The crystal structure of C176A mutated [Fe]-hydrogenase suggests an acyl-iron ligation in the active site iron complex

Takeshi Hiromotoa, Kenichi Atakab, Oliver Pilak, Sonja Vogta, Marco Salomone Stagnic, Wolfram Meyer-Klaucce, Eberhard Warke, Rudolf K. Thauer, Seigo Shima, Ulrich Ermlerd

aMax-Planck-Institut für Terrestrische Mikrobiologie, Karl-von-Frisch-Straße, D-35043 Marburg, Germany
bBielefeld University, Department of Chemistry, Universitätsstraße 25, D-33615 Bielefeld, Germany
cEMBL Hamburg, Notkestraße, 85, D-22603 Hamburg, Germany
dMax-Planck-Institut für Biophysik, Max-von-Laue-Straße 3, D-60438 Frankfurt/Main, Germany

A R T I C L E  I N F O

Article history:
Received 20 November 2008
Revised 30 December 2008
Accepted 5 January 2009
Available online 20 January 2009

Edited by Hans Eklund

Keywords:
Hydrogenase
Iron–guanylylpyridinol-cofactor
Methanogenic archaea
Iron complex
X-ray crystal structure
X-ray absorption spectroscopy

A B S T R A C T

[Fe]-hydrogenase is one of three types of enzymes known to activate H2. Crystal structure analysis recently revealed that its active site iron is ligated square-pyramidally by Cys176-sulfur, two CO, an "unknown" ligand and the sp2-hybridized nitrogen of a unique iron–guanylylpyridinol-cofactor. We report here on the structure of the C176A mutated enzyme crystallized in the presence of dithiothreitol (DTT). It suggests an iron center octahedrally coordinated by one DTT-sulfur and one DTT-oxygen, two CO, the 2-pyridinol's nitrogen and the 2-pyridinol's 6-formylmethyl group in an acyl-iron ligation. This result led to a re-interpretation of the iron ligation in the wild-type.

© 2009 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

1. Introduction

The reversible oxidation of molecular hydrogen, which serves many microorganisms as electron supplier or electron sink in their energy metabolism, is catalyzed by hydrogenases from which three basic types have been discovered [1]. [NiFe]- and [FeFe]-hydrogenases use a binuclear [NiFe] and [FeFe] center for H2 activation, respectively, and several Fe/S clusters for electron delivery (H2 = 2H+ + 2e−) [2]. [Fe]-hydrogenase contains a mononuclear iron center that somehow facilitates the generation of a hydride (H2 = H+ + H−) that is abstracted by methyltetrahydromethanopterin (methyl-H4MPT) [3]. Despite their different protein architectures and not being phylogenetically related the active sites reveal amazing similarities as a result of a convergent evolutionary process presumably provoked by special chemical requirements for H2 activation [1,4,5]. In all three types the iron (in [FeFe]-hydrogenase the iron proximal to the 4Fe–4S cluster) is redox-inactive, low-spin, presumably present in the oxidation state II, and ligated by at least one sulfur and three unusual π-accepting ligands such as CO, cyanide and/or a pyridinol that are arranged similarly in a square-pyramidal or octahedral geometry [1]. However, why these ligands and this arrangement are applied for the heterolytic cleavage of H2 is not well understood. Large efforts are undertaken to elucidate the catalytic mechanism because H2/H− interconversion is considered as one of the major reactions in future energy storage/transformation processes [3].

[Fe]-hydrogenase catalyzes a reaction involved in many methanogenic archaea in CO2 reduction to methane by reducing methenyl-H4MPT to methylene-H4MPT at the expense of H2 [3]. The enzyme consists of a homodimer of 38 kDa per monomer built up of the C-terminal segments of both subunits forming a central helical unit and of two peripheral N-terminal domain units adopting a Rossmann-like fold [6] (Fig. 1A). Each of the latter domains binds at the C-terminal end of their parallel β-sheet a unique iron–guanylylpyridinol (FeGP)-cofactor whose catalytically competent iron complex points into the reaction site located inside a...
cleft between the central and the peripheral units [1]. Based on the interpretation of IR, Mössbauer, extended X-ray absorption fine structure (EXAFS) and X-ray crystallographic data from the holoenzyme (apoenzyme reconstituted with FeGP-cofactor) the iron complex consists of a low-spin iron square-pyramidally ligated by Cys176-sulfur of the polypeptide chain, two CO, an "unknown" ligand and the sp2-hybridized nitrogen of the cofactor’s pyridinol nitrogen [1,7–11]. The sixth position appears to be occupied by a hydrogen-bonded solvent molecule, however, too distant to be a direct ligand (Fig. 1B and C).

We report here on the crystal structure at 1.95 Å resolution of the holoenzyme of [Fe]-hydrogenase from Methanocaldococcus jannaschii in which the active site Cys176 was mutated to an alanine. It revealed that the Cys176 sulfur and the “unknown” ligands of the iron complex of the wild-type enzyme are replaced in the C176A enzyme by the dithiothreitol (DTT) present in the crystallization solution. The obtained electron density prompted us to re-interpret the iron ligation structure recently published for the wild-type enzyme [1]. A better fit was obtained when the 2-pyridinol moiety contributes two rather than one ligands to the iron, the sp2-hybridized nitrogen and the 6-formylmethyl group, the latter in an acyl-iron ligation. The modified iron-ligation pattern in the C176A mutated enzyme is compatible with the previously obtained IR and EXAFS data of the mutated and wild-type enzymes.

2. Materials and methods

2.1. C176A holoenzyme preparation, crystallization and X-ray structure determination

The C176A mutant from M. jannaschii was prepared as described previously [7]. The apoenzyme was overproduced in Escherichia coli BL21(DE3) cells, purified and reconstituted with FeGP-cofactor [12]. The resulting catalytically inactive holoenzyme was crystallized and diffraction data were collected at the Swiss Light Source beamline PXII (Villigen). The data were processed and scaled with the programs HKL [13] and XDS [14] (Table 1). The obtained isomorphous crystals allowed a straightforward structure determination using the wild-type enzyme model solved previously [1]. Model refinement was performed with REFMAC5 [15].

Fig. 1. Iron-ligation structure of wild-type [Fe]-hydrogenase and of its iron-guanylylpyridinol-cofactor as proposed previously [1]. (A) Ribbon diagram shows that the dimeric enzyme is composed of one central (orange) and two peripheral (blue) units. The two active sites are located within the two clefts between these units. (B) Structure of the FeGP-cofactor when it covalently binds to the enzyme via Cys176 as published [1]. (C) Schematic representation of the iron square-pyramidally ligated to the Cys176-sulfur, an “unknown” ligand, two CO and the pyridinol’s sp2-hybridized nitrogen. A solvent is associated to the iron trans to the pyridinol’s nitrogen. Bond length was derived from the X-ray crystallographic data [1].
and model building/manual inspections with COOT [16]. The $R_{\text{work}}$ and $R_{\text{free}}$ factor converged to 16.5% and 20.5% in the resolution range 1.95–40.0 Å (Table 1). Figs. 1A–3A are produced by PYMOL (DeLano Scientific). The coordinates of the C176A mutated enzyme model and the re-refined wild-type enzyme model were deposited at the RCSB Protein Data Bank under the PDB accession codes 3F46 and 3F47.

2.2. Infrared spectroscopy

Attenuated total reflection infrared (ATR-IR) spectroscopy was performed using Bruker VERTEX 70 IR spectrometer with Liquid nitrogen cooling system for the MCT detector equipped with Sili-

Table 1
Data collection and refinement statistics.

<table>
<thead>
<tr>
<th>Data set</th>
<th>[Fe]-hydrogenase C176A</th>
<th>[Fe]-hydrogenase wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Data collection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>0.992</td>
<td>0.992</td>
</tr>
<tr>
<td>Space group</td>
<td>14.22</td>
<td>14.22</td>
</tr>
<tr>
<td>Unit cell parameter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$a$, $c$ (Å)</td>
<td>96.4, 166.6</td>
<td>95.9, 165.8</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>41.7–1.95 (2.0–1.95)</td>
<td>26.2–1.75 (1.80–1.75)</td>
</tr>
<tr>
<td>Redundancy (%)</td>
<td>7.9 (7.7)</td>
<td>5.2 (3.6)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>96.9 (95.8)</td>
<td>95.5 (81.6)</td>
</tr>
<tr>
<td>$R_{\text{merge}}$ (%)</td>
<td>6.4 (70.1)</td>
<td>5.6 (50.8)</td>
</tr>
<tr>
<td>I/σ(I)</td>
<td>17.2 (4.2)</td>
<td>16.0 (2.6)</td>
</tr>
<tr>
<td>B. Refinement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolution limit (Å)</td>
<td>40.0–1.95</td>
<td>25.0–1.75</td>
</tr>
<tr>
<td>$R_{\text{work}}/R_{\text{free}}$ (%)</td>
<td>16.5/20.5</td>
<td>17.3/20.5</td>
</tr>
<tr>
<td>Number of Residues</td>
<td>345</td>
<td>344</td>
</tr>
<tr>
<td>Number of solvent molecules</td>
<td>169</td>
<td>238</td>
</tr>
<tr>
<td>Rmsd bond lengths (Å)</td>
<td>0.016</td>
<td>0.016</td>
</tr>
<tr>
<td>Rmsd bond angles (°)</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Cruickshank’s DPI (Å)</td>
<td>0.13</td>
<td>0.10</td>
</tr>
<tr>
<td>Average B (Å$^2$) protein/FeGP/ solvent, DTT</td>
<td>37.5/28.2/46.4, 40.2</td>
<td>32.8/26.7/43.2</td>
</tr>
</tbody>
</table>

2.3. X-ray absorption data re-analysis

The extracted iron K-edge (mutants: 25–680 eV; wild-type: 25–800 eV) EXAFS data were converted to photoelectron wave vector k-space and weighted by $k^3$. The spectra were refined with EXCURV98 [17]. The program calculated the theoretical EXAFS for defined structural model. In addition to single scattering contributions, multiple scattering linear units were defined for Fe–C=O. The potential acyl group in the vicinity of the iron has been modelled by a single carbon ion, because its other atoms do not contribute to multiple scattering by a linear orientation towards the iron and therefore they are not identifiable in the EXAFS. Parameters of each structural model, namely the atomic distances ($R$), the Debye–Waller factors ($2\sigma^2$), and a residual shift of the energy origin, were optimized, minimizing the fit index.

3. Results

3.1. Iron-coordination in the C176A holoenzyme

The overall structure of the C176A enzyme is highly similar to the wild-type enzyme, reflected in the root-mean-square (r.m.s.) deviation of 0.2 Å using 344 $C_\alpha$ atoms. Significant differences were exclusively found around the mutated residue and the FeGP-cofactor whose occupancy is nearly 100%.

The structure of the iron complex is primarily characterized by the ligation of DTT (present in the crystallization solution) in a bidentate manner (Fig. 2). The eliminated iron-coordinating

![Fig. 2.](image-url)
Cys176-sulfur is replaced by the 1-sulfur of DTT and its 2-hydroxyl group also ligates to the iron from that site which was occupied in the wild-type enzyme by the “unknown” ligand (Fig. 1) [1]. The entire molecule of DTT is clearly visible in the electron density and its temperature factor is only moderately higher than that of the polypeptide (Table 1). The 3-hydroxyl group of DTT is anchored to the protein matrix via a hydrogen bond to the hydroxyl group of Thr13 and the 4-thiol group of DTT interacts with hydrophobic regions of the surrounding residues.

The binding of DTT to the iron creates a serious problem in that the 2-hydroxyl group of DTT severely interferes with the 6-carboxymethyl substituent of the pyridinol ring if assumed to be in the conformation of the wild-type enzyme (Fig. 1B) [1]. This prompted us to rotate the pyridinol ring of FeGP cofactor by 180° compared to the orientation reported for the wild-type enzyme. The shape of the electron density in the rotated conformation, however, did not allow the incorporation of a carboxylate group with an oxygen as the iron ligand but it did allow the modeling of an acyl group with an iron-ligating carbon. In this arrangement the acyl oxygen is linked to the polypeptide chain by a hydrogen bond with the amide group of Ala176. In the rotated conformation the pyridinol’s hydroxyl group points towards the cleft between the central and peripheral unit. The hydroxyl group fits well into the electron density and interacts with the imidazole group of His14 and the 2- and 3-hydroxyl group of DTT.

While one of the intrinsic CO-binding sites corresponds to that reported for the wild-type enzyme the second CO-binding site now appears to be positioned trans to the pyridinol’s nitrogen in the C176A enzyme (Fig. 2A). ATR-IR measurements before and after crystallization of the C176A enzyme verified the existence of the two CO ligands in an angle of 90° as previously found in the wild-type (Supplementary Fig. S1). A minor improvement is possible if the occupancy of one CO ligand of the C176S and C176A enzymes is lowered by 0.25 or 0.5, respectively. The slightly better refinement value points towards a labile ligand. Thus the re-analysis of EXAFS data from C176 mutants supports the presence of a carbon as iron ligand which was not considered in former models.

The interactions between the protein and the acyl group are identical in the C176A- and wild-type enzymes, the pyridinol’s hydroxyl group of the wild-type enzyme is not hydrogen-bonded to His14 but to a solvent molecule that is anchored to the protein matrix via Thr13.

Fig. 3. Re-interpreted iron-ligation structure of wild-type [Fe]-hydrogenase (A) F_o–F_c omit electron density map around the iron complex contoured at the 2.8σ level (in blue), shown in the same orientation as in Fig. 2A. (B) Schematic representation of the iron octahedrally surrounded by the pyridinol’s nitrogen, the pyridinol’s formylmethyl-acyl carbon, one CO, and the Cys176 sulfur ligands as well as by the “unknown” and “solvent” binding sites. According to the current data the second CO more likely sits at the “solvent” binding site (see Sections 3.2. and 4). Bond length was derived from the EXAFS data (see Table 2).
The previous interpretation of the electron density was biased by the lack of imagination concerning the possibility of an acyl group as iron ligand and on the subsequent conclusion that the orientation of a negatively charged carboxylate group towards the rather unpolar protein interior is unlikely. Moreover, the hydroxyl group of the pyridinol ring in van der Waals contact to the iron-ligating CO group is also compatible with IR spectroscopic data as the FeGP-cofactor in the active enzyme shows CO stretching frequencies at 2116 cm$^{-1}$ and 2044 cm$^{-1}$ in water [8] and at 1996 cm$^{-1}$ and 1928 cm$^{-1}$ in the dried state (Supplementary Fig. S1). These CO stretching frequencies are similar to those from ($\eta$5-cyclopentadienyl)dicarboxylacyliron complexes in organic solvents or in the dried state [24,25]. Apparently, the electronic state of iron in both complexes is related arguing for an acyl-iron ligation in the FeGP cofactor of [Fe]-hydrogenase. Therefore, from now on acyl-iron complexes should be included in the list of possible biomimetic compounds for [Fe]-hydrogenase. Interestingly, an acyl group as metal ligand (to nickel) in an enzyme has so far only been reported for the acetyl-CoA synthase/decarboxylase reaction as possible intermediate in the formation of acetyl-CoA from CO and a methyl group [26,27].

The positions of the Cys176-sulfur and of the intrinsic CO arranged trans to the Cys176-sulfur are not changed by the re-interpretation. This leaves the positions of the “unknown” ligand and of the “solvent” in the previous wild-type enzyme model (Fig. 1 C) as possible binding sites for the second intrinsic CO (Fig. 3). While the C176A structure favors the “solvent” binding site for the second intrinsic CO its position in the wild-type enzyme cannot be identified on a structural basis. The electron density at the “solvent” binding site, although compatible with a (partially bound) intrinsic CO is not clearly connected to that of the iron and the weaker electron density of the “unknown” binding site is not sufficiently shaped.

A definitive assignment of the second intrinsic CO-binding site is difficult since the C176A enzyme structure is not active and that both of the mutant and the wild-type structure might be modified during the structure determination process especially at the solvent-exposed “unknown” ligand and “solvent” binding sites because of the instability of the FeGP-cofactor.

Acknowledgements

This work was supported by the Max Planck Society, the Fonds der Chemischen Industrie and the BMBF (BioH2 project). We thank Hartmut Michel for continuous support and the staff of PXII at the SLS for help during data collection.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.01.017.

References
