Extended X-ray absorption fine structure of the [Fe]-hydrogenase Hmd active site

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Abstract. Hydrogenases are enzymes that catalyze the reversible oxidation of molecular hydrogen. Although their structure and catalytic mechanism are of considerable applied interest as models for the development of efficient catalysts for hydrogen fueled processes, the understanding of how hydrogenases react with H₂ is only in its infancy. Two of the three known types of hydrogenases are iron-sulfur proteins that contain a dinuclear metal center, either [NiFe] or [FeFe]. In contrast, [Fe]-hydrogenase is the only mononuclear hydrogenase and thus a perfect system for studying the structural and electronic determinants of these enzymes. Here we summarize recent improvements in modeling based on the EXAFS signal and the geometric structure of this metalloenzyme in its as isolated or reconstituted form. The individual contributions to the EXAFS resulting in two different structural models are presented and discussed. Inspired by the new crystal structure, we show an advanced EXAFS model for the enzyme from Methanothermobacter marburgensis.

1. Introduction

Many microorganisms utilize the reversible oxidation of molecular hydrogen as an electron supply or electron sink. This reaction is catalyzed by hydrogenases of which three basic types have been discovered [1, 2]. [NiFe]- and [FeFe]-hydrogenases use a dinuclear active site for activation of H₂ and typically a chain of Fe/S clusters for electron delivery (H₂ ⇌ 2H⁺ + 2e⁻) [2]. In contrast, [Fe]-hydrogenase is the only mononuclear hydrogenase and thus a perfect system for studying the structural and electronic determinants of these enzymes. Here we summarize recent improvements in modeling based on the EXAFS signal and the geometric structure of this metalloenzyme in its as isolated or reconstituted form. The individual contributions to the EXAFS resulting in two different structural models are presented and discussed. Inspired by the new crystal structure, we show an advanced EXAFS model for the enzyme from Methanothermobacter marburgensis.
Mononuclear [Fe]-hydrogenase shares several features with these enzymes: the initial EXAFS characterization of the enzyme isolated from Methanothermobacter marburgensis (mHmd) identified a single sulfur ligand bound to the iron ion of the FeGP-cofactor. Site directed mutagenesis studies on all three cysteine residues of [Fe]-hydrogenase from Methanocaldococcus jannaschii (jHmd) (i.e.: C10A, C176A, C176S, C250A), together with EXAFS studies on these mutants and on a Se-Cys form, determined that the sulfur donor is provided by Cys176 [4]. In addition, two CO-donor groups were clearly visible in the EXAFS and the corresponding Fourier transform in line with earlier IR studies [6]. Interestingly, the contribution of the remote oxygen ion from carbon monoxide to the EXAFS is in jHmd as well as in mHmd much stronger than that observed for [NiFe]-hydrogenase HoxC [7]. Following a conservative approach for ab initio EXAFS data analysis, the remaining FT signal bracketed by the CO and sulfur contributions were refined as oxygen groups, because the backscattering potentials are highly similar for the donor types oxygen and nitrogen.

Recent structural information obtained by EXAFS and protein crystallography allows an advanced analysis of these data: In the C176A-mutant of jHmd in addition to the nitrogen donor of the pyridinol group of the FeGP-cofactor its acyl-carbon donor is modeled to be bound to the iron ion. Re-analysis of the jHmd wild type EXAFS shows that this model better fits both crystallographic electron density

Figure 1. Fe-coordination for the Fe ion proximal to the FeS-clusters in [FeFe]-hydrogenase (left), the Fe-ions in [NiFe]-hydrogenase (center) and [Fe]-hydrogenase (right). The position of the CO ligand trans to N, and O trans to acyl-C in [Fe]-hydrogenase are not assigned unambiguously in the crystal structure.

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Figure 2. EXAFS and corresponding Fourier transform for mHmd and wild-type jHmd isolated under different conditions and refined with different structural models (black: measurements): mHmd(1): Hmd from *M. marburgensis* modelled as tetrahedral Fe-coordination as initially published [4]; jHmd(2): Hmd apoenzyme from *M. jannaschii* heterologously produced in *E. coli* and reconstituted with FeGP-cofactor with pentacoordination as published in [4]; jHmd(3): a sample similar to jHmd(2) but of higher quality and metal concentration with tetrahedral structural model for Fe-coordination [5]; jHmd(4): the protein under crystallization conditions as published in [5]; jHmd(5): advanced, octahedral structural model for jHmd(3) as published in [1]. Note, the sensitivity of jHmd to different buffer conditions.
and EXAFS data [1]. Here, we show that distinguishing between individual contributions of light ligands can improve the fit quality considerably.

2. Material and Methods

The jHmd samples from *M. jannaschii* and mHmd from *M. marburgensis* were prepared as described previously [6, 4, 5]. Whereas the wild type form of Hmd from *M. marburgensis* has been isolated from the organism, for jHmd and its mutants the apoenzyme was overproduced in *Escherichia coli* BL21(DE3) cells, purified and reconstituted with the FeGP-cofactor [8].

All Fe-K edge XAFS spectra were recorded at the EMBL Hamburg EXAFS beamline (DESY, Germany) in fluorescence mode. The extracted iron K-edge EXAFS data were converted to photoelectron wave vector k-space by KEMP [9] and weighted by \( k^3 \). The spectra were refined with EXCURV98 [10]. The program calculated the theoretical EXAFS for defined structural models. In addition to single scattering contributions, multiple scattering linear units were defined for Fe-C=O and Fe-C-N. The acyl group 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 (\( \text{R} \)), the Debye-Waller factors (2\( \sigma^2 \)), and a residual shift of the energy origin, were optimized, minimizing the fit index (Table 1).

3. Results and Discussion

In our initial study we identified the cysteinic sulfur ligand as well as two CO groups in Hmd from *M. jannaschii* and from *M. marburgensis*. The remaining backscattering contributions at about 2 Å were refined by 1 or 2 oxygen donor atoms, respectively, as shown in Figure 2. The spectrum obtained for the enzyme from *M. jannaschii* overexpressed in *E. coli* and reconstituted with the FeGP-cofactor differs considerably from the mHmd spectrum. This has been modeled by an additional oxygen.

![Figure 3](image-url)

**Figure 3.** EXAFS and corresponding Fourier transform for reconstituted jHmd(3) (black: measurement, red: refinement) with individual components contributing to tetrahedral EXAFS model (green: one out of two CO, blue: S, magenta: O). Due to the multiple scattering within the CO unit the contribution by the oxygen ions is strongly enhanced. No destructive interference at lower photoelectron wave-vectors (yellow area) for sulphur and oxygen backscattering is observed. At higher wavenumber vectors the oxygen signal is strongly damped due to its nature and the Debye-Waller factor obtained in the refinement (Table 1).
Preparations of jHmd with different buffer conditions yielded different EXAFS traces much more similar to mHmd. This spectrum, jHmd(3), was initially refined as a tetracoordinated Fe-ion (see below) [4]. In the crystal structure of jHmd one ligand could not clearly be identified, prompting the speculation that it might be caused by the buffer conditions. This has been tested by EXAFS on jHmd under such conditions (jHmd(4)). Here, only small changes occur and no additional ligand can be identified by EXAFS [5]. Prompted by the crystal structure of the C176A-mutant of jHmd [1] an octahedral Fe-coordination has been modeled in the corresponding EXAFS as well, assuming for the first time binding of the acyl-carbon to the metal ion. This resulted in an improvement of the EXAFS fit [1]. Based on this structural model the fit for jHmd(3) improves slightly as well.

The individual components contributing to the EXAFS of jHmd using the tetrahedral model are shown in Figure 3: The backscattering from the two CO groups, of which only one is shown, and the sulfur donor are dominating. As visualized in the Fourier transform the oxygen from CO contributes even stronger to the EXAFS than the carbon ion.

This is caused by the focusing of the photo electron wave by the carbon ion. The signals for sulfur and oxygen represent each a single contribution at an average distance with a disorder defined by the Debye-Waller factors. For small photo electron wave vectors these signals are in phase. At higher wave vectors when they get out of phase the oxygen contribution is small, reflecting of the higher Debye-Waller factor obtained in the fit (Table 1).

![Figure 3](image3.png)

**Figure 3.** The individual components contributing to the EXAFS of jHmd using the tetrahedral model are shown in Figure 3: The backscattering from the two CO groups, of which only one is shown, and the sulfur donor are dominating. As visualized in the Fourier transform the oxygen from CO contributes even stronger to the EXAFS than the carbon ion.

![Figure 4](image4.png)

**Figure 4.** EXAFS and corresponding Fourier transform for reconstituted jHmd(5) (black: measurement, red: refinement) with individual components contributing to octahedral EXAFS model (green: CO, blue: S, orange: N, magenta: O, light blue: (acyl-)carbon). The destructive interference of the carbon ascribed to the acyl-group and the N/O contribution at lower photoelectron wave-vectors (yellow area) results in a quasi-cancellation of these two contributions in this energy range. At higher energies mainly interference with the sulphur contribution can be observed. Here, the O and N contributions are very small due to their higher Debye-Waller factor that we ascribe to a weaker binding of the oxygen donor. In the Fourier transform magnitude the negative interference is not visible.
Table 1: EXAFS parameters for models shown in this paper. The numbers ($n$) of ligand atoms (L) to the iron ion, their distance to the iron ion ($R$), the Debye-Waller factor ($2\sigma^2$), the $C-O$, the Fermi energy for all shells ($EF$), and the fit index ($\Phi$), indicating the quality of the fit are shown.

| $n$ | Fe | L | $R$ (Å) | $2\sigma^2$ | $R_{\text{Cu}}$ (Å) | $EF$ (eV) | $\Phi$ |
|-----|----|----|--------|-------------|----------------|----------|------|
| mHmd (1) | 2 | Fe | C$^a$ | 1.801 (4) | 0.0084 (8) | 1.121 (8) | -9.0 (4) | 0.01196 |
| 1 | Fe | O | 2.034 (6) | 0.007 (1) |
| 1 | Fe | S | 2.308 (3) | 0.0051 (6) |
| 2 | Fe | O$^a$ | 2.922 (4) | 0.0083 (5) |
| jHmd (2) | 2 | Fe | C$^a$ | 1.813 (5) | 0.007 (1) | 1.100 (13) | -7.3 (4) | 0.4825 |
| 2 | Fe | O | 2.004 (5) | 0.007 (1) |
| 1 | Fe | S | 2.34 (1) | 0.008 (1) |
| 2 | Fe | O$^a$ | 2.923 (8) | 0.013 (1) |
| jHmd (3) | 2 | Fe | C$^a$ | 1.792 (4) | 0.0077 (9) | 1.142 (8) | -8.9 (4) | 0.2005 |
| 1 | Fe | O | 2.040 (9) | 0.013 (2) |
| 1 | Fe | S | 2.321 (4) | 0.0071 (8) |
| 2 | Fe | O$^a$ | 2.934 (4) | 0.0081 (5) |
| jHmd (4) | 2 | Fe | C$^a$ | 1.795 (5) | 0.007 (1) | 1.12 (1) | -8.3 (7) | 0.3643 |
| 1 | Fe | O | 2.03 (1) | 0.008 (2) |
| 1 | Fe | S | 2.351 (6) | 0.007 (1) |
| 2 | Fe | O$^a$ | 2.921 (7) | 0.0098 (9) |
| jHmd (5) | 2 | Fe | C$^a$ | 1.769(5) | 0.0050(7) | 1.170 (8) | -10.6(5) | 0.1664 |
| 1 | Fe | C | 1.88(1) | 0.0020(1) |
| 1 | Fe | O | 2.052(9) | 0.014(2) |
| 1 | Fe | N | 2.052(9) | 0.014(2) |
| 1 | Fe | S | 2.335(4) | 0.0064(7) |
| 2 | Fe | O$^a$ | 2.939(3) | 0.0025(4) |

In Figure 4 the individual contributions are shown for the octahedral Fe-coordination. Note that the major contributions to the EXAFS (cysteinic sulfur, carbon and oxygen of the carbon monoxide ligands) differ only marginally from the one for tetrahedral coordination (Figure 3). The residual signal has initially been modeled only by one oxygen contribution, which is based on its small size fully justified. When additional information became available more complex models were considered, allowing for partially destructive interference of these components. Here, the carbon and the oxygen contribution nearly cancel out at small wave-vectors and the carbon backscattering contributes to a much better fit at higher wave-vectors.

This is as well visualized by the corresponding Fourier transform: Measurement and fit match much nicer than for the tetrahedral model. The values resulting from this fit are given in table 1 showing that the other contributions are only marginally affected. Based on these considerations we re-analysed the mHmd (1) data and compared them to the new structural model for the Hmd active site. As shown in table 1 the model again improves slightly. This is inline with the optical representation (Figure 5) and allows concluding that the active sites in mHmd and jHmd are virtually identical.

Moreover, this work highlights the need for additional criteria in XAS data analysis. Here, the XANES as well as methods considering target values for bond lengths, bond valance sum, Debye-Waller factors and Fermi energy shift will play an important role [11, 12].
Figure 5. EXAFS and corresponding Fourier transform for mHmd (black: measurement, red: refinement) assuming an octahedral Fe-coordination as indicated in Figure 1.

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