reduced at around -370 mV . Therefore, hydride formation and concomitant oxidation of the $[2\text{Ru}]$ core increases the

redox potential of the [4Fe-4S]_H sub-cluster by approximately 100 mV. This signifies that the strong electronic contact between the two sub-clusters is retained in the HydA1-[2Ru]_H-ADT H-cluster analogue.

In summary, deep mechanistic insight is provided when the ligands of the [2Fe]_H cofactor in [FeFe]-hydrogenase are left intact but the metals are changed. This work exploits the stability characteristic of ruthenium hydrides.^[16] Interestingly, both protonated and unprotonated precursors mature apo- [FeFe]-hydrogenase and produce identical [2Ru]_H analogues. This μ -hydride→terminal hydride transformation demonstrates the profound influence of the protein on the stereochemistry of the hydride, an effect yet to be replicated in models.^[8] Although the [4Fe-4S]_H sub-cluster functions normally in these ruthenated proteins, the [2Ru]_H is redoxinactive, hence catalysis does not proceed. Thus, the high activity of FeFe-based active sites reflects the advantages of the mild redox potentials of the Fe^I/Fe^{II} couple. Finally, the work allows the first interrogation of a protein metal hydride with and without the ADT cofactor, showing that the amine constitutes a critical proton relay.^[2,3]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Scheme of potential catalytic states of the H-cluster in [FeFe]-hydrogenases, highlighting protonation and hydride formation at the reduced [2Fe]_H sub-site in combination with a redox event at the [4Fe-4S]_H cluster. The two terminal CO and two CN⁻ ligands are omitted for clarity.

Figure 2. FTIR of $[\text{Ru}_2(\text{ADT})(\text{CO})_4(\text{CN})_2]^{2-}$ and $[\text{Fe}_2(\text{ADT})(\text{CO})_4(\text{CN})_2]^{2-}$ in DMSO (A) and in H_2O (B), where the complex is protonated to form a bridging hydride (μH) (light blue trace).

Figure 3.

FTIR spectra of HydA1-[2Ru]_H-PDT. A) The oxidized state at pH 8 adjusted with a 1:1 ratio of protein/thionin. B) The reduced state is shown as prepared with three-fold excess of sodium dithionite at pH 8 and 6 respectively. C) The effect of H/D exchange is demonstrated through artificial maturation in D₂O with 2.0% H₂ atmosphere or 2.5% D₂ atmosphere at pD 8. All FTIR spectra are taken at room temperature with a spectral resolution of 2 cm^{-1} .

Figure 4.

FTIR spectra of HydA1-[2Ru]_H-ADT. A) The oxidized state at pH 6 (black)/8 (blue)/10 (green) adjusted with thionin in a 1:1 ratio. B) The samples were all reduced with a three times excess of dithionite at pH 6/8/10, indicating two pH-dependent reduced states. The bands of the hydride state are colored while the bands of the reduced state are indicated by closed (high pH) or open (low pH) bullets. C) Two oxidized samples, one matured in H₂O (light blue) and the other in D₂O (dark blue) at pH/pD 8. All FTIR spectra are taken at room temperature with a spectral resolution of 2 cm⁻¹.

Scheme 1.
Synthesis of $[\text{Ru}_2(\text{ADT})(\text{CO})_4(\text{CN})_2]^{2-}$.