**How the structure of the large subunit controls function in an oxygen-tolerant [NiFe]-hydrogenase**

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**INTRODUCTION**

_Salmonella enterica_ serovar Typhimurium is a Gram-negative γ-Proteobacterium and an opportunistic animal pathogen. _S. enterica_ is a facultative anaerobe and as such has a remarkably flexible metabolic capability. The bacterium shows optimal growth under aerobic conditions, but in the absence of O₂ can rapidly switch to anaerobic respiration in order to utilize alternative electron acceptors [1,2]. Molecular hydrogen (H₂) is an important respiratory electron donor for a number of pathogenic bacteria and _H₂_ respiration is known to contribute to the virulence of _S. enterica_ [3–5].

H₂ oxidation (or ‘uptake’) is catalysed in _S. enterica_ by three [NiFe]-hydrogenases encoded by the _hyaABCDEF_ (STM1786–STM1791), _hybOABCEDFG_ (STM3150–STM3143), and _hydABCDEF_ (STM1539–STM1531) operons. Two of these (hya and hyb) encode homologues of the _Hyd-1_ and _Hyd-2_ [NiFe]-hydrogenases found in _E. coli_ and _hybOABCEDFG_ operons are up-regulated during fermentation and anaerobic respiration respectively [18,19], the _hyd_ operon is optimally expressed under aerobic conditions [6]. This finding led to an initial biochemical study of _Hyd-5_ that revealed that _Hyd-5_ belongs to a class of aerobically expressed ‘O₂-tolerant’ hydrogenases, i.e. [NiFe]-hydrogenases, that can sustain _H₂_ oxidation in the presence of O₂ [10].

The _O₂-tolerant_ [NiFe]-hydrogenases are of special interest with regard to their potential biotechnological applications, in particular their utilization in platinum- and membrane-free enzymatic _H₂_ fuel cells [20]. A key feature of the _O₂-tolerant_ enzymes is the special _Fe–S_ cluster located proximal to the [NiFe] active site. This proximal cluster comprises an unusual [4Fe–3S] structure stabilized by six conserved cysteine residue ligands [21,22]. Unlike other _Fe–S_ centres, this cluster is able to change conformation and reach a super-oxidized state, and it is thought that the resultant capability of releasing two electrons towards the active site is important during _O₂_ attack, since it will assist in the reduction of inhibitory _O₂_ to water [15,23]. As a result of this _O₂-reducing ‘rescue mechanism’, following reaction with _O₂_ the _O₂-tolerant_ enzymes form a Ni(III) state described as the Ni-B ‘ready’ state, comprising a bridging OH– between the Ni²⁺ and Fe³⁺ ions. The ‘ready’ label indicates that upon one-electron reduction of Ni(III) to Ni(I) the enzyme is reactivated rapidly back to a catalytically active form. By led to a loss in the ability of _Hyd-5_ to oxidize hydrogen in air. Furthermore, the H229A variant was found to have lost the overpotential requirement for activity that is always observed with oxygen-tolerant [NiFe]-hydrogenases. It is possible that His73 has a role in stabilizing the super-oxidized form of the proximal cluster in the presence of oxygen, and it is proposed that Glu73 could play a supporting role in fine-tuning the chemistry of His229 to enable this function.

Key words: hydrogen metabolism, iron–sulphur cluster [NiFe]-hydrogenase, oxygen-tolerance, protein film electrochemistry (PFE), _Salmonella enterica_.

Salmonella enterica is an opportunistic pathogen that produces a [NiFe]-hydrogenase under aerobic conditions. In the present study, genetic engineering approaches were used to facilitate isolation of this enzyme, termed _Hyd-5_. The crystal structure was determined to a resolution of 3.2 Å and the hydrogenase was observed to comprise associated large and small subunits. The structure indicated that His229 from the large subunit was close to the proximal [4Fe–3S] cluster in the small subunit. In addition, His229 was observed to lie close to a buried glutamic acid (Glu73), which is conserved in oxygen-tolerant hydrogenases. His229 and Glu73 of the _Hyd-5_ large subunit were found to be important in both hydrogen oxidation activity and the oxygen-tolerance mechanism. Substitution of His229 or Glu73 with alanine led to a loss in the ability of _Hyd-5_ to oxidize hydrogen in air. Furthermore, the H229A variant was found to have lost the overpotential requirement for activity that is always observed with oxygen-tolerant [NiFe]-hydrogenases. It is possible that His73 has a role in stabilizing the super-oxidized form of the proximal cluster in the presence of oxygen, and it is proposed that Glu73 could play a supporting role in fine-tuning the chemistry of His229 to enable this function.

Abbreviations: _BV_, Benzyl Viologen; IMAC, immobilized metal-ion-affinity chromatography; PFE, protein film electrochemistry; _scc_, standard cubic m;
SH, standard _H₂_ electrode; TM, transmembrane domain

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Co-ordinates for the _Hyd-5_ crystal structure have been deposited in the PDB under accession code 4C3O.
contrast, standard O₂-sensitive [NiFe]-hydrogenases have a [4Fe–
4S] cubane as a proximal cluster and rapidly form an inactive Ni–A
‘unready’ state upon O₂ attack. In the absence of O₂, all [NiFe]-
hydrogenases form the Ni-B state when exposed to sufficiently
oxidizing potentials.

In the present study, genetic engineering approaches have
been used to overproduce S. enterica Hyd-5 in situ. A strong promoter
was placed upstream of the native hydABCD EF GHI operon on the
S. enterica chromosome, and a stretch of sequence encoding
the small subunit TM domain was replaced with DNA encoding
an affinity tag. This allowed isolation and characterization of
a water-soluble active variant of Hyd-5. The crystal structure of
Hyd-5 is presented at 3.2 Å resolution and reveals that a
histidine residue side chain from the large subunit (His²²⁹)
is in close proximity to the special [4Fe–3S] cluster within
the small subunit. In addition, a buried glutamic acid (Glu⁷³),
which is peculiar to O₂-tolerant hydrogenases, is noted within
close proximity of His²²⁹. Electrochemical analysis of the Hyd-5
H₂₂⁹A and E7₃₃A variants revealed that these substitutions have
dramatic effects on the catalytic properties and O₂-tolerance of
the enzyme. In the case of H₂₂⁹A, this includes removal of the
overpotential requirement for H₂ oxidation. The present study
highlights the co-operation required between both subunits of a
[NiFe]-hydrogenase in controlling reactivity of the enzyme with
H₂ and O₂.

MATERIALS AND METHODS

Bacterial strains

To construct S. enterica strain LB03 (Pₜ₅, hydÄ₃–H₅), DNA encoding the TM and extreme C-terminus of HydA
(from the codons for Gly3¹⁴ to Lys6⁶⁷) was deleted from the
strain SFTH06 (Pₜ₅, hydÄ₃) [10] as follows. DNA covering
part of the hydÄ gene up to codon Gly3¹⁴ was amplified by the
oligonucleotide primers HyaATMfor (5′-CCGG TCTAG AGAT
GGT TTT GCC TAC TTGG CTTG-3′) and HyaATMrev (5′-CCGG
GA TCCC GGCGT GTTG AAAAAG-3′), digested with XbaI and KpnI
and cloned independently into similarly digested pBluescript KS
+ [24]. This plasmid was then excised from pBluescript KS
+ and cloned into the previous pBluescript KS
+ plasmid. The entire
hydÄ construct as a BamHI-KpnI fragment. The entire
hydÄ gene was placed upstream of the native
hydABCDEFGHI operon
was assayed as described in [25].

Hydrogenase assays

For whole-cell hydrogenase activity assays, Duran bottles
containing 500 ml of low-salt (5 g/l) LB medium were incubated
for 16 h anaerobically, without agitation, at 3⁰C. Cells were
harvested, washed and resuspended in 50 mM Tris/HCl (pH 7.5)
before BV (Benzyl Viologen)-dependent H₂ oxidation activity
was assayed as described in [25].

Rocket immunoelectrophoresis

A Hyd-5 antiserum was raised in rabbits against enzyme
purified from SFTH06 [10] and was commercially produced
by Eurogentec. Duran bottles containing 500 ml of low-salt LB
medium were inoculated with 0.5 ml of pre-culture and incubated
for 16 h anaerobically, without agitation, at 3⁰C. Cells were
harvested, washed and resuspended in a 50 mM Tris/HCl (pH 7.5)
containing 40 % (w/v) sucrose (10 ml/g of cells). EDTA (5 mM)
and lysozyme (0.6 mg/ml) were added and the suspension was
incubated, without shaking, at 3⁰C for 30 min. The sphaeroplasts
were harvested by centrifugation (17000 g for 15 min at 4⁰C) and
the supernatant was saved as the periplasmic fraction. Protein
samples (2 µl) were added to small wells of a 1 % (w/v)
agarose gel-containing electrophoresis buffer [20 mM sodium
barbitone/HCl (pH 8.6) and 0.1 % Triton X-100] and Hyd-5

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antiserum (7.5 μl/3 ml of gel). Samples were electrophoresed at 2 mA per plate for 16 h at 4°C. Plates were then removed, immersed in 50 mM Tris/HCl (pH 7.5) buffer containing BV and Tetrazolium Red, and incubated under an atmosphere of 100% H2 for 16 h. Hyd-5 activity was detected as intense red precipitin arcs.

### Preparation of proteins

Chromosomally encoded Hyd-5<sub>ΔTM−His</sub> was purified from the S. enterica LB03 (Ptet, hydA<sub>ΔTM−His</sub>) strain that had been cultured anaerobically in 10 litres of LB (low-salt) medium with no other additives. After inoculation with a 5 ml pre-culture, two 5 litre Duran bottles were incubated for 16 h without shaking at 37°C. Cells were harvested, washed and resuspended to a final concentration of 10 ml/g of cells in 50 mM Tris/HCl (pH 7.5, 150 mM NaCl and 75 mM imidazole (buffer A). Next, cells were lysed under pressure using the Emulsiflex-C3 homogenizer and cell debris was removed by centrifugation at 17 400 g for 15 min at 4°C. The supernatant was loaded directly on to a 5 ml HisTrap HP column (GE Healthcare) equilibrated previously with buffer A. The column was washed subsequently with 15 column volumes of buffer A before bound protein was eluted by a linear gradient of 75–500 mM imidazole in the same buffer. Fractions containing Hyd-5<sub>ΔTM−His</sub> were identified following SDS/PAGE (12% gel) analysis and InstantBlue staining.

Protein concentrations were determined using the method of Lowry et al. [26], whereas SDS/PAGE was by the method of Laemmli [27] and Western immunoblotting by the method of Dunn [28].

### Crystallization

Following IMAC (immobilized metal-ion-affinity chromatography), the Hyd-5 protein was purified further by size-exclusion chromatography using a Superdex 200 26/60 column (GE Healthcare) equilibrated with 20 mM Tris/HCl (pH 7.5) and 150 mM NaCl. The sample was then dialysed into 10 mM Tris/HCl (pH 7.8) and 50 mM NaCl, then concentrated using a Vivaspin 20 (Sartorius) to 6 mg/ml. This was the stock solution for crystallization.

Hyd-5 was crystallized at 20°C by the hanging-drop vapour-diffusion method using 0.75 μl of protein solution mixed with 0.75 μl of reservoir containing 19% (w/v) PEG4000, 0.1 M Mes (pH 6) and 0.24 M lithium sulphate. Brown monoclinic blocks, with the approximate dimensions 80 μ × 80 μ × 40 μ, grew over 2–3 days. The crystals were prepared for data collection with cryoprotection using the mother liquor adjusted to include 5% (v/v) glycerol and then plunged into liquid N2. Diffraction properties were characterized in-house using a Rigaku HFM007 rotating anode X-ray generator coupled to a Saturn 944HG + CCD (charge-coupled device) detector.

### X-ray data collection, processing, structure solution and refinement

Single-wavelength diffraction data were measured on beamline I03 of the Diamond Light Source (Harwell, U.K.) using a PILATUS 6M pixel detector. Data were indexed and integrated using XDS [29] and scaled using SCALA [30]. Molecular replacement was performed using the search model of MOLREP [31], and a search model of the membrane-bound [NiFe]-hydrogenase from Ralstonia eutropha (PDB code 3RGW) [15], which shares 67% overall sequence identity for the α-subunit and 80% overall sequence identity for the β-subunit. Three αβ dimers were identified within the asymmetric unit and they are labelled A and B for the large and small subunits, and then CD and EF respectively. The model was built using electron and difference density maps and the geometry was improved in COOT [32], before refinement in REFMAC5 [33]. Local NCS (non-crystallographic symmetry) restraints were used throughout the refinement, along with TLS (Translation–Libration–Screw-rotation) refinement [34] in the latter stages. Each cycle of refinement consisted of electron and difference density map inspection and model manipulation together with incorporation of cofactors, waters and ions, followed by REFMAC5 refinement. Owing to the modest resolution of the diffraction data, only 114 water molecules were included in the model. Several Mg2+ ions bound at the C-terminus were included on the basis of a strong feature on the electron density maps that was consistent with metal ions found in related structures determined at higher resolution. The model of the core heterodimers was restrained to be similar with a Cα average RMSD of just 0.08 Å for copies in the asymmetric unit, therefore only the AB combination is detailed in the following discussions.

### Crystallographic statistics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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</thead>
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<td>Data collection</td>
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<tr>
<td>Space group</td>
<td></td>
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<tr>
<td>Wavelength (Å)</td>
<td>0.9763</td>
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<tr>
<td>Unit cell parameters</td>
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<tr>
<td>a, b, c (Å)</td>
<td>115.5, 122.2, 227.8</td>
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<tr>
<td>β (°)</td>
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<tr>
<td>Resolution range (Å)</td>
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<tr>
<td>Unique reflections (n)</td>
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<tr>
<td>Completeness (%)</td>
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<tr>
<td>Favored, allowed, disallowed (%)</td>
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<tr>
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<tr>
<td>Mean B-factor</td>
<td>56.9</td>
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<tr>
<td>Subunit L, S, A, B, C, D</td>
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<tr>
<td>FeS centres (heterodimers: LS and AB)</td>
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<tr>
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<td>52.5, 56.4, 53.1</td>
</tr>
<tr>
<td>FXS (medial 3Fe–4S cluster)</td>
<td>55.7, 55.7, 55.7</td>
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<tr>
<td>NFU ([NiFe] active site)</td>
<td>53.2, 58.5, 55.7</td>
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<tr>
<td>Magnesium, chlorides, sulfates, water</td>
<td>49.4, 74.9, 114, 46.6</td>
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<tr>
<td>Ramachandran plot</td>
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<tr>
<td>Favoured, allowed, disallowed (%)</td>
<td>92.1, 7.6, 0.3</td>
</tr>
</tbody>
</table>

The crystallographic statistics are presented in Table 1. Co-ordinates except calculated using the 5% of the data that are not included in any refinement calculations.
PFE (protein film electrochemistry)

All PFE was performed in an anaerobic glove box (O₂ < 2 p.p.m.) filled with N₂. A gas-tight glass cell housed the three electrode configuration used. Within the water-jacketed main body of the cell, the graphite working electrode was located along with a platinum wire that acted as the counter electrode. A saturated calomel reference electrode was located in a reference side arm, connected to the main cell via a Luggin capillary. A 100 mM NaCl solution was used to fill the side arm, which remained at ambient temperature. The quoted potentials have been converted into values compared with the SHE (standard H₂ electrode) by the addition of the correction factor 0.241 V. Mixed buffer solutions [36] were prepared with purified water and were placed inside the cell at sufficient levels to cover all electrode connections. The gases were supplied by BOC and were flowed as the correct mixtures at a constant total rate of 100 scc/min (where scc is standard cubic cm) by use of mass flow controllers (Smart-Trak; Sierra Installations) connected to the electrochemical cell.

The graphite electrode surface (electrodes manufactured in-house) was prepared for enzyme application by sanding with Norton P1200 abrasive sheets directly before 3500 rev./min to allow removal of the TM by truncation of HydA at Gly314 while maintaining incorporation of a C-terminal His₆ tag. The resultant S. enterica strain was designated LB03 (P₅₅, hydA_TM⁻₆₆₆).

The LB03 (P₅₅, hydA_TM⁻₆₆₆) strain was cultured at a small scale under anoxic conditions and whole cells were assayed for BV-linked hydrogenase activity (Figure 2A). The LB03 (P₅₅, hydA_TM⁻₆₆₆) strain displayed increased hydrogenase activity compared with the parent strain (Figure 2A). Next, the LB03 (P₅₅, hydA_TM⁻₆₆₆) strain was cultured at a larger scale (10 litres) and IMAC was employed, in the absence of detergents or anaerobic precautions, to isolate any water-soluble His-tagged proteins from a crude cell extract. Following SDS/PAGE, co-purification of the Hyd-5 large subunit (HydB, α-subunit) together with a truncated small subunit (HydA, β-subunit) was observed (Figure 2B). No other co-purifying proteins were detected and the isolated enzyme retained hydrogenase activity with BV as the artificial electron acceptor (results not shown). From this it can be concluded that up-regulation of the S. enterica hyd operon at its native locus, together with genetic removal of the HydA TM, results in the recovery of an active water-soluble processed and assembled [NiFe]-hydrogenase.

The crystal structure of S. enterica Hyd-5

The isolated Hyd-5 enzyme was subjected to size-exclusion chromatography before being concentrated and entered into crystallization experiments under standard aerobic conditions.
The S. enterica Hyd-5 isoenzyme

Figure 3  The structure of a Hyd-5 \( \alpha_2 \beta_2 \) heterotetramer

(A) The large (\( \alpha \)) subunits are shown as red and green ribbons, the small (\( \beta \)) subunits as yellow and cyan. The van der Waals surface of the protein is depicted as semi-transparent form. The spheres identify positions of the Fe–S clusters in the small subunits and the [NiFe] cluster in the large subunits. (B) In similar fashion, a single ‘core’ hydrogenase enzyme comprising an \( \alpha \beta \) heterodimer is shown.

residue. The Hyd-5 structure also has strong feature in the electron density that would correspond to a hydrated Mg\(^{2+}\) ion.

Each Hyd-5 core heterodimer was observed to contain at least four metal cofactors at 8–11 Å separation from each other (Figure 3B). The Fe–S clusters are typical of an O\(_2\)-tolerant hydrogenase, with a medial [3Fe–4S] cluster co-ordinated by three cysteine residues, and a distal cubane [4Fe–4S] cluster co-ordinated by three cysteines and one histidine. In the \( \alpha_2 \beta_2 \) assembly (Figure 3A), the two distal [4Fe–4S] clusters from the two \( \beta \)-subunits (small subunits) lie in close proximity, less than 15 Å apart (Figure 3A).

The special proximal [4Fe–3S] cluster of the small subunit is co-ordinated by six cysteine residues (Figure 4). In this structure, the backbone amide of the small subunit Cys\(^{20}\) is approximately 3.0 Å from Fe and at this resolution it is impossible to claim this provides a ligand for the metal ion, as has been suggested in alternative studies [14]. Indeed, the placement of a nearby glutamate (Glu\(^{76}\) in the small subunit), with which there is a hydrogen bond, would argue against that. Strikingly, however, a conserved histidine residue (His\(^{229}\)) of the large subunit comes close to, and points directly at, the proximal [4Fe–3S] cluster (Figure 4). The distance between the large subunit His\(^{229}\) NE2 and Fe of the cluster is 3.3–3.5 Å, which is not a direct co-ordination of the metal, but certainly close enough to potentially have an effect on the properties of the cofactor (Figure 4).

The roles of large subunit residues His\(^{229}\) and Glu\(^{73}\) in the catalytic cycle

The large subunit His\(^{229}\) is completely conserved in all [NiFe]-hydrogenases (Figure 5A). In addition, the structure of Hyd-5 indicates that a buried glutamic acid (Glu\(^{73}\) of the large subunit) is close to His\(^{229}\) (Figure 5B). The Glu\(^{73}\) residue is highly conserved
in O₂-tolerant [NiFe]-hydrogenases and a glutamine residue is most commonly found at this position in standard O₂-sensitive hydrogenases (Figure 5A). In the Hyd-5 structure, the Glu73 side chain appears to donate a hydrogen bond to the carbonyl of Pro230 in the large subunit, and this indicates that it is protonated.

In order to assess whether His229 or Glu73 of the Hyd-5 large subunit have important roles in hydrogenase activity in general, it was decided to genetically remove these side chains and replace them with alanine. The *S. enterica* LB03 (P₅, hydAΔ₅–His, hydB) strain was modified to yield two new strains, LB03 H229A (P₅, hydAΔ₅–His, hydB) and LB03 E73A (P₅, hydAΔ₅–His, hydB). To ascertain whether the variant enzymes were capable of H₂-oxidation activity, the LB03 H229A and LB03 E73A strains were cultured anaerobically and the H₂-oxidizing activity of whole cell samples assessed using the artificial electron acceptor BV (Figure 2A). The strains retained good levels of H₂-dependent BV reduction activity (Figure 2A). In addition, periplasmic fractions were prepared from the same strains and samples analysed by rocket immunoelectrophoresis followed by activity staining using BV and Tetrazolium Red under H₂-saturated conditions (Figure 2A). This technique reports on the relative levels of enzyme in different samples and also gives a non-quantitative indication of enzyme activity. No Hyd-5 activity arcs were observed in the periplasm of the wild-type strain LT2 (Figure 2A), which has no soluble periplasmic hydrogenases. However, data for the LB03 H229A and LB03 E73A mutants suggest that these enzyme variants are active and aqueous-soluble in the periplasm, albeit at slightly less abundance than the enzyme from their parent strain LB03 (Figure 2A). The Hyd-5 H229A and LB03 E73A variants were purified by IMAC and analysed by SDS/PAGE (Figure 2B). Both variant enzymes appeared physically stable with little contaminating proteins observed (Figure 2B).

Next, purified water-soluble native Hyd-5Δ₅–His, and the H229A and E73A variants, were applied separately to graphite electrodes and analysed by PFE. Figure 6 shows cyclic voltammograms of the three enzymes under differing H₂ concentrations. At all of the percentages of H₂, studied the response of the native Hyd-5 enzyme and the E73A variant were concurrent,
The S. enterica Hyd-5 isoenzyme

Figure 6  H2 oxidation electrocatalysis

Comparing the catalytic response to H2 of (A) native S. enterica Hyd-5 and the (B) E73A and (C) H229A variants at pH 6.0, 37 °C. Cyclic voltammograms were performed under different percentages of H2, as indicated. A N2 carrier gas was used to give a total gas mixture flow rate of 100 scc/min. The potential was increased from −0.56 to +0.24 V compared with the SHE, and then the scan direction was reversed all at a rate of 5 mV/s. The electrode was rotated at 4000 rev/min in all experiments. (D) To emphasize differences in catalytic onset potentials the voltammograms measured at 10 % H2 for each enzyme are compared. The current during the forward and back potential sweep has been averaged and then normalized relative to the current at 0 V compared with the SHE.

with onset potentials (where a current increase marks the electrode potential where H2 oxidation begins) that were independent of H2 concentration and with a similar shape (accounting for differential adsorption of enzyme on to the electrode) (Figure 6). However, the Hyd-5 H229A variant shows a Nernstian shift in the H2 oxidation onset potential as a function of the percentage of H2 (−0.31 V compared with the SHE at 1 % H2; and −0.34 V compared with the SHE at 10 % H2, pH 6) (Figure 6). The relationship between Hyd-5 H229A onset potential and the concentration of H2 used in the assay indicates that this variant lacks the overpotential requirement seen for native Hyd-5, the E73A variant and many other O2-tolerant [NiFe]-hydrogenases [21,38]. At more positive potentials (greater than −0.02 V), there was also a significant fall in current for the H229A variant, where the native enzyme activity remains steady under these conditions (Figure 6). This decline in activity at higher potentials is indicative of anaerobic inactivation (formation of the Ni-B state) occurring more readily in the H229A variant (Figure 6).

The roles of large subunit residues His229 and Glu73 in tolerance to O2 attack

The lack of an overpotential requirement for H2 oxidation activity observed in the present study for Hyd-5 H229A is reminiscent of the behaviour of native O2-sensitive [NiFe]-hydrogenases. Thus the effect of O2 exposure on the H2-oxidation activities of the native Hyd-5 and the H229A and E73A variants was examined. Both cyclic voltammetry and chronoamperometry experiments, which compared activity before, during and after exposure to 3 % O2 in the presence of 3 % H2, were employed (Figure 7). Prolonged exposure of the native Hyd-5 enzyme to 3 % O2 had little effect on catalysis, with retention of 80 % of the pre-O2 catalytic current at −0.059 V compared with the SHE (Figure 7), thus corroborating the O2-tolerance of the native enzyme [10]. Any inactivation that did occur for the native enzyme under O2 was probably due to formation of the Ni-B state, as the activity profiles in both the chronoamperometry and cyclic voltammetry experiments show that, following exposure to O2, the enzyme recovers full activity rapidly once anaerobic conditions are re-established (Figure 7). Interestingly, relative to the native Hyd-5, the application of aerobic assay conditions had a far greater effect on the catalytic activity of both the H229A and E73A variants (Figure 7). Specifically, in cyclic voltammetry the zero-current response at potentials more positive than approximately 0 V compared with the SHE shows that under these oxidizing conditions activity of the H229A and E73A variants was fully inhibited by O2 exposure. Additionally, in chronoamperometry at −0.059 V compared with the SHE, under prolonged exposure to 3 % O2 the Hyd-5 H229A variant showed a continual decline in H2 oxidation activity, whereas at this potential the E73A variant exhibited stable activity at approximately 50 % of the pre-O2 catalytic current (Figure 7). A proportion of the aerobic inactivation of the H229A and E73A variants was probably due to formation of the Ni-A species as full catalytic activity was never regained for either variant after O2 inhibition (Figure 7). Taken altogether, it can be concluded that both the H229A and the E73A variants are compromised with regards to O2-tolerance, although the effect of O2 exposure has the most negative effect in the H229A variant.
Figure 7  O2 tolerance

Comparing the response to O2 of native Hyd-5 and the variants H229A and E73A at pH 6.0, 37 °C. Cyclic voltammetry (upper panels) and chronoamperometry experiments (lower panels) compare activity before (i), during (ii) and after (iii) O2 exposure. Cyclic voltammetry was measured at 5 mV/s, and the potential was first swept in a positive direction, up to 0.24 V, before the scan direction was reversed. For chronoamperometry, the potential was maintained at −0.059 V compared with the SHE throughout the experiment. A N2 carrier gas was used to give a total gas mixture flow rate of 100 scc/min and the exact gas compositions at each stage comprised (i) 3 % H2 only, (ii) 3 % H2 and 3 % O2, and (iii) 3 % H2 (recovery stage). The electrode was rotated at 4000 rev./min.

DISCUSSION

Although the S. enterica genome encodes only four [NiFe]-hydrogenases, the aerobically expressed uptake hydrogenase has been termed Hyd-5 to avoid confusion with the Hyd-4 of E. coli [10]. Two of the three uptake hydrogenases in S. enterica (Hyd-1 and Hyd-5) are predicted to generate a transmembrane proton electrochemical gradient by a redox loop mechanism (similar to that suggested for respiratory formate dehydrogenases [39]) and the energy conserved by the oxidation of H2 is crucial for pathogenesis within the host [4]. Indeed, genetic removal of the uptake hydrogenases from S. enterica results in an avirulent strain incapable of invading the spleen or liver tissue [3].

Hyd-5 is of great interest as it may represent a new class of O2-tolerant enzyme in enteric bacteria; not only is Hyd-5 tolerant to O2 attack, but it is also actually expressed and assembled under aerobic conditions [38]. Understanding the molecular basis behind O2-tolerance, and what processes are involved in enzyme biosynthesis, are therefore important research objectives.

**His229** of the large subunit has an intimate relationship with the proximal cluster

In the present study, new insight into O2-tolerance in [NiFe]-hydrogenases has been gained. The crystal structure of S. enterica Hyd-5 classifies it firmly as a member of the O2-tolerant [NiFe] membrane-bound hydrogenases [38]. The small subunit contains a proximal [4Fe–3S] cluster co-ordinated by six cysteine residues. However, in contrast with the other available O2-tolerant hydrogenase structures, the S. enterica Hyd-5 structure reveals a large subunit histidine residue (His229) very close to the proximal [4Fe–3S] cluster, with a distance of approximately 3.3 Å between the His229 NE2 and an Fe3+ ion. In the [NiFe]-hydrogenase family, this histidine residue is conserved to the point of invariance, even amongst O2-sensitive hydrogenases (Figure 5), and was noted to be close to the proximal cluster in the first ever report of a [NiFe]-hydrogenase crystal structure [40]. The role of this conserved histidine residue in hydrogenase activity has remained largely unexplored, however, most probably because the distance between the histidine and the proximal cluster is seen to vary between structures. In the E. coli Hyd-1 structures, for example, the distance between the large subunit His229 and an Fe3+ of the proximal cluster is 4.2 Å, which was considered too long to be a ligand to the metal [14].

The respiratory NADH dehydrogenase (Complex I) shares an evolutionary relationship with [NiFe]-hydrogenases [41], to such an extent that Complex I contains an equivalent to the [NiFe]-hydrogenase proximal cluster called the N2 cluster, which is present in the Nqo6 subunit. Moreover, the adjacent Nqo4 subunit of Complex I has an equivalent side chain to Hyd-5 His229 that is close enough to the N2 cluster in Nqo6 to hydrogen bond to it [42]. The side chain is termed His169 in Nqo4 and Kashani-Poor et al. [43] found that when the equivalent of the Nqo4 His169 was mutated to alanine it resulted in the loss of the characteristic
N₂ spectroscopic signal and a concomitant reduction in enzyme activity. Furthermore, substitution of the histidine residue with methionine lowered the potential of the cluster and removed any pH dependence [44].

In the present study, substitution of the Hyd-5 large subunit His₂²⁹ with alanine did not disrupt assembly of the enzyme, but instead had a profound effect on its catalytic properties and O₂-tolerance, including removal of the overpotential requirement for H₂ oxidation, which is an effect not seen in any previous studies of O₂-tolerant hydrogenases. The native proximal cluster is known to be held at an unusually high potential in O₂-tolerant [NiFe]-hydrogenases, and it is possible that the Hyd-5 alanine residue variant displays a lower cluster potential that leads to both the greater extent of anaerobic inactivation and loss of the H₂ oxidation overpotential requirement observed in the present study.

The chemistry of O₂-tolerant and O₂-sensitive [NiFe]-hydrogenases does not only differ under aerobic conditions. Anaerobically, PFE has been used to demonstrate that both classes of hydrogenase do form the Ni-B state, but the potential at which this occurs is different for each, with O₂-tolerant enzymes being electrochemically identifiable by formation of the inactive state at higher potentials. The O₂-sensitive enzymes have also been shown to lack the overpotential requirement for the onset of H₂-oxidation activity that is seen in O₂-tolerant enzymes. Although hydrogenase variants have been described that display compromised O₂ tolerance, to date no variant has been observed to lose the overpotential requirement for activity, which is an important problem in the search for a H₂ production catalyst that functions in air. The loss of the overpotential requirement displayed by the S. enterica Hyd-5 H₂C₉A enzyme is therefore significant.

The role of Glu⁷³ of the large subunit

The presence of the large subunit His₂²⁹ residue in all [NiFe]-hydrogenases, and Complex I, is difficult to reconcile with the clear negative effect that an His₂²⁹ substitution has on the specialist property of O₂ tolerance. One possible explanation is that the chemistry surrounding and influencing the His₂²⁹ imidazole group is subtly different in O₂-tolerant hydrogenases. The large subunit Glu⁷³ is highly conserved in O₂-tolerant hydrogenases and essentially never present in standard O₂-sensitive enzymes, being always replaced by glutamine residue except for in the thermophilic *Aquifex* enzyme. The Glu⁷³ side chain lies close to the [NiFe] active site, the His₂²⁹ residue and the proximal [4Fe–3S] cluster. Although the Glu⁷³ residue is pointing away from His₂²⁹ in the ‘resting’ structure described in the present paper, it would only take a small conformational alteration for the side chain to adopt a different rotamer and form a hydrogen bond with His₂²⁹. Such a conformational change would not be unusual or unreasonable to predict, and this could push His₂²⁹ closer to the proximal cluster, or indeed pull it further away. Note that it is highly probable that His₂²⁹ may be able to reposition itself due to the flexible region it occupies, which is rich in conserved proline and glycine residues. Interactions between glutamate/aspartate and histidine residues are very common in Nature, for example in the formation of catalytic dyad/triad charge relay systems used by some proteases [45]. It is conceivable that Hyd-5 Glu⁷³ could induce or stabilize a charge-transfer network between His₂²⁹ and the oxidized proximal Fe–S cluster in a dynamic three-way interaction. In this way, generation of a new ‘super-His’ ligand could be used to fine-tune the redox properties of the special proximal cluster.

Conclusion

Historically, overexpression of [NiFe]-hydrogenases has been complicated by the lack of co-ordinated expression of various biosynthetic genes required for cofactor insertion and other post-translational modifications. In the present study, these issues have been largely overcome by using a genetic engineering approach to up-regulate the native operon and affinity tag a water-soluble version of the native enzyme. It was also possible to generate point mutations at the native locus and isolate fully assembled variants.

This approach has led to fresh insight into the structure and mechanism of an O₂-tolerant [NiFe]-hydrogenase. Previous mutagenic studies have sought to understand the biochemical influence of the small subunit side chains and the Fe–S clusters, whereas studies of large subunit variants have focussed largely on the gas channel rather than the cofactor environments. Under an O₂ atmosphere the significantly compromised O₂ tolerance of both variants tested in the present study suggests that His₂²⁹ may have a stabilizing interaction with the open ‘super-oxidized’ form of the proximal cluster; however, spectroscopic analyses will be required to test this hypothesis. Understanding the precise role of Glu⁷³ will also require further experimentation, including production of an E73Q variant followed by further spectroscopic and enzymatic comparisons with O₂-sensitive enzymes. It is tempting to speculate, however, that deprotonation of Glu⁷³ could release it from its resting position and the resultant conformational change facilitate an interaction with nearby His₂²⁹, which could, in turn, modulate the position of His₂²⁹ relative to the proximal cluster during the catalytic cycle.

AUTHOR CONTRIBUTION

Lisa Bowman, Lindsey Flanagan and Paul Fyfe performed the research; Lisa Bowman, Lindsey Flanagan, Paul Fyfe, Alison Parkin, William Hunter and Frank Sargent designed the research, analysed the data and wrote the paper; and Alison Parkin, William Hunter and Frank Sargent supervised the research.

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