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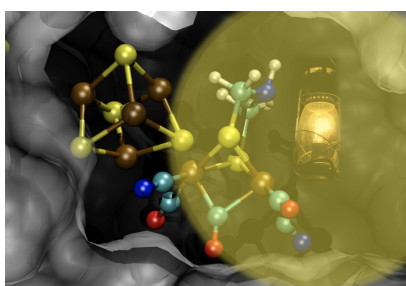
Photochemistry and photoinhibition of the H-cluster of FeFe hydrogenases

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Abstract

Hydrogenases are enzymes that catalyze the oxidation and production of molecular hydrogen. For about fifteen years, there have been many reports about the successful connection of these enzymes to photosensitizers with the aim of designing H₂ photoproduction systems, but relatively little attention has been paid to whether and why illumination may affect the catalytic properties of the enzyme. In all hydrogenases, hydrogen activation occurs at an inorganic active site that includes at least one Fe-carbonyl motif, which may make it sensitive to irradiation. Here we review the evidence that hydrogenases are indeed photosensitive. We focus mainly on the so-called FeFe hydrogenases; their active site, called the H-cluster, consists of a [4Fe4S] cluster that is bound by a cysteine sulfur to a diiron site. The iron atoms of the binuclear cluster are coordinated by carbonyl and cyanide ligands and an azadithiolate group. We describe the effects of UV-visible light irradiation on the enzyme under cryogenic or turnover conditions and the photoreactivity of model complexes that mimic the diiron site. We emphasize the dependence of the photochemical processes on wavelength, and warn about FeFe hydrogenase photoinhibition, which should probably be considered when attempts are made to use FeFe hydrogenases for the artificial photosynthesis of solar fuels. We also underline the relevance of studies of synthetic mimics of the H-cluster for understanding at atomistic level the photochemical processes observed in the enzyme.



TOC summary: the photosensitivity of the active site of hydrogenases is an opportunity for studying its reactivity. However, the photoinhibition of certain hydrogenases may have a negative impact on H₂ photoproduction.

Introduction

Dihydrogen is a very common energy vector in Nature, as most microorganisms either oxidize or produce H₂ in relation to their energy metabolism. These reactions are catalyzed by enzymes called hydrogenases¹ and nitrogenases.

H₂ oxidation occurs in relation to bacterial respiration: it is coupled to the reduction of a number of electron acceptors such as sulfate in sulfate reducing bacteria or O₂ in Knallgas bacteria, and the free energy of the reaction is used for producing chemical energy in the form of ATP.

The dihydrogen that is consumed by this process can be produced in a number of ways, either in the same microorganism or in a microorganism from the same consortium.

The most prominent H₂-producing metabolic pathway is dark-fermentation², as e.g. occurs in strictly anaerobic Clostridial species³ or in enteric bacteria such as *Escherichia coli*².

H₂ is also a byproduct of the reduction of N₂ to NH₃, a key step in the global nitrogen cycle which occurs e.g. in cyanobacteria: the latter reaction is catalyzed by the enzyme nitrogenase, which evolves at least one molecule of H₂ per N₂ reduced^{4,5}.

Biological splitting of water into O₂ and H₂ can also be observed in photosynthetic microorganisms such as green algae and cyanobacteria⁶, but the combination of O₂ evolution and H₂ production is not a natural physiological pathway⁷, and it is severely limited by the oxygen sensitivity of the enzymes involved in H₂ production: hydrogenase or nitrogenase^{6,8-11}. Efforts have been made to genetically engineer the enzymes¹²⁻¹⁴ or the strains¹⁰ of these photosynthetic microorganisms, aiming for continuous, light-driven biological H₂ production⁹⁻¹⁵. Another strategy to work around the O₂ sensitivity problem in microalgae consists in alternating aerobic and H₂ production phases by regulating the activity of Photosystem II; reducing equivalents are stored in the form of starch during the aerobic phase and then fed into hydrogenase via Photosystem I^{11,16}. Alternatively, *spatial* separation of water oxidation and H₂ production can also prevent nitrogenase inhibition by O₂ in cyanobacteria¹¹.

Biological, photosynthetic H₂-production is therefore not easily achieved¹⁷, but chemists have long worked towards the goal of water-splitting-based hydrogen production by designing original semi-artificial systems that interface hydrogenases or hydrogenase mimics with either photoactive materials or molecular photosensitizers, including the photosystems involved in natural photosynthesis¹⁸⁻²⁶.

Regarding the hybrid devices that include a photosynthetic enzyme, limitations may arise from the photodamage of the photosystems, which has been thoroughly studied by the photosynthesis community²⁷⁻³⁰. *In vivo*, the effect of the photodamage is balanced by *de novo* synthesis and repair mechanisms, but *in vitro* the damage decreases the stability of the hybrid systems^{31,32}.

An Achilles' heel of the artificial photoproduction systems that has received much less attention is the electron acceptor side: there is published evidence that some hydrogenases are sensitive to light irradiation, but this was rarely considered as a potential weakness^{25,33,34}.

Nature makes three distinct enzymes to heterolytically split molecular H₂. The so-called "NiFe hydrogenases" and "FeFe hydrogenases" both catalyze H₂ oxidation and production at a binuclear metal center and have been considered in relation to the design of artificial photosynthesis systems. In contrast, "Fe hydrogenases" catalyze hydride transfer from H₂ to methenyl-tetrahydromethanopterin, an organic substrate involved in microbial methane production. All three types of active sites contain at least one Fe-carbonyl bond³³, and are sensitive to light but in a different manner, and according to mechanisms that have not been fully identified.

The active site of Fe hydrogenases³⁴ is inactivated by exposure to UV-A (320-400 nm) or blue-light (400-500 nm), as a result of Fe release from the cofactor³⁵. The enzyme is protected from light-induced inactivation by the binding of the inhibitor CO³⁵ and, in the particular case of the enzyme from *Methanothermobacter marburgensis*, by its oligomerization³⁵. A key aspartate residue and some protein rearrangements are involved in the Fe hydrogenase protection from light-induced decomposition of the active site³⁶.

The active site of NiFe hydrogenases exists in the dark in a number of forms, some of which (the so-called Ni_U-A, Ni_r-B, Ni-C and Ni_a-L states) are EPR active. Ni_U-A and Ni_r-B correspond to oxidized, inactive states of the enzyme that disappear upon reductive activation of the enzyme. The Ni_U-A³⁷ and Ni_a-C³⁸⁻⁴¹ states and the EPR-silent Ni_a-S^{42,43} and Ni-S-CO⁴⁴⁻⁴⁷ states are all altered upon irradiation with visible light under cryogenic conditions. Ni_a-C is an intermediate in the catalytic cycle, which bears a bridging hydride. Ni_a-C is photoconverted under cryogenic temperatures into Ni_a-L, where the electrons are formally stored on the nickel ion. Additionally, this photoconversion results in the change of the protonation state of a terminal Ni-coordinated Cys residue⁴⁸. The photoproduct Ni_a-L can also be formed in the dark at ambient temperature⁴⁹. The CO-inhibited Ni_a-S state is light-sensitive: the extrinsic Ni-bound CO dissociates under illumination at cryogenic temperature (<120 K) restoring the catalytically relevant Ni_a-S intermediate⁴⁴⁻⁴⁷. Illumination with blue light accelerates the reactivation of the Ni_r-B state of the enzyme from *Aquifex aeolicus* at room temperature⁵⁰. A mechanism for the removal of the bridging OH ligand from the Ni_r-S inactive ready state upon irradiation has been suggested⁵¹. Re-activation can also be triggered by light under cryogenic conditions in the protein crystals⁵². We are not aware of any evidence that NiFe hydrogenases are damaged upon irradiation.

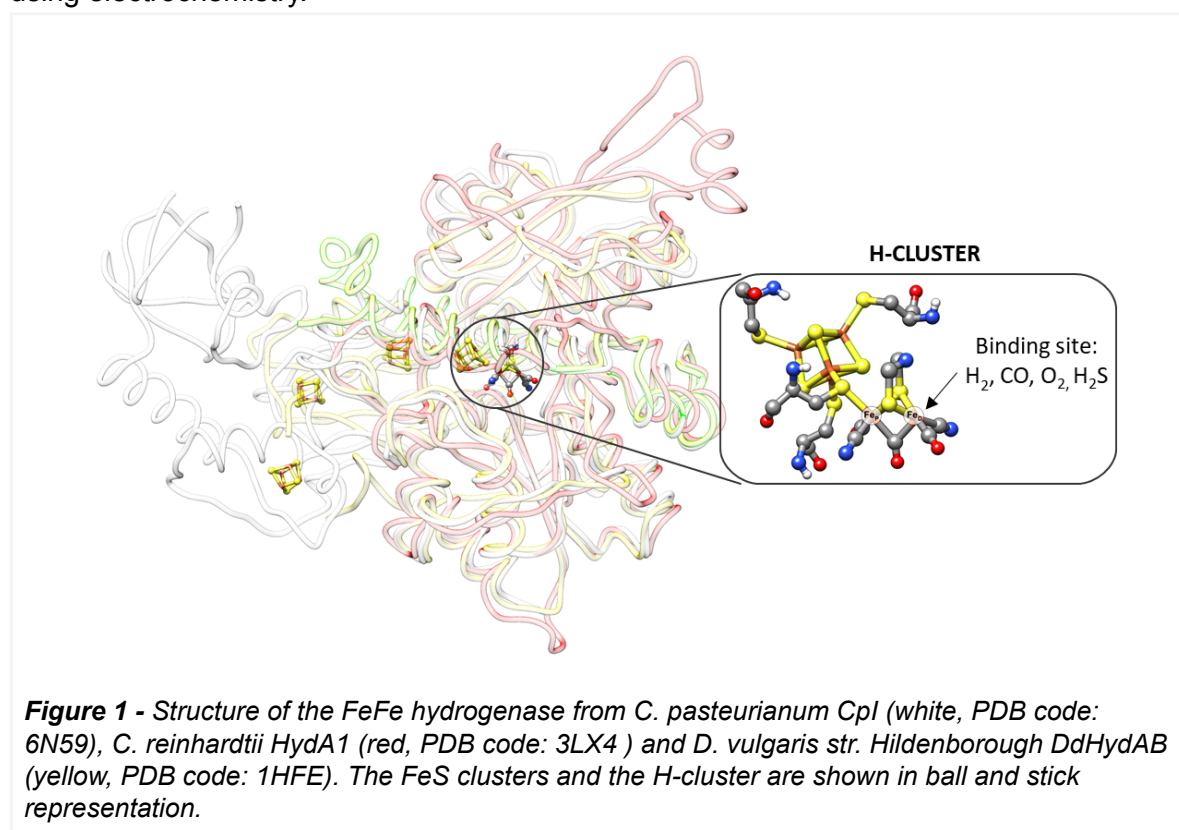
In the context of designing H₂ photoproduction devices, FeFe hydrogenase and their synthetic mimics are preferred because they are deemed more active for H₂ production^{53,54}. In this review we focus on FeFe hydrogenases and their biomimetic models, and we discuss the various effects that have been observed upon irradiation.

The structure of FeFe hydrogenases

The photochemical properties of FeFe hydrogenases are determined by the electronic structure of their inorganic cofactors. The active site of FeFe hydrogenases⁵⁵, called the H-cluster, consists of two subclusters bridged and attached to the protein matrix by a cysteine residue: a canonical [4Fe4S]_H cluster and a [2Fe]_H site; the two Fe ions are called proximal, Fe_P, and distal, Fe_D, in reference to the distance to the [4Fe4S]_H subcluster. Each

iron in the $[2\text{Fe}]_{\text{H}}$ site binds a CO and a CN^- ligand and the two Fe are bridged by a further CO and an azadithiolate (adt) group (figure 1).

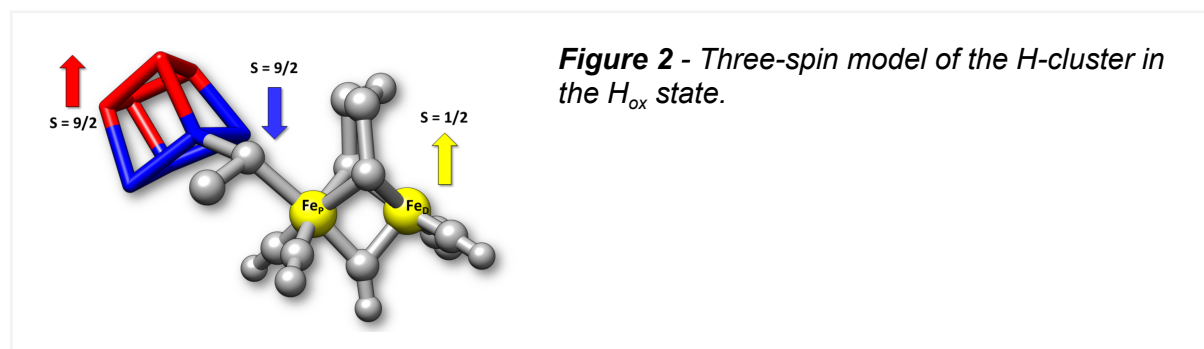
Although many states of the H-cluster have been observed using EPR and FTIR spectroscopy^{55–66}, most investigations of the photochemistry of FeFe hydrogenases have focused on the so-called H_{ox} , $\text{H}_{\text{ox}}\text{-CO}$ and H_{inact} states. In the $\text{H}_{\text{ox}}\text{-CO}$ and H_{inact} states, extrinsic CO or HS^- ligands, respectively, coordinate the Fe_{D} apical site^{67,68} (Figure 1). Both CO and sulfide binding prevent the damage of the enzyme by O_2 ^{69–71}, showing that they target the same binding site as O_2 ^{68,72}. One-electron reduction of H_{ox} results in the accumulation of two redox species (H_{red} and $\text{H}_{\text{red}}\text{H}^+$) related by proton coupled electron redistribution⁷³. More reduced states, characterized by the presence of a terminal hydride ligand on Fe_{D} , are observed under certain conditions, in particular when proton transfer is blocked^{64,74–76}. The enzyme photochemistry under reducing conditions was also investigated using electrochemistry.



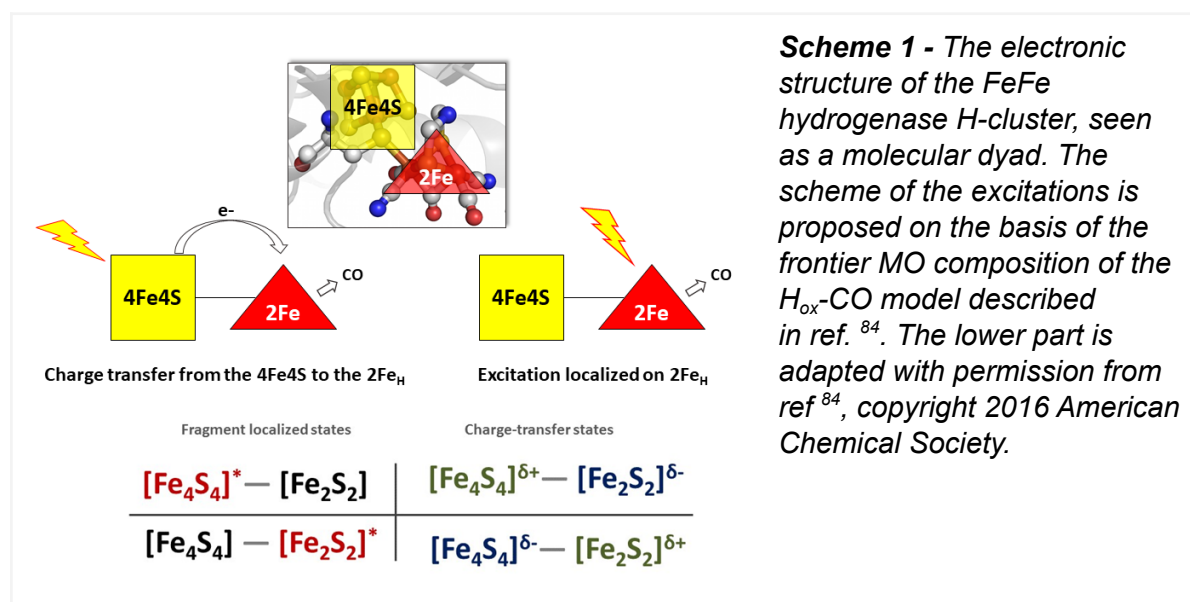
Investigations on the photochemical properties of the H-cluster aimed at understanding the effect of light irradiation and the nature and reactivity of the photoproducts. Fast or ultrafast processes are difficult to observe, but the dynamics of the excited states can be studied using quantum chemistry with time-dependent DFT (TDDFT).

In general, electronic excited states are always calculated from the ground state wave-function, whose characterization is required before the effect of excitation can be investigated. The ground state electronic structure of the H-cluster in the H_{ox} and $\text{H}_{\text{ox}}\text{-CO}$ states can be described as a three-spin system: the $[2\text{Fe}]_{\text{H}}$ site has a spin of $1/2$, each $[2\text{Fe}2\text{S}]$ layer of the $[4\text{Fe}4\text{S}]_{\text{H}}$ cluster has $S = S(2) + S(5/2) = 9/2$ (figure 2). The iron atoms of the binuclear subcluster, in both H_{ox} and $\text{H}_{\text{ox}}\text{-CO}$ states of the H-cluster, are in the $\text{Fe}^{\text{I}}\text{Fe}^{\text{II}}$ redox configuration. The attribution of the redox state to Fe_{P} and Fe_{D} is not trivial: FTIR^{66,67},

EPR⁷⁷ and DFT⁷⁸ studies suggest that Fe_D is the Fe^I while ENDOR, HYSCORE⁷⁹ and FTIR spectroelectrochemistry^{61,65,80} data support the paramagnetic Fe^I nature of Fe_p. The [4Fe4S]_H²⁺ cluster consists of two [2Fe2S] layers coupled antiferromagnetically, each formed by one Fe^{II} and one Fe^{III} high-spin ions, coupled ferromagnetically, giving an overall low-spin state^{58,78,81,82}.



The frontier molecular orbitals (FMOs) of the H-cluster in the ground state (GS) obtained from DFT calculation are localized on the [4Fe4S]_H cluster and/or the [2Fe]_H site, depending on the state of the H-cluster^{81,83}. We have calculated the frontier molecular orbitals (FMOs) of H_{ox}-CO in the ground state⁸⁴. In this case, single occupied HOMO and LUMO are localized on the [4Fe4S]_H and on the [2Fe]_H respectively. We described the H-cluster as a dyad system that consists of two fragments covalently bound to one another: the [4Fe4S]_H cluster and the [2Fe]_H site. As shown in scheme 1, the excited states decay of this system can be described in terms of a charge-transfer (CT) or a localized mono-electronic transition depending on the orbital composition of the FMOs involved^{85,86}.



The above-described H-cluster defines the large family of FeFe hydrogenases. These enzymes are present in many microorganisms. They differ in terms of protein sequences, quaternary structures and cofactor content^{87,88}. The immediate environment of the H-cluster is mostly conserved, although some variations have been observed and may induce unusual catalytic properties^{55,85,86}. Non conserved residues that are remote from the active site may

also affect active site chemistry, as demonstrated using site-directed mutagenesis in the particular case of *Clostridium Beijerincki* FeFe hydrogenase⁸⁹. They embed a variable number of accessory FeS clusters which are used to mediate long range intramolecular electron transfer: there are 2 accessory clusters in the FeFe hydrogenases from *D. desulfuricans* (DdHydAB), *D. vulgaris* (DvHydAB) and *M. elsdenii* (MeHydA) FeFe hydrogenases, 4 in the FeFe hydrogenases from *Clostridium acetobutylicum* (Cal), and *Clostridium pasteurianum* (Cpl), and none in the CrHydA1 enzyme from *Chlamydomonas reinhardtii* (figure 1).

The effect of light on the CO-inhibited state of FeFe hydrogenases

The reactivation of the CO-inhibited FeFe hydrogenase by light has been studied since the 1930s, initially to learn about the metal content and structure of the active site and the binding site of hydrogen. More recent investigations of the effect of irradiation have focused on understanding the properties and the reactivity of the active site (Table 1).

Early functional studies of the effect of light on CO-inhibited hydrogenases

In 1933, Kempner and Kubowitz showed that the production of H₂ by *Clostridium butyricum* is inhibited by CO and illumination reverses the inhibition⁹⁰. At that time neither the structure of the enzyme nor the presence of nickel and/or iron in the active site had been established; the flash photolysis of CO had been used to study the reaction of myoglobin with small ligands^{91,92} and the light-dependent CO-inhibition of hydrogenases was taken as an indication of the presence of an iron atom at the active site, as in myoglobin.

The reversal of CO inhibition by white light was also observed with intact cells and cell-free extracts of other bacteria⁹³⁻⁹⁷. Some of these early observations are ambiguous because some of the bacteria that were investigated, e.g. *D. desulfuricans*, produce FeFe and NiFe hydrogenases, both of which are light-sensitive in the CO-inhibited state. The effect of irradiation was probably also modulated by the permeability of the biological membranes.

Rudolf K. Thauer and coworkers performed the first kinetic study of FeFe hydrogenases inhibition by CO *in vitro* and also observed the reversal of the inhibition by light⁹⁸. The authors qualitatively observed that after exposure to CO, the H₂ oxidation activity of the enzyme is ten times greater under the light of a halogen lamp than in the dark, which was interpreted as evidence of the photolysis of the CO-enzyme complex. The effect of light was fully reversible. The rates of binding and release of CO under illumination were not measured.

Spectroscopic and crystallographic investigations

EPR and FTIR spectroscopic techniques have later been used to show that illumination affects the structure of the H-cluster in the H_{ox}-CO state. Under cryogenic conditions, one CO ligand is photo-dissociated, but the structure of the photoproduct depends on temperature. At room temperature the illumination allows the exchange between intrinsic and extrinsic CO, demonstrating the dynamics of the [2Fe]_H site.

Low temperature

Peters and Lemon obtained the first crystallographic structure of the FeFe hydrogenase of *Clostridium pasteurianum* (Cpl)⁹⁹, and in 2000 they reported the effect of irradiating the crystal of the CO-inhibited state with a red laser (635.8 nm) under a liquid nitrogen stream¹⁰⁰. The exogenous CO binds on the axial site of Fe_D and partial dissociation is observed after irradiation. The difference between the electron density maps evidences an increased distance between the S of a conserved methionine residue and the bridging CO, resulting from an increased electron density on the bridging CO after the photolysis of the apical CO¹⁰⁰.

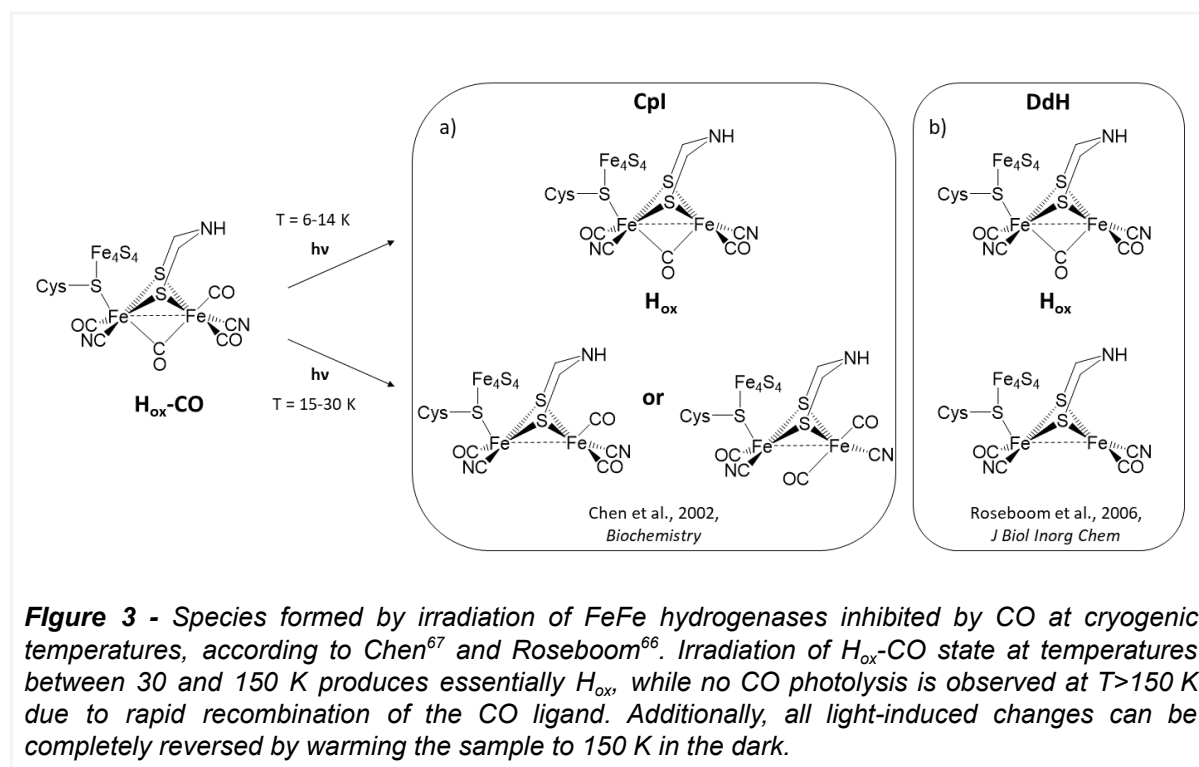
In the late 1980's, Patil¹⁰¹, Kowal¹⁰² and coworkers had used EPR to examine the effect of light on the enzymes from *D. vulgaris* (DvHydAB) and *C. pasteurianum* (Cpl), respectively, under cryogenic conditions. Patil and colleagues observed that the H_{ox}-CO EPR signal at $g = 2.06$ is converted to the rhombic $g = 2.10$ signal, assigned to H_{ox}, upon irradiation with a 200 W mercury arc lamp at 9 K. Heating the sample to 200 K gives back the H_{ox}-CO signal, showing that the CO ligand remains in the environment of the H-cluster and rebinds if the temperature is high enough that the corresponding energy barrier can be overcome. According to Kowal *et al.*, irradiation at 8 K produces H_{ox}, but irradiation at 30 K produces a signal at $g = 2.26, 2.12$ and 1.89, falsely suggesting the presence of a low spin Fe^{III} center, at a time when the structure of the H-cluster was unknown.

Changes in H-cluster structure have also been detected using FTIR spectroscopy. Peters, Bagley and coworkers observed that the irradiation of Cpl H_{ox}-CO with a 300 W xenon lamp at 6-14 K produces the H_{ox} state (which decays back to H_{ox}-CO above 150 K), but a second species is produced upon irradiation above 20 K; it is characterized by the loss of the bridging CO band at 1809 cm⁻¹ and decays above 80 K by CO rebinding⁶⁷. The authors hypothesize that the later species gives the $g = 2.26$ rhombic signal in the experiments of Kowal *et al.*^{101,102}, and that the photodissociation involves the bridging or apical CO depending on temperature (see fig. 3). Working with *D. desulfuricans* hydrogenase (DdHydAB), Roseboom *et al.* also observed by FTIR that illuminating H_{ox}-CO at 15–30 K with an halogen lamp or with a more intense light (xenon lamp), causes the disappearance of both the bands of extrinsic CO and of the bridging CO, or only of the bridging CO in molecules where the extrinsic CO is already photo-dissociated⁶⁶. The removal of the bridging CO from H_{ox}-CO at cryogenic temperatures has no effect on the frequencies of the other intrinsic COs and on the charge densities of the irons, again in contrast with the results in ref ⁶⁷. It is unclear if the observed differences result from the enzymes from *C. pasteurianum* and *D. desulfuricans* behaving differently, or the different irradiation times (10 minutes versus 20 minutes to hours) or light power (300 W vs 150 W) or lamp emission spectra. Based on an EPR and ENDOR investigation, Lubitz and coworkers concluded that the "second" photoproduct (H_L) is characterized by an unpaired spin density localized on the diiron site, in particular over the CN⁻ ligands, resulting from a structural rearrangement which cannot be merely the dissociation of bridging CO¹⁰³. In all cases, all the signals obtained after irradiation decay upon warming the sample in the dark at 150 K.

Katz *et al.* also recently observed by FTIR the photo-dissociation of exogenous CO from the H-cluster of CrHydA1 after blue light irradiation at 460 nm and 80 K, mimicking the resonance raman spectroscopy conditions, under which only the H_{ox} and H_{red} states result¹⁰⁴.

Laser irradiation at low temperature of an H₂-treated *C. reinhardtii* HydA1 sample induces an intramolecular electron transfer from the dinuclear cluster to the [4Fe4S]_H subsite¹⁰⁴.

Lorent *et al.* recently studied the effect of light on the production of hydride-bound states under cryogenic conditions, in absence of CO¹⁰⁵. The authors use photoactivation in combination with cryogenic infrared (IR) and electron paramagnetic resonance (EPR) spectroscopy to study the [FeFe]-hydrogenase from *C. reinhardtii* (*CrHydA1*), discovering two new terminal hydride species.



Room temperature

In experiments carried at room temperature, Albracht *et al.* observed that the illumination with an halogen lamp has no effect on the EPR and FTIR spectra of the H_{ox}-CO and H_{inact} states, but in samples prepared with ¹³CO illumination shuffles the two distal CO ligands bound to Fe_D^{59,66}.

Stripp *et al.* used this effect to study the dynamics of the [2Fe]_H site by isotope exchange with real-time attenuated total reflection FTIR (ATR-FTIR)¹⁰⁶. The exposure of CrHydA1 in the H_{ox}-CO state to exogenous ¹³CO results in a shift of the IR bands that is ascribed to the exchange of one of the intrinsic CO ligands on Fe_D with the labelled ligand. Subsequent irradiation with red light (640 nm) and then blue light (460 nm) induces the formation of a cluster binding four labeled ¹³CO ligands. A difference is observed between *C. pasteurianum*, *D. desulfuricans* and *C. reinhardtii* hydrogenases: red light illumination exchanges the bridging CO of Cpl and DdHydAB hydrogenases, whereas blue light is required to obtain the same effect in the case of CrHydA1. Stripp *et al.* propose the structures of the different forms from the IR spectra using a DFT model of the H-cluster with truncated cysteines, and larger models that include amino acids around the active site. Regarding the H_{ox}-CO species, these calculations support the presence of the distal CN-

ligand in the apical position, instead of the consensual equatorial position. In contrast, other spectroscopic¹⁰⁷ and DFT studies^{108–110} have considered unlikely the coordination of the distal CN⁻ on the apical binding site.

Kinetics

The kinetics of CO binding or release has been examined using time-resolved IR spectroscopy, to focus on rebinding of CO after photodissociation, and also under turnover conditions by protein film voltammetry, to examine how irradiation affects the kinetics of inhibition by CO.

Regarding the rebinding experiments, Mirmohades *et al.*¹¹¹ irradiated a *C. reinhardtii* FeFe hydrogenase 1 sample in the H_{ox}-CO state by flashing a UV laser (10 times 10 ns flash, $\lambda = 355$ nm), at room temperature, and used time-resolved nanosecond mid-infrared spectroscopy to detect CO photo-dissociation and rebinding. The quantum yield of conversion is around 10% for this enzyme, and around 8% of the total sample was converted into H_{ox} after flashing. The kinetics of rebinding was not 1st-order, suggesting that the dissociated CO may reach several binding sites in the protein before rebinding to Fe_D, and the half-life of the rebinding of CO was 13 ± 5 ms.

In protein film voltammetry, the enzyme is adsorbed onto an electrode, and the electrons produced upon H₂ oxidation are directly transferred to the electrode. The resulting current is proportional to the turnover frequency, and the variations in current report on any changes in activity^{112–114} which may result from CO binding or release. A light source can be directed towards the electrode that supports the enzyme, to examine how irradiation affects the activity or the inhibition kinetics^{112,113}.

Parkin¹¹⁵, Goldet¹¹⁶ and coworkers in the Armstrong group have first used this approach to qualitatively evidence the effect of light on the recovery of activity of the CO-inhibited enzyme. The illumination of the enzyme with the white light from a halogen lamp (10 W and 20 mW/cm² in ref ¹¹⁵, 150 W in ref ¹¹⁶) enhances the recovery of activity, but only under oxidizing conditions; no effect is seen at low electrode potential, when the enzyme is evolving H₂. No difference was noted between *C. acetobutylicum* HydA1 and *C. reinhardtii* HydA1. The rate of the process was not determined.

We also used electrochemistry to examine how light affects the CO binding and release kinetics, aiming for a quantitative description of the photoreactivity. We irradiated the FeFe hydrogenases from *M. elsdenii*, *C. acetobutylicum* and *C. reinhardtii* (HydA1) adsorbed onto electrodes with monochromatic lasers, varying the wavelength (405, 450, 532 and 635 nm) and the incident power density (from 0 to 60 μ W/cm²). We obtained the values of the CO binding ("in") and release ("out") rate constants using previously developed methods^{14,117–119}. We found that irradiation has no effect on the CO binding rate, but blue or violet light efficiently dissociates the exogenous CO. From the slope of the linear change in k_{out} against light power, we estimated the value of the molecular absorption cross-section relative to the photodissociation of CO, which appears to be similar to the values deduced from the extinction coefficient of H-cluster biomimetic complexes; this shows that most of the absorbed blue-violet light leads to the dissociation of CO.

We combined the experimental results with the conclusions from TDDFT calculations to obtain an electronic and mechanistic description of the process. The CO inhibited H_{ox}-CO

form of the H-cluster is an oversaturated organometallic complex since one of the two Fe atoms formally counts 19 valence electrons. Our TDDFT calculations¹²⁰ show that H_{ox} -CO absorbs at a lower wavelength than H_{ox} , and evidence a resulting CO photolysis pathway. Indeed, starting from the electronic structure of the H-cluster⁸³, the H_{ox} -CO calculated spectra showed that only the $Fe_4S_4 \rightarrow Fe_2S_2$ charge-transfer and Fe_2S_2 localized excitations in the visible light range are active toward CO dissociation, in agreement with the experimental results. The exploration of the potential energy surfaces of the excited states as a function of the Fe-CO bond length clearly evidenced a non-radiative pathway characterized by a series of internal conversions (IC) that end with the release of the CO in the apical position. Using TDDFT geometry optimizations we also simulated one of these possible pathways for the dissociation of the apical CO⁸⁴.

Photoinhibition

Early FTIR and EPR measurements showed DdHydAB is damaged by the sunlight or by the white light from a Xe lamp, but not a halogen lamp. In contrast, recent PFV and FTIR studies of *D. desulfuricans* hydrogenase showed that inactivation occurs upon irradiation with a halogen lamp under oxidizing turnover conditions. Regarding Cal and CrHydA1 hydrogenases, no inactivation was observed upon illumination with a halogen lamp or lasers in the visible range, but UV-B irreversibly inactivates the enzyme under both oxidizing and reducing conditions (Table 1).

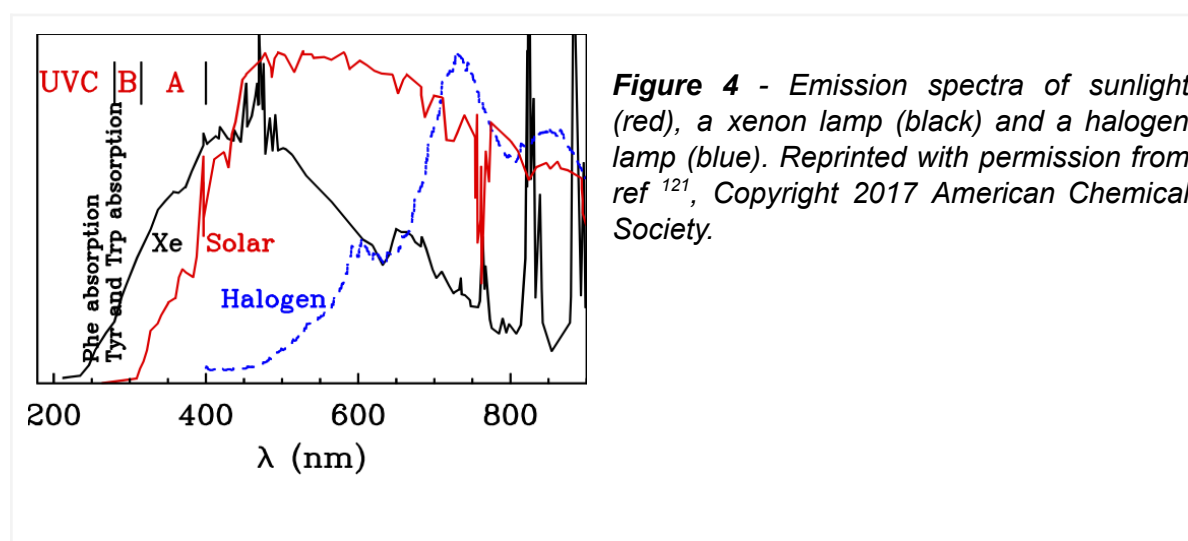


Figure 4 shows the xenon and halogen lamps emission spectra. The halogen lamp spectra has an overall higher emission in the visible range above 600 nm than the xenon lamp but the latter has some emission peaks in the near-infrared, and higher UV components than the halogen lamp. The power density actually received by the sample also certainly matters⁸⁴, since saturation of light absorption only occurs when the absorption cross section is very large; however, this information is rarely available in research papers.

The first evidence of the detrimental effect of light on FeFe hydrogenase was published by Albracht and coworkers in 2006⁵⁹, in an IR and EPR investigation of *D. desulfuricans* hydrogenase (HydAB). At room temperature and upon irradiation with a UV-filtered xenon lamp, the EPR and FTIR⁶⁶ spectra of *D. desulfuricans* HydAB initially in the H_{ox} state show

contributions from the H_{ox}-CO state, which is a signature of H-cluster degradation: CO ligands are released from some degraded H-cluster and bind to other, still-intact enzyme molecules. This “cannibalization” process is also observed when the enzyme is exposed to O₂¹²². DdHydAB in the H_{red} state was less sensitive to light. Under cryogenic conditions, illumination of the *D. desulfuricans* hydrogenase samples in the H_{ox}-CO state with a xenon lamp leads to the disappearance of both the extrinsic and bridging CO bands from the FTIR spectra, whereas only the release of the extrinsic CO is observed using a halogen lamp. The specific effect of the two lamps suggests that the differences between the emission spectra of the halogen and xenon lamp are crucial. Xenon lamps have a UV component, which is almost completely absent in halogen lamps, but the authors used a “glass light guide” that eliminated “most of the UV contribution”⁶⁶. We can only speculate that the different effects of the lamps are due to the higher intensity of the emission of the xenon lamp in the visible range or to a small fraction of not filtered UV light.

In 2016, Rüdiger and coworkers¹²³ also observed the light sensitivity of *D. desulfuricans* hydrogenase at room temperature, using protein film voltammetry and FTIR. The FTIR spectra of *D. desulfuricans* HydAB after irradiation presented the typical H_{ox}-CO bands previously observed by Albracht *et al.*^{59,66} In electrochemistry experiments, the authors illuminated the protein film with a 50 W halogen lamp positioned under the electrochemical glass cell, which we expect should block UV light. Under oxidizing conditions, constant illumination induces an inactivation followed by a partial recovery of the activity; another partial reactivation is observed when the light source is switched off. No such effect is observed under reducing conditions (figure 2b in ref ¹²³). The authors propose that the photoinhibition of the enzyme on the electrode is due photodamage and cannibalization, and the subsequent reactivation would result from the dissociation of CO. However, and as pointed out by the authors themselves, the experimental conditions do not allow the small amount of CO resulting from enzyme degradation to bind to nearby enzyme molecules on the electrode surface. The two-step reactivation, under illumination and after light removal, is not explained.

In the above described experiments performed with *D. desulfuricans* hydrogenase, the role of the emission spectra of the lamp is not clear. Rüdiger *et al.* observed the inactivation of DdHydAB using a halogen lamp, whereas Albracht *et al.* observed the photodamage of the same enzyme upon irradiation with a xenon lamp but not with a halogen lamp. In independent electrochemistry experiments, Parkin *et al.* also saw no photoinhibition of *D. desulfuricans* hydrogenase by irradiating with a halogen lamp¹¹⁵ (whose power was lower than that used by Rüdiger *et al.*).

Regarding the photoinhibition of the enzymes from *C. acetobutylicum* (HydA1) and *C. reinhardtii* (HydA1), Goldet and collaborators detected no photoinhibition upon irradiation with a halogen lamp; they only observed that irradiation slightly increases magnitude of the current and do not comment on whether or not it is possible that an increase in activity would result from irradiation heating the graphite electrode¹¹⁶. We consider it unlikely that irradiation would significantly perturb *the catalytic cycle* of hydrogenase: because of the small value of the absorption cross section of the enzyme in the visible range, photon absorption is a very rare event, unless a high power laser is used.

Reference	Enzyme	source	λ	P	T
Thauer et al, 1974 ⁹⁸	Cpl	halogen		250 W	RT
Patil et al, 1986 ¹⁰¹	DvHydAB	halogen		300 W	Cryo
Kowal et al, 1989 ¹⁰²	Cpl	xenon		200 W	Cryo
Lemon et al, 1999 ⁹⁹	Cpl	laser	632.8 nm		Cryo
Chen et al, 2002 ⁶⁷	Cpl	xenon		300 W	Cryo
Albracht et al, 2006, Roseboom et al, 2006 ^{59,66}	DdHydAB	halogen, xenon		150 W	Cryo and RT
Parkin et al, 2006 ¹¹⁵	DdHydAB	halogen		10 W	RT
Goldet et al, 2009 ¹¹⁶	CrHydA1, Cal	halogen		150 W	RT
Silakov et al, 2011 ¹⁰³	DdHydAB	laser	355 nm		Cryo
Katz et al, 2016 ¹⁰⁴	CrHydA1	LED	460 nm		Cryo
Senger et al, 2016 ¹⁰⁶	Cpl	laser	460 nm, 640 nm		RT
Mirmohades et al, 2016 ¹¹¹	CrHydA1	laser	355 nm		RT
Sensi et al, 2016 ⁸⁴	CrHydA1, Cal, MeHydA	laser	405, 450, 532, 635 nm	10 mW	RT
Rodriguez-Macia et al, 2016 ¹²³	DdHydAB	halogen		50 W	RT
Sensi et al, 2017 ¹²¹	CrHydA1, Cal	xenon	UVA, UVB, white (using filters)	150 W	RT
Lorent et al, 2020 ¹⁰⁵	CrHydA1	LED	460 nm		Cryo

Table 1. Summary of the literature about the effect of light on distinct FeFe hydrogenases, under various conditions of irradiation: wavelength λ , power P, temperature (room temperature, 'RT', or cryogenic conditions, 'Cryo').

On our side, we also used direct electrochemistry to look for evidence of photoinhibition of Cal and CrHydA1. We saw no detrimental effect of laser irradiation at 405, 450, 532 or 635 nm, but using a xenon lamp and various filters, we concluded that UV-B slowly and irreversibly inactivates the enzymes; this occurs under both H₂ oxidation and H⁺ reduction conditions when the enzyme is adsorbed onto an electrode, and also when the enzyme is stored in solution. According to time-dependent DFT (TDDFT) calculations, the H_{ox} UV-visible spectrum is characterized by the presence of charge-transfer excited states

involving the intrinsic CO ligands that can be populated upon absorption in the UV domain. Exploring the H_{ox} excited states potential energy surfaces (PES) as a function of the Fe-CO and Fe-S bonds lengths of the $[2Fe]_H$ subcluster, we observed *in silico* the dissociation of the terminal CO ligands. The most stable photo-product has an all-terminal geometry without the bridging CO and with two equatorial COs, irrespective of the redox state of the initial species. The reduced form of this photo-product could easily form a stable dead-end with a bridging hydride, which we considered the likely structure of the inactive photoproduct¹²¹.

The reason why DdHydAB is apparently sensitive to white light, unlike homologous hydrogenases, is unknown¹²¹.

Photochemistry of mimics of the H-cluster

The hydrogenases scientific community has invested enormous efforts in the search of structural and functional analogues of the H-cluster. Many models have been synthesized, considering the diiron $[Fe_2(CO)_6(pdt)]$ model as the parent^{124,125,126}. Photochemical activation is a common tool for ligand substitution in this family of complexes¹²⁴. Figure 5 shows three general mechanisms by which Fe_2S_2 models can produce H_2 .

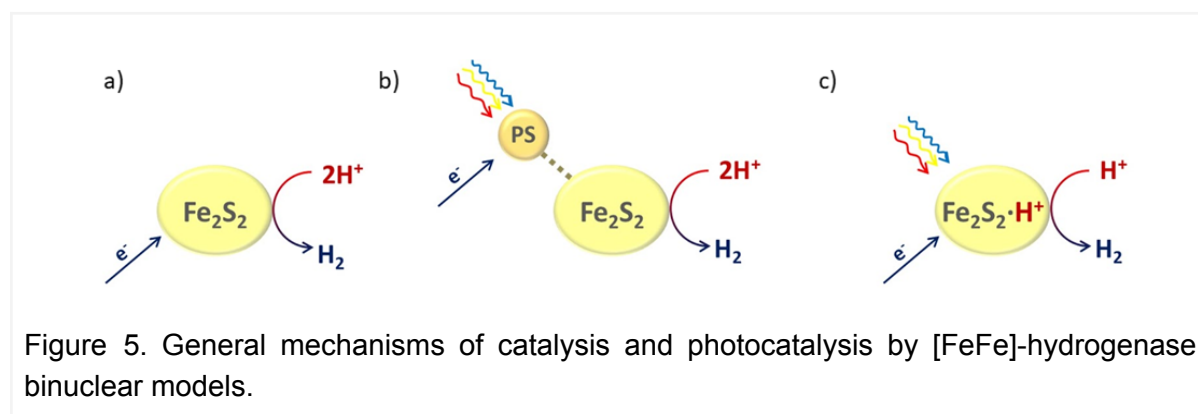


Fig 5a shows H_2 production by an Fe_2S_2 model in an acidic solution and in the presence of an electron donor.

A photocatalytic diad system is obtained when this cluster is conjugated to a photosensitizer (PS), such as a classical Ru(II) or Pt(II) complex with bis- or tris-dentate pyridine or porphyrins ligands (Fig 5b).^{25,127} H_2 can then be produced in the presence of a less reducing electron donor, since the free energy for H_2 evolution comes in part from an electron or energy transfer process from PS to the cluster¹²⁸.

An alternative approach consists in using a protonated Fe_2S_2 model that can itself absorb light energy (Fig 5c). The advantage is to avoid the sensitization and all issues related to the chemical PS-model conjugation and the quantum yield of the PS-model quenching. The first photocatalyst of this type was the protonated $Fe_2(pdt)(\mu-H)(CO)_4(dppv)$ (where $dppv = cis-1,2-C_2H_2(PPh_2)_2$) investigated by Rauchfuss *et al.*¹²⁹⁻¹³¹ In this case, light absorption induces a loss of the bridging character of the hydride, which is reminiscent of the behavior of the $\mu-CO$ in the CO-inhibited form of the H-cluster¹²⁹⁻¹³¹. The formation of a terminal hydride is favored by the presence of electron-rich phosphine ligands¹³².

Reference	λ (nm)	$\text{Fe}_2(\text{L})_6$ (bridging ligand)	
		Ligands	bridging ligand
Ridley <i>et al.</i> , 2008 ¹³³	350	CO	pdt
Brown-McDonald <i>et al.</i> , 2009 ¹³⁴	365	CO	pdt
Bertini <i>et al.</i> , 2009 ¹³⁵	-	CO	pdt
Stewart <i>et al.</i> , 2010 ¹³⁶	350	CO	pdt
Kaziannis <i>et al.</i> , 2010 ¹³⁷	355,435	CO	pdt
Marhenke <i>et al.</i> , 2011 ¹³⁸	365	CO	pdt
Stromberg <i>et al.</i> , 2011 ¹³⁹	-	CO	pdt,edt
Bingaman <i>et al.</i> , 2012 ¹⁴⁰	266,355, 532	CO	pdt
Muhammad <i>et al.</i> , 2012 ¹⁴¹	355	CO	pdt
Kania <i>et al.</i> , 2012 ¹⁴²	355	4CO, 2PMe ₃	pdt
Johnson <i>et al.</i> , 2013 ¹⁴³	355,532	4CO, 2PMe ₃	pdt
Bertini <i>et al.</i> , 2014 ¹⁴⁴	-	CO	pdt
Caplins <i>et al.</i> , 2014 ¹⁴⁵	400	CO	bdt
Thornley <i>et al.</i> , 2015 ¹⁴⁶	330, 400	CO	pdt
Goy <i>et al.</i> , 2016 ¹⁴⁷	-	CO	fluorene; silafluorene
Hunt <i>et al.</i> , 2016 ¹⁴⁸	365	4CO, 2CN ⁻	pdt
Bertini <i>et al.</i> , 2018 ¹⁴⁹	-	4CO, 2PMe ₃	edt
Meyer <i>et al.</i> , 2018 ¹⁵⁰	266,355, 532	4CO, 2PMe ₃	edt
Stromberg <i>et al.</i> , 2018 ¹⁵¹	266, 400	4CO, 2CN ⁻	pdt
Thornley <i>et al.</i> , 2019 ¹⁵²	200, 525	4CO, bipy/phen	pdt
Bertini <i>et al.</i> , 2021 ¹⁵³	-	CO, μ CO	pdt
Meyers <i>et al.</i> , 2021 ¹⁵⁴	400	mono substituted CN ⁻	edt,pdt

Table 2 - Summary of the literature about the photochemistry of the Fe_2S_2 model complexes (pdt=propandithiolate ; edt=etandithiolate ; bipy=2,2'-bipyridine ; phen=1,10-phenanthroline ; PMe₃=trimethylphosphine).

Besides the design and investigation of photocatalytic systems, an interest has grown in photochemistry of the simple Fe_2S_2 model. Starting with the investigation of $\text{Fe}_2(\text{CO})_6(\text{pdt})$ in 2008,¹³³ many models with the general formula $\text{Fe}_2(\text{CO})_4(\text{L})_2(\text{bridging ligand})$ have been studied (Table 2). The synthetic $\text{Fe}(\text{CO})_4(\text{CN})_2(\text{adt})$ compound (used for the in vitro reconstitution of [FeFe]-hydrogenases) is catalytically inert^{155,156}, but the closely related $\text{Fe}(\text{CO})_4(\text{CN})_2(\text{pdt})$ is active in H_2 photoproduction using $[\text{Ru}(\text{bpy})_3]^{2+}$ as photosensitizer and sodium ascorbate as sacrificial electron donor, upon irradiation with visible light ($\lambda > 400$ nm)^{156,157}(Artero et al. 2015).

The main focus of the photochemical studies was on the ultrafast (< 1 ns) photodynamics of the low-energy excitation, which is mainly dominated by CO photolysis, as is usual in metal-carbonyl complexes. The aim was to understand the effect of the ligands on the electronic properties of the model in relation to the nature of the photoproducts. The photostability of the models is crucial, if the goal is to wire them to photosensitizers for H_2 photoproduction. Flash photolysis kinetics, ultrafast pump/probe time-resolved IR (TRIR) spectroscopy and quantum chemistry calculations have been used to characterize the non-radiative transitions that occur upon UV-Vis light absorption.

The TRIR investigation of Fe_2S_2 18-electron models evidenced at least one ultrafast CO dissociation process, with the formation of an 16-electron intermediate species, whose life time depends on the chemical nature of the ligands (e.g. 25 ps in the case of a phen substituted model¹⁵² and much longer for a cyano-functionalized model¹⁵¹). TDDFT calculations¹⁴⁴ suggests that dissociation after excitation in the UV domain results from the population of a dissociative $\text{Fe} \rightarrow \text{CO}$ charge transfer (CT) state, which involves an equatorial CO ligand.

When $\text{Fe}_2(\text{CO})_4(\text{L})_2(\text{bridging ligand})$ models are considered, ligand isomerization is observed, which is due to the concomitant CO dissociation and Fe-S bond breaking during the non-radiative decay of the first excited state to the ground state^{149,150}. The latter result is reminiscent of the photo-inhibition of the enzyme, where photochemical channels are populated when the enzyme is irradiated in the UV range. After CO photolysis, the formation of a long lived solvent adduct is observed¹⁴¹, as in the case of the mono cyano substituted models¹⁵⁴, and geminate recombination gives back the initial model compound.

The excited state properties of the CO inhibited models have been investigated only theoretically, because over-saturated 19 electron complexes are not stable enough to be synthesized (see however ref. ¹⁵⁸). According to TDDFT calculations, the $\text{Fe}_2(\text{CO})_6(\mu\text{-CO})(\text{pdt})$ complex, a model of the $\text{H}_{\text{ox}}\text{-CO}$ form of the H-cluster, photo-releases a terminal CO after excitation in the visible domain in a lower energy range than observed with H_{ox} models¹⁵³.

Conclusions

The investigation of the photochemistry of the H-cluster biomimetic models has been important not only to design a better and efficient system for hydrogen photo-production, but also to shed light on the functionality of the active site itself. The spectroscopic, electrochemical and DFT studies on the photochemistry of FeFe hydrogenases contributed

not only to improve the development of hydrogen photo-production but also to obtain a fine description of the H-cluster electronic structure.

Our survey of H-cluster biomimetic model photochemistry has revealed that most of the complexes are photosensitive in the UV domain, as are the enzymes, and undergo ultrafast photodissociation of a terminal CO ligand. Following this, the unsaturated models form a solvent adduct that slowly decays to the initial model. The scrambling of the ligands is often observed along the vibrational cooling pathway, this process being favored by the transient vacancy of the CO ligand and the weakening of a Fe-S bond. The main difference between the photodynamics of the H-cluster and of its models comes from the ability of the latter to form a solvent adduct, whereas the H-cluster is shielded from the solvent.

The investigation on CO inhibited models has shown that the presence of an exogenous CO ligand lowers the energy range of the MLCT bands and of the CO photolysis channels. For this reason it appears that the ligand CO protects the $[2Fe]_H$ cluster since it is able to capture visible light, after which the system decays through photo-dissociative channels that involve a CO ligand, thus preventing irreversible photo-damage.

The literature on the photochemistry of the enzymes is related to photo processes that occur on longer time scales, and is relevant to the practical use of hydrogenases in H_2 photoproduction systems.

Overall, the literature is not fully consistent and difficult to assess because of the lack of precise report of experimental design and control experiments, regarding e.g. heating and photoelectric effects, or the details about the light sources and power, and the nature of the transparent materials the containers and electrochemical cells are made of. However, clear differences in photosensitivity have been observed between homologous FeFe hydrogenases, the enzyme from *D. desulfuricans* being particularly fragile in this respect. It is remarkable that this enzyme is also more O_2 sensitive than e.g. clostridial FeFe hydrogenases¹⁴, and sensitive to inhibition by sulfide whereas Cpl hydrogenase apparently is not⁷⁰. These variations are puzzling, considering that all these enzymes embed the same H-cluster, whose environment is very conserved. These observations demonstrate that structural details must influence the resistance to photochemical stress of FeFe hydrogenases. Such long distance effects are difficult to investigate. Regarding oxidative stress, we recently demonstrated that residues that are remote from the active site may change the flexibility of the protein and the reactivity in the 1st coordination sphere of the distal Fe.⁸⁹

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