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Biochemical and Biophysical Research Communications 339 (2006) 277–283
BBRC
www.elsevier.com/locate/ybbrc

Functional insights from the structural modelling of a small Fe-hydrogenase

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Received 29 October 2005
Available online 10 November 2005

Abstract

Recently, a novel Fe-hydrogenase from a high rate of hydrogen producing *Enterobacter cloacae* strain IIT-BT08 was identified and partially characterized. This 147 residue protein was found to be much smaller than previously known Fe-hydrogenases, yet retaining a high catalytic activity. We predicted the structure of this protein and found it to be structurally similar to one of the two sub-domains containing the catalytic H-cluster so far jointly present in all other Fe-hydrogenases. This novel architecture allows a tentative explanation of protein function with the high rate of catalytic activity being due to a missing regulatory sub-domain, presumably allowing higher enzymatic activity at the cost of greater exposure to oxygen inactivation. This new insight may improve our understanding of the molecular and functional organization of other, more complex Fe-hydrogenases.

Keywords: Biohydrogen; *hydA*; H-cluster; Fold recognition; Function prediction

Hydrogen metabolism is one of the most ancient processes of life. In many prokaryotes and in several eukaryotes, it is mediated by a general class of metalloenzymes collectively called hydrogenases (official name: ferredoxin hydrogenase, EC 1.12.7.2), which catalyze the reversible reduction of protons to molecular hydrogen (H_2). Since the pioneering discovery and characterization of hydrogenases in 1931 [1], their importance in natural sciences and their potential for technological applications such as production of a non-polluting fuel have commanded extensive research efforts in this field.

With reference to the metal composition in their active center, hydrogenases have been divided in four classes: (i) NiFe-hydrogenases [2], (ii) NiFeSe-hydrogenases [3], (iii) Fe-hydrogenases [4], and (iv) hydrogenases devoid of metal atoms, which were found only in archaea [5]. As a general rule, NiFe-hydrogenases catalyze H_2 uptake, whereas Fe-hydrogenases catalyze preferentially H_2 evolution [6].

NiFe-hydrogenases are the most extensively studied and the three-dimensional structures of several proteins belonging to this group have been solved [2]. These enzymes are found in a variety of anaerobic and facultative archaea, eubacteria, and cyanobacteria, and they usually consist of two different polypeptide chains extensively interacting with each other, defined as large and small subunits [7,8]. Structural data collected by X-ray crystallography have shown a catalytic site containing a NiFe-binuclear center deeply buried in the large subunit [6] and interconnected with the molecular surface by a network of hydrophobic hollows and channels which allow hydrogen gas exchange with the outside. The less studied Fe-hydrogenases are found in strictly anaerobic bacteria, such as *Clostridium pasteurianum* and *Desulfovibrio desulfuricans*, in some unicellular green algae, such as *Chlamydomonas reinhardtii* and *Scenedesmus obliquus*, and in several eukaryotic protists containing hydrogenosomes, such as *Trichomonas vaginalis* [7]. All Fe-hydrogenases, for which the sequence

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.11.012

has been determined, share a conserved domain of about 320 amino acids in the C-terminal region which embodies the active site, the so-called H-cluster (hydrogen cluster). Spectroscopic studies have indicated that this moiety is markedly different from the FeS clusters previously observed in other proteins with redox activity, containing six iron atoms organized as a [4Fe-4S] cubane covalently bridged to a [2Fe-2S] sub-cluster by a single cysteinate thiol [9]. The H-cluster is coordinated to the polypeptide chain by four highly conserved cysteine residues. Instead, the N-terminal portion of Fe-hydrogenases from different organisms is rather variable but it usually contains additional [4Fe-4S] or [2Fe-2S] clusters, named F-cluster, organized in a ferredoxin-like domain and probably involved in the electron transfer from the donor (ferredoxin) to the H-cluster. The algal Fe-hydrogenases are singular in this respect as they miss the entire N-terminal domain and are presumably characterized by a direct electron transport pathway from ferredoxin to the H-cluster. Also, a hydrophobic channel links the dinuclear center to the molecular surface, thereby mediating the gas exchange between the two compartments. The active site contains the diatomic π-acceptor ligands CO and CN^- coordinated to the iron atoms. These non-proteic ligands are supposed to play a role in hydrogen activation by stabilizing the metals in their low-oxidation state. A further key feature of most hydrogenases known to date is their exquisite sensitivity to molecular oxygen, which is higher for the Fe-only enzymes.

Interestingly, the catalytic activity of Fe-hydrogenases is 10–100 times higher than that of NiFe-hydrogenases, making them the most efficient H_2 production catalysts known to date. Indeed, the ability of unicellular green algae to release H_2 gas upon illumination has been considered as a phenomenon of great scientific and technological interest as it holds the promise of generating energy from nature's most plentiful resources, light and water. In spite of this, the gene coding for *C. reinhardtii* Fe-hydrogenase has been isolated only recently [10] and the three-dimensional structure of the corresponding protein is still unknown. However, as outlined above, the three-dimensional structures of Fe-hydrogenase from *D. desulfuricans* and *C. pasteurianum* have been determined by X-ray crystallography to 1.6 and 1.8 Å resolution, respectively [11,12]. Since all Fe-hydrogenases share a high degree of sequence similarity, especially in the region involved in the catalytic mechanism, these data can provide useful hints for the structure of the algal enzyme. Moreover, a synthetic metallosulfur cluster core, analogue to the H-cluster, has been recently built up, through linking of a di-iron subsite to a [4Fe-4S] cluster [13], and this gives a unique opportunity to improve our understanding of the Fe-hydrogenase catalytic machinery. Two novel proteins required for assembly and maturation of an active Fe-hydrogenase, called HydG and HydEF, have been recently found in *C. reinhardtii* [14] and are strictly conserved in organisms containing this enzyme. HydG and HydEF belong to the radical *S*-adenosylmethionine (also indicated as "Radical SAM") protein superfamily [15], commonly involved in several biochemical reactions including sulfur insertion, radical formation, and anaerobic oxidation. It has been proposed that these accessory proteins play a role in the mobilization of iron for assembly of the Fe-hydrogenase H-cluster.

The smallest Fe-hydrogenase known to date has been found in *Enterobacter cloacae* IIT-BT 08, a gram negative, facultative anaerobe and high yield hydrogen producing bacterial strain. The gene coding for this enzyme has been recently cloned [16] and the nucleotide sequence is found to be similar to those of several known Fe-hydrogenases, mainly in the C-terminal region, that includes the active site. On the other hand, nothing is known about the structure of this novel, small protein able to catalyze hydrogen activation, even when overexpressed in *Escherichia coli*, which is not hydrogen producing per se. In the following, we analyze the structural features of this particular protein and highlight a novel single domain architecture, which allows us to make a hypothesis on the structure–function relationship in Fe-hydrogenases.

Materials and methods

A bioinformatics approach using sequence and domain database searches was employed, combining and cross-validating results from different fold recognition servers. The protein sequence from *E. cloacae* was taken from the original publication and confronted with the one deposited in the NCBI non-redundant database [17] with Accession No. AAU11811. At the time of writing, three slightly different revisions of this sequence had been deposited in the database, each time correcting the previous one in only a few residues. A multiple alignment of these revisions is available as supplementary material (Web Fig. 1). The revision closest to the published sequence [16] was used throughout this work. The implications of the other two variants will be discussed later. The databases InterPro [18], Pfam [19], and PROSITE [20] were searched for similar sequences. A PSI-BLAST [21] search with default *E* value cut-offs was also performed. The secondary structure of the target protein was predicted using the consensus method of Albrecht et al. [22]. The fold recognition meta server, which collects single predictions taken from different state-of-the-art servers for fold recognition available on the web [23], was used to derive fold class predictions. Among the meta server predictions, the 3D-Jury consensus prediction [24] and FFAS03 [25] were found particularly useful. The MANIFOLD [26] fold recognition program was used to include functional information in the form of the enzyme code (EC) classification for fold recognition. This method combines PSI-BLAST [21] searches and secondary structure element alignments [27]. Structural similarity among the predicted fold classes was based on both the SCOP [28] and CATH [29] classification schemes since these differ in some cases. The multiple alignment was constructed with T-COFFEE [30] and manually improved due to the non-contiguous nature of the domain. The structural model was constructed using the HOMER server (URL: http://protein.cribi.unipd.it/homer/), where 3D coordinates of conserved template positions were copied, while insertions and deletions were modelled using a fast divide and conquer method [32]. Side chains were placed using SCWRL [33] and the final model was evaluated with FRST [34].

Results and discussion

Fold recognition

Database searches with the *E. cloacae* sequence in InterPro, Pfam, and Prosite did not generate any hits.

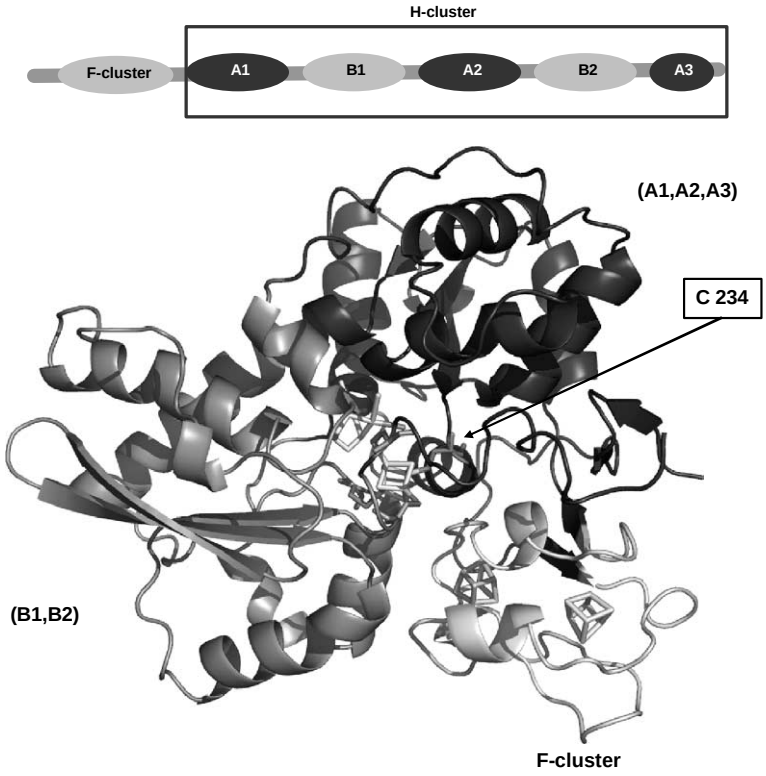

Fig. 1. CATH domain structure of the *D. desulfuricans* Fe-hydrogenase (PDB code 1hfe, chain L). The three domains (F-cluster, H-cluster A and B) are highlighted in different shades of gray. A schematic representation (top) is presented to give an idea of the structural arrangement (bottom). The iron/sulfur clusters and four interacting cysteines of the H-cluster are shown as sticks. An arrow indicates the non-conserved cysteine 234. See text for details.

PSI-BLAST identified a couple of hits against the PDB database [35], but all were above the confidence threshold. The first hit, PDB code 1qmh (chain A, SCOP class d.68.2), had an *E* value of 0.14 and 30% sequence identity with 89 (out of 147) target residues close to the N-terminus aligned. The remaining five hits had *E* values in the range 3.4–7.7. Interestingly, one of these hits is PDB code 1hfe (chain L, SCOP class c.96.1), one of the few known Fe-hydrogenase structures (the one from *D. desulfuricans*). Only the C-terminus was aligned for 57 residues, with an *E* value of 5.9 and also 29% sequence identity over the aligned part. Since the predicted secondary structure did not match well with the first PSI-BLAST hit, we continued the search for a structural template with fold recognition methods. The meta server analysis also did not produce significant hits, with the highest 3D-Jury score being 34.0. The most recurring SCOP classes were a.168.1, e.29.1, c.4.1, and d.110.1. Among the individual server results, a Fe-hydrogenase (SCOP class c.96.1) was ranked sixth by FFAS03, with a *Z* score of 6.8 compared to 7.3 for the first hit. It is worth noting that, so far, the used methods rely entirely on sequence and secondary structure information only. MANIFOLD revealed the significance of the Fe-hydrogenase fold (PDB code 1hfe, chain L) through its associated functional information. In addition to the weak sequence similarity in the C-terminal part and compatible secondary structure arrangement, the EC code similarity made this template clearly stand out from the rest.

Modelling

The domain structure of the Fe-hydrogenase from *D. desulfuricans* (PDB code 1hfe) is described in SCOP as two domains, with the region interacting with the H-cluster as a single domain of about 320 residues and a second domain surrounding the F-cluster. On the other hand, CATH divides this region carrying the H-cluster in two sub-domains of about 140 and 200 residues, and also recognizes the portion encompassing the F-cluster as a separate domain. A closer inspection reveals that CATH actually recognizes the domain referring to the H-cluster as being composed of five sequence fragments A1, B1, A2, B2, and A3 where (A1, A2, A3) and (B1, B2), respectively, form the two discontinuous sub-domains (see Fig. 1). So far, the distinction between SCOP and CATH had been purely hypothetical, as no structure with the single CATH domain was known. Our fold recognition results suggest the *E. cloacae* sequence to be structurally similar to that of the 140 residue domain only, corresponding to the B1-B2 sub-domain. This structural arrangement is not unheard of, as similar situations have already been described in other proteins [36–38]. Due to the inherent difficulty in using automated sequence alignment methods in the presence of non-contiguous domains, the T-COFFEE alignment (Fig. 2) had to be improved by hand. The smaller N-terminal part was manually adjusted to fall in register with the B1 sub-domain. This alignment was used to

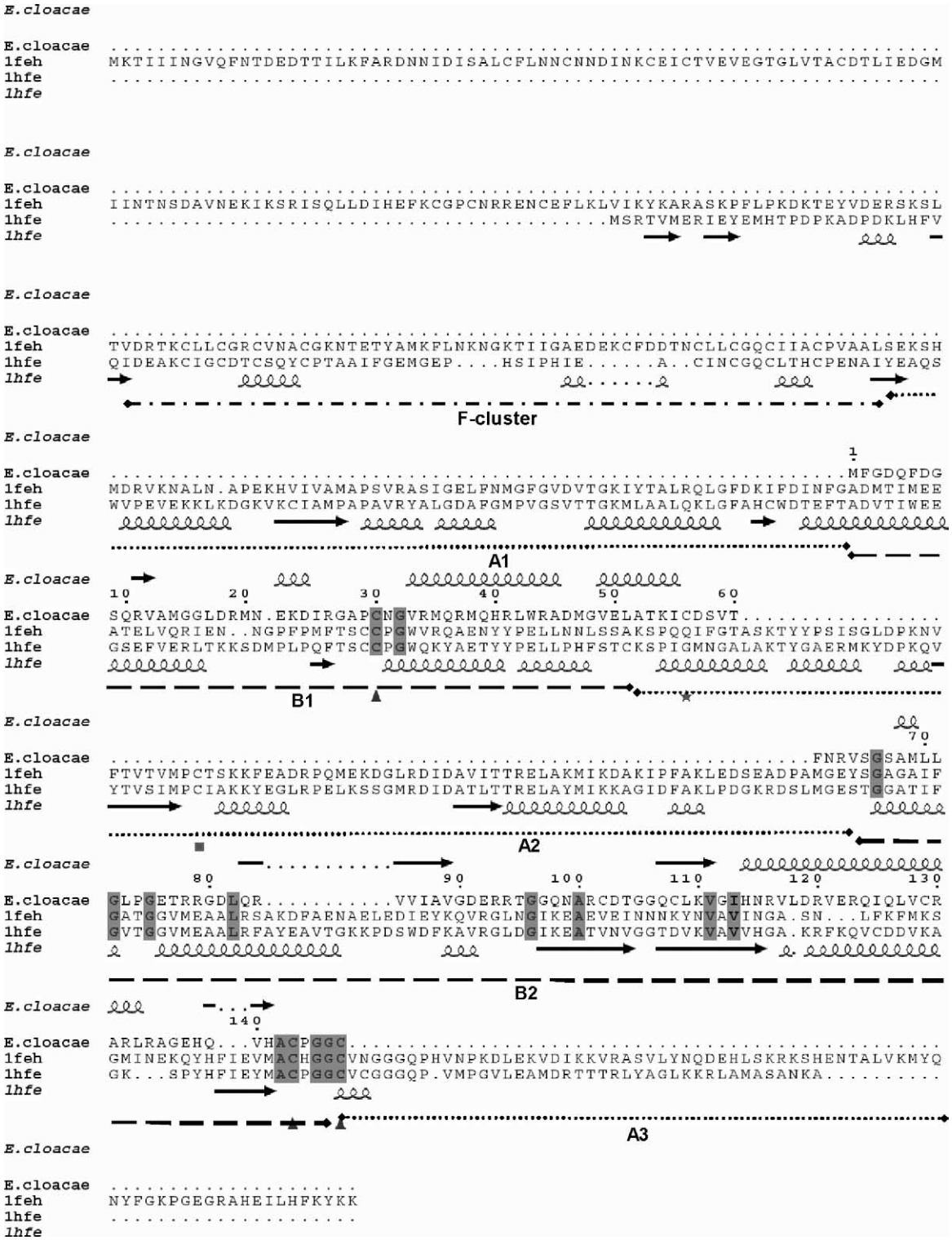

Fig. 2. Sequence alignment of the *E. cloacae*, *D. desulfuricans* (PDB code 1hfe, chain L), and *C. pasteurianum* (PDB code 1feh) Fe-hydrogenase sequences. The consensus predicted secondary structure of the *E. cloacae* protein is shown above and the DSSP secondary structure of the *D. desulfuricans* protein below the sequence. The CATH domain structure (see text for details) for the F-cluster and H-cluster sub-domains A and B is represented with dashed and dotted lines. Three conserved active site cysteine residues are indicated by triangles. The fourth cysteine, residue 234 in *D. desulfuricans*, is indicated with a small box and the structurally compatible fourth cysteine, residue 56 in *E. cloacae*, with a star. Conserved positions are highlighted in gray.

generate the structural model shown in Fig. 3. Although overall sequence identity is rather low, with 23 out of 147 (16%) conserved residues, several key structural features are maintained. For comparison, the sequence identity between the two known Fe-hydrogenase structures is 37.1%. The C-terminal part, corresponding to sub-domain

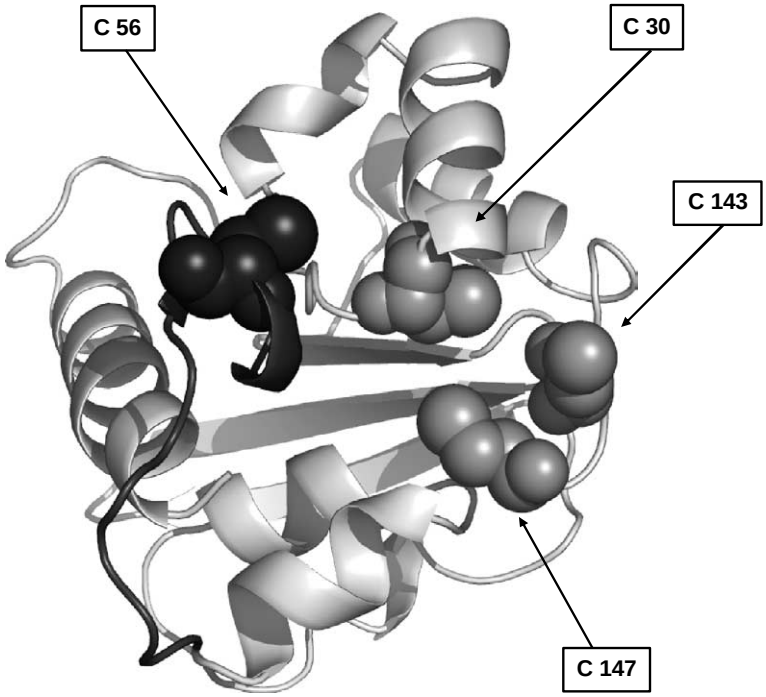

Fig. 3. Structural model of the *E. cloacae* protein. The three conserved active site cysteine residues are drawn as spheres. The non-conserved cysteine residue 56 and the surrounding flexible loop are drawn in dark gray. The structure is rotated with respect to Fig. 1.

B2, is better conserved (24% sequence identity over 85 residues) and the active site well conserved. In fact, three of the four cysteine residues coordinating the iron/sulfur cluster appear strictly conserved in the alignment. In particular, the C-terminal cysteine from the *E. cloacae* sequence is aligned with the cysteine known to form a covalent bond with the iron/sulfur cluster [11] and to bridge the [4Fe-4S] cubane to the [2Fe-2S] cluster. The cysteine residue missing from the template is located on the other domain (residue 234 in PDB 1hfe), which is not aligned. Instead, a new cysteine appears in a structurally compatible position (residue 56) in a flexible loop segment. Due to the low sequence identity in the loop region, it is not possible to make a reliable prediction of the exact conformation of the cysteine residue. It is reasonable to believe that this new cysteine may replace the missing one in coordinating the iron/sulfur cluster, inducing a modification of the local environment at the active site. However, the claimed hydrogen evolving activity of the *E. cloacae* protein when expressed in *E. coli* [16] suggests that overall geometry of this catalytic site may be slightly different from the H-cluster previously characterized in other Fe-hydrogenases. Indeed, it could be more similar to the usual FeS clusters, that *E. coli* is able to synthesize even in the absence of the accessory HydG and HydEF proteins essential to produce a true H-cluster such as that of *C. reinhardtii* [14].

Implications for the database sequence

The C-terminal cysteine from the *E. cloacae* sequence appears to have a central role in the function of this enzyme. The slight differences between the three deposited sequences (Web Fig. 1) consist in a deletion of this crucial residue in two cases and some other variations in functionally less important regions. Our results therefore suggest the second revision of the sequence in the database, which corresponds to the published sequence [16], to be the correct one, as far as it ensures the presence of the essential terminal cysteine residue. The two other sequence variants may hardly code for a functional protein, as the iron/sulfur cluster cannot be correctly coordinated.

A novel catalytic sub-domain

Many microorganisms are able to produce hydrogen from mono- and disaccharides, starch, and (hemi)cellulose under anaerobic conditions. In theory, fermentation of one mole of glucose yields 12 molecules of H_2. However, in practice a maximum of 4 H_2 are gained, while the other two-thirds are still enclosed in the produced metabolites (e.g., acetate). With mesophiles about 1–2 moles of H_2/mole of glucose is normally obtained, while thermophiles display a yield of $\geqslant 3$ moles of H_2/mole of glucose [39]. Interestingly, hydrogen yield from *E. cloacae* IIT-BT 08 is found to be 6 moles of H_2/mole of sucrose under anaerobic conditions [40]. It is not yet known which metabolic pathway could yield such favorable stoichiometry and whether this small hydrogenase is characterized by a particularly high activity. We may speculate that the lack of a second sub-domain surrounding the active site allows a faster electron transfer from the donor substrate, thereby allowing a high activity. The second sub-domain present in other enzymes would then be primarily responsible

for regulating the activity of the enzyme and/or partially protecting it from inactivation. It is to be expected that the lack of this sub-domain would presumably make the *E. cloacae* enzyme particularly sensible to oxygen inhibition, requiring strict anaerobiosis for the enzyme to remain active. This is likely an adaptation of the bacteria to survive in highly anaerobic environments, where it is more important to have efficient fermentation and proton reduction than resistance to oxygen. This view seems to be in agreement with a recent report on the unusual oxygen tolerance of the Fe-hydrogenase from Thermotogales (e.g., *Thermotoga neapolitana* and *Thermotoga maritima*), a large class of thermophilic bacteria [41]. In fact, although no three-dimensional structure is available for Fe-hydrogenases of these microorganisms, it clearly appears from the amino acid sequence that oxygen tolerance is associated with a higher complexity of these enzymes in terms of quaternary structure [42].

Conclusion

The structural similarity between a recently characterized Fe-hydrogenase from *E. cloacae* and other Fe-hydrogenases has been highlighted. Our modelling data confirm the presence of the two distinct sub-domains predicted by CATH analysis in the subunit encompassing the active site of other Fe-hydrogenases and proposes, for the first time, a model for their function, where a catalytic sub-domain is complemented by a regulatory sub-domain. To date, after more than 70 years of related research, the mutually exclusive nature of oxygen and hydrogen production has not been overwhelmed and still represents a critical hindrance slowing the progress and the achievements of a H_2-based biotechnology in the field of renewable energies. Our results open up new working perspectives aimed to elucidate the functional role of the regulatory sub-domain, in terms of both hydrogen production and oxygen inhibition, in Fe-hydrogenases. Moreover, the availability of a 3-D structure of this simple hydrogenase makes it an ideal system for electron paramagnetic and infrared spectroscopy studies aimed to elucidate the molecular mechanism and the electronic intermediates involved in proton reduction catalysis.

Acknowledgments

The authors are grateful to Giovanni Giacometti for stimulating discussions, to Mario Albrecht and Giuseppe Zanotti for critical comments on the manuscript. S.T. is funded by a 'Rientro dei cervelli' grant from the Italian Ministry for Education, University and Research (MIUR). This work was supported by grants PRIN (2003), FIRB (2001), and FISR (2005) from MIUR to G.M.G.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.11.012.

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