

Molecular Hydrogen: Redox Reactions and Possible Biological Interactions

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Short title: Hydrogen gas and redox

Abstract

Molecular hydrogen (H₂), either as a gas or as hydrogen-rich water (HRW), is suggested to be a useful treatment for a range of human diseases and also to improve agricultural output. It is often posited that H₂ accomplishes its biological action, in part, through its antioxidant effects, including reacting with hydroxyl radicals ($\cdot\text{OH}$) and peroxynitrite (ONOO⁻), however, this direct reaction has been questioned. The antioxidant effects of H₂ are also often mediated by heme oxygenase (HO-1), although the exact mechanism remains elusive. Alternatively, it has been proposed that H₂ can propagate its effects through the reduction of Fe³⁺ in various redox-active proteins, which is the focus of this review. It is suggested that a systematic experimental analysis of proteins containing heme prosthetic groups would help elucidate the biological mechanisms of H₂ and its development as a medical and restorative therapeutic.

Keywords: Antioxidants; Heme oxygenase; Hydrogen gas; NADPH oxidase; Nitric oxide; Redox; Reactive Oxygen Species; Soluble guanylyl cyclase

Introduction

Molecular hydrogen (H₂) is now recognized as a molecule which can have significant effects in a range of organisms, from plants to animals. H₂ and its biological reactions have had a significant influence on evolution [1]. Along with several other small redox-active compounds, H₂ can be adopted for positive reasons by cells [2]. H₂ is also produced in significant quantities by algae such as *Chlamydomonas* [3], so much so that it has been suggested to be used for biofuel production [4]. In animals, including humans, the use of H₂ in treatments has been suggested for a range of diseases, including sepsis [5], cancer [6] and COVID-19 [7,8]. In plants, H₂ has been shown to be beneficial when growth is subject to stress, including that caused by salinity [9], UV-A irradiation [10] and heavy metals [11].

Although H₂ was considered to be a relatively insoluble and biologically inert gas, it is now recognized as being biologically important [12,13]. It is relatively easy to administer exogenously. In cells, H₂ can move through membranes easily, unlike other reductants, such as NADH, and is unlikely to be easily compartmentalized. Therefore, it can have targets at multiple locations in cells, including in organelles. It also appears to have, at least at present, no identified harmful effects on cells. However, as has been argued before [14], it is hard to envisage how H₂ can be perceived by cells using a classical receptor-type mechanism or via direct post-translational modification of proteins. Therefore, the question remains: what are the direct targets of H₂ in cells? Here, some of the interactions which have been suggested are reviewed, with a focus on the interaction of H₂ with Fe³⁺.

Antioxidant effects of H₂: are they real?

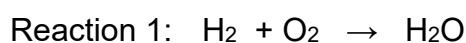
One of the most reported effects of H₂ in cells is as an antioxidant [13,15]. This could manifest itself in three ways: i) H₂ acts as a direct scavenger of reactive species, ii) it increases the antioxidant capacity of cells downstream, for example by increasing superoxide dismutase (SOD) or catalase (CAT) presence or activity, and/or iii) decreases ROS production via its signal modulating effects. In a treatise of the scavenging effects, it was reported that H₂ was selective against the hydroxyl radical ($\cdot\text{OH}$) but was not a direct scavenger of other reactive oxygen species (ROS) [16]. Conversely, some have reported that H₂ can reduce the levels of measured H₂O₂ [17], a significant signaling molecule in plants and animals. Whilst others have suggested that the action of H₂ are mediated by its ability to reduce levels of peroxynitrite (ONOO⁻) [16,18], a mechanism that would influence the signaling from ONOO⁻ and therefore from nitric oxide (NO) metabolism.

Penders *et al.* [19] disputes the direct scavenging claims. They report that the reaction of H₂ and $\cdot\text{OH}$, as determined using pulse radiolysis, is too slow to be of physiological relevance. Reactions of $\cdot\text{OH}$ with other biomolecules is probably more likely than with H₂, meaning that H₂ would have little chance to influence the biological effects of $\cdot\text{OH}$ generation in cells. The authors further claim that there is no influence of H₂ on ONOO⁻ either, suggesting that the effects of H₂ are more likely mediated by other mechanisms, potentially involving Fe³⁺ ions. However, using myoglobin, cytochrome P450 and putidaredoxin, they argue there was no reduction of heme or iron-sulfur clusters. Below, the notion of H₂ reduction of Fe³⁺ is explored further.

Redox of H₂ and its possible downstream consequences

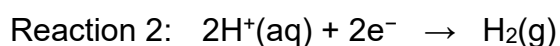
The effects of H₂ on animal and plant cells are only recently becoming well recognized. However, H₂ metabolism in bacteria has been known for a long time [for example, 20]. To illustrate, *Desulfovibrio* species, which have hydrogen as a central part of their energy metabolism, are known to be part of the human gut microbiome [21], this may be important for the action of the H₂ ingested as hydrogen-rich water (HRW).

Knallgas bacteria may use H₂ as a source of electrons in what is referred to as the Knallgas reaction (Reaction 1):



This reaction in cells is facilitated by hydrogenases [22,23]. Algae also engage in hydrogen metabolism. In a recent paper it was reported that there was a bi-directional energy transfer between H₂ and NADPH in *Chlamydomonas reinhardtii*, a process involving ferredoxin-NADPH oxidoreductase (FNR) [24]. Therefore, a question which may be asked is: are any of the precedents or principles in lower organisms able to be translated to higher plants and animals?

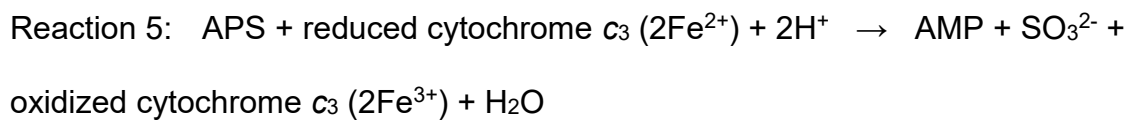
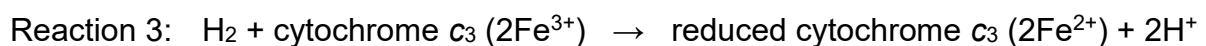
Biological redox potentials are quoted against the reference of the Standard Hydrogen Electrode (SHE) [25], which has the redox half cells as shown in Reaction 2:



A SHE is created by having a platinum electrode in 1M H⁺ solution and hydrogen gas at 1 bar (100 kPa), which has a potential defined as 0 mV. At pH 7, this translates to the H₂/H⁺ half-cell having a potential of -414 mV (Table 1). This means, that theoretically, hydrogen should be able to pass electrons and reduce biomolecules with an E_o' (mV) more positive than -414 mV. As can be seen in Table 1, reduction of

ferredoxin from spinach would not be possible, with an E'_o of -432 mV. Further, *Chlamydomonas* hydrogenase could not reduce spinach ferredoxin [26], probably as it is not likely to be a thermodynamically favorable reaction.

A report of particular note here is the treatise on the hydrogen-mediated reduction of sulfate in *Desulfovibrio desulfuricans* [27], which makes a good example of how H_2 can reduce Fe^{3+} . The summary of the paper details the following reactions (Reactions 3-5):



This shows two compelling effects. Firstly, the prosthetic heme Fe^{3+} can be reduced to Fe^{2+} by H_2 , albeit facilitated by the presence of the enzyme, so altering the kinetics. Secondly, the reduced heme can catalyze downstream reactions, in this case the generation of SO_3^{2-} .

It may be important to note here that the report also states that at pH 6.0 the final product is likely to be hydrogen sulfide. H_2S is a compound known to be involved in cell signaling events [28] and so has an influence on cell function.

The research [27] concludes that cytochrome c_3 has a mid-point potential of -205 mV, and so can be reduced by H_2 , but it also shows that heme prosthetic groups, as part of a protein, can become reduced by H_2 , with the Fe^{3+} as the target, just as previously suggested [19]. Therefore, even though neither myoglobin nor cytochrome P450 could be reduced [19], there may be other cytochromes in plants and animals which could be H_2 targets. Some of the possible targets and their mid-point potentials are listed in Table 1.

H₂ is often found to have effects during stress responses, that can arise from either biotic or abiotic challenges [29,30]. A family of enzymes which are often associated with such stress responses are the NADPH oxidases [31,32]. In animals there are seven members of the NADPH oxidase protein family: Nox1-5, Duox 1-2. These can be grouped into three categories. The original NADPH oxidase was characterized from the phagosome and plasma membranes of neutrophils. It was noted that this catalytic enzyme required NADPH as a co-factor and that its role was to transfer two electrons in a reaction that produced superoxide (O₂^{•-}) on the outside of the cell. Activation of the NOX complex requires the assembly of several polypeptides: gp91-*phox* (in the membrane), p22-*phox* (in the membrane) and three cytosolic polypeptides (p47-*phox*, p67-*phox* and p40-*phox*), along with a monomeric G proteins Rac. A factor of pertinence here may be that Nox 1, Nox 2 and Nox 3 have the same compositional characteristics. Whilst Nox 5 and the Duox proteins are independent of the cytosolic proteins, can produce H₂O₂ as well as O₂^{•-}, and are regulated by Ca²⁺ ion interplay. Nox 4 seems to be particularly different in that it is intracellular, controlled by gene expression with constitutive activity, is independent of the cytosolic subunits, and generates H₂O₂. The activities and roles of NADPH oxidase proteins were recently reviewed [33]. In plants the oxidase enzymes are referred to as respiratory burst oxidase homologues (RBOHs). They produce superoxide anions, are controlled by a variety of mechanisms including by G proteins and membrane lipids, and they are involved in a wide range of plant mechanisms including development and stress responses [34]. With a range of characteristics, and their roles in stress responses, it is tempting to suggest that this family of proteins could be good targets for H₂.

NADPH oxidase is a cytochrome *b* containing enzyme. This chromophore has an absorbance at 558 nm, and is referred to as cytochrome *b*₅₅₈, but alternatively as cytochrome *b*₂₄₅, after the original mid-point potential reported for this enzyme [35]. However, subsequent analysis has shown that the gp91-*phox* subunit contains two nonidentical heme groups, with mid-point potentials of -225 mV and -265 mV [36], both of which are more positive than the biological H₂ potential of -414 mV. Therefore, thermodynamically, reduction is possible, and with some isoforms not needing an activation step, e.g., Nox 4, it is tempting to suggest that reduction of this cytochrome in different cell types is worth exploring. However, because the cytochrome uses oxygen as a terminal electron acceptor, the original redox measurements were carried out in an oxygen-free argon atmosphere, otherwise the steady-state reduction could not be recorded. Therefore, any future experiments with such cytochromes should also be carried out under similar conditions. This would not be easy if the proposed reduction is from a gaseous molecule, i.e., H₂. Of course, as was carried out with the original NADPH oxidase cytochrome characterization [37], the kinetics of any reaction would also need to be determined in order to show that it would have physiological relevance.

The activity of membrane bound NADPH oxidase is reported to be electrogenic, in that there has to be an H⁺ compensation to allow the electron transfer across the membrane from NADPH to oxygen [38]. This is because NADPH is oxidized on the cytoplasmic side of the membrane, whilst the electrons reduce O₂ on the other side. H₂ oxidation would also yield H⁺, but if this is directly at the heme groups it is possible that this would not require the trans-membrane movement of H⁺. If an H⁺ is generated directly at one of the hemes the proton may already be on the correct side of the membrane. The terminal heme must have access to the far side

of the membrane for O₂ to interact and the product to be generated on the conformational side of the membrane. Therefore, reduction by H₂ may be less complicated than by NADPH. It should also be considered that the product, if there is a reaction and a product generated, would be either superoxide anions or hydrogen peroxide, and both could partake in ROS signaling, and not necessarily lead to oxidative stress, as the accumulation of ROS can be compartmentalized in cells [39]. Therefore, it could be speculated that in animals, Nox 4 may have a pivotal role in H₂ biochemistry because it is intracellular, does not require cytosolic subunits for activity, and is able to directly generate H₂O₂ [33], the main ROS signal in cells. Accordingly, it is proposed here that the influence of H₂ on Nox enzymes, and in particular Nox 4, ought to be explored.

Other Fe³⁺/heme containing enzymes which may provide an insight into H₂ activity include those located in the electron transport chain of mitochondria or in chloroplasts, with their function being essential for ATP production. If any components of the electron transport chains of such organelles are found to be reduced by H₂, there are at least two possible outcomes. The electrons can either enter the pathway and be used to drive expected activity – in mitochondria this would be H⁺ pumping and O₂ reduction – or alternatively, electron leakage from such chains, leading to increased ROS [40] or nitric oxide [41], which could result in downstream signaling and altered cellular activity. Reduction of any such electron transfer components would be worth investigating, if only to rule this out as an action of H₂.

However, some caution needs to be exercised here as although some cytochromes, such as cytochrome *c*, (E_o' +254 mV) do not autoxidize, Complex IV (cytochrome *a*: E_o' +290 mV) also has oxygen as a terminal electron acceptor, so

such experiments may again need to be carried out under oxygen-free conditions. Even so, as listed in Table 1, these heme centers are theoretically more thermodynamically capable of being reduced by H₂ than NADPH oxidase, so may be worth exploring. Although it was previously shown that neither myoglobin nor cytochrome P450 interacted with H₂ [19], even though experiments were carried out under a nitrogen atmosphere, this does not rule out that other heme centers could not be reduced by H₂.

Other cell signaling heme proteins

Two heme proteins of note which may be considered because they are so instrumental in cell signaling pathways are soluble guanylyl cyclase (sGC) and nitric oxide synthase (NOS).

Ravi [42] reported that the heme group of sGC can be oxidized to the Fe³⁺ state by ONNO[•]. This is of significance as oxidation renders the sGC insensitive to NO[•], which is one of the primary modes of activation of this enzyme. It would be interesting to determine if H₂ could reverse this removal of this control mechanism by reconvert the Fe³⁺ back to Fe²⁺, and so enabling NO[•] regulation to be restored. However, if found to be the case, this may not account for the effects of H₂ in plants, where the downstream events associated with NO[•] are thought to be distinct from those in animals, and that classical cGMP signaling is not involved [43].

Nitric oxide synthase (NOS), an enzyme system with similarities to cytochrome P450, especially in the reductase domain [44], may also be of interest when assessing H₂ redox interactions. Even though the heme in cytochrome P450 was not found to be reduced by H₂, this does not rule out NOS as a possible target, as the heme here has a different function to P450 and there are significant

differences in the enzyme systems. Therefore, the redox state of NOS would be worth investigating, although, as NOS has not been found in higher plants, either at the genetic or protein levels, likewise with sGC, this almost certainly cannot be relevant when accounting for the H₂ effects seen in plants [45]. Nevertheless, similar redox-type reactions also occur in plants, and H₂ may function in a similar way in those redox-active complexes.

Heme oxygenase and H₂ spin states

Further to the discussion above, there are other mechanisms suggested for H₂ action in cells. Heme oxygenase (HO-1) has been reported to mediate the effects of H₂ treatments in both animals [46] and plants [47]. Jin *et al.* [48] demonstrated that when the common crop alfalfa (*Medicago sativa* L.) was supplied with exogenous H₂ or the heme oxygenase-1 (HO-1) inducer, hemin, enhanced tolerance to oxidative stress occurs. The experimental results show that H₂ pre-treatment can augment levels of *MsHO-1* mRNA transcription, of the subsequent encoded protein, and amplify HO-1 cellular activity. However, the exact role H₂ has in influencing this mechanism is not well understood.

Lastly, H₂ can exist in two spin states, *ortho* and *para*, and to be able to switch between them [49,50]. It has been suggested that the spin state of H₂ may be able to influence the actions of biomolecules used in cell signaling events [51], although to date there is no evidence of this. Consequently, along with the possible influence on Fe redox states, there are other avenues that will need to be evaluated before a full understanding of the complex effects and primary targets of H₂ administration can be resolved.

Conclusions and the future

There is no doubt that H₂ has effects on cellular activities in both animals and plants, and it has been suggested to be a future treatment for human disease [5-8] and for improving agricultural practices [9-11]. It has been proposed that these effects are mediated by a range of actions (Figure 1), including H₂ acting as a scavenger of reactive molecules, ·OH and ONOO⁻ [16,52], and induction of heme oxygenase [46-48], although there has been doubt cast on the physiological relevance of the former [19]. It has also been suggested that H₂ may interact with Fe³⁺ [19], perhaps in heme prosthetic groups. Although the original report showed no experimental evidence for this, only looking at the reduction states of myoglobin and cytochrome P450.

Theoretically, using the known mid-point potentials for many heme containing proteins, the reduction of Fe³⁺ to Fe²⁺ should be thermodynamically possible.

Certainly, there is precedence, as seen in prokaryotic systems (e.g., *Desulfovibrio desulfuricans*) [27]. Some enzymes, such as NADPH oxidase homologues [33], are known to be involved in disease and stress responses in both plants and animals, so these also may be targets worth exploring. However, the pro-oxidant capacity of formed Fe²⁺ should not be dismissed, as further downstream events may potentially be propagated [54].

In conclusion, the authors advocate for a thorough investigation into the reduction of heme prosthetic groups in proteins to be carried out in both animals and plants, establishing whether an H₂/Fe³⁺/Fe²⁺ mechanism may account for some of the diverse effects seen in biological systems.

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Table 1: Some biologically relevant redox couples at 25°C, pH7 [27,36,53].

Half cell: redox reaction	E° (mV)
Ferredoxin (Fe ³⁺) + e ⁻ → ferredoxin (Fe ²⁺) - spinach	- 432
2H ⁺ + 2e ⁻ → H ₂	- 414
NAD ⁺ + H ⁺ + 2e ⁻ → NADH	- 320
Cytochrome <i>b</i> ₅₅₈ (Fe ³⁺) + e ⁻ → cytochrome <i>b</i> ₅₅₈ (Fe ²⁺) N.B. two non-identical heme groups in gp91- <i>phox</i>	- 245 (average) (- 225 & -265)
S + 2H ⁺ + 2e ⁻ → H ₂ S	- 243
Cytochrome <i>c</i> ₃ (Fe ³⁺) + e ⁻ → cytochrome <i>c</i> ₃ (Fe ²⁺)	- 205
Cytochrome <i>b</i> (Fe ³⁺) + e ⁻ → cytochrome <i>b</i> (Fe ²⁺)	+ 77
Ubiquinone + 2H ⁺ + 2e ⁻ → ubiquinol	+ 45
Cytochrome <i>c</i> ₁ (Fe ³⁺) + e ⁻ → cytochrome <i>c</i> ₁ (Fe ²⁺)	+ 220
Cytochrome <i>c</i> (Fe ³⁺) + e ⁻ → cytochrome <i>c</i> (Fe ²⁺)	+ 254
Cytochrome <i>a</i> (Fe ³⁺) + e ⁻ → cytochrome <i>a</i> (Fe ²⁺)	+ 290
Cytochrome <i>f</i> (Fe ³⁺) + e ⁻ → cytochrome <i>f</i> (Fe ²⁺)	+ 365

Figure 1: A schematic summarizing possible downstream targets of H₂.

