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

Lisa BOWMAN*†, Lindsey FLANAGAN†, Paul K. FYFE‡, Alison PARKIN†, William N. HUNTER‡ and Frank SARGENT*‡

*Division of Molecular Microbiology, College of Life Sciences, University of Dundee, Dundee DD1 5EH, Scotland, U.K.
†Department of Chemistry, University of York, Heslington, York YO10 5DD, England, U.K.
‡Division of Biological Chemistry and Drug Discovery, College of Life Sciences, University of Dundee, Dundee DD1 5EH, Scotland, U.K.

**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 His229 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.

---

**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), hybABCDEDFG (STM3150–STM3143), and hydBACDEFGHI (STM1539–STM1531) operons. Two of these (hya and hyb) encode homologues of the Hyd-1 and Hyd-2 [NiFe]-hydrogenase enzymes found in *Escherichia coli* [6–8] and in the present study the *E. coli* nomenclature will be applied to these *S. enterica* enzymes. The third uptake isoenzyme, encoded by the hyd operon (Figure 1), has no equivalent in *E. coli* and its existence was revealed following assembly of the *S. enterica* genome [3,9]. The enzyme product of *hyd* has been named Hyd-5 [10]. All three *S. enterica* H2-uptake enzymes share similar ‘core’ structures with a large subunit (α-subunit) containing the [Ni–Fe–CO–2CN−] catalytic centre, and a small subunit (β-subunit) containing three Fe–S clusters. The αβ heterodimers often oligomerize into larger αβ2 heterotetramers [11–15]. The small subunits each contain single C-terminal TMs (transmembrane domains) that anchor the αβ heterodimers to the periplasmic face of the inner membrane [16]. The three *S. enterica* H2-uptake enzymes can therefore be subcategorized as ‘membrane-bound’ [NiFe]-hydrogenases.

*S. enterica* Hyd-1, Hyd-2 and Hyd-5 were found to be differentially expressed and utilized at different stages of infection [17]. Whereas the *hya* and *hyb* 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 in 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 residues [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(II) the enzyme is reactivated rapidly back to a catalytically active form. By
used to delete the TM domain by homologous recombination as described in [24], finally generating LB03.

The derivatives of LB03 encoding the HydB E73A and H229A variants were generated as follows. DNA surrounding the Glu 73 codon was amplified using the oligonucleotides HydBGlufor (5′-GGCGCTCTAGACATGACAAATGTTACC-3′) and HydBGlurev (5′-GGCGCGTAATCTCGTCAAAATGTTAGG-3′), whereas DNA surrounding the His 229 codon was amplified using the oligonucleotides HydBHisfor (5′-GGCGCTCTAGACATGACAAATGTTACC-3′) and HydBHisrev (5′-GGCGCGTAATCTCGTCAAAATGTTAGG-3′). The resulting PCR products were digested with XbaI and KpnI and cloned independently into similarly digested pBluescript KS+.

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 37°C. 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 S. enterica [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 37°C. 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 37°C for 30 min. The sphaeroplasts were harvested by centrifugation (17000 g for 15 min at 4°C) 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

Figure 1 The S. enterica hyd operon encodes Hyd-5
The structure of the hyd operon (STM1539–STM1531). S. enterica was genetically engineered to facilitate isolation of Hyd-5. The strong Ts promoter from the pQE series of expression vectors (Qiagen) was inserted immediately upstream of hydA (STM1539) on the S. enterica chromosome. In addition, a section of the hydA gene encoding a TM was deleted and replaced by the sequence for a His₆ affinity tag. The roles of specific gene products are indicated.
Chromosomally encoded Hyd-5<sup>ATM</sup> –<sub>H<sub>2</sub>O</sub> was purified from the *S. enterica* LB03 (*hydA<sup>ATM</sup> –<sub>H<sub>2</sub>O</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 17400 × 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<sup>ATM</sup> –<sub>H<sub>2</sub>O</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 % (v/v) PEG4000, 0.1 M Mes (pH 6) and 0.24 M lithium sulphate. Brown monoclinic blocks, with the approximate dimensions 80 × 40 × 40 µm, grew over 2–3 days. The crystals were prepared for data collection with cryoprotection using the mother liquor adjusted to include 20 % (w/v) glycerol and then plunged into liquid N<sub>2</sub>. 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 package MOLEP [31], and a search model of the membrane-bound [NiFe]-hydrogenase from *Ralstonia eutrophia* (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 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 C<sub>α</sub>-RMSD from ideal geometry of 55.0, 56.5, 59.2, 59.6, 56.0, 59.2 for the large and small subunits, and then CD and EF respectively. The S. enterica Hyd-5 isoenzyme

### Table 1 Crystallographic statistics

Values in parentheses refer to the highest resolution bin of approximate width 0.1 Å. 

| Parameter         | Value               |
|-------------------|---------------------|
| Data collection   |                     |
| Space group       | P2<sub>1</sub>       |
| Wavelength (Å)    | 0.9763              |
| a, b, c (Å)       | 115.5, 122.2, 227.8 |
| β (°)             | 95.6                |
| Resolution range (Å) | 29.5–3.2         |
| Unique reflections (n) | 51975          |
| Completeness (%)  | 99.6 (98.6)          |
| <λ(σ(l)>          | 9.7 (1.8)           |
| Multiplicity (%)  | 3.8 (3.8)           |
| Rmerge (%)        | 10.4 (58.1)         |
| Refinement        |                     |
| Resolution range (Å) | 29.5–3.2         |
| Number of used reflections | 49122     |
| R<sub>work</sub>, R<sub>free</sub> | 15.9, 20.6     |
| Protein atoms     | 19918               |
| Waters, chlorides, magnesium, sulfates | 114,7,3,2 |
| RMSD from ideal geometry |                     |
| Bond lengths (Å)  | 0.011               |
| Bond angles (°)   | 1.523               |
| Thermal parameters (k<sup>2</sup>) |                   |
| Wilson B-factor   | 83.8                |
| Mean B-factor     | 56.9                |
| Subunit L, S, A, B, C | 55.0, 56.5, 59.2, 59.6, 56.0, 59.2 |
| FeS centres (heterodimers: LS, AB and CD) | |
| F4S (proximal 4Fe–3S cluster) | 82.0, 82.1, 82.3 |
| FXS (medial 3Fe–4S cluster) | 52.5, 56.4, 53.1 |
| SFW (distal 4Fe–4S cluster) | 55.7, 52.7, 52.59 |
| NFU ([NiFe]<sub>2</sub> active site) | 53.2, 58.5, 55.7 |
| Magnesium, chlorides, sulfates, water | 49.4, 74.9, 114, 46.6 |
| Ramachandran plot |                     |
| Favoured, allowed, disallowed (%) | 92.1, 7.6, 0.3 |

The S. enterica Hyd-5 crystal structure have been deposited in the PDB under accession code 4C3O.
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 3 μl of enzyme was pipetted on to the surface and adsorbed for a period of approximately 30 s. The electrode was then inserted into the electrochemical cell. This ‘working’ electrode was rotated using an Origatrod rotator (Origalys) at >3500 rev./min to allow an adequate supply of substrate and removal of product. A CompactStat potentiotstat (Ivium Technologies) and the IviumSoft program were used to control the experiment.

RESULTS

Isolation of S. enterica Hyd-5

The Hyd-5 [NiFe]-hydrogenase is anchored in the periplasm by the small subunit C-terminal helix which spans the inner membrane. In the present study, the SFT06 (Pt5, hydAHis) strain that overproduces active Hyd-5 [10] was modified 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 (Pt5, hydATM−His).

The LB03 (Pt5, hydATM−His) strain was cultured at a small scale under anoxic conditions and whole cells were assayed for BV-linked hydrogenase activity (Figure 2A). The LB03 (Pt5, hydATM−His) strain displayed increased hydrogenase activity compared with the parent strain (Figure 2A). Next, the LB03 (Pt5, hydATM−His) 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 the SFTH06 (Pt5, hydATM−His). LB03 H229A, LB03 E73A and the parental strain LT2a. The arrow shows the direction of protein migration. (B) SDS/PAGE (12% w/v gel) of pooled IMAC fractions of proteins isolated from strains SFTH06 (Pt5, hydATM−His), LB03 (Pt5, hydATM−His) (ΔTM), LB03 H229A and LB03 E73A. Images taken from different gels are separated by black borders.

A single crystal form was obtained that displayed space group I2 with unit cell lengths a = 115.5, b = 122.2, c = 227.8 Å and β = 95.6°. The structure was solved by molecular replacement using the structure of the membrane-bound [NiFe]-hydrogenase from R. eutropha (PDB code 3RGW) and refined to 3.2 Å resolution (Figure 3). Hyd-5 crystallized with three core hydrogenase αβ heterodimers in the asymmetric unit, and two of the three hydrogenase dimers associated with each other (Figure 3A) as an αβ,β heterotetramer. This αβ2 arrangement is common and had been observed for other [NiFe]-hydrogenases [10–14]. The third heterodimer in the asymmetric unit self-associates about a crystallographic 2-fold axis to produce the same αβ2 arrangement.

The C-terminus of the large subunit is well buried within the structure, which is to be expected as [NiFe]-hydrogenase large subunits are known to be C-terminally processed and locked in position before docking on to their small subunit partners [37]. In addition, available [NiFe]-hydrogenase large subunit structures have an Mg2+ ion that interacts with the extreme terminal histidine
The S. enterica Hyd-5 isoenzyme

Figure 3  The structure of a Hyd-5 α2β2 heterotetramer

(A) The large (α) subunits are shown as red and green ribbons, the small (β) 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 αβ heterodimer is shown.

Residue. The Hyd-5 structure also has strong feature in the electron density that would correspond to a hydrated Mg2+ 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 O2-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 α2β2 assembly (Figure 3A), the two distal [4Fe–4S] clusters from the two β-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 Cys20 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 (Glu76 in the small subunit), with which there is a hydrogen bond, would argue against that. Strikingly, however, a conserved histidine residue (His229) of the large subunit comes close to, and points directly at, the proximal [4Fe–3S] cluster (Figure 4). The distance between the large subunit His229 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 His229 and Glu13 in the catalytic cycle

The large subunit His229 is completely conserved in all [NiFe]-hydrogenases (Figure 5A). In addition, the structure of Hyd-5 indicates that a buried glutamic acid (Glu73 of the large subunit) is close to His229 (Figure 5B). The Glu73 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, hydAΔ⁷₅Δ₁TM−His) strain was modified to yield two new strains, LB03 H229A (P⁵, hydAΔ⁷₅Δ₁TM−His, hydB H229A) and LB03 E73A (P⁵, hydAΔ⁷₅Δ₁TM−His, hydB E73A). 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-5Δ⁷₅Δ₁TM−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  H$_2$ oxidation electrocatalysis

Comparing the catalytic response to H$_2$ 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 H$_2$ as indicated. A N$_2$ 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% H$_2$ 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 H$_2$ oxidation begins) that were independent of H$_2$ 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 H$_2$ oxidation onset potential as a function of the percentage of H$_2$ ($-0.31$ V compared with the SHE at 1% H$_2$; and $-0.34$ V compared with the SHE at 10% H$_2$, pH 6) (Figure 6). The relationship between Hyd-5 H229A onset potential and the concentration of H$_2$ used in the assay indicates that this variant lacks the overpotential requirement seen for native Hyd-5, the E73A variant and many other O$_2$-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 His$^{229}$ and Glu$^{73}$ in tolerance to O$_2$ attack

The lack of an overpotential requirement for H$_2$ oxidation activity observed in the present study for Hyd-5 H229A is reminiscent of the behaviour of native O$_2$-sensitive [NiFe]-hydrogenases. Thus the effect of O$_2$ exposure on the H$_2$-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% O$_2$ in the presence of 3% H$_2$, were employed (Figure 7). Prolonged exposure of the native Hyd-5 enzyme to 3% O$_2$ had little effect on catalysis, with retention of 80% of the pre-O$_2$ catalytic current at $-0.059$ V compared with the SHE (Figure 7), thus corroborating the O$_2$-tolerance of the native enzyme [10]. Any inactivation that did occur for the native enzyme under O$_2$ 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 O$_2$, 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 O$_2$ exposure. Additionally, in chronoamperometry at $-0.059$ V compared with the SHE, under prolonged exposure to 3% O$_2$ the Hyd-5 H229A variant showed a continual decline in H$_2$ oxidation activity, whereas at this potential the E73A variant exhibited stable activity at approximately 50% of the pre-O$_2$ 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 O$_2$ inhibition (Figure 7). Taken altogether, it can be concluded that both the H229A and the E73A variants are compromized with regards to O$_2$-tolerance, although the effect of O$_2$ 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 His¹⁹⁹ in Nqo4 and Kashani-Poor et al. [43] found that when the equivalent of the Nqo4 His¹⁹⁹ 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 surrounding and influencing the His²²⁹ imidazole group is subtly different in O₂-tolerant hydrogenases. The large subunit 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 H229A 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 glutamate 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.

ACKNOWLEDGEMENTS

We thank Professor Fraser Armstrong (University of Oxford, Oxford, U.K.), Professor Tracy Palmer (University of Dundee, Dundee, U.K.), Dr Conny Pinske (University of Dundee, Dundee, U.K.) and Dr Jen McGowall (formerly University of Dundee, now University of Bath, Bath, U.K.) for useful discussions and advice. We acknowledge staff at the Diamond Synchrotron, beamline I03, for access to the facility and excellent support.

FUNDING

This work was supported the Biotechnology and Biological Sciences Research Council (BBSRC) via a Targeted Priority Studentship [grant number BB/G016690/1], Tennessen Scotland [grant number T12/48], the Wellcome Trust via grants [grant numbers 082596, 094090 and 100476] and a Ph.D. studentship (WT095024MA to L.F.), and the Engineering and Physical Sciences Research Council (EPSRC) [grant number EP/K031589/1].

REFERENCES

1. Winter, S. E., Thiennimitl, P., Winter, M. G., Butler, B. P., Huseby, D. L., Crawford, R. W., Russell, J. M., Bevinis, C. L., Adams, L. G., Tsolis, R. M. et al. (2010) Gut inflammation provides a respiratory electron acceptor for Salmo nella. Nature 467, 426–429

2. Winter, S. E., Winter, M. G., Xavier, M. N., Thiennimitl, P., Poon, V., Koestra, A. M., Laughlin, R. C., Gomez, G., Wu, J., Lawhon, S. D. et al. (2013) Host-derived nitrate boosts growth of E. coli in the inflamed gut. Science 339, 709–711

3. Maier, R. J., Olczak, A., Maier, S., Soni, S. and Gunn, J. (2004) Respiratory hydrogen use by Salmo nella enterica serovar Typhimurium is essential for virulence. Infect. Immun. 72, 6294–6299

4. Maier, R. J. (2005) Use of molecular hydrogen as an energy substrate by human pathogenic bacteria. Biochem. Soc. Trans. 33, 83–85
