Introduction:
Hydrogenases (H\textsubscript{2}ases) are a unique class of metalloproteins that catalyze the reversible oxidation of molecular hydrogen\textsuperscript{1}. H\textsubscript{2}ases consist of three classes: [Fe]-H\textsubscript{2}ases, [NiFe]-H\textsubscript{2}ases, and metal-free H\textsubscript{2}ases\textsuperscript{2}. The [Fe]-H\textsubscript{2}ases enzymes employ the biologically distinct ligands CO and CN which are bound to an active site termed the H-cluster. The H-cluster from the [Fe]-H\textsubscript{2}ase, whose structure has been elucidated via X-ray crystallography for both \textit{Clostridium pasteurianum} I (CpI)\textsuperscript{3} and \textit{Desulfovibrio desulfuricans}\textsuperscript{4}, consists of the aforementioned CO and CN ligands terminally bound to the active site, which contains unique Fe-S clusters (Figure 1). These Fe-S clusters mediate the electron transport processes that are critical to the function of this enzyme and work in concert with the CO and CN ligands, which are believed to stabilize the low iron oxidation and spin states that are accepted as a criterion for activity\textsuperscript{5}. The ability for this finely tuned H-cluster to efficiently catalyze the production of hydrogen at high reaction rates (\textmu mol min\textsuperscript{-1} g\textsuperscript{-1}) has resulted in significant scientific interest.

Biosynthesis:
Recent work has shown that four proteins are necessary for the \textit{in vivo} formation of active [Fe]-H\textsubscript{2}ase from \textit{Chlamydomonas reinhardtii}, expressed heterologously in \textit{Escherichia coli}\textsuperscript{6}; these proteins are designated HydA, HydE, HydF, and HydG. The HydA protein is believed to serve as the primary structural protein required for enzyme activity. The HydE and HydG are accessory proteins that are members of the radical S-
adenosylmethionine (SAM) family of enzymes while the HydF protein is a GTP/ATP-ase with a domain consistent for iron sulfur cluster binding. Recent work has shown that the activation of the apo-hydrogenase can be accomplished in vitro by combining crude cell extracts from the inactive HydA protein with co-expression of the three inactive accessory proteins HydEFG (Figure 2). Recent work performed on these accessory proteins has led to the hypothesis that the two radical SAM proteins (HydE and HydG), in concert with HydF, enact precedent chemistry upon a central metabolite or amino acid that serves to assemble the H-cluster. Details for this possible mechanistic pathway are shown in Schematic 1 of the Appendix.

**Analysis:**

Determining the properties of hydrogenase that allow this protein to efficiently and rapidly catalyze the production of hydrogen gas has been a central research question in this field. The 4Fe-4S cluster (Figure 1, above) consists of Fe atoms containing a 2+/3+ mixed oxidation state. This group is attached via a cysteinal linkage to a 2Fe-2S cluster with the two Fe atoms containing a +1/+2 mixed valent state, making this active site biologically distinct. The low oxidation state Fe atoms on the 2Fe-2S cluster are believed to bind protons directly to the metal, converting the proton (H+) to a hydride (:H−).
The hydride ligand, exposed to the aqueous milieu available in the cell, readily reacts with water to produce hydrogen gas. The unusual mixed valence state on the metal is maintained by the strong field, low-spin, CO and CN ligands. These ligands provide a σ donor to the metal while the Fe d orbitals back-donates electron density into the CO and CN π* orbitals. These bonding interactions allow for the low spin and low valence state in this smaller 2Fe-2S cluster. In contrast, the 4Fe-4S cluster exists as a cubane unit with approximate tetrahedral (T₄) geometry. Each 2+/3+ Fe atom on the 4Fe-4S cluster is bound to three adjacent sulfur atoms; the higher oxidation states are believed to provide an electron transport pathway that readily directs electrons towards the 2Fe-2S cluster, enabling the rapid production of H₂ gas.

Methods:
As mentioned above, Fe-only H₂ases contain unique physical properties on the active site that endow this protein to be probed with various types of spectroscopy. Fourier Transform Infrared Spectroscopy (FT-IR), Electron Paramagnetic Resonance (EPR), and Mossbauer Spectroscopy are three types of spectroscopy capable of providing structural insight into the active site. FT-IR spectroscopy utilizes infra-red radiation to identify the vibrational energies of many organic groups and ligands. EPR spectroscopy uses microwave radiation to detect the resonant absorption of unpaired electrons contained in metal ions or organic radicals. Mossbauer spectroscopy use γ radiation targeted at complexes with specific isotopes i.e. ⁵⁷Fe to detect metal hyperfine splitting and quadrupole interactions that provide structural information for certain complexes. In addition to probing the H cluster of Fe-only H₂ase, these spectroscopic tools can be used to detect components of the active site in the accessory proteins (HydA, HydE, HydF, and HydG). Detecting CO and CN ligands, unusual spin states etc., in the individual proteins can be useful in determining the mechanism for Fe-only H₂ase biosynthesis.

**FT-IR Spectroscopy:**

FT-IR spectroscopy uses IR radiation to measure the vibrational energies of CO and CN ligands in the H cluster. This method can detect the number of bound CO, CN, and thiolate ligands, and the type of bonding interaction (terminal or bridging) that these
ligands experience with the metal. Larger vibrational frequencies correlate with stronger CO and CN bond energies. Characteristic stretching frequencies include 2080 cm\(^{-1}\) for terminal CN ligands, 1950-2000 cm\(^{-1}\) for terminal CO groups, and approximately 1800 cm\(^{-1}\) for bridging CO ligands. In addition, the H cluster can exist in different forms that vary in their level of activity. A “CO-inhibited” form eliminates hydrogen production and occurs when an extra carbonyl ligand attaches to an Fe atom on the 2Fe-2S cluster. The binding of this exogenous CO raises the stretching frequencies of adjacent CO and CN groups by reducing the \(\pi^*\) back-donation that the Fe contributes to the ligands. The reduced \(\pi\) back-donation of the metal weakens the Fe-C bonds; consequently, the stretching frequencies of the CO and CN ligands increases. Therefore, the IR spectra of the H cluster in its native and CO-inhibited form can be used to gain structural and mechanistic insight into how this protein serves to catalyze hydrogen production.

Electron Paramagnetic Resonance:

EPR spectroscopy is a ground state resonant absorption technique that uses the paramagnetic spin properties of unpaired electrons to provide structural insight into many types of complexes. EPR detects the differences in energy obtained when the degeneracy of a paramagnetic electron system is lifted by electron-Zeeman and electron-nuclei splitting interactions. In transition metal complexes, hyperfine structure arises from the metal ion while superhyperfine structure is associated with the ligands\(^{12}\). Due to the small energy differences in these interactions, EPR spectroscopy requires extremely sensitive detectors and the resulting absorption spectra are often displayed as the first derivative with respect to the applied magnetic field. The locations of the peaks, or “g-values”, are compared with tabulated values to identify the groups of interest. The paramagnetic properties of the H cluster allow it to be probed with EPR spectroscopy. For example, the oxidized state of the H cluster (Figure 1) contains a \(S = \frac{1}{2}\) paramagnetic ground state corresponding to a rhombic EPR signal. Upon addition
of CO, the rhombic signal is replaced with an axial signal and hydrogen activity is eliminated. Subsequent sparging with argon reverts the reduced, CO inhibited species, back to the native oxidized state\(^1\). This example demonstrates that in analogous fashion to IR spectroscopy, EPR studies can also provide insight into the mechanism of hydrogen production through carbon monoxide binding and subsequent inhibition.

**Mossbauer Spectroscopy:**

Nuclear transitions between an unstable atomic state to a more stable atomic state causes the emission of \(\gamma\)-radiation. The energy of the emitted radiation is equal to the energy of the nuclear transition and the recoil energy of the nucleus. Mossbauer spectroscopy exploits the “Mossbauer effect”, which shows that a fraction of \(\gamma\)-rays emitted from nuclei have extremely small, or non-existent recoil energies. These recoil free \(\gamma\)-rays can be absorbed by a nucleus of the same type; this process is known as “resonant absorption”. The energy of the atoms emitting the \(\gamma\)-rays (the source) can vary by acceleration through a linear motor. Consequently, a Mossbauer spectrum simply shows the intensity of the \(\gamma\)-rays plotted as a function of source velocity. When the \(\gamma\)-ray source emits radiation without recoil, and this radiation is absorbed by nuclei of the same type with high frequency, a peak is observed on the spectra. The locations of these resonant absorptions are compared with tabulated values, and can be used to obtain quadrupole splitting, metal hyperfine interactions, and isomer shifts for many complexes. However, the Mossbauer effect imposes two inherent limitations on the types of systems that can be studied\(^15\): the \(\gamma\)-rays of the source must be close to the nuclear transitions being probed; in addition, the \(\gamma\)-ray energies should be small so that a larger fraction of recoil free emissions are observed. \(^{57}\)Fe, \(^{129}\)I, \(^{119}\)Sn, and \(^{121}\)Sb are the most common sources of \(\gamma\)-radiation in Mossbauer spectroscopy that meet these criteria, with \(^{57}\)Fe by far the most common element used. Thus, \(^{57}\)Fe Mossbauer spectroscopy is a useful technique to probe the H cluster of hydrogenase. The \(^{57}\)Fe hyperfine structure of the active site varies in the oxidized and CO-reduced forms of the \([4Fe-4S]\)^{+x} cluster. In the absence of CO, the 4Fe-4S cluster occurs in the diamagnetic 2+ state while the dithionite-reduced hydrogenase exists in the paramagnetic +1 state\(^14\). This +1 paramagnetic state in the 4Fe-4S cluster
arises from an exchange interaction between excited and ground states of the cluster. As a result, the unique spin structures of these Fe-S clusters allow them to be probed with Mossbauer spectroscopy.

**Conclusions:**
Hydrogenases catalyze the reversible oxidation of molecular hydrogen into protons. [Fe]-H$_2$ases enzymes employ iron-sulfur clusters that are believed to control the electron transport processes that are critical to the function of this enzyme. CO and CN ligands are attached to the 2Fe-2S subclusters and serve to maintain the low iron oxidation and spin states that enable the active H cluster to efficiently produce hydrogen. Numerous spectroscopic probes can elucidate both structural and mechanistic information about the H cluster. FT-IR spectroscopy is used to determine the positions and types of bonding for CO and CN groups to the active site. EPR spectroscopy reveals the spin properties of the paramagnetic systems applied to the H cluster. Mossbauer spectroscopy reveals quadrupole splitting, hyperfine structure, and isomer shifts that provide insight into oxidation and spin states of systems containing $^{57}$Fe. A future research goal in this field includes using these spectroscopic probes to monitor both the active H cluster and the active Fe-S and/or CO-CN components in the independent accessory proteins that serve to assemble the H cluster. Understanding this complex biosynthesis may enable this system to be exploited to provide a clean source of hydrogen gas for future societal needs.

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**Schematic 1**

- **I**: Formation of dithiolate ligand by HydE/HydG from substrate amino acid or glycollate intermediate.

- **II**: Glycine radical formation by HydG/HydE and translocation by HydF.

- **III**: Glycine decomposition to CO/N=C=CH.

- **IV**: Cluster translocation and insertion by HydF.

- **V**: Schematic representation of the protein structure.