Understanding Hydrogen: lessons to be learned from physical interactions between the inert gases and the globin superfamily

John T. Hancock[1]*, Grace Russell[1], Tim J. Craig[1], Jennifer May[1], H. Ruth Morse[1], Jonathan S. Stamler[2]

1 School of Applied Sciences, University of the West of England, Bristol, BS16 1QY, UK
2 Institute for Transformative Molecular Medicine, Department of Medicine, Case Western Reserve University School of Medicine, Cleveland, OH, 44106, USA.

* Correspondence: john.hancock@uwe.ac.uk Tel.: +44(0)1173282475

Abstract: Hydrogen gas (molecular hydrogen, H_2) has significant effects in a range of organisms, from plants to humans. Many inert gases have been reported to have similar effects, and such responses may be most pronounced when cells are stressed. Xenon (Xe), for example, is a well-known anesthetic. The direct targets of these gases, in most cases, remain elusive. Myoglobin and hemoglobins are known for their roles in the transport of gases through coordinate interactions with metals (O_2, NO, CO) and covalent modifications of thiols (NO, H_2S) and amines (CO_2). These are well exemplified in biotrophic reactions of NO with heme iron (to form iron nitrosyl heme) and cysteine (to form bioactive *S*-nitrosothiols) essential for tissue oxygenation. Here we consider an alternative "third mode" of gas transport in what have been dubbed 'Xenon pockets', whereby inert gases may have functional effects. Many proteins have similar cavities, and possible effects include alterations in allosteric properties of proteins (potentially altering protein hydration). Here, it is suggested that like other inert gases, H_2 also has biological effects by utilizing these protein structures. This ought to be investigated further, in a range of species, to determine if this is the mode of action of H_2.

Keywords: Argon; hemoglobin; hydrophobic cavities; inert gases; molecular hydrogen; myoglobin; xenon.

1. Introduction

There has been an escalating interest in the effect of hydrogen gas (molecular hydrogen: H_2) on biological systems, and it has been suggested that H_2 can be used as a therapeutic in biomedicine [1] and in agriculture [2]. For example, it has been suggested that H_2 treatment may be useful for mitigating the effects of neurodegenerative disease [3] and as a treatment for COVID-19 [4,5]. In plant sciences, field trials show that H_2 can improve the quality of rice [6].

H_2 can be supplied to biological systems in a variety of ways. For treatment in hospital, it can be given as a gas, often in combination with oxygen in what is referred to as oxy-hydrogen (HHO: H_2/O_2) [7]. Alternatively, H_2 gas can be bubbled into water to create a solution which is enriched in H_2, referred to as hydrogen-rich water (HRW), as used by Lin *et al*. [8] when studying fatty liver caused by alcohol in mice. A variation of this is hydrogen-rich saline (HRS) [for example: 9.10]. Other variations include the use of hydrogen nanobubble water (HNW) [11], which is reported to have more hydrogen dissolved and to retain the H_2 in solution for longer. One of the issues with many H_2 treatments is that the H_2 gas quickly moves into the atmosphere and therefore has limited biological activity. It tends to give a bolus effect. HNW may go some way to making this more physiological, having a slower and longer H_2 release into biological materials. It appears that in humans H_2 can be administered to the lungs [12], or as a drink [13]. It can even be used

as a topical treatment [14]. For plants HRW can be supplied to the soil [15], the feed water [16] or foliage [17], and the atmosphere can be augmented with H_2 gas [18]. Alternatively, H_2 can be supplied to biological tissues via a donor molecule, which will release H_2 in the location needed. One such donor is magnesium hydride (MgH_2), as used by Li *et al*. [19]. Such donors, in similarity with HNW, allow for prolonged diffusion, and therefore greater/sustained physiological exposure of the organism or tissue to H_2.

With so many ways to treat biological materials with H_2 a range of physiological effects have been reported but the underlying mechanisms remain somewhat controversial [20]. It has been widely reported that H_2 increases the antioxidant capacity of cells [21-23]. Enzymes such as superoxide dismutase (SOD), ascorbate peroxidase and catalase (Cat) have increased gene expression and increased activity [23], for example, whilst changes in glutathione (GSH) metabolism have also been noted [24]. However, the direct actions of H_2 which lead to such changes in the endogenous antioxidants of the cell, and therefore the intracellular redox status, have not been defined.

Others have noted that there are changes in the activity of heme oxygenase during H_2 treatment. This was reported in cucumber adventitious root development [25] and in treatment of inflammatory bowel disease [26]. In the latter paper, HRW induced gene expression of HO-1, as well as lowering oxidative stress, endoplasmic reticular stress, and inhibiting the immune response, all mitigating the effects of the disease [26]. However, as before, the direct action of H_2 to bring these changes about was not unraveled.

It has been suggested that H_2 acts directly as an antioxidant, particularly scavenging the hydroxyl radical ($^{\cdot}OH$), a reactive oxygen species (ROS), and peroxynitrite ($ONOO^-$), a reactive nitrogen species (RNS). However, other ROS and RNS which can act in cell signaling roles, such as the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and nitric oxide ($^{\cdot}NO$), are all arguably more important in controlling cell function than $^{\cdot}OH$ or $ONOO^-$ [27,28]. However, even the action of H_2 against $^{\cdot}OH$ or $ONOO^-$ has been disputed. With a more in-depth look at the kinetics involved Penders *et al*. [29] argue that these reactions of H_2 are not significant under physiological conditions.

If it is not known how H_2 controls gene expression, the antioxidant capacity of the cell, or the activity of heme oxygenase, and the direct interaction with ROS and RNS is questioned, alternative mechanisms of H_2 action are required.

It has been suggested that the redox midpoint potential for the H_2/H^+ couple is low enough to drive the change of the redox status of some biomolecules [30], perhaps those containing heme and those involved in mitochondrial function. There appears to be a precedent of this in bacterial systems, with cytochrome c_3 being reduced by H_2 [31]. There is no experimental evidence of similar reactions taking place in animals or plants, but it seems premature to rule this out.

An alternative mechanism was mooted because of the spin states of H_2 [32]. Again, there is no experimental evidence given for this mode of action of H_2, but the idea of atomic states of inert gases will be revisited below, when discussing Xe, so perhaps we should not rush to rule this out. Interestingly, the Fe of hemoglobin (Hb) has been known for a long time to have paramagnetic properties, as more recently discussed 33]. According to the original paper the oxygen molecule undergoes “a profound change in electronic structure” when it interacts with the hemoglobin [34]. Recently it has been suggested that it is possible for H_2 to have a direct interaction with the Fe in the heme of hemoglobin [35]. In this work the authors theoretically explore the manner in which the heme may alter the electronic configuration of the H_2 molecule, depending on whether the interaction is symmetrical or asymmetrical. They further suggest that there is the possibility for the production of hydrogen radicals ($^{\cdot}H$), which then would have the capacity to react with hydroxyl radicals or peroxynitrite, and hence this may be the mechanism by which these reactive compounds are scavenged from the cell. However, as intriguing as this is, it does pose many questions. It is not reported here if H_2 binding to the hexa-position of the Fe^{2+} in deoxyhemoglobin alters the overall capacity for O_2 transport *in vivo*. When oxygen binds to deoxyhemoglobin, the Fe transitions to the Fe^{3+} state with the oxygen being a bound superoxide anion. Kim *et al*. [35] also suggest loss of negative

charge on the Fe on H_2 binding, akin to the mechanism seen with oxygen. It would be interesting to see a full set of visible wavelength spectra for hemoglobin when various forms of the protein are treated with hydrogen gas. If H_2 can bind to heme prosthetic groups as suggested by Kim *et al*. [35], it would be interesting to know how this could be extrapolated to a whole range of other heme-containing proteins. Some of such proteins also bind oxygen, such as nitric oxide synthase (NOS) and NADPH oxidase. Others do not react with oxygen, such as cytochrome *c*. In some proteins the heme is covalently bound to the polypeptide (e.g. cytochrome *c*) whilst in others it is not, such as cytochrome *b*. Such questions are pertinent not just to animals, but to plants too, where a true hemoglobin does not exist, although homologues do [36]. Will they be able to undergo a similar mechanism as mooted by Kim *et al*. [35]? Therefore, this work is interesting and may inform a range of experiments in the near future.

It is clear, therefore, that H_2 has biological effects, but it is still unclear how it is acting. However, several other inert gases also have biological effects, including argon (Ar), Xe, helium (He), krypton (Kr) and neon (Ne). It is unlikely that such gases have chemical effects on biological materials, as being noble gases they are chemically inert, but they may have physical effects. Here, it is proposed that the effects of H_2 may be similar, and the biological responses to H_2 may be downstream of how H_2 interacts with proteins, and this may have direct comparisons with the action of the noble gases. Several significant reactive signaling molecules, including the gas $^{\cdot}NO$, have effects through the covalent modification of proteins and other bio-molecules. Such protein modifications include oxidation (by ROS) [37], *S*-nitrosylation (RNS. *S*-nitrosylation is alternatively referred to as *S*-nitrosation) [38], nitration (by RNS) [39] or persulfidation (by hydrogen sulfide (H_2S)) [40]. However, it is very unlikely that noble gases partake in such reactions, and it is also not likely that H_2 could be involved in the catalysis of such biomolecule alterations. Therefore, if direct chemical reactions are unlikely, a more physical interaction may be responsible for the action of H_2 and other inert gases. Below the evidence is reviewed.

2. Biological Effects of Argon

Ar is an inert gas. It is a single atom, with an atomic number of 18, and atomic mass of 39.948. It is the third most abundant gas in the atmosphere, at just under 1% (9340 ppm) of the composition of air. Even though it is described as "extremely inert" [41], being a noble gas, it has still been shown to have bioactivity. Ar has been shown to have anesthetic properties at high pressure – interestingly, Kr shows the same properties [42]. Ar effects have been reported in an ischemic stroke model and to have neuro-protective effects [43,44]. Ar also gave protection to the myocardium against infarction [45], but failed to protect kidney tissues if they are deprived of oxygen and glucose [46]. Ar was explored as a gas useful for diving in the 1930s, but was discarded because of its anesthetic effects [47].

Binding of Ar to deoxyhemoglobin was suggested in a study of nitrogen (N_2) binding [48]. It was thought that Ar should be able to bind the same hydrophobic pockets as N_2 in the proteins, and that this may have some influence on O_2 binding, but because of likely differences in 2,3-diphosphoglycerate (2,3-DPG) concentrations in the experiments of others it was hard for the authors to be confident on the exact effects.

Very recently, effects of argon in plants have been reported [41]. In this study, argon was delivered to the plants as argon-rich water (ARW) which had been created by bubbling 99.9% pure argon into distilled water. Once diluted this gave a range of argon concentrations up to 0.750 mmol L^{-1}. This argon-containing solution increased germination rates and seedling growth when the plants (alfalfa; *Medicago sativa* L. "Victoria") were under salinity stress. NaCl-induced lowering of α/β-amylase activities were abolished by argon, and gene expression studies showed that relevant genes were affected, for example ARW increased the level of transcripts for *NHX1*, a Na^+/H^+ antiporter. Interestingly, the authors also noted an increase in the antioxidant capacity of the plants once treated with ARW, an effect often reported with HRW.

Clearly, therefore, Ar has biological effects in a range of organisms. Being inert, it is unlikely that argon is involved in the direct chemical alteration of biological molecules, but more likely there is a physical interaction which is mediating the effects seen. Therefore, it is proposed here that H_2 may also have a similar physical interaction and in order to understand what might be happening, turning to what is known about Xe may be a way forward.

3. Biological Effects of Xenon

Xe is also a noble gas, with an atomic number of 54 and an atomic mass of 131.293. Even though it is an inert gas it has been shown to have a range of bioactivities.

Xe has long been known to be an anesthetic agent [49]. Such ideas were being reported by J.H. Lawrence and colleagues in the 1940s [50]. Xenon is known to have effects as an antagonist of the N-methyl-D-aspartate (NMDA)-type glutamate receptor, and as such has been studied for its neuro-protective effects [51]. Xe has also been found to elevate hypoxia-inducible factor-1α (HIF-1α) and hence vascular endothelial growth factor (VEGF), as well leading to an increase in expression of inducible nitric oxide synthase (iNOS) [52]. Other biological effects include the inhibition of the migration of breast adenocarcinoma cells and decreased release of 'regulated on activation normal T cell expressed and secreted' (RANTES), a pro-angiogenesis factor [53]. In endothelial cells Xe decreased Ca^{2+} signaling during the cell cycle [54]. Xenon has also been shown to have protective effects during hypoxia [55] and during hypothermic conditions [56], although when such work was repeated more recently the authors state "Xenon gas did not affect cell function" [57], however, the conditions of cell growth used here were not as harsh as used by others. Finally, Xe has been shown to have anti-apoptotic effects, maintain mitochondrial integrity and inhibit the activity of caspase-3 [58].

Xenon effects have been seen in plants too. Chlorophyll content, as well as the properties of membranes and vesicle trafficking in root cells was affected by the treatment of xenon gas (80% Xe, 20% O_2) [59] – chlorophyll was reduced but using root epidermal cells, it was found that Xe treatment increased the size of Brefeldin A-induced compartments (Brefeldin A inhibits vesicle recycling).

Although not an exhaustive list of the biological effects of xenon, clearly it does have an influence on cell function and is likely to impinge on cell signaling mechanisms in a wide range of organisms, from plants to humans.

4. Biological Effects of Other Noble Gases

Other inert gases also have biological effects. He and Ne for example, give cardioprotection [45].

He is a non-anesthetic gas [60], but it is used for the treatment of airway obstruction and for other ventilation problems [61]. He is known to reduce ischemia-reperfusion damage, as well as have effects on lung tissues, blood vessels and on the immune system [62]. Cell signaling components implicated in having such effects include ion channels, kinases, ROS and NO. Others too have listed signaling molecules downstream of He effects. In cardiac tissue this included extracellular signal-regulated kinase 1/2 (ERK-1/2), p38 mitogen activated protein kinase (p38 MAPK), protein kinase C-epsilon (PKC-ε), and heat shock protein 27 (HSP27) [63].

Not all studies are supportive of noble gases having positive effects. In a study using neuronal cultures from mice, neither Kr nor Ne gases had any protective effects, and He was found to be detrimental to cells [64]. Argon was found to be the best noble gas tested when it came to cellular protection following O_2 and glucose deprivation. As mentioned above, Ar failed to protect renal cells from deprivation of oxygen/glucose, and Kr and Ne had similar null effects [46]. Even so, it can be seen that a range of inert gases do have biological effects, and for all of them direct chemical reactions with biomolecules are unlikely. Therefore, do they have similar modes of action and even similar targets in cells?

5. Bioactivity Action of Noble Gases.

As discussed, there are a range of noble gases which have biological effects and there are a wide range of proteins involved in mediating the downstream effects. For example, the action of He in cardioprotection was suggested to be mediated by kinases, in particular inhibition of PI3K, Erk1/2, and p70s6K [45], and to have effects through inhibition of the mitochondrial permeability transition pore (mPTP**).** Interestingly, the authors said that they had not looked at the "biochemical actions of helium" on the proteins which they had identified as important for mediating the effects seen. Rizvi *et al*. [46] in their work on human renal cells (HK2), found that Xe caused an increase in phospho-Akt (p-Akt), hypoxia-inducible factor-1α (HIF-1α) and Bcl-2 levels, the latter instrumental in one of the apoptosis initiation pathways. In rat heart tissue, Xe effects were reported to be mediated by protein kinase C (PKC)-ε and p38 mitogen-activated protein kinase (MAPK), but downstream of these signaling components it was found that MAPK-activated protein kinase-2 (MAPKAPK-2) and HSP27 were involved, leading to signaling to the actin cytoskeleton [65]. Winkler *et al*. [66] highlight the vast array of proteins which have been identified *in silico* as potentially able to bind to five noble gases, that is, He, Ne Ar, Kr, Xe. The authors downloaded 127,854 protein structures from the Protein Data Bank, and then used a computational approach to estimate how the noble gases might interact with the polypeptide structures. Their analysis included the solvent-accessible surface area (SASA) of the gas atoms and how this matched the hydrophobicity of the protein contact points. In a further paper the emphasis was not only focused on binding strength, but also where it was thought that there would be an alteration of protein function, where such functional difference would also have clinical relevance [67]. Some notable examples of proteins pulled out of the analysis include kinases (both serine/threonine and tyrosine, including MAPKs), phosphatases, carbonic anhydrase, phosphodiesterases, caspases, and nitric oxide synthase. Therefore, a wide range of cell signaling components are potentially altered by the presence of noble gases. It would be interesting to take this approach with H_2 as well.

It has been suggested that the spin state of H_2 may have an influence on how it interacts with biological molecules, be that small signaling components such as NO or larger entities like proteins [32]. A similar mechanism has been proposed for Xe. Xu et al. [68] looked at nuclear Overhauser effects and interactions with lipids, and the authors suggested that this may account for the molecule's anesthetic action. Smith *et al*. [69] suggest that the nuclear spin of Xe can influence other radical electron pairs, so hinting at a possible mechanism. Investigating spin polarization–induced nuclear Overhauser effect (SPINOE), it was suggested that the induced spin polarization of Xe and He could be transferred to other nuclei [70]. How significant any of these physical effects are to biological systems has yet to be determined, but the literature suggests that it is worth exploring and not instantly dismissing by assuming they are inert and therefore inactive.

6. Xenon Pockets in the Globins

Kim *et al*. [35] suggests that H_2 has a direct interaction with hemoglobin through the Fe^{2+} of the heme prosthetic group; this manifests itself as scavenging hydroxyl radicals and peroxynitrite, and hence cellular effects are seen. Both hydroxyl radicals [71] and peroxynitrite [72] are reactive small molecules, so any influence in their accumulation and action can lead to cellular effects. However, this is not the only manner in which hemoglobin will interact with gases.

Hemoglobin is a tetrameric protein responsible for the transport of oxygen in many species, including humans, as well as buffering the blood and acting as a reservoir for NO, and influencing NO metabolism, in animals and plants [73-76]. There is significant structural homology between humans and other higher order species in key areas of the globin chains responsible for binding oxygen and NO [76], as well as homologues in many

other organisms, including plants [36]. Hemoglobin is one of the most abundant proteins in humans, accounting for approximately 1% of total body weight [77].

The Fe^{2+} of the heme prosthetic group (protoporphyrin IX) of the hemoglobin subunits are hexa-coordinate. Four of the coordinates are used to hold the iron atom to the porphyrin rings, whilst a fifth is coordinated to a histidine in the polypeptide chain. However, this allows the protein to bind and release O_2 at the sixth coordinate position – it here that Kim *et al*. [35] suggest that H_2 may bind. The binding of O_2 is allosteric [78], affected by many factors such as oxygen, carbon dioxide (CO_2) and proton concentrations (hydrogen, chloride), as well as blood pH, blood flow, ATP and 2,3-DPG concentrations. Upon binding oxygen, hemoglobin undergoes conformational changes to increase oxygen affinity as hemoglobin subunits progressively bind, leading to a sigmoidal oxygen dissociation curve. For a review on hemoglobin see [79].

The heme group can exist in different electronic states, including oxygenated (R, relaxed form), deoxygenated (T, tense form) and methemoglobin forms (oxidation of heme iron to Fe^{3+} : MetHb). Each of these states has a different absorbance spectrum in the visible wavelengths making them relatively easy to study [80]. O_2 is not the only gas which can have significant effects on hemoglobin structure and therefore function, with CO_2, NO and CO also impacting. CO, for example, stabilizes Hb in the oxygenated R form, and has 200 times greater affinity for Hb than O_2. CO_2 has perhaps the most significant impact on oxygen binding affinity of Hb through the Bohr effect. CO_2 in humans is transported via binding the terminal amino groups in the alpha chains, with increasing CO_2 concentration shifting the oxygen dissociation to the right, decreasing oxygen binding affinity [81]. The Bohr effect therefore maximizes binding capacity of oxygen in the lungs, whilst also optimizing delivery of O_2 to tissues with greatest need. The importance of regulating the oxygen dissociation curve via mechanisms such as CO_2 concentration can be seen by the clinical significance of conditions affecting this, such as the hemoglobinopathies, of which over 1000 variants have been reported [82]. Clinically, drugs such as Voxelotor are employed to treat conditions such as sickle cell anemia, shifting the HbO_2 dissociation curve, however, many hemoglobinopathies are still much more effectively treated by regular RBC replacement via transfusion [77].

In addition to impacting Hb individually, many gases work cooperatively and competitively to bind Hb and affect its function. For example, circulating levels of NO bound to Hb are dependent on Hb oxygen saturation, with binding of NO to cysteine residues mediating physiological response of vasodilation in hypoxic conditions [75,76]. MetHb is thought to be able to carry hydrogen sulfide (H_2S) in the vasculature, and H_2S is another gas which has significant signaling roles, including regulating blood flow. Therefore, understanding how gases affect hemoglobin is important.

NO can bind both hemes in hemoglobin and covalently modify cysteine residues. But whereas heme sequesters NO, cysteine 93 on β-globin (βCys93) is *S*-nitrosylated, creating a vasodilatory *S*-nitrosothiol (SNO) [82]. This has the potential to alter blood flow and hence O_2 delivery, while serving to transport NO around the body through the vascular system. However, as mentioned, H_2 is unlikely to be involved in this type of chemistry.

It has been known for a long time that Xe can associate with hemoglobin, with an association curve being published about sixty years ago [83]. Others followed with the solubility of both Xe and Kr being reported in the presence of hemoglobin, and albumin [84]. Therefore, the interactions of these noble gases with proteins needed to be understood.

Using Metmyoglobin as a model, cavities in the polypeptide chain were resolved to 1.9 Angstroms [85, and correction of this paper, 86]. Using X-ray crystallographic techniques on sperm whale myoglobin at 7 atm Xe, it was found that there were four Xe binding sites. The authors concluded that these bindings were in polypeptide cavities, that such structures were probably present in native protein, and that there could be an influence on protein conformation. The same research group went on to investigate what they described as the “energies of xenon binding” to myoglobin. They suggest that there

is in fact a fifth potential binding where water would normally coordinate the iron and describe a "connecting network of channel-like pathways through the static protein structure". They also suggest that there is an easy route from internal binding cavities to the protein surface [87]. As Xe can bind myoglobin, ^{129}Xe NMR chemical shifts could be used by others to determine the differences between the MetHb from pig and horse. These authors said that the Xe binds with a 1:1 ratio, with respect to the protein, and that the binding constant was such that the Xe freely exchanges with the free Xe on the outside of the protein structure. It was also reported that the Xe-Fe distance in the two proteins was different: xenon–iron distance, 7.4 Å for pig and 5.3 Å for horse [88].

Therefore, the existence of Xe cavities in protein structures can be seen also in proteins related to myoglobin, i.e. hemoglobin. This was reported over sixty years ago [83,89]. More recently, Savino *et al*. [90] looked at the Xe binding of deoxygenated wild-type human hemoglobin, as well as mutant variants. They also compared the data with that found with sperm whale myoglobin. It was reported that the binding in the a and b subunits of hemoglobin was different, with the b subunit being most similar to myoglobin. Tilton and Kuntz had previously noted differences of Xe between the a and b subunits of hemoglobin [91]. They also said that: "One of the binding sites in metmyoglobin is associated with a cavity on the proximal side of the porphyrin ring, opposite the O_2 binding site" (the authors cite Schoenborn here [89]). It has also been suggested that the ^{129}Xe chemical shift in myoglobin is dependent on the oxidation state and spin state of the iron in the heme of myoglobin [91,92]. Leading on from this, and perhaps of more pertinence to the argument here are the effects of Xe on oxygen transport of hemoglobin. In the a subunit O_2 may be leaving through a Xe binding cavity, and the treatment of hemoglobin with Xe decreases the efficiency of O_2 leaving the molecule. The same was not true for the b subunits [93]. However, it has been suggested that the binding of ^{129}Xe can be used as a probe for the measure of the oxygenation of blood [92].

7. Hydrophobic Cavities in Other Proteins

It seems clear from the work on the globins that inert atoms, such as Xe, can interact with proteins and have an influence on their activity. Can this be extended to other proteins too? And if so, can H_2 be acting through interaction with such cavities?

Prangé *et al*. [94], using X-ray diffraction, showed the interaction of Xe with a range of proteins, including elastase, subtilisin, cutinase, collagenase, lysozyme, urate oxidase and nuclear retinoid-X receptor. These were sourced from a range of organisms from *Bacillus licheniformis* to humans. Although Xe was used at above atmospheric pressures, (8 to 20 bar), they concluded that Xe could bind within discrete pockets and channels embedded in proteins. Using ^{129}Xe NMR, and a range of proteins including metmyoglobin, methemoglobin, lysozyme, and lipoxygenase, the binding of Xe to protein structures was further reported [95]. Lipoxygenase was found to bind gas molecules with the highest affinity, therefore more than the globins.

Using X-ray diffraction, the serine protease, subtilisin, was shown to bind Xe in the protein's active site. It was suggested, because the active site is a common feature of this class of polypeptide as it is relatively conserved. [96]. However, no major structural changes were seen on Xe binding. In a study also using X-ray diffraction on monooxygenase hydroxylase (MMOH), from *Methylococcus capsulatus*, it was concluded that hydrophobic cavities that could bind Xe may have a functional role in sequestering and making available the substrates for subsequent catalytic activity [97].

Xenon was used as probe to investigate the dioxygen binding of copper amine oxidase [98]. The rationale given included the knowledge that Xe can bind to hydrophobic cavities in proteins, but also that it is similar in size to O_2 but easier to detect. One binding pocket was common to three oxidases studied and it was suggested that this O_2 binding region was important for catalysis. With a focus on anesthesia, four proteins (urate oxidase, lysozyme, neuroglobin, myoglobin) were studied by X-ray diffraction to compare the binding of Xe and nitrous oxide. The authors concluded that hydrophobicity alone

could not account for the binding of the gases to the proteins, and that volume needs to be considered, and that the gases could bind in a fully reversible manner [99].

Recently, two different methods were used to study Xe binding to proteins [100]. These were stability of proteins from rates of oxidation (SPROX) and limited proteolysis (LiP). Using a proteomic approach, focused on the yeast genome, SPROX identified 31 novel proteins, while LiP identified 60. Further bioinformatic analysis showed that many of these proteins were involved in mechanisms which involved ATP. These include ATPase pumps and ATP synthase. Therefore, Xe seems to have a potential influence on ATP metabolism and cellular energy supplies.

There are clearly a range of proteins which can interact with Xe, even though it is inert. Many of the reports are based on using Xe at above atmospheric pressures, but it still shows that such interactions are possible, and may account for changes in cellular activity. Oxygen binding and ATP metabolism may be affected, for example. The conclusion of the work with Xe binding to proteins and the investigations of hydrophobic cavities and channels on polypeptide structures is the question of whether other inert molecules can interact with proteins in the same manner, and here the real question is: can H_2 interact in this way?

It is known that H_2 can increase O_2 saturation in blood and therefore be beneficial for exercise routines [101]. Perhaps, therefore, the work by Kim *et al*. [35] where H_2 may sterically hinder O_2 binding makes little sense, but on the other hand, if Xe, and H_2 by extension, decreases the O_2 release from hemoglobin [93] then this may account for higher O_2 saturation in red cells.

8. Conclusions, and Future

There is no doubt that H_2 has profound effects in a range of organisms, from plants to humans, and its application has been suggested for both medical purposes [1] and for agriculture [2]. However, even though there are a multitude of effects, the direct action of H_2 remains somewhat elusive. Many of the effects reported, such as increases in the cell's antioxidant capacity (e.g. increased SOD or CAT), or increased gene expression, would need to have an increase in the activity of signaling components, but this would need some direct interaction of H_2 with those signaling molecules, most likely proteins. As H_2 is not able to directly facilitate post-translational modifications (PTMs) of proteins, such as *S*-nitrosylation, there must be a different interaction involved. Rather than PTMs having an effect directly on polypeptide topology, very recent work on the hydration of proteins, such as hemoglobin, suggests that the interaction with water may have effects on the allosteric nature of the polypeptides, and hence their function [102]. It is possible that inert gases such as Xe (or H_2?) may have an influence on such hydration by occupying polypeptide cavities. In another recent paper by the same group, the migration of Xe was studied in Mb [103]. Here, the transition energies were dependent on the occupation state of other cavities in the polypeptide. Certain key amino acids, such as Phe138, were suggested to gate the migrations of Xe too. The authors suggested that the work shows that Mb is in fact an allosteric protein. Therefore, it appears that inert gases can influence the structure, or the dynamic nature of topological changes, they could have an influence on function, which may influence downstream signaling or gene expression if having effects on the right proteins. The question here is can H_2 partake in such interactions as suggested for Xe? Even if H_2 disrupted the interaction of other gas molecules with polypeptides it could be significant. It certainly seems worth investigating, perhaps from a theoretical/mathematical perspective, as well as eventually using an experimental approach.

There is potential for H_2 to act as a direct antioxidant, and it is thought that it can scavenge both hydroxyl radicals and peroxynitrite [27]. This has been disputed, mainly because of the kinetics of the reactions [29]. More recently, a mechanism whereby the H_2 molecule interacts with the Fe of heme prosthetic groups may account for the direct scavenging activity of H_2, mediated by the generation of a hydrogen radical [35]. However, the kinetics of the reaction, and how widespread this may be, accounting for the

wide range of effects seen, needs to be determined – the model protein used was hemoglobin which does not exist in plants *per se*, for example, although homologues do exist. This possible action of H_2 would be worth further investigation in a range of organisms, including plants, nematodes, and higher animals.

With the above in mind, a different mechanism, considering the relative inert nature of H_2, would be a direct interaction with the amino acid chain of a protein. As discussed, precedents for this can be seen in the action of noble gases on proteins. Biological effects of noble gases are widely known. Xenon is a well-known anesthetic, for example, whilst argon, neon, helium and krypton have all been studied for their effects. Should H_2 be added to this group? Not all the noble gases have the same effects, but their mode of action may be informative to those studying H_2 effects. The work by Winkler and colleagues [66,67] highlighted how *in silico* approaches can be used to investigate how inert molecules can interact directly with proteins. Therefore, it would be interesting to see this approach used for H_2 too.

Here, like the work of Kim *et al*. [35], it is suggested that hemoglobin may be used as a model for investigating how H_2 may have a physical interaction with proteins. As well as oxygen, hemoglobin can interact with a range of gases, including CO, CO_2, H_2S and NO. Again, should H_2 be added to this mix? Different gases interact with hemoglobin in different ways. Kim *et al*. [35] suggest that H_2 takes the hexa-position of the Fe^{2+} in lieu of oxygen. Here, we are suggesting that the manner in which Xe interacts with proteins such as hemoglobin could be a good model for understanding the direct action of H_2. As well as gases interacting with proteins through the classical manners of binding to heme or causing PTMs such as *S*-nitrosylation, a third way could be through "Xenon pockets", and this may account for why H_2 seems to have such wide-ranging effects in a variety of organisms, from plants to humans. Future investigations could take an *in silico* approach, as used by Winkler *et al*. [66,67], but needs to be subject to experimentation, especially as hemoglobin is so tractable, having distinct spectra.

It is not suggested here that a direct physical interaction with proteins is the only mechanism of H_2 action in cells. The work by Kim *et al*. [35] may open the door on our understanding to the scavenging effect of H_2. The redox poise of the H_2 couple may allow a direct mechanism on some heme -containing proteins [30]. In different cellular environments, where the redox state or pH are different may dictate different modes of H_2 action, but it seems timely for this aspect of H_2 biology to be thoroughly investigated.

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