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Hyperthermostable and oxygen resistant hydrogenases from a hyperthermophilic bacterium *Aquifex aeolicus*: Physicochemical properties

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Abstract

The discovery of hydrogenases in hyperthermophiles has important ramifications not only in microbial physiology and evolution but also in biotechnologies. These organisms are the source of extremely stable enzymes (regarding temperature, pressure, and O_2). *Aquifex aeolicus* is a microaerophilic, hyperthermophilic bacterium containing three [NiFe] hydrogenases. It is the most hyperthermophilic bacterium known to date and grows at 85 °C under a $H_2/CO_2/O_2$ atmosphere. The *Aquificales* represent the earliest branching order of the bacterial domain indicating that they are the most ancient bacteria. Two *Aquifex* hydrogenases (one membrane-bound and one soluble) have been purified and characterized. In contrast to the majority of the [NiFe] hydrogenases, the hydrogenases from *A. aeolicus* are rather tolerant to oxygen. The molecular basis of the oxygen resistance of *Aquifex* hydrogenases has been investigated. The great stability of *Aquifex* hydrogenases with respect to oxygen and high temperatures make these enzymes good candidates for biotechnological uses.

Keywords: *Aquifex aeolicus*; Hydrogenase; Thermal stability; Oxygen resistant

1. Introduction

Microorganisms having the remarkable property of growing at temperatures near and above 100 °C have been isolated from shallow submarine and deep-sea volcanic environments over the last 20 years. The majority of these hyperthermophilic microorganisms belongs to the domain of the Archaea and is considered to represent the most slowly evolving forms of life [1–3]. The discovery of hyperthermophiles has important ramifications, not only in microbial physiology and evolution, but also in biotechnology [4]. Whereas numerous hyperthermophilic Archaea are known, only very few hyperthermophilic organisms from the domain of the bacteria have been discovered so far. *Aquifex* is the most hyperthermophilic bacterium growing at 85 °C under a $H_2/CO_2/O_2$ atmosphere in a medium containing only inorganic compounds. The *Aquificales* represent the earliest branching order of the bacterial domain indicating that they are the most ancient bacteria known to date. Similar to most of the *Aquificales*, *Aquifex aeolicus* is a hydrogen-oxidizing, microaerophilic, obligate chemolithoautotrophic bacterium [5]. It gains energy for growth from an uncommon electron transfer, from hydrogen to oxygen, and uses the reductive TCA

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(tricarboxylic acid) cycle as CO_2-fixing mechanism. Stimulated by the exceptional phylogenetic and physiological properties of *A. aeolicus*, as well as by its potential as a source of extremely stable enzymes, we have set out to study its hydrogen metabolism. Hydrogen metabolism in organisms is mediated by hydrogenases which are usually sensitive to inhibition by oxygen, and therefore predominantly occurs in anoxic environments. Depending on the metabolic requirements of the parent organism, hydrogenases catalyze either hydrogen uptake or hydrogen evolution, according to $H_2 \Leftrightarrow 2H^+ + 2e^-$. Operons encoding three distinct [NiFe] hydrogenases have been annotated in the *Aquifex* genome sequence and referred to as hydrogenases I, II and III in keeping with the annotated names mbhSL1, mbhSL2 and mbhSL3 [6]. We have purified two of these enzymes to homogeneity and analysed the phylogenetic positioning of all three hydrogenases [7]. The operon including the genes coding for the membrane-bound hydrogenase I has a typical organization of the group 1 of [NiFe] hydrogenases as defined by Vignais et al. [8]. In contrast, the order of hydrogenases II genes in operon is quite unusual and has been found only in very few other microorganisms. Hydrogenases I and II are membrane-attached and periplasmic whereas hydrogenase III is soluble in the cytoplasm. We have shown that sequence features of these three hydrogenases are homologous to those of the [NiFe] hydrogenase from *Desulfovibrio gigas* [7]. The presence, in the same bacterium, of hydrogen andoxygen metabolism offers the possibility to obtain enzymes more resistant to oxygen and to understand the reason of this resistance. In this work, we report the biochemical and biophysical characterization of the soluble and one membrane-attached [NiFe] hydrogenases from *A. aeolicus* regarding their stability to high temperatures and inhibition by oxygen gas.

2. Experimental section

2.1. Growth conditions of A. aeolicus

A. aeolicus was routinely grown at 85 °C in 2 l bottles under 1.4 bars of H_2/CO_2 in SME medium modified according to [5] at pH 6.8 and harvested in the late exponential growth phase. Typical yields were in the range of 400 mg of cell material per liter of culture.

2.2. Enzyme purification

Hydrogenases from *A. aeolicus* were purified at room temperature under semi-anaerobic conditions as described in [7]. Fractions containing hydrogenase activity were concentrated and stored in liquid N_2 in the presence of 10% glycerol.

2.3. Hydrogenase activity assays

Hydrogenase activity was routinely determined at 80 °C, as previously described [7], by the hydrogen consumption assay using methylviologen ($\varepsilon_{604} = 13\,600\,M^{-1}\,cm^{-1}$) as the electron acceptor and hydrogen as the electron donor.

2.4. Temperature dependence, thermal stability and oxygen inhibition

The temperature dependence of the enzyme activity was measured between 20 and 100 °C in 50 mM Hepes, pH 7. Thermodynamic parameters for the rate-determining step were obtained from the Arrhenius plot for hydrogenase activity.

$$\ln(k) = \ln A - Ea/RT,$$

where Ea is the activation energy, A the intercept of the Arrhenius equation, R the gas constant and T the absolute temperature.

The thermo stability of the purified enzyme was tested in sealed vials, which were incubated at 80 °C. The vials were then cooled on ice for 5 min, and the remaining enzyme activity was assayed at 80 °C. The oxygen tolerance of the purified enzyme was tested using sealed vials, which were incubated at 0, 37 or 80 °C in presence of 20% oxygen. The remaining enzyme activity was assayed at 80 °C.

2.5. EPR spectroscopy and protein film voltammetry

Electron paramagnetic resonance (EPR) spectra were recorded on a Bruker ELEXSYS E500 X-band spectrometer fitted with an Oxford Instruments ESR900 liquid He cryostat. Experiments were performed with 70 μM of proteins in Tris-HCl 50 mM buffer, pH 7.6. Protein film voltammetry experiments were carried out as described in [9].

2.6. Sequence analysis

Multiple sequence alignments were performed using ClustalW [10] at http://pbil.univ-lyon1.fr/.

3. Results and discussion

3.1. Sequence analysis

We have previously shown that hydrogenase I and hydrogenase II from *A. aeolicus* clearly fall into group 1 hydrogenases which mostly contains enzymes involved in electron transfer associated with transmembrane proton translocation [7]. Hydrogenase III, in contrast, cannot be straightforwardly attributed to one of the four major groups but the phylogenetic analysis showed that it is close, in amino acid sequence, to the sensory and cyanobacterial uptake hydrogenases [7]. Regulatory hydrogenases function as hydrogen sensor for the regulation of hydrogenase expression, are less active and oxygen-insensitive compared to the *Desulfovibrio* [NiFe] hydrogenases [11]. H_2 sensors have been characterized from *Rhodobacter capsulatus*, *Ralstonia eutropha* and *Bradyrhizobium japonicum* [12–14]. By sequence comparisons, it has been proposed that hydrophobic cavities, likely providing pathway for H_2 diffusion between the molecular surface and the buried active site, are significantly narrower in this group of proteins limiting access of larger molecules like O_2 [15]. Amino acid residues lining the putative gas channels of standard [NiFe] hydrogenases have been identified by crystal structures and amino acid sequences analyses. Two of them, valine and leucine located close to the active site and highly conserved in the large subunit of [NiFe] hydrogenases, are replaced by the more bulky residues isoleucine and phenylalanine, respectively, in the sensory hydrogenases leading to a decreased accessibility of oxygen gas to the active site [16]. Fig. 1 shows that *A. aeolicus* large subunit hydrogenase III possesses, like the hydrogen sensors, Ile and Phe residues in position 61 and 110, respectively, (corresponding to positions 74 and 122 in the large subunit of [NiFe] hydrogenase from *Desulfovibrio fructosovorans* (*Df*) [16]). The size of gas channel in hydrogenase III could prevent inhibition by oxygen for this enzyme. In contrast, hydrogenases I and II contain, like standard [NiFe] hydrogenases, the Val and Leu residues at these positions indicating that the gas cavities in these *Aquifex* enzymes might be classical (Fig. 1).

Concerning the regulatory hydrogenase of *Ralstonia eutropha*, no evidence for the presence of [4Fe–4S] and [3Fe–4S] clusters are available [17]. Buhrke et al. have recently proposed the existence of two [2Fe–2S] clusters in this enzyme and pointed to conserved histidine residues, present only in the sequences of hydrogen sensors, that could be involved in Fe/S clusters binding [18]. Multiple sequences alignment using

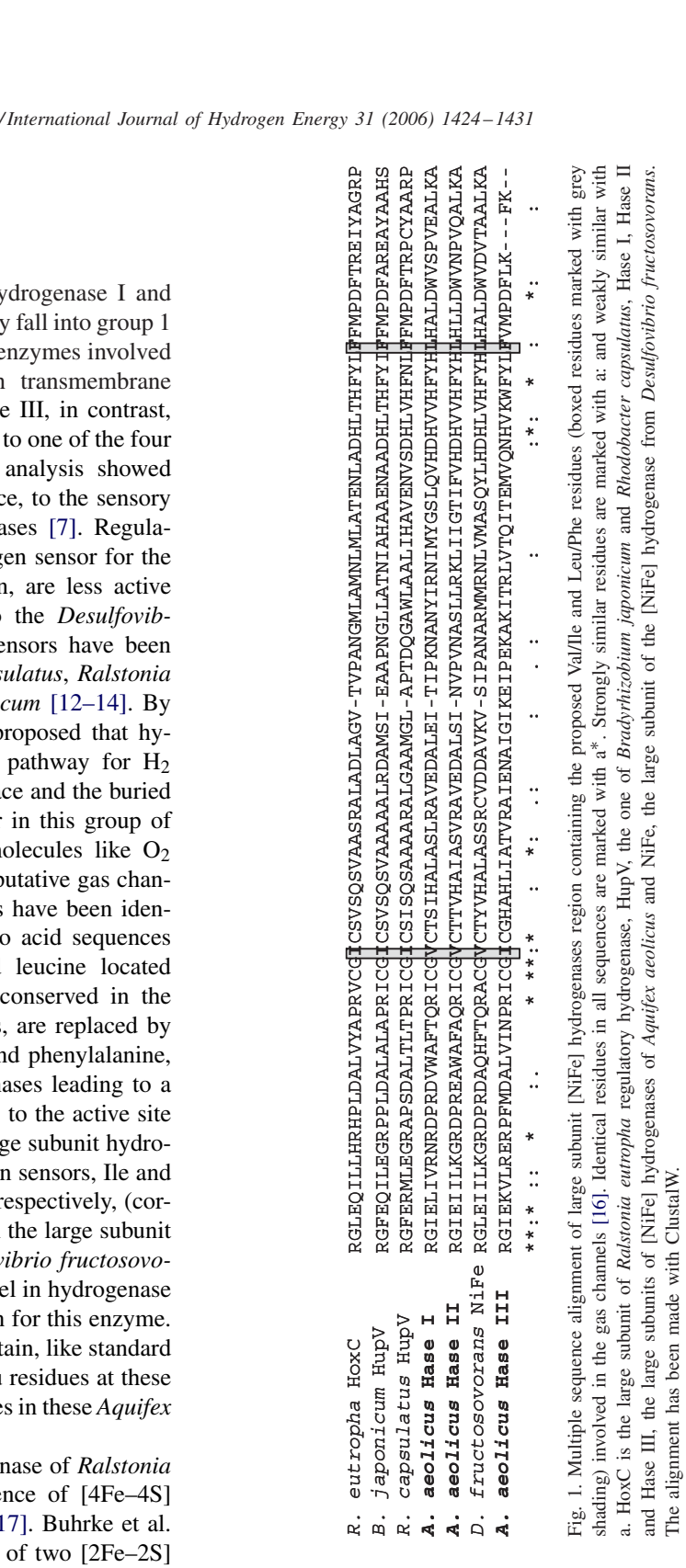

Fig. 1. Multiple sequence alignment of large subunit [NiFe] hydrogenases region containing the proposed Val/Ile and Leu/Phe residues (boxed residues marked with grey shading) involved in the gas channels [16]. Identical residues in all sequences are marked with a*. Strongly similar residues are marked with a: and weakly similar with a. HoxC is the large subunit of *Ralstonia eutropha* regulatory hydrogenase, HupV, the one of *Bradyrhizobium japonicum* and *Rhodobacter capsulatus*, Hase I, Hase II and Hase III, the large subunits of [NiFe] hydrogenases of *Aquifex aeolicus* and NiFe, the large subunit of the [NiFe] hydrogenase from *Desulfovibrio fructosovorans*. The alignment has been made with ClustalW.

Table 1
Thermal stability of hydrogenases from *Aquifex aeolicus*

	Hydrogenase III	Hydrogenase I	Hydrogenase I–cytchrome *b*
Yield for 50 g of cells	3.5 mg	3 mg	4.5 mg
Specific activity (U/mg)	100	4	13
Thermostability (80 °C)	> 4 h	< 2 h	> 4 h

Table 2
Thermodynamic parameter for the rate-limiting step of methylviologen reduction

	Hydrogenase III	Hydrogenase I	Hydrogenase I–cytochrome *b*
E_{act} (kJ/mol)	50	44 67	94

sequences from small subunits of hydrogenases used for Fig. 1 showed the absence of these four His residues in all three *A. aeolicus* hydrogenases (data not shown). This result indicates that hydrogenase III, although being phylogenetically closer to hydrogenases group 2 (H_2 sensor and cyanobacterial hydrogenases [8]), does not contain Fe–S clusters such as found in regulatory hydrogenases. Moreover, we have already pointed out the presence of a [3Fe–4S] cluster in this enzyme by EPR [7].

3.2. Properties of the purified hydrogenases from *A. aeolicus*

The membrane-bound hydrogenase I, the soluble hydrogenase III and the hydrogenase I–cytochrome b^I complex were purified to homogeneity [7].

3.2.1. Optimum temperature and thermal stability

The purified enzymes are active between 20 and 90 °C with an optimal temperature at 80 °C and do not require reductive activation. Hydrogenase I is rather unstable since no activity is detected after 2 h at 80 °C in anaerobiosis (Table 1). In contrast, 85% of activity still remained after 4 h of incubation at this temperature for hydrogenase I in interaction with the membrane-bound cytochrome b^I. Hydrogenase III shows also a high stability against thermal inactivation (up to 4 h) [19].

From the Arrhenius plot, the activation energies were calculated (Table 2). Hydrogenase I alone exhibits a biphasic Arrhenius plot. Such biphasicity in the energy of activation for the hydrogenase has not been reported so far and may be explained by the existence of two conformational states of the enzyme above and below the break point, each with a different catalytic competence.

3.2.2. Oxygen tolerance

Recent works have shown that some hydrogenases are very insensitive to oxygen. This is the case for the CO-linked hydrogenase from *Rubrivivax gelatinosus*, which is tolerant to O_2 and catalytically active in oxygen [20] and two different [NiFe] hydrogenases from *Ralstonia eutropha* [15,21].

3.2.2.1. Catalytic activity. The *A. aeolicus* [NiFe] hydrogenases are rather tolerant to oxygen. Soluble hydrogenase III is more sensitive than hydrogenase I with a loss of activity after 2 h of exposure to 20% O_2 at 0 °C. Hydrogenase I has a high stability since 50% of the initial activity is still measurable after exposure to O_2 for 24 h at the same temperature. In contrast to hydrogenase I alone, the hydrogenase–cytochrome *b* complex was more sensitive to O_2 inhibition as all the activity disappeared after 30 min in presence of O_2. To test if lipids take a role in the stability of this complex, we have reconstituted liposomes with hydrogenase I—cytochrome *b* complex. Liposomes were immobilized on a BIAcore sensor-chip SA. The presence of hydrogenase in the liposomes was verified by injection and binding of anti-hydrogenase antibodies (data not shown). The complex embedded in liposomes became more oxygen tolerant as 50% of the activity was recovered after 3 days in the presence of 20% oxygen at 30 °C.

As hyperthermophilic proteins are less flexible at 0 °C than at physiological temperatures, we have tested if O_2 can have access to the active site of hydrogenase I at 37 and 80 °C. We have pre-incubated the enzyme for 2 h in the presence of O_2 and then the methyl viologen reducing activity was tested. Whatever the temperature used, no inactivation was detected.

As the activity measurements have been done in presence of H_2 and in absence of O_2, we used protein film voltammetry to determine how the oxygen sensitivity of hydrogenase I compares to that from the prototypical [NiFe] hydrogenase from *Df* [9]. In this technique, no mediators are used, as the enzyme receives directly electrons from an electrode which is poised to maintain the enzyme in the oxidized state. The catalytic activity is directly measured as a current, whose change against time reveals the rate of activation of inactivation of the enzyme. In our experiments, the two enzymes were found to differ in that *Df* [NiFe] hydrogenase needs to be poised under reductive

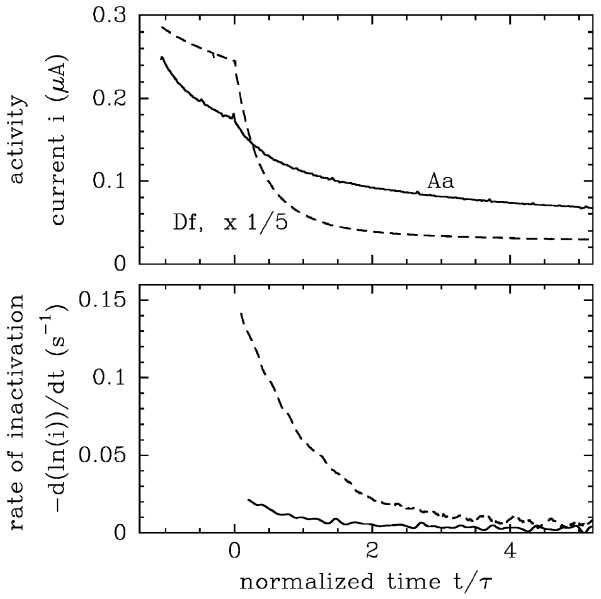

Fig. 2. Activity (top) and rate of inactivation (bottom) against time, in protein film voltammetry experiments with *A. aeolicus* hydrogenase I (Aa) and *D. fructosovorans* [NiFe] hydrogenase (*Df*) adsorbed at an electrode surface. The air-saturated solution was injected at $t = 0$. Electrode potential set to 200 mV vs. SHE. 40 °C. pH 7. Electrode rotation rate 2 krpm.

condition for full activity to develop (this is known to result from the slow reduction of the inactive state Ni-A), whereas the activity of *A. aeolicus* hydrogenase I was not increased following such reductive treatment. The top panel in Fig. 2 shows how the activities of hydrogenase I (plain line) and *Df* [NiFe] hydrogenase (dashed line) change following the injection at time $t = 0$ of a small amount of aerated buffer ($x = 0.016$, using the notations as in [9]). The transform of the current in the bottom panel shows how the pseudo-first order rate of inactivation decreases with time (this results from the oxygen concentration decreasing with time like $\exp(-t/\tau)$). All things being equal, the rate of aerobic inactivation was five times smaller for *A. aeolicus* hydrogenase I than for *Df* [NiFe] hydrogenase (8 ± 2 vs. $38 \pm 4\,s^{-1}$/atm of O_2).

3.2.2.2. Spectroscopic studies. A sample of *as prep* purified hydrogenase I of *A. aeolicus* in 50 mM Hepes buffer pH 7 was studied by EPR spectroscopy. The EPR spectra showed the same characteristics as those already described [7]: an isotropic signal centred at $g = 2.01$ arising from the oxidized $[3Fe4S]^{1+}$ centre (Fig. 3A) and a rhombic signal at 2.29, 2.16 and 2.01 corresponding to the Ni-B state of the [NiFe] centre (Fig. 3E). In contrast with the spectrum given by other *as prep* [NiFe] hydrogenases, no Ni signal corresponding to the Ni-A form of the [NiFe] centre was observed. This indicates that the enzyme is entirely in the 'ready form' that can be rapidly activated upon reduction. This result is in line with the fact that hydrogenase I does not require an activation step. These spectra did not change after incubation in presence of O_2 (20%) at 20 °C.

Two hypothesis for the oxygen resistance have been proposed: (i) the hydrophobic cavities serving as gas channels being narrow enough to block O_2 access to the active site [15–17] or (ii) supernumerary (compared to prototypical [NiFe] hydrogenases) CN ligands modulating the properties of the active site regarding hydrogen catalysis and reaction with oxygen [21,22]. That no significant difference between the Ni EPR signals of hydrogenase I and standard [NiFe] enzymes was observed, suggests that oxygen resistance is not due to the coordination sphere of the Ni ion being different.

To study the structural effect of exposure to oxygen, a redox cycling experiment was performed as follows. In step I, the enzyme was incubated at 85 °C for 10 min and then rapidly frozen in a cold ethanol bath (−70 °C). This procedure led to the disappearance of the $[3Fe–4S]^{1+}$ signal and to the appearance of a complex spectrum in the range $g = 2.06–1.75$, arising from the reduced $[4Fe4S]^{1+}$ centres being coupled by spin–spin magnetic interactions. Fig. 3F shows that the signals coming from the [NiFe] centre almost disappeared. These results show that a simple incubation at high temperature can lead to the reduction of the enzyme. This can be explained by the presence of H_2 molecules probably trapped in the gas channels of the enzyme during the purification process. In step II, the same incubation procedure (85 °C for 10 min) was followed by the injection of 10 μL of oxygen-saturated buffer in the EPR sample (150 μL). The recovery of the $[3Fe–4S]^{1+}$ signal showed clearly that the enzyme was re-oxidized. More interestingly, the spectrum arising from the [Ni–Fe] centre is composite with a mixture of Ni-B signal and of another rhombic signal ($g = 2.29, 2.23, 2.01$) which can be attributed to the Ni-A species (Fig. 3G) by comparison with g-values reported for mesophile [NiFe] hydrogenases [23]. Thus, the appearance of the Ni-A species is clearly consequent on the oxidative stress undergone by the enzyme at high temperature. This demonstrates that only at high temperature oxygen can reach the active site and react with it. In step III, the sample was reduced again by incubation under an atmosphere of H_2 for 20 min at 85 °C. This led to the reduced state of the enzyme, characterized by the signals arising from the

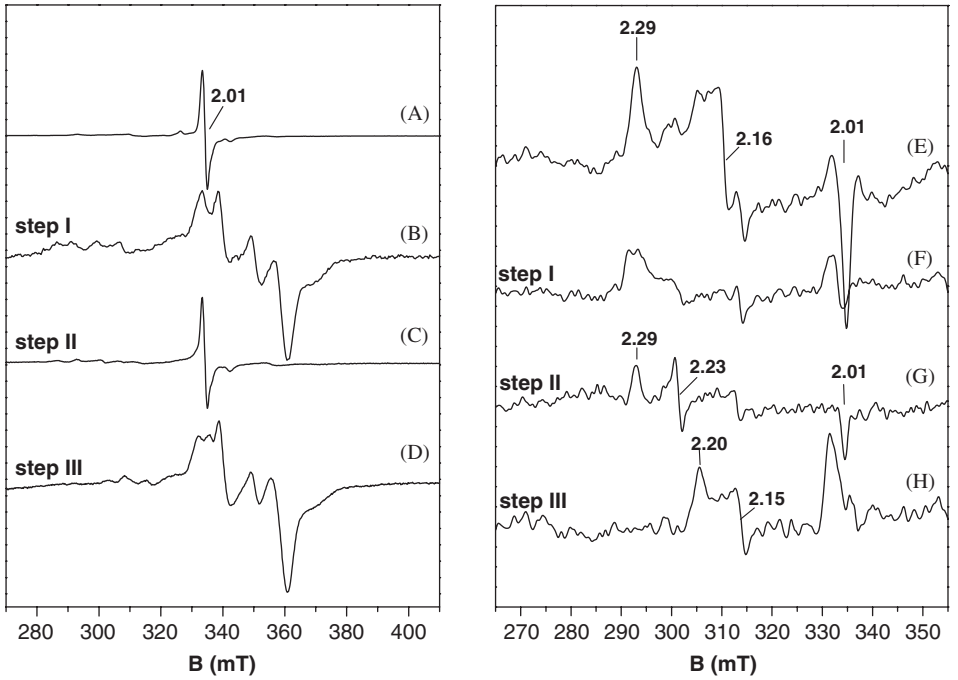

Fig. 3. Hydrogenase I EPR spectra. [Fe–S] centre signals (left panel) and zoom on the [Ni–Fe] centre signals (right panel). Step I: incubation (85 °C, 10 min). Step II: incubation (85 °C, 10 min) followed by the injection of 10 μL of buffer saturated in oxygen. Step III: Incubation under H_2 (85 °C, 20 min). Experimental conditions: $T = 15$ K (A,C), 6 K (B,D), 70 K (E,F,G,H); microwave power at 9.42 GHz, 1 mW (A,C), 10 mW (B, D–H); modulation amplitude, 1 mT at 100 kHz. Trace G is the spectral difference between step I and step II.

$[4Fe4S]^{1+}$ (Fig. 3D) and Ni-C ($g = 2.20$, 2.15, 2.01, Fig. 3H). Thus, even after the formation of the Ni-A species upon exposure to oxygen, hydrogenase I can be rapidly activated under hydrogen, and this is likely to be related to the oxygen tolerance of the enzyme.

4. Conclusion

We have shown that the membrane-bound hydrogenase I possesses the molecular properties of and belongs to group 1 [NiFe] hydrogenases [7]. This group includes the membrane-associated respiratory uptake hydrogenases, i.e. the enzymes capable of supporting growth with hydrogen as an energy source. They typically transfer electron from H_2 to the quinone pool through a membrane-integral cytochrome *b*. *In vivo*, the electrons are probably transferred to the cytochrome bc^1 complex and then to the cytochrome oxidase to reduce molecular oxygen [19]. Hydrogenase II also belongs to group 1 hydrogenases [7], indicating a similar role as hydrogenase I in energy conservation. Phylogenetic analysis showed that hydrogenase III from *Aquifex* is close to H_2-sensing and cyanobacterial hydrogenases [7]. A role for H_2-signalling hydrogenase has been previously proposed [8], but the specific activity (100 units/mg) and the cellular abundance of hydrogenase III (3.5 mg/50 g cells) are not in favour of such function for this enzyme. We proposed a role in the CO_2-fixing pathway through the reductive TCA cycle [7] as previously suggested for the soluble [NiFe] hydrogenase from *Hydrogenobacter thermophilus*.

Molecular hydrogen is considered as an important clean energy carrier for future technologies. At least two modes of application of hydrogen-metabolizing protein catalysts are being discussed: (1) hydrogenases may be used as catalysts in hydrogen production by coupling oxygenic photosynthesis to biological hydrogen production; (2) hydrogenases could be used directly in biological fuel cells. The oxygen sensitivity of hydrogenases is one of the major problems in designing a stable system. To date, only a few hydrogenases displays tolerance to oxygen and various studies using mutagenesis techniques to generate oxygen-tolerant hydrogenases have been developed. Hydrogenases from extremophilic organisms, which are naturally tolerant to various extreme environments, could provide inspiration for the development of biotechnological processes. In consequence, *A. aeolicus* hydrogenases are good candidates for

biotechnological applications due to their high resistance with respect to aerobic and thermal inactivation. The literature suggests that oxygen insensitivity could be due to: (i) the gas channels being significantly narrower compared to oxygen-sensitive hydrogenases to block O_2 access to the active site [15] or (ii) a peculiar structure of the active site (with the presence of a Ni-bound cyanide group) [21,22]. This latter mechanism may actually be operational in the soluble [NiFe] hydrogenase from *Ralstonia eutropha*: Bleijlevens et al. [22] have recently shown that the *hypX* gene of this bacterium is specifically involved in the biosynthetic pathway that delivers the nickel-bound cyanide. To date, this gene has been found only in eight bacteria, which grow exclusively or preferentially under aerobic conditions, including *A. aeolicus* (annotated *hoxX*). This prompted us to use EPR spectroscopy to attest the perturbations in the electronic structure of the active site that are expected to result from the presence of such additional ligands. However, the high similarity between the Ni EPR signals of hydrogenase I and those observed in hydrogenases from mesophilic organisms indicates that the Ni ion environment is the same in the two kinds of enzymes. Our spectroscopic results do not support the presence of additional CN ligands in the coordination sphere of the Ni in hydrogenase I, although we cannot completely exclude that such a coordination change could lead to EPR-silent Ni–Fe species.

We demonstrated that upon exposure to oxygen, hydrogenase I is indeed inactivated, albeit slowly and transiently (Fig. 2), and that the active site then transforms into an inactive Ni-A species similar to that observed in prototypical hydrogenases (Fig. 3), but which can be reduced quickly. In contrast to hydrogenase III which possesses the Ile and Phe residues in its large subunit that might confer the resistance to oxygen as in the regulatory hydrogenase, hydrogenase I presents the classical residues (Val, Leu). Our results suggest that the oxygen tolerance of the enzyme cannot result from oxygen being unable to reach the active site or to react with it. Instead, it may result from both the kinetic of inactivation being slower and the kinetics of reactivation being much faster than in standard hydrogenases. Experiments are in progress in our laboratory to test this hypothesis by characterizing further the kinetics of interconversion between active and inactive states of the enzyme.

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