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[FeFe]-Hydrogenase Maturation: HydG-Catalyzed Synthesis of Carbon Monoxide

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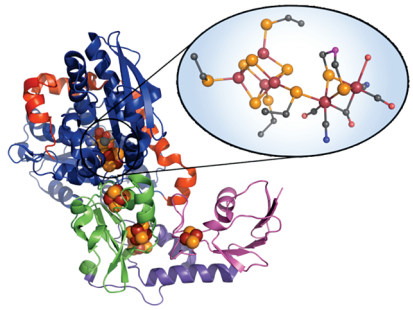
Abstract: Biosynthesis of the unusual organometallic H-cluster at the active site of the [FeFe]-hydrogenase requires three accessory proteins, two of which are radical AdoMet enzymes (HydE, HydG) and one of which is a GTPase (HydF). We demonstrate here that HydG catalyzes the synthesis of CO using tyrosine as a substrate. CO production was detected by using deoxyhemoglobin as a reporter and monitoring the appearance of the characteristic visible spectroscopic features of carboxyhemoglobin. Assays utilizing ^{13}C-tyrosine were analyzed by FTIR to confirm the production of HbCO and to demonstrate that the CO product was synthesized from tyrosine. CO ligation is a common feature at the active sites of the [FeFe], [NiFe], and [Fe]-only hydrogenases; however, this is the first report of the enzymatic synthesis of CO in hydrogenase maturation.

The organometallic metal centers at the active sites of all known hydrogenases are unique in biology. In addition to containing unusual nonprotein ligands such as carbon monoxide (CO) and cyanide (CN^-), these organometallic centers serve as the catalytic sites for the reversible reduction of protons, a reaction central to both early and present-day microbial life and future bioenergy applications. The unique H-cluster of the [FeFe]-hydrogenase is comprised of a [4Fe-4S] cluster bridged by a cysteinyl residue to a 2Fe cluster coordinated by three CO, two CN^-, and a bridging dithiolate (Chart 1).[1] Three accessory proteins, two radical *S*-adenosylmethionine (AdoMet) enzymes (HydE and HydG) and a GTPase (HydF), are necessary for the synthesis of the 2Fe subcluster of the H-cluster.[2] HydF serves as a carrier for an H-cluster precursor that is transferred to the structural protein to yield an active hydrogenase.[3] HydG has been shown to catalyze the cleavage of tyrosine to produce *p*-cresol,[4] and we have recently demonstrated that this tyrosine cleavage leads to formation of CN^-.[5] We report here that HydG also catalyzes the formation of CO, monitored via binding of CO to deoxyhemoglobin. These results provide the first example of enzymatic production of CO in the maturation of any hydrogenase.

HydG was heterologously produced in *Escherichia coli* BL21 using the plasmid pCDF::HydG-his.[3] The protein was purified in an anaerobic chamber (Coy). Anaerobically purified HydG contains up to 2.8 Fe/protein and exhibits visible and electron paramagnetic resonance (EPR) spectroscopic properties consistent with the presence of iron−sulfur clusters, with the EPR spectra suggestive of the presence of both [4Fe-4S] and [2Fe-2S] clusters [Supporting Information (SI)]. Reconstitution of this as-isolated enzyme with iron and sulfide (SI) produced protein containing up to 8.7 ± 0.7 Fe/protein and was absolutely necessary in order to observe the catalytic activity described in this Communication. These iron numbers, together with the conservation of two cysteine motifs in HydG, suggest that holo-HydG contains two iron−sulfur clusters, a possibility that has also been suggested by others.[6] Our reduced reconstituted HydG shows a single fast-relaxing signal (g = 2.03, 1.92, 1.90) characteristic of $[4Fe\text{-}4S]^+$ clusters (Figure 1) and similar to a recently reported EPR spectrum of HydG.[7] Addition of AdoMet to the reduced reconstituted enzyme splits this signal into two distinct rhombic signals ($g_{cluster1}$ = 2.02, 1.93, 1.91 and $g_{cluster2}$ = 2.00, 1.87, 1.83), presumably arising from two different iron−sulfur clusters on HydG. Both signals have a temperature dependence characteristic of $[4Fe\text{-}4S]^+$ rather than $[2Fe\text{-}2S]^+$ clusters (Figure 1 and SI).

Chart 1. Overall Structure and H-Cluster of the [FeFe]-Hydrogenase from *Clostridium pasteurianum*



Assays for HydG-catalyzed CO formation were carried out in an anaerobic chamber (Mbraun, O_2 < 1 ppm) and in sealed 1.4 mL anaerobic cuvettes. Reconstituted HydG was assayed for CO production using deoxyhemoglobin (deoxyHb) as a reporter. Assays contained HydG (20−65 μM) in buffer (50 mM HEPES, pH 7.4, 0.5 M KCl, 5% glycerol) containing 1−4.8 mM dithionite. DeoxyHb was added to a final concentration of ∼10 μM heme. UV−visible difference spectra before and after addition of deoxyHb were used to quantify deoxyHb, which shows characteristic λ_{max} values at 430 (Soret band) and 555 nm (visible band).[8] Tyrosine was then added, and the assay was initiated by addition of AdoMet. The results (Figure 2) reveal formation of HbCO, dependent on the presence of HydG, tyrosine, and AdoMet. The formation of HbCO is evidenced by the shift in the Soret band (430 to 419 nm) and by the shift and splitting of the 555 nm band, which together

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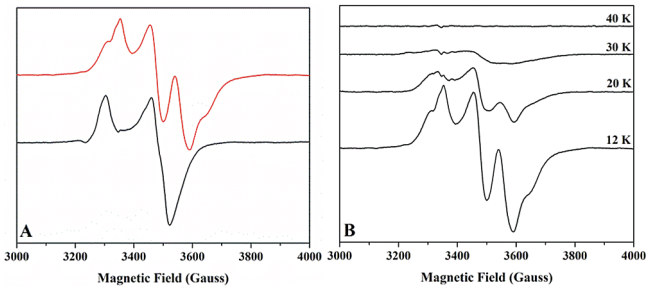

Figure 1. X-band EPR spectra of reduced reconstituted HydG. (A) Reduced HydG in the absence (65 μM enzyme, 8.7 $\pm$ 0.7 Fe/protein, black line) and in the presence (65 μM enzyme, 8.7 $\pm$ 0.7 Fe/protein, red line) of 1 mM AdoMet. EPR parameters: 12 K, 9.37 GHz, 1.84 mW. (B) Temperature dependence of reduced HydG in the presence of AdoMet. EPR parameters as in (A) with temperatures indicated.

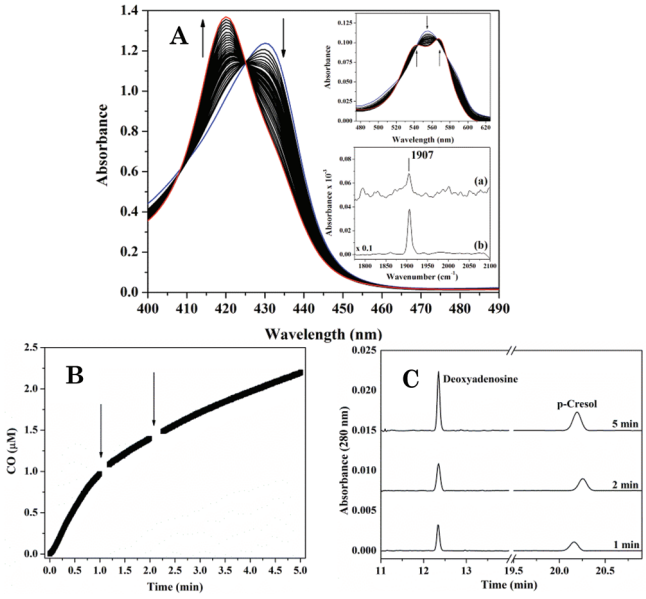

Figure 2. HydG-dependent CO formation. (A) Time-dependent conversion of deoxyHb to HbCO as monitored by changes in the Soret and visible bands (top inset). Reaction contained 26 μM HydG (6.4 $\pm$ 0.2 Fe/protein), 4 mM dithionite, 1 mM Tyr, 1.1 mM SAM, and 9.2 μM heme (as Hb). Bottom inset shows FTIR spectra of HbCO formed from ^{13}C-tyrosine (a) and a control spectrum (b) in which ^{13}CO was added to Hb (see SI for details). (B) Single-wavelength (419 nm) kinetics illustrating the biphasic nature of HbCO formation at 30 °C. Arrows in panel B represent where single wavelength kinetics scan was paused and aliquots were removed for HPLC analysis (C and SI). Reaction contained 45 μM HydG (7.2 $\pm$ 0.2 Fe/protein), 10 μM heme, 1 mM AdoMet, and 500 μM tyrosine.

give rise to a visible spectrum characteristic of HbCO.[8] Tyrosine was confirmed as the source of CO by observing the appropriate isotopic shifts of the HbCO FTIR spectra for reactions using U-^{13}C-tyrosine as a substrate (Figure 2 and SI).

Reconstituted HydG with 8.7 $\pm$ 0.7 Fe/protein provided the greatest levels of HbCO formation. Single-wavelength kinetic experiments at 419 nm showed biphasic HbCO formation, with a linear burst phase corresponding to k_{cat}= 11.4($\pm$0.09) $\times$ 10^{-4} s^{-1} and a slow phase with k_{cat} = 1.71($\pm$0.01) $\times$ 10^{-4} s^{-1} (Figures 2 and S4 in SI). Although it was not possible to quantify both CN^- and CO under identical conditions in a single assay due to the particular requirements of each assay, the rate of the fast phase of HbCO formation is consistent with the rates we have previously reported for CN^- formation (k_{cat} = 20 $\times$ 10^{-4} s^{-1} at 37 °C), given the lower temperatures utilized in the current assays.[5] The total amount of CO detected is limited by the quantity of deoxyHb present in the assay (~10 μM heme). Although attempts were made to utilize higher concentrations of deoxyHb (in shorter path length cuvettes due to the high extinction coefficient of the Soret band), we were unable to significantly improve the quantity of CO detected. Further, HPLC analysis reveals the formation of 5′-deoxyadenosine and *p*-cresol concomitant with CO (Figure 2). The quantities of these organic products are consistent with our previously reported CN^- assay but always exceed the detected quantities of CO, suggesting perhaps that, under our current assay conditions, CO synthesized by HydG is partially sequestered within the protein. Our CN^- assays utilized protein denaturation prior to CN^- analysis, which may account for the differences in total amounts of product detected.[5] Efforts are underway to improve the yields of CO in these assays.

Although the three known types of hydrogenases ([FeFe]-, [NiFe]-, and [Fe]-) all contain CO ligands at their active sites, the work reported herein is the first demonstration of enzymatically catalyzed CO synthesis in the maturation of a hydrogenase. The results presented here, when coupled with our recent report that HydG catalyzes the formation of CN^- from tyrosine,[5] point to a novel radical AdoMet reaction in which generation of an amino acid radical on tyrosine leads to decomposition into diatomic ligands; an analogous reaction was proposed in our original hypothesis for H-cluster assembly.[9] The radical-mediated interconversion between small molecules (CO and CN^-) and an amino acid is potentially an interesting link between biochemistry and prebiotic chemistry. The stoichiometric formation of *p*-cresol in the HydG-catalyzed reaction[5] suggests that dehydroglycine may be an intermediate that undergoes chemically precedented decarbonylation[10] to produce CO and CN^-.

The diatomic ligands synthesized by HydG are presumably transferred to HydF, which has been shown to contain CO and CN^- ligands when expressed in the presence of HydE and HydG ($HydF^{EG}$).[7,11] Further, we have shown that $HydA^{\Delta EFG}$, which can be activated by $HydF^{EG}$, contains a [4Fe-4S] cluster but lacks the 2Fe subcluster of the H-cluster.[3,12] The cumulative data thus point to a mechanism for H-cluster assembly in which a 2Fe precursor to the H-cluster is assembled on HydF by the activities of HydE and HydG and is subsequently transferred to HydA.

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Supporting Information Available: Experimental procedures; spectroscopic and HPLC data. This material is available free of charge via the Internet at http://pubs.acs.org.

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