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Structural differences of oxidized iron–sulfur and nickel–iron cofactors in O_2-tolerant and O_2-sensitive hydrogenases studied by X-ray absorption spectroscopy

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ABSTRACT

The class of [NiFe]-hydrogenases comprises oxygen-sensitive periplasmic (PH) and oxygen-tolerant membrane-bound (MBH) enzymes. For three PHs and four MBHs from six bacterial species, structural features of the nickel–iron active site of hydrogen turnover and of the iron–sulfur clusters functioning in electron transfer were determined using X-ray absorption spectroscopy (XAS). Fe-XAS indicated surplus oxidized iron and a lower number of ~2.7 Å Fe–Fe distances plus additional shorter and longer distances in the oxidized MBHs compared to the oxidized PHs. This supported a double-oxidized and modified proximal FeS cluster in all MBHs with an apparent trimer-plus-monomer arrangement of its four iron atoms, in agreement with crystal data showing a [4Fe3S] cluster instead of a [4Fe4S] cubane as in the PHs. Ni-XAS indicated coordination of the nickel by the thiol group sulfurs of four conserved cysteines and at least one iron–oxygen bond in both MBH and PH proteins. Structural differences of the oxidized inactive [NiFe] cofactor of MBHs in the Ni-B state compared to PHs in the Ni–A state included a ~0.05 Å longer Ni-O bond, a two times larger spread of the Ni–S bond lengths, and a ~0.1 Å shorter Ni–Fe distance. The modified proximal [4Fe3S] cluster, weaker binding of the Ni–Fe bridging oxygen species, and an altered localization of reduced oxygen species at the active site may each contribute to O_2 tolerance.

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1. Introduction

Hydrogenases (H_2ases) are enzymes which catalyze effective and reversible hydrogen (H_2) formation or cleavage at protein-bound metal centres [1–3]. They are of high interest in the contexts of renewable energy generation, biotechnology, and chemical catalysis. An obstacle for using H_2ases in applications is the rapid inactivation of many enzymes by oxygen (O_2) [4,5]. However, H_2ases from various bacterial species have been identified, which exhibit high O_2-tolerance of their H_2-turnover activity [6–9]. Understanding this unusual catalytic behaviour may lead to improved enzymes as well as to novel synthetic catalysts.

The known O_2-tolerant H_2ases all belong to the [NiFe] type, meaning that their active site contains one nickel and one iron atom [10]. In particular, members of the phylogenetically related subclass of O_2-tolerant membrane-bound [NiFe]-H_2ases (MBHs) have attracted much research effort [11–13]. They consist of a large subunit binding the [NiFe] active site of H_2 turnover and a small subunit with three iron–sulfur clusters in proximal, medial, and distal positions to the [NiFe] site, functioning as an electron transfer relay, and are anchored to the periplasmic side of the cytoplasmic membrane via a *b*-type cytochrome. The standard type of O_2-inhibited periplasmic [NiFe]-H_2ases (PHs) shows similar subunits and cofactor complements, but a soluble cytochrome-*c* is the electron transfer partner in many cases (Fig. 1). Hydrogenase II from

Abbreviations: EPR, electron paramagnetic resonance spectroscopy; EXAFS, extended X-ray absorption fine structure; FTIR, Fourier-transform infrared spectroscopy; H_2ase, hydrogenase; MBH, membrane-bound [NiFe] H_2ase; PH, periplasmic [NiFe]-H_2ase; TXRF, total-reflection X-ray fluorescence analysis; XANES, X-ray absorption near edge structure; XAS, X-ray absorption spectroscopy

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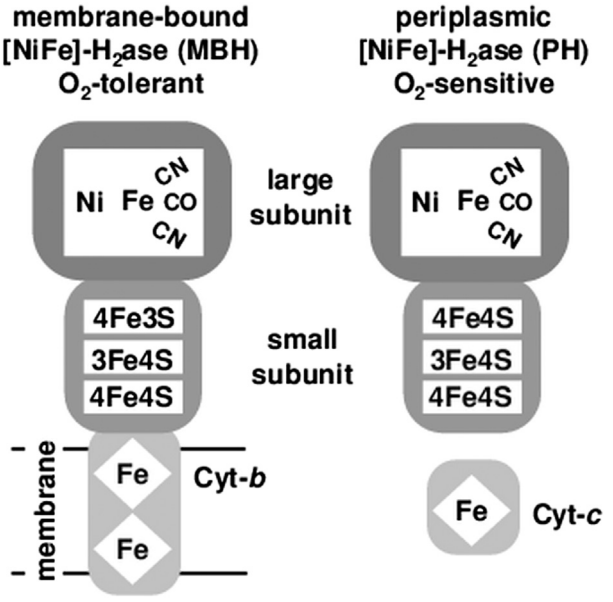

Fig. 1. Schematic organisation of [NiFe]-hydrogenases of the MBH and PH types. Our enzyme preparations lacked the membrane and the cytochrome-*b* or -*c* heme proteins. A proximal [4Fe3S] cluster in the MBHs was identified in crystal structures [14–16,66]. Notably, the *E. coli* hydrogenase II is a membrane associated enzyme not using cyt-*c*, but here grouped among the PHs because of the lack of the two additional cysteines in its small subunit, the presence of which is characteristic for the MBHs.

Escherichia coli (denoted as *Ec*2) is included here as a PH, although it is associated with the cytoplasmic membrane and does not use a *c*-type cytochrome as electron-transfer partner, because it resembles other standard type hydrogenases in terms of its catalytic properties and sensitivity to O_2.

Protein crystallography has revealed the general organisation of the metal cofactors in MBHs [14–16] and PHs [17–20]. In both enzyme types, the [NiFe] site is coordinated by four conserved cysteine residues, two thiolate sulfur atoms of which bridge the Ni and Fe atoms and the other two ligate the nickel, while the iron carries one carbon monoxide (CO) ligand and two cyanide (CN^-) groups [9,21,22]. However, the amino acid sequence accounting for the [NiFe] binding site in the large subunit is not strictly conserved among the various MBH and PH proteins from different organisms [23]. By treatment with various oxidants or reductants, the [NiFe] site can be poised in spectroscopically defined redox states, two of which are associated with reversibly inactivated enzyme, namely the so-called Ni-A and Ni-B states, containing Ni(III) and Fe(II) ions and an oxygen species in the metal-bridging position [24]. This species likely is a hydroxyl (OH^-) in Ni-B whereas for Ni-A the structural assignment is controversial [24,25]. Inactivation of PHs under O_2 or under anoxic conditions and oxidizing potentials favours formation of the Ni-A state, requiring extensive reduction procedures to be reactivated, and Ni-B usually is a minor species [26]. In the MBHs, similar conditions favour Ni-B formation and Ni-A is not observed in most enzymes [9,27,28]. Reactivation of Ni-B under reducing conditions is much faster compared to Ni-A in particular in the MBHs, possibly related to fine-structural differences at the [NiFe] site [29].

The O_2-tolerant MBHs are further distinguished from the PHs by the presence of two additional cysteine residues in the amino acid sequence of the small subunit, located in the vicinity of the binding site for the proximal FeS cluster [11,30,31]. Crystal structures of MBH proteins from *Ralstonia eutropha* [14], *Hydrogenovibrio marinus* [16], and *E. coli* [15] have revealed that these cysteines indeed coordinate three of the four iron atoms of the proximal cluster, transforming it from a cubane [4Fe4S] cluster as in the PHs to a novel [4Fe3S] cluster species. Mutagenesis and spectroscopic studies have shown that the modified proximal cluster is crucial for the O_2-tolerant catalytic behaviour of the MBHs [28,30]. This may be due to the surplus electron donation capacity of the modified cluster to the [NiFe] site, leading to full reduction of metal-bound oxygen species and thereby to rapid reactivation of the enzymes under O_2 [6,7,31]. Vice versa, it may be expected that the not yet crystallized MBHs with the two cysteines in their sequence contain a similar [4Fe3S] cluster.

X-ray absorption spectroscopy (XAS) facilitates detection of FeS cluster variations and determination of the nickel coordination at the active site in [NiFe]-H_2ases [29,32–35]. Here, we used XAS to obtain structural parameters of the FeS and [NiFe] cofactors in three O_2-sensitive PHs and four O_2-tolerant MBHs from six organisms. The data suggested similar FeS clusters in the four MBHs, in particular a proximal cluster differing from that in the PHs. The Ni site overall was similar in both enzyme types, but showed increased coordination disorder and weaker nickel–oxygen bonding in the MBHs (Ni-B) compared to the PHs (Ni-A).

2. Materials and methods

2.1. Protein sample preparation

Purified H_2ase proteins, i.e. MBHs from *R. eutropha* HF649 [29], *Aquifex aeolicus* (H_2ase I) [9], *E. coli* (*Ec*1) [36], and *Hydrogenophaga* spec. AH24 [37], and PHs from *Desulfovibrio gigas* [38], *Desulfovibrio fructosovorans* [39], and *E. coli* (*Ec*2) in their oxidized states were prepared following established procedures as described elsewhere [9,28,29,37,39] and concentrated to 0.5–1 mM. The enzymes were purified under microaerobic reducing conditions and poised thereafter mainly in the Ni-B state (MBHs) or Ni-A state (PHs) using air oxidation, as verified by the characteristic and well-known EPR and FTIR spectral signatures (not shown) of the protein preparations [9,22,27–29,36,37,40–42]. This revealed contents ≥65% of the Ni-B state in the MBHs and the Ni-A state in the PHs. Protein solutions (~20 μl) were filled into sample holders for XAS and frozen in liquid nitrogen. Aliquots of the protein samples were used for TXRF to determine the metal contents.

2.2. Metal content quantification

Ni and Fe contents of H_2ase proteins were determined by total-reflection X-ray fluorescence analysis (TXRF) [43] on a PicoFox instrument (Bruker) after adding a gallium elemental standard (Sigma) to the protein solutions (v/v 50:50).

2.3. X-ray absorption spectroscopy

XAS experiments were performed at beamlines KMC-1 of BESSY (Helmholtz Zentrum Berlin, Germany), D2 of HASYLAB (DESY, Hamburg, Germany), 16.5 of SRS (Daresbury, UK), and at the XAS beamline of ANKA (Karlsruhe Institut für Technologie, Karlsruhe, Germany) using standard set-ups (including a double-crystal monochromator) and procedures [44]. Kα fluorescence-detected XAS spectra at the Ni and Fe K-edges were collected for samples held in liquid-helium cryostats at 20 K with energy-resolving multi-element Ge detectors (Canberra or Ortec). XAS spectra were averaged (4–16 scans) after energy calibration of each scan using the spectra of Ni or Fe metal foils as standards normalized, and EXAFS oscillations were extracted as described elsewhere [29,44,45]. The energy scale of XAS spectra was converted to a wavevector (k) scale using E_0 values of 8333 eV (Ni) and 7112 eV (Fe); E_0 was refined to ~7120 eV (Fe) and ~8336 eV (Ni) in the spectral simulations. Unfiltered k^3-weighted EXAFS spectra were used for least-squares curve-fitting simulations with the in-house programme SimX [44] and phase-functions calculated with FEFF8 [46] and Fourier-transform (FT) calculation (S_0^2 values of 0.9 (Ni) and 0.85 (Fe)). The simulation quality was judged by calculation of the filtered R-factor (R_F) [44].

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