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Investigation of hydrogenase enzymes and the presence of orthologs in the human proteome

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Abstract

Hydrogenase enzymes catalyze the reversible oxidation/reduction of hydrogen (H_2) and play a crucial role in microbial energy metabolism, with significant implications for human immunity. H_2, produced by gut microbes during fermentation or administered exogenously, is vital in modulating oxidative stress and inflammation. In the gastrointestinal tract, microbial H_2 production can reach up to 13 L/day, with approximately 71% of commensal bacteria capable of metabolizing H_2. By interacting with complex I, particularly the NDUFS7 subunit, H_2 may reduce mitochondrial electron leakage and limit the generation of reactive oxygen species (ROS). Excessive ROS can trigger pro-inflammatory signaling and impair immune responses. This study investigated the presence of hydrogenase orthologs in the human proteome, particularly within mitochondrial complex I, and their potential role in immune function. This novel research highlights a possible evolutionary link between microbial hydrogenases and human immunity, suggesting that microbial-derived H_2 may support immune homeostasis by mitigating oxidative stress and inflammation. Although human homologs of nickel/iron hydrogenases, such as NDUFS2 and NDUFS7, likely lack classical hydrogenase activity, sequence similarities between NDUFS7 and hydrogenase subunits in Asgard archaea and δ-proteobacteria indicate the conservation of potential redox-active sites. Redox activity, occurring at the N2 iron-sulfur cluster in NDUFS7, may influence mitochondrial oxidative stress responses, which are integral to immune regulation. These findings open new avenues for exploring the therapeutic potential of H_2 in immune regulation.

Keywords: Complex I; Evolution; Hydrogen; Hydrogenases; Redox activity; NDUFS7; Oxidative stress

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1. Introduction

The intestinal microbiome plays a fundamental role in regulating immune function and mitigating oxidative stress, serving as a critical interface between host systems and the external environment. Comprising a vast array of microorganisms, the gut microbiome not only modulates innate and adaptive immune responses but also defends against pathogens while maintaining immune homeostasis.[1] A key mechanism through which the microbiome influences immune health is the production of short-chain fatty acids

(SCFAs), such as butyrate, acetate, and propionate, during the fermentation of indigestible dietary fibers. SCFAs strengthen the gut epithelial barrier, thereby reducing the translocation of bacterial endotoxins that can trigger systemic inflammation and immune dysregulation.[2] Furthermore, SCFAs exhibit potent anti-inflammatory properties and are known to regulate regulatory T cells, which play a crucial role in maintaining immune tolerance and preventing excessive immune responses.[3]

In addition to the favorable effects of microbial metabolites on adaptive immunity, the intestinal microbiome is pivotal in reducing oxidative stress, a major contributor to inflammation and cellular damage. The immune system relies on a delicate balance of oxidative and antioxidative processes to function effectively. Oxidative stress, a phenomenon that can lead to erratic immune signaling, occurs when the production of reactive oxygen species (ROS) exceeds the body's ability to detoxify them. ROS, including superoxide and hydroxyl radicals, are highly reactive molecules that participate in a network of signaling pathways. Furthermore, ROS are known to influence cellular stress responses, including the expression of proinflammatory chemokines and cytokines, as well as apoptosis.[4] While ROS signaling is essential for immune responses, such as the destruction of pathogens, excessive levels can damage healthy cells and tissues, leading to inflammation and impaired immune responses.[1,2] Disruption of this balance may result in either an overactive immune response, contributing to autoimmune diseases, or a weakened somatic response, increasing susceptibility to infections. Many microbial metabolites, including hydrogen (H_2) and glutathione, act as antioxidants, either by directly neutralizing ROS and preventing oxidative damage or by activating signaling pathways, such as nuclear factor erythroid 2-related factor 2, which promotes the expression of antioxidant enzymes and enhances cellular defenses against oxidative stress.[5]

The gastrointestinal tract hosts more than 10^{12} microorganisms, collectively referred to as the gut microbiota. This diverse array of archaea, bacteria, bacteriophage, fungi, and viruses contributes to various physiological processes, including immune function.[1,6] During the fermentation of undigested carbohydrates by commensal bacteria, H_2 gas is produced as a metabolic byproduct. This H_2 can be absorbed into the bloodstream and expelled via the lungs, or it can be utilized by methanogens and sulfate-reducing bacteria through a process known as interspecies H_2 transfer.[7-9] It is estimated that the intestinal microbiome can produce approximately 13 L of H_2 each day, with around 71% of commensal bacteria capable of metabolizing H_2,[7] indicating the potential significance of H_2 in regulating intestinal immune function.

Emerging research suggests that H_2 acts as an effective antioxidant and anti-inflammatory agent, with numerous studies showing that H_2, whether produced endogenously by microbes or administered exogenously, can reduce oxidative stress, inflammation, and modulate immune responses,[10-14] For instance, H_2 has been shown to stimulate the production of butyrate, an essential SCFA known for its anti-inflammatory properties,[8] The ability of the gut microbiota to modulate H_2 production and utilization is crucial for maintaining gut health. *Methanobrevibacter smithii*, for example, uses H_2 to reduce carbon dioxide into methane (CH_4), a less reactive byproduct, thus preventing excess accumulation of H_2.[9] Interspecies H_2 transfer plays a key role in sustaining gut microbial diversity and contributes to overall gut health. In addition, recent studies indicate that H_2 positively affects mitochondrial structure and function, enhancing adenosine triphosphate production, mitigating oxidative stress, and stabilizing membrane potential.[15-18] These findings suggest that H_2 supports favorable energy dynamics in somatic cells.

Over the past decade, H_2 has gained interest as a modulator of oxidative stress and inflammation,[10-12,16] with several studies showing that H_2 can attenuate inflammation in various models of intestinal diseases, including colitis and inflammatory bowel disease. For example, a study conducted by Song *et al.*[19] demonstrated that H_2-rich water, which mimics the effects of microbial-produced H_2, significantly reduced colonic inflammation in a mouse model of ulcerative colitis by decreasing levels of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α). Significant increases in glutathione concentration and inhibition of pathogenic bacteria, including *Enterococcus faecalis*, *Clostridium perfringens*, and *Bacteroides fragilis*,[19] were also noted. As a non-polar, electrochemically neutral, and lightweight (molecular weight: 2.016 g/mol) diatomic molecule, H_2 can traverse biological membranes and target intracellular compartments, playing a crucial role in the interaction between intestinal microbes and the immune system.

Many reports describe the anti-inflammatory effects of H_2 treatments, supported by scientific evidence indicating that H_2 suppresses biological markers of oxidative stress and pro-inflammatory peptides (for example, TNF-α), interleukins (IL; for instance, IL-6 and IL-1ß), and nuclear factor kappa B (NFκB).[10-12,16] These protective mechanisms involve not only interactions with multiple cellular processes, as described above but also the regulation of p38 mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase signaling cascades responsible for initiating the biosynthesis of proinflammatory cytokines.[20,21]

To understand the origins of these complex regulatory pathways, the ancient environmental conditions that may have contributed to the development of early biological membranes should be examined. Given the volatile chemistry of the Hadean Earth, circa 4 billion years ago, it is plausible that the first membranes were formed not from organic materials but from minerals such as mackinawite.[22-27] These semi-permeable, inorganic protomembranes could form in the vicinity of submarine alkaline hydrothermal vents, where reductive, electron-rich volcanic detritus is exposed to the acidic, proton-rich oceanic waters, potentially providing the chemical disequilibrium and minerals necessary for organic life.[22,23] As the Earth's early atmosphere was likely composed of reducing gases such as carbon monoxide, H_2, and CH_4, it has been suggested that the ability to utilize iron (Fe)- induced catalysis of H_2 as a means of supplying electrons and protons for energy production, evolved billions of years ago.[24] Preiner *et al.*[27] propose that minerals such as mackinawite and magnetite could serve as prebiotic hydrogenases, facilitating organic reactions. Therefore, from an evolutionary standpoint, it is possible that H_2 was one of the first reducing agents exploited in early energy metabolism, a phenomenon that now predominantly occurs in the mitochondria of animals.

Hydrogenase enzymes catalyze the reversible oxidation/reduction of H_2 ($H_2 \leftrightarrow 2H^+ + e^-$) and are found in all single-celled organisms and many multicellular organisms;[28-30] however, they are not known to exist in animals and humans. Recent research by Lu.[31] indicates that complex I may have retained hydrogenase-type activity. Hydrogenases can be categorized into specific phylogenic groups, namely (i) iron only, (ii) iron-iron, and (iii) nickel-iron ([Fe], [FeFe], and [NiFe], respectively).[28,29] Among these, the [NiFe] hydrogenases are the most commonly occurring, present in a wide range of microbial species.

[NiFe] hydrogenases can be further divided into four classes: (i) membrane-bound hydrogenases (Mbh), (ii) nitrogen-fixing cytoplasmic hydrogenases, (iii) cytoplasmic hydrogenases that utilize 8-hydroxy-5 deazaflavin (coenzyme F420) as a low-potential redox co-factor, and (iv) oxygen (O_2)-sensitive, membrane-bound, energy-converting hydrogenases.[28,29] Of these subgroups, membrane-bound group iv hydrogenases exhibit activity resembling that of mitochondrial complex I,[28,31] making this group particularly relevant to the present discussion.

The interaction between H_2, commensal microbes, and the immune system forms a complex network with significant implications for health and immune function. A deeper understanding of this relationship could lead to novel therapeutic strategies for gastrointestinal and immune-related diseases. Within the context of innate immunity, the evolution of complex I is central to immune regulation. It is conceivable that H_2 influenced the development of immune defense mechanisms by modulating the metabolic pathways responsible for ROS generation.

The syntrophy theory of evolution[32,33] emphasizes H_2 as a crucial intermediary in metabolic exchanges, particularly involving an H_2-consuming, sulfate-reducing δ-proteobacterial host, an H_2-releasing Asgard archaeon, and a sulfide-oxidizing α-proteobacterium. Therefore, it is reasonable to propose that H_2 metabolism, facilitated by hydrogenase enzymes, played a key role in the early bioenergetic processes essential for the evolution of complex life.

The primary objective of this research was to investigate the potential evolutionary connection between hydrogenases and the functional subunits of complex I across various microorganisms that may have contributed to the evolution of eukaryotic cells. This study sought to elucidate how hydrogenases might have influenced the development of bioenergetic systems critical to the evolution of complex cellular and somatic functions, including the modulation of cellular redox potential and immune responses.

2. Methods

To explore the potential evolutionary link between hydrogenases and the functional subunits of complex I, this study examined three microorganisms implicated in the syntrophic hypothesis of evolution: a δ-proteobacterium (*Desulfovibrio carbinolicus*), an Asgard archaeon (*Candidatus Heimdallarchaeota*), and an α-proteobacterium (*Rhodobacter sphaeroides*).

Mbh investigated in this study include: (i) *Ca. Heimdallarchaeota* MbhJ (Uniprot# A0A1Q9PFW3) and MbhL (Uniprot# A0A1Q9PFM5), (ii) *D. carbinolicus* with subunits Hyd494 (Uniprot# A0A4P6HTH3) and Hyd258 (Uniprot# A0A4P61469), and (iii) *R. sphaeroides* HupL (Uniprot# Q3J0L7), HupS (Uniprot# O86467), HupU (Uniprot# O86466), and HupV (Uniprot# Q3J0M0).

2.1. Basic local alignment search tool (BLASTp) analysis

To assess whether the hydrogenase proteins of interest retained homology within the human proteome, the BLASTp was employed to analyze the [NiFe] hydrogenase enzyme sequences from all three species.[34]

2.2. Dot plot analysis

Using the [NiFe] sequences, a bioinformatic matrix analysis was conducted using the EMBOSS program to

identify regions of similarity between complex I subunits and [NiFe] hydrogenases.[35]

2.3. Clustal Ω

Prokaryotic sequences displaying similarities with the human proteins NDUFS2 and NDUFS7 were then aligned and analyzed using the Clustal Ω program.[36]

2.4. ScanProsite

Using selected motifs of interest, an analysis was performed with ScanProsite to identify predicted sites of post-translational modifications, including N-glycosylation, N-myristoylation, and phosphorylation, known to support pro-oxidative and proinflammatory signaling pathways. This investigation focused on pinpointing redox-sensitive sites that could potentially facilitate H_2-driven antioxidant and anti-inflammatory signaling activity.[37]

3. Results

3.1. BLASTp

The BLASTp analysis identified the NDUFS2 subunit as most comparable to the large membrane-bound subunits of [NiFe] hydrogenases, which are responsible for H_2 catalytic activity, and the NDUFS7 module as most similar to the non-catalytic small subunit. With 27% sequence identity, the homology between the human protein NDUFS2 and the large MbhL subunit of *Ca. Heimdallarchaeota* hydrogenase is higher than that between NDUFS2 and Hyd494 of *D. carbinolicus* (23%) and *R. sphaeroides* (<20%).

Exceeding the 25% significance threshold,[38] the BLASTp analysis of *Ca. Heimdallarchaeota* showed a 35% similarity with NDUFS7. Notably, the BLASTp search indicated 35% homology between the [NiFe] hydrogenases of *D. carbinolicus* and the NDUFS7 subunit of mitochondrial complex I. Conversely, the BLASTp search indicated <20% homology between the [NiFe] hydrogenases of *R. sphaeroides* and the NDUFS7 subunit.

3.2. Dot plot analysis

Consistent with previous findings,[29] the BLASTp analysis confirmed that NDUFS2 is most similar to the large membrane-bound subunits of [NiFe] hydrogenases responsible for hydrogenase activity. To further explore whether other regions of [NiFe] hydrogenases may be represented in NDUFS2, a similarity matrix – referred to as a dot plot analysis – was created to visualize sequence homology (Figure 1).

In accordance with previous research, this analysis also confirmed that NDUFS7 is most similar to the small membrane-bound subunits of [NiFe] hydrogenases. To further explore whether other regions of [NiFe] hydrogenases may be represented in NDUFS7, a similarity matrix was created (Figure 2).

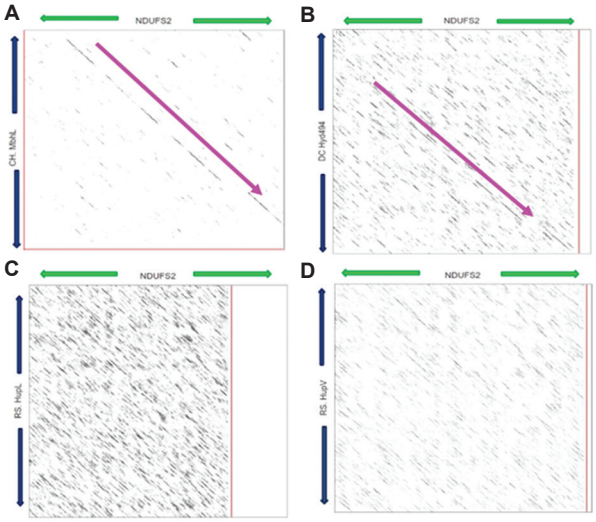

Figure 1. Similarity matrix analyses of NDUFS2 and hydrogenases from (A) *Candidatus Heimdallarchaeota* MbhL B (418aa), (B) *Desulfovibrio carbinolicus* Hyd494 (494aa), (C) *Rhodobacter sphaeroides* HupL (596aa), and (D) *Rhodobacter sphaeroides* HupV (475aa)
Notes: Dot plot analysis of the NDUFS2 protein (463aa) sequence which is on the X-axis (green arrows indicate sequence direction). The hydrogenase of interest is listed on the Y-axis (blue arrows indicate sequence direction). Clear alignments denote areas of homology. The horizontal red line indicates the amino acid sequence length of NDUFS2. The vertical red line represents the amino acid sequence length of hydrogenases. Regions of interest are marked with pink arrows. (A) identifies a close similarity between NDUFS2 and the MbhL protein of *Candidatus Heimdallarchaeota* (CH), as evidenced by a clear diagonal pattern. (B) displays some similarities between NDUFS2 and the large [NiFe] hydrogenase subunit of *Desulfovibrio carbinolicus* (DC; Hyd494). In contrast, (C and D), derived from the sequences of *Rhodobacter sphaeroides* (RS) HupL and HupV, respectively, show minimal homology with NDUFS2.

3.3. Clustal Ω analysis

To determine whether the catalytic site is well-conserved, a Clustal alignment was performed between NDUFS2 and the hydrogenases of interest (Figure 3).

To examine whether the small uptake subunit of hydrogenases is well-conserved, a further Clustal alignment was performed between NDUFS7 and the hydrogenases of interest (Figure 4).

4. Discussion

The anti-inflammatory effects of H_2 gas produced by the gut microbiota have gained significant attention in recent years.[39-42] This microbial-derived H_2 is a byproduct of the fermentation processes carried out by specific gut bacteria, particularly from the phylum *Bacteriodetes* and *Firmicutes*.[43] These bacteria generate H_2 during the

anaerobic metabolism of dietary fibers that are indigestible by the human body. H_2 produced by commensal bacteria can be utilized by other microbial populations, particularly methanogenic archaea, sulfate-reducing bacteria, and acetogenic bacteria, which employ H_2 as an electron donor in their respective metabolic pathways.[8] This symbiotic relationship between H_2 producers and consumers helps maintain a balanced intestinal environment. Imbalances, such as an overproduction of H_2, can lead to gastrointestinal disturbances like small intestine bacterial overgrowth.[44] In contrast, insufficient H_2 utilization, which is a more common occurrence, may impair microbial metabolic efficiency, leading to an increase in ROS and subsequent inflammation.

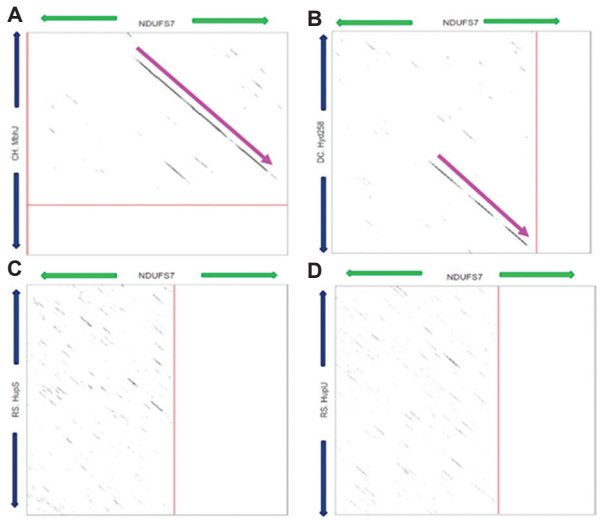

Figure 2. Similarity matrix analyses of NDUFS7 and hydrogenases from (A) *Candidatus Heimdallarchaeota* MbhJ (155aa), (B) *Desulfovibrio carbinolicus* (DC) Hyd258 (258aa), (C) *Rhodobacter sphaeroides* (RS) HupS (369aa), and (D) *Rhodobacter sphaeroides* (RS) HupU (330aa)
Notes: Dot plot analysis of the NDUFS7 protein (213aa) sequence. X-axis (green arrows) indicates NDUFS7 sequence direction. Y-axis (blue arrows) indicates the sequence direction of the hydrogenase of interest. Pink arrows denote regions of clear homology. The horizontal red line marks the truncation of the amino acid sequence of the hydrogenase. The vertical red lines indicate the truncation of the NDUFS7 amino acid sequence.

The gut microbiome is well established as a modulator of systemic inflammation through various mechanisms, including the regulation of immune cell activity,[45] cytokine production,[46] and the maintenance of intestinal epithelial integrity.[47] A healthy microbiome, enriched in H_2-utilizing microbes, is critical for preventing leaky gut syndrome, which can lead to systemic inflammation by allowing bacterial endotoxins to enter the bloodstream.[48] Furthermore, microbial-produced H_2 has been shown to promote the growth of beneficial bacteria that outcompete pathogenic species linked to inflammation.[43] For instance, butyrate-producing bacteria benefit from the H_2 economy in the intestinal environment, as their metabolic pathways help regulate inflammation and enhance intestinal barrier function.[8] Thus, the role of H_2 in gut health and immune function highlights an underexplored yet critical intersection between the intestinal microbiome, metabolic byproducts, and inflammatory regulation.

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NDUFS2      -------------NDVDPPKDTIVKNITLNFGPQHPAAHGVLRLVMELSGEMVRKCDPHI    113
MBHL        -------------TLTDGSDSPPGADHHIFIGPQHPWAEEPAHFIIHLKGERVVEADIRI    57
494         GCPARPEEPRPAPGVTDHFRLRGEEAHEVAVGPVHAGIIEPGHFRFQCSGEDVYHLEISL    170
HupV        ------------------------MTRLVVGPFN-RVEGDLEVHLEVAEGA-VTAARVN    33
HupL        -----------MVATPNGFNL-DNSGRRIVVDPVT-RIEGHMRCEVNVDDQGIIRNAVST    47
                                     : ..*        .  ..

NDUFS2      GLLHRGTEKLIEYKTYLQALPYFDRLDYVSMMCNEQAYSLAVEKLLNIRP--PPRAQWIR    171
CH.MbhL     GFNLRGIEKAMENRTWRQNTMLVPRACGICSAVHQNVYVRVVEKLAGVEDQISERARLIR    117
DC.Hyd494   GFQHRGIEARLIGGPDKRTIHFMETLAGDTTIGHSLAHAALVEALT--ETAVPARGRAIA    228
RS.HupL     GTMWRGLEVILKGRDPRDAWAFTERICGVCTGTHALTSVRAVEDALGISI--PDNANSIR    105
RS.HupV     APLYRGFERMLEGRDPRDALTITPRICGICSISQSVAAARALGAAMGLAP--APAGERVA    91
            .   ** *  :                        :  .    :           .. :

NDUFS2      RMHAAYIRPGGVHQDLPLGLMDDI-----------------YQFSKNFSLRLDELEELL    262
MBHL        RVNPALMLPGGVKRDIPKDKADKA-----------------RPMLNKLIKQVEYHKKVF    208
494         RFGRGLVRPGGVAFDLDKPTIREL-----------------LSRLELTRRAAFGAAELL    319
HupV        WPHTLAVQPGGVTRA--------PG--AAERMRILSSLRSFRRHLERTLFGGPLEAFAAL    211
HupL        NPHPNWLV-GGVPCPINIDGVGAVGAINMERLNLVSSIIDQCIQFTNNVYLP-----DVV    279
                  :  ***                                 .           .
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Figure 3. Short sections of the Clustal alignment between NDUFS2 and the large hydrogenase subunits
Notes: Histidine 88 is highlighted in bold and underlined. Blue highlights denote the motifs and conserved residues of interest. Grey highlights indicate the -HXXAHXVLR- motif and its conserved residues. Italics show the region surrounding the active site of hydrogenases. Pink highlights tyrosine 151 (human). Asterisks "*" identify identical residues across all sequences. Colons ":" represent strongly similar residues. Full stops "." denotes weakly similar residues.
Abbreviations: *CH: Candidatus Heimdallarchaeota; DC: Desulfovibrio carbinolicus; RS: Rhodobacter sphaeroides.*

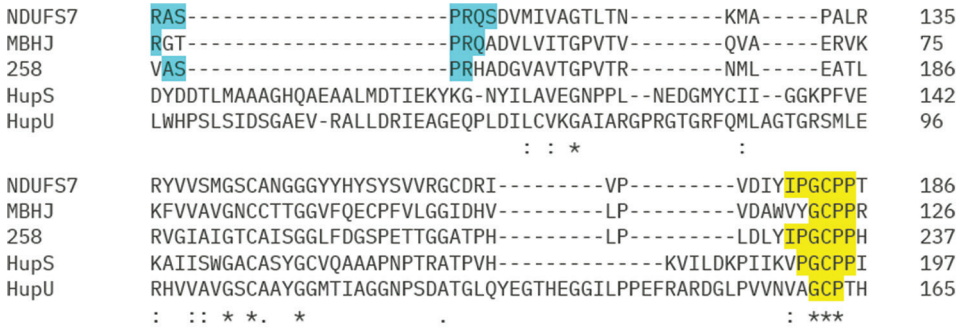

Figure 4. Short sections of the Clustal alignment between NDUFS7 and the small hydrogenase subunits
Notes: Blue highlights indicate conserved residues within the protein kinase C phosphorylation site -RASPRQS-. Yellow highlights mark the -IPGCPP- N-myristoylation site. Asterisks "*" identify identical residues across all sequences. Colons ":" show similar residues. Full stops "." denotes weakly similar residues.

As previously noted, the inflammatory response is significantly influenced by the redox status of cells and their compartments, with ROS playing essential roles in both normal cellular functions and pathological conditions. In healthy cells, ROS participate in signaling pathways essential for homeostasis and immune responses. Nevertheless, during inflammation, ROS production is often elevated. Overproduction of ROS or deficiencies in antioxidant defense mechanisms can lead to oxidative stress, damaging cellular components such as lipids, proteins, and DNA, thus exacerbating inflammatory responses.[49,50] ROS acts as a signaling molecule that activates transcription factors, including NFκB and activating protein-1, which are responsible for upregulating proinflammatory cytokines such as TNF-α and various ILs (for instance, IL-6 and IL-1β), thereby amplifying the inflammatory cascade.[51,52] While ROS is necessary for cell signaling and pathogen defense, their prolonged presence can lead to chronic inflammation and tissue damage, contributing to aging and various health conditions. Therefore, maintaining a balance between ROS production and antioxidant defenses is vital for regulating inflammation and preventing disease progression.

Due to its nearly constant redox activity and abundant electron supply, complex I serves as a prominent source of superoxide within the mitochondria. There are potentially two sites within complex I where O_2 can accept electrons from the nicotinamide adenine dinucleotide co-factor: (i) the flavin mononucleotide (FMN) module and (ii) the ubiquinone binding site.[53] Duong *et al.*[54] identified through *in silico* modeling that the FMN module is likely the putative site for ROS production, concluding that ROS generation is further stimulated by the absence of ubiquinone at the ubiquinone/complex I interface. In the absence of ubiquinone, the intraprotein channel becomes accessible to O_2, exposing it to a region where electrons are concentrated, thus enhancing the potential for ROS formation. In addition, incomplete reduction of ubiquinone at the Q module can lead to the production of semiquinone, a negatively charged intermediate capable of contributing to oxidative stress and proinflammatory signaling.[55] The redox midpoint potential of the ubiquinone/semiquinone couple (−0.163 V)[56] is similar to that of the oxygen/superoxide couple (−0.16 V),[57] suggesting that semiquinones may also significantly contribute to ROS formation in this context. Notably, reports indicate that the subunits of complex I that form the ubiquinone docking channel may have originated from hydrogenase enzymes.[28,29,31]

Numerous empirical and pre-clinical studies have identified H_2 as an effective redox mediator and regulator of the immune response.[58-64] In single-celled organisms, H_2 is metabolized by hydrogenase enzymes that catalyze the reversible oxidation/reduction of H_2. Among the various hydrogenase groups, [NiFe] hydrogenases are the most prevalent, found in diverse microbiota, fungi, and plants.[65-67] All characterized [NiFe] hydrogenases comprise a large subunit containing the active H_2 deprotonation site and a smaller subunit housing up to nine iron-sulfur (FeS) clusters. Similarly, complex I of the mitochondrial electron transport chain also relies on a series of FeS clusters to transfer electrons to the terminal N2 cluster, where they reduce ubiquinone to ubiquinol.

In the present study, initial BLASTp analysis identified NDUFS2 as the most similar to the catalytic units of the [NiFe] hydrogenases investigated. Supporting these findings, matrix analysis (Figure 1) identified a relatively strong correlation between NDUFS2 and NDUFS7 with the [NiFe] hydrogenases from *Ca. Heimdallarchaeota* (27% and 35%, respectively) and δ-proteobacterium *D. carbinolicus* (23% and 35%, respectively). In contrast, the α-proteobacterium *R. sphaeroides* showed less than 20% homology, indicating greater evolutionary distance.

Given the potential homology between hydrogenases and complex I subunit proteins, the active site is particularly interesting, as similar sequence alignments could indicate retention of form and function. Notably, the -RGXE- motif in the catalytic site (Figure 3) is conserved across all sequenced proteins, suggesting a distant evolutionary relationship between NDUFS2 and the large subunits of [NiFe] hydrogenases. The conserved glutamic acid residue (glutamic acid 119 in NDUFS2) may be pertinent to proton transfer.[65]

While this study identified limited homology between the aligned regions of NDUFS2 and microbial hydrogenase active sites, the redox-sensitive tyrosine 151 residue may be involved in the electron transfer chain, conserved only in *Ca. Heimdallarchaeota*. This is significant, as a crystal structure analysis of NDUFS2, conducted by Kampjut and Sazanov,[68,69] demonstrated that the ubiquinone molecule docks within 4.5 Å of tyrosine 108 in the ovine NDUFS2 module, corresponding to the human equivalent, tyrosine 151. In NDUFS2, the redox-sensitive tyrosine 151 residue may be involved in redox-regulated reactions central to the complex's function. Given that tyrosine 95 in MbhL is also conserved (Figure 2), this could indicate a similar role in electron transport within *Ca. Heimdallarchaeota*. Furthermore, tyrosine residues often contribute to protein stability through hydrophobic interactions and H_2 bonding.[70,71] Therefore, preventing the oxidation of these conserved tyrosines may be critical for H_2 in maintaining the protein's structural integrity, especially in regions vital for its function.

The structural analysis of NDUFS2 further identifies histidine 59 and asparagine 160 (in ovine), corresponding to histidine 112 and asparagine 182 (in humans), as potential candidates for proton translocation.[69] It has been proposed that a proton shuttling mechanism among this triad of residues (asparagine, histidine, and tyrosine) could create a negative charge, enhancing ubiquinone binding potential and lowering the redox potential of the N2 FeS cluster, thereby facilitating electron transfer.[69] Therefore, although the catalytic ability may have been lost through evolution, H_2 could still influence complex 1 dynamics through interactions with redox-sensitive residues.

Wirth *et al.*[72] further suggest that redox functionality likely exists within the β1–β2 helices of NDUFS2 at positions 88 – 96 (human), showing the -HPXAHXVLR- arrangement (Figure 3). Histidine 88 and histidine 92 of this sequence are situated in close proximity to both ubiquinol and the terminal 4Fe-4S cluster (N2) of NDUFS7, which may provide the redox sensitivity required for electron transfer to ubiquinone. However, this region is not well conserved across the species studied. Nonetheless, the alignment does reveal conserved homology immediately preceding the -HPXAHXVLR- motif. The preceding -GPQHP- sequence, positions 85 – 91 (human), contains histidine 88 and two redox-active proline residues (positions 86 and 89), likely close to ubiquinol, which could be key in the partial-to-full reduction of ubiquinone and downstream immune signaling responses.

Further along the sequence, at positions 228 – 232, there is another conserved motif, -RPGGV-. Although it has not yet been identified as a site for protein modification, its conservation throughout the examined hydrogenases suggests that it may play a critical role in protein structure and function. Although speculative, the idea that NDUFS2 serves as a key oxygen-sensing module and a regulator of complex I activity[73] suggests that these residues may be relevant as sites of H_2 activity. H_2 could protect these sites from autoxidation, thereby facilitating optimal protein function.

The structural relationship between the proton-transferring NDUFS2 and the FeS (N2)-containing NDUFS7 subunit indicates that this specific region of the ubiquinone binding module is likely responsible for electron transfer. By reducing electron leakage and subsequent ROS formation through structural maintenance, H_2 could have significant downstream cellular effects. For example, the protein kinase C (PKC) phosphorylation site (-RASPRQS-) in the smaller subunit shares the most sequence identity among species (Figure 4). If H_2 modulates phosphorylation in this region, it could influence the cellular signaling cascades that regulate the expression of proinflammatory factors such as NFκB and TNF-α in mammalian physiology. Interestingly, H_2 is noted to influence other protein kinase pathways, including MAPK.[20,21] Therefore, this raises the question of whether H_2 could influence the activity of this relatively well-conserved region, which is likely crucial for understanding H_2 bioactivity in humans.

Recent investigations utilizing a combination of mass spectroscopy and *in silico* modeling of the NDUFS7 unit identified that bovine arginines 108 and 112 (arginine 111 and 115 in humans) within the highly conserved C-terminal -RASPRQ- motif were integral for retaining ubiquinone in the hydrophobic cavity.[74] Nevertheless, it remains unclear whether direct electron transfer activity occurs at either of these moieties. If H_2 stabilizes this region or prevents oxidative damage, it is likely that a steady supply of electrons would be available for the complete reduction of ubiquinone to ubiquinol, thereby enhancing the immediate antioxidant potential of the mitochondria. This could have significant downstream effects, including an increased membrane potential and reduced cellular stress response.[75,76]

Further along the sequences (at positions 180 – 185 in humans; Figure 4), there is a well-conserved - IPGCPP- motif rich in redox-active proline residues, along with a cysteine thiol residue at position 183. The abundance of conserved residues associated with redox chemistry suggests an important conserved function in this region. Notably, the -IPGCPP- motif in *D. carbinolicus* is fully conserved, raising further questions about whether this region is integral to the function of hydrogenase remaining and potentially linking it to the electron transfer function of NDUFS7. In addition, the -IPGCPP- site is significant due to N-myristoylation, a post-translational modification crucial for regulating innate immune responses, including toll-like receptor-dependent inflammatory reactions.[77] Consequently, H_2 might modulate N-myristoylation and thereby influence the innate immune response, although further research is needed to validate this hypothesis.

Figure 4 also illustrates that the PKC phosphorylation site -RASPRQS-, as identified by Yoga *et al.*,[74] is relatively well conserved. Notably, the phosphorylation target, serine residue 113 in humans, is represented by threonine in *Ca. Heimdallarchaeota*, suggesting a retained function, as threonine can also be phosphorylated.[78] There is no correlation between the phosphorylation target -RASPRQS- in *R. sphaeroides*, indicating divergence in form and function within this region. The correlation between the smaller subunit of the [NiFe] hydrogenase (Hyd258) and NDUFS7 also reveals the retention of the serine residue -RASPRQS- motif. This suggests that in δ-proteobacteria, this segment may be significant for understanding the influence of H_2 on protein phosphorylation and cell signaling events.[79] Understanding whether H_2 has any influence on the molecular activity of this conserved region could be crucial for advancing our knowledge of its biological effects.

5. Future perspectives

The interplay between hydrogenase enzymes, microbial H_2 metabolism, and immune function represents a promising area of research, particularly regarding the role of H_2 in modulating oxidative stress and inflammation. Future studies should explore several key areas to enhance our understanding of the evolutionary links between microbial hydrogenases and human immune regulation.

One significant avenue for future research is to further investigate the potential redox activity of human complex I subunits, particularly NDUFS2 and NDUFS7, and their interactions with microbial-derived H_2. In accordance with Lu,[31] who posits that complex I may exhibit hydrogenase-like activity, our findings suggest that while classical [NiFe] hydrogenase activity has been lost, NDUFS7 may retain significant redox functionality, with the potential to influence mitochondrial oxidative stress responses. Therefore, one of the initial steps should involve determining the crystal structures of the identified human hydrogenases NDUFS2 and NDUFS7 using *in silico* modeling and X-ray crystallography, followed by probing their functional analyses with techniques such as cryo-electron microscopy[80] and redox-sensitive fluorescent probes.[81] These investigations could clarify whether the NDUFS2 and NDUFS7 subunits directly interact with H_2 and how this interaction affects mitochondrial electron flow and ROS production in human cells.

As evidence increasingly highlights the gut microbiota's role in shaping systemic immune responses,[82] it is essential to investigate the broader immunological effects of microbial H_2 production within the gastrointestinal system. Given the significant H_2 output from commensal bacteria and its potential impact on mitochondrial redox states, research should determine whether microbial-derived H_2 directly influences immune cells or operates through the gut-liver or gut-brain axes. Longitudinal studies using germ-free and hydrogen-supplemented animal models, such as those demonstrated by Yang *et al.*,[83] alongside human clinical trials, could elucidate the immunomodulatory effects of H_2 in both health and disease contexts.

Another key avenue for research lies in exploring the therapeutic potential of H_2 in managing immune-related conditions. While studies have demonstrated its anti-inflammatory and antioxidant effects,[10-17] the precise mechanisms remain unclear. For instance, investigating how H_2 modulates the phosphorylation and N-myristoylation of mitochondrial proteins, such as NDUFS7, could provide valuable insights into its role in reducing electron leakage and mitigating ROS production. Further clinical studies in autoimmune diseases, inflammatory disorders, and age-related immunosenescence could offer practical applications for harnessing these benefits.

Finally, extending research into microbial hydrogenases beyond the intestinal microbiome – specifically in the skin, oral, and respiratory microbiomes – may uncover additional pathways through which H_2 influences immune function. Understanding the interplay between hydrogenase activity, microbial H_2 metabolism, and immune regulation offers a promising frontier for therapeutic exploration. Such investigations could unlock novel strategies to leverage microbial interactions for maintaining immune homeostasis and mitigating inflammation.

6. Conclusion

This report provides evidence that the closest human homologs of [NiFe] hydrogenases are unlikely to possess

classical hydrogenase activity due to the absence of a functional Ni-Fe di-metal core. A detailed analysis of protein sequences reveals stronger similarities between the human proteins NDUFS2 and NDUFS7 – key components of the ubiquinone binding channel in complex I – and their homologous proteins in the *Ca. Heimdallarchaeota*, with hydrogenase similarity percentages of 27% and 35%, respectively. In contrast, similarities with the α-proteobacterium *R. sphaeroides* are less than 20%, and those with the δ-proteobacterium *D. carbinolicus* are 23% and 35%. These findings suggest a potential evolutionary link[38] between archaeal hydrogenases and complex I subunits, although further research is necessary to confirm this hypothesis.

Of particular relevance to microbial and immunological research, this study identifies two motifs within the NDUFS7 subunit – the PKC phosphorylation site (-RASPRQS-) and the N-myristoylation site (-IPGCPP-) – as potential sites for H_2 activity. If H_2 is found to support the structure and function of these motifs, it could mitigate electron leakage, reduce the formation of ROS, and prevent oxidative damage. Such outcomes would have significant implications for maintaining redox balance and limiting pro-inflammatory signaling, thereby highlighting a promising therapeutic avenue for regulating immune responses and microbial interactions in human cells.

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Conflict of interest

This work was part-funded by Water Fuel Engineering, a manufacturer of oxy-hydrogen inhalation devices. Grace Russell is the Guest Editor of this special issue but was not involved in the editorial or peer-review processes for this paper, either directly or indirectly.

Author contributions

This is a single-authored manuscript.

Ethics approval and consent to participate

Not applicable.

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Availability of data

The data supporting the findings of this study are available from the corresponding author on request.

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