Reduction of Unusual Iron-Sulfur Clusters in the H₂-sensing Regulatory Ni-Fe Hydrogenase from Ralstonia eutropha H16*

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The regulatory Ni-Fe hydrogenase (RH) from Ralstonia eutropha functions as a hydrogen sensor. The RH consists of the large subunit HoxC housing the Ni-Fe active site and the small subunit HoxB containing Fe-S clusters. The heterolytic cleavage of H₂ at the Ni-Fe active site leads to the EPR-detectable Ni-C state of the protein. For the first time, the simultaneous but EPR-invisible reduction of Fe-S clusters during Ni-C state formation was demonstrated by changes in the UV-visible absorption spectrum as well as by shifts of the iron K-edge from x-ray absorption spectroscopy in the wild-type double dimeric RHWT [HoxBC]₂ and in a monodimeric derivative designated RHstop lacking the C-terminal 55 amino acids of HoxB. According to the analysis of iron EXAFS spectra, the Fe-S clusters of HoxB pronouncedly differ from the three Fe-S clusters in the small subunits of crystallized standard Ni-Fe hydrogenases. Each HoxBC unit of RHWT seems to harbor two [2Fe-2S] clusters in addition to a 4Fe species, which may be a [4Fe-3S-3O] cluster. The additional 4Fe-cluster was absent in RHstop. Reduction of Fe-S clusters in the hydrogen sensor RH may be a first step in the signal transduction chain, which involves complex formation between [HoxBC]₂ and tetrameric HoxJ protein, leading to the expression of the energy converting Ni-Fe hydrogenases in R. eutropha.

Ni-Fe hydrogenases represent an important class of metalloenzymes that catalyze the reversible cleavage of molecular hydrogen into electrons and protons (reaction H₂ ⇌ 2H⁺ + 2e⁻) (1). The chemolithoautotrophic β-proteobacterium bacterium Ralstonia eutropha H16 houses three different Ni-Fe hydrogenases that are physiologically active in the presence of O₂ (2, 3). The membrane-bound and soluble NAD-reducing enzyme is involved in energy conversion (4, 5).

The regulatory Ni-Fe hydrogenase (RH) belongs to a particularly interesting type of Ni-Fe hydrogenase functioning as hydrogen sensors (6). Hydrogen sensors have also been described in Bradyrhizobium japonicum (7) and Rhodobacter capsulatus (8). Upon the interaction of the RH with molecular hydrogen, a complex signal transduction cascade is initiated that leads to the expression of the energy-converting hydrogenases (9).

The RH consists of the large subunit HoxC that harbors the hydrogen-activating Ni-Fe site and the small subunit HoxB that contains iron-sulfur clusters (6). Several unusual properties of the RH (3, 10, 11) are remarkably different from those of the so-called standard Ni-Fe hydrogenases from inter alia, Desulfovibrio gigas and Allochromatium vinosum (1, 12, 13). In contrast to the dimeric standard Ni-Fe hydrogenases, the RH forms a double dimer [HoxBC]₂ (Fig. 1A) that is connected to a tetramer of the HoxJ protein (14). The N-terminal input module of HoxJ containing a PAS domain is required for the formation of the RH-HoxJ complex, whereas the C-terminal domain of HoxJ has histidine protein kinase activity (14). The RH cleaves H₂ only at extremely low rates (3, 10). In contrast to standard hydrogenases, which can exist in up to nine different redox states (1, 13), in the RH only two states of functional relevance have been detected (10). After aerobic isolation the enzyme is in its oxidized state containing Ni(II). This state does not need to be activated but is always ready to bind hydrogen, a prerequisite for the sensor function (10, 11). In the presence of H₂ it is rapidly converted to a state revealing a typical EPR-signal, termed Ni-C, due to a Ni(III)-H⁻ species formed during heterolytic H₂ cleavage (15). In standard Ni-Fe hydrogenases the nickel is coordinated by four conserved cysteine residues. X-ray absorption spectroscopy (XAS) investigations on the RH, however, revealed that nickel may be coordinated by less than four cysteines (11). The iron atom of the RH active site, on the other hand, carries two cyanides and one CO molecule, similar to standard Ni-Fe hydrogenases (3, 10).

Although information has become available about the sequence of events that occur at the Ni-Fe active site upon interaction of the RH with H₂ (10, 11, 15), it is unclear whether electron transfer out of the Ni-Fe site takes place during H₂ cleavage and to where these electrons are transferred. Information on these points is expected to contribute to the understanding of the H₂-sensing mechanism of the RH-HoxJ complex (14).

In standard Ni-Fe hydrogenases of the D. gigas type, the small subunit contains three Fe-S clusters, two [4Fe-4S] and one [2Fe-2S].
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one [3Fe-4S] (16), which are bound via conserved cysteines and one histidine residue found in all Ni-Fe hydrogenase sequences. During hydrogen turnover these clusters become reduced as detected by EPR spectroscopy (1, 17). The RH small subunit HoxB also contains these conserved cysteines. Therefore, it was postulated that it might also harbor three Fe-S clusters (6). EPR investigation of the RH, however, did not show reduced Fe-S clusters when the Ni-C EPR signal was formed under H₂ (3, 10, 15). It has been proposed that a non-Fe-S cofactor may be involved in electron transfer instead (10).

In this work the double dimeric wild-type RH (RHWT, Fig. 1A) and a derivative denoted as RHstop (Fig. 1B), which forms a monodimer due to mutational truncation of the C-terminus of HoxB (14), was compared. It has been suggested that the RHstop may lack the putative non-Fe-S cofactor (14). In both preparations, for the first time reduction of Fe-S clusters in the presence of H₂ was clearly detected in UV-visible spectra and by XAS at the iron K-edge. Support for reduction of a non-Fe-S cofactor was not obtained. Seemingly, the Fe-S clusters of the RH differ from those of standard hydrogenases.

MATERIALS AND METHODS

**Bacteria Growth and Enzyme Purification**—Strains with the initials HF were derived from *R. eutropha* H16 (DSM4428, ATCC 17699). Large scale cultivation of *R. eutropha* strains, cell harvesting, cell disruption, and preparation of soluble protein extracts were published before (10, 14). RHWT (Fig. 1A) was purified from the RH-overproducing strain *R. eutropha* HF371(pGE378) as described in Bernhard et al. (10). Starting with 50 g of cells (wet weight) yielded 3.7 mg of RH with a specific activity of 1.6 units/mg of protein. The RHstop protein (Fig. 1B) was purified from *R. eutropha* HF574(pGE567) as a Strep-tag II fusion protein.² Starting with 18 g of cells yielded 1.5 mg of RHstop with a specific activity of 1.6 units/mg of protein.

The homogeneity of the respective protein preparations was investigated by SDS-PAGE analysis and subsequent Coomassie staining. The amount of impurities was quantified by using the Gelscan Professional V5.1 software (BioSciTec, Frankfurt, Germany). After background subtraction, the sum of the percent differential integrated density of the HoxC- and HoxB-specific bands was correlated to the sum of the percent differential integrated density from the contaminating proteins.

**Assays of Hydrogenase Activity**—H₂-oxidizing activity was quantified by an amperometric H₂ uptake assay as in Pierik et al. (3) using a H₂ electrode with methylene blue as an electron acceptor. One unit of HoxB methylene blue oxidoreductase activity was the amount of enzyme that catalyzed the consumption of 1 μmol of H₂/min. Protein concentrations were determined according to the protocol of Bradford (18).

**Analysis of Metal Contents**—Atomic absorption spectroscopy (AAS) and total reflection x-ray fluorescence analysis (TXRFXA) (19) were used for quantification of nickel and iron. For AAS, three aliquots of each RH preparation were solubilized overnight in concentrated HNO₃ (65%, Suprapur, Merck), then diluted with ultrapure water (Millipore) to 1–5% HNO₃, and further diluted for measurements using the transversely heated graphite furnace technique with longitudinal Zeeman-effect background correction on a PerkinElmer Life Sciences Analysist 800 spectrometer equipped with an autosampler and WinLab32 software.

² T. Buhrke, O. Lenz, and B. Friedrich, manuscript in preparation.
yielded on the average about 0.6 mol of nickel in the RHWT and tents by AAS in combination with protein determination close to 1 mol of nickel in the RHstop (Table I). The relatively arbitrary units.

of the C terminus of HoxB.

tions of RHWT contained sizable amounts of copurified proteins, the preparations. SDS-PAGE analysis indicated that preparations were homogenous (Fig. 3). The respective error ranges represent

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FIG. 2, A, FTIR spectra of oxidized RHWT and RHstop (normalized on the CO bands). B, normalized Ni-C EPR spectra due to Ni(III)-H - (15) in the H2-reduced samples later used for XAS. EPR conditions: temperature 30 K, microwave power 250 microwatts, modulation amplitude/ frequency 2 milliTesla/100 KHz. Quantification of measured Ni-C spectra in combination with nickel determination by AAS revealed that >90% of the nickel was present as Ni(III) in RHWT and RHstop, arbitrary units.

(Ni(III)-H - ) of the Ni-Fe active site with the hydride in a bridging position between nickel and iron (15). The Ni-C EPR signals (Fig. 2B) were similar in RHWT and RHstop. Signal quantification revealed that greater than 90% of the nickel was converted to Ni(III) in both preparations after incubation with H2 for 10 min, implying similarly effective heterolytic hydrogen cleavage. However, EPR signals from singly reduced Fe-S clusters were completely absent in the Ni-C state. Reduced Fe-S clusters were also not detected when temperatures between 6 and 50 K and microwave power variations between 0.01 and 10 milliwatt were applied (data not shown).

In summary, RHWT and RHstop appeared to be similar with respect to the coordination of the iron of the Ni-Fe site both in the oxidized state and in the Ni-C state, which was nearly quantitatively formed in both cases in the presence of hydrogen. Moreover, both preparations showed identical hydrogenase activities (see “Materials and Methods”). The catalytic properties of RHstop were, thus, not affected by the truncation of the C terminus of HoxB.

Determination of Metal Contents—Analysis of the nickel contents by AAS in combination with protein determination yielded on the average about 0.6 mol of nickel in the RHWT and close to 1 mol of nickel in the RHstop (Table I). The relatively low nickel content in RHstop can be explained with impurities in the preparations. SDS-PAGE analysis indicated that preparations of RHWT contained sizable amounts of copurified proteins, whereas preparations of RHstop were homogenous (Fig. 3). The amount of impurities in the RHWT sample was quantified by calculating the intensities of HoxC- and HoxB-specific protein bands and the sum of the band intensities derived from the contaminating proteins. According to this analysis the RHWT sample contained about 16% of impurities, whereas the RHstop sample was pure. Therefore, assuming that each HoxBC unit of

FIG. 3. SDS-PAGE analysis of purified RH samples. Purified samples of RHWT (left lane) and RHstop (right lane) were separated by SDS-polyacrylamide gel electrophoresis (12% gels; 5 μg of protein per lane) and subsequently stained with Coomassie brilliant blue (Serva). The RH subunits HoxC and HoxB are indicated by arrows. Due to the truncation of its C-terminal 55 amino acids, the HoxBstop band from the RHWT sample was pure. Therefore, assuming that each HoxBC unit of

TABLE I

Metal contents of RH preparations

Ni4Fe(HoxBC) values represent the average over values from AAS and protein determinations from five independent preparations of both RHWT and RHstop. Mean iron/nickel ratios were derived from the AAS values and from concentrations determined by TRXFA on two preparations of both RHWT and RHstop. The respective error ranges represent S.D.

|          | Nickel/[HoxBC] | Iron/[HoxBC] | Iron/Nickel (AAS, TRXFA) |
|----------|----------------|--------------|--------------------------|
| RHWT     | 0.56 ± 0.09    | 4.53 ± 0.95  | 8.09 ± 1.04, 8.83 ± 1.16 |
| RHstop   | 1.03 ± 0.21    | 4.83 ± 2.17  | 4.69 ± 2.38, 6.12 ± 0.24 |

Based on sequence homologies (see “Discussion”) one would expect HoxB to contain three [4Fe-4S] clusters (6). Taking also the iron atom of the Ni-Fe site into account, one HoxB monodimer might contain 13 Fe/Ni. Thus, the experimentally determined iron content of RHWT was significantly lower than expected. Furthermore, HoxBWT seemed to contain more iron than HoxBstop. The double dimeric RHWT may, thus, comprise iron species that are absent in monodimeric RHstop.

Detection of Fe-S Cluster Reduction by UV-Visible Spectroscopy.—The optical absorption spectra of the oxidized RHWT and RHstop showed, in addition to a peak at 280 nm due to the absorption of the aromatic amino acid residues of the protein, a broad shoulder around 410 nm, presumably due to the presence of Fe-S clusters (Fig. 4, solid lines). That both preparations were fully oxidized was apparent from the absence of any Ni-C EPR signal (data not shown). The extinction coefficients were calculated at 390 and at 420 nm and compared with data from the literature (Table II). The ε(420 nm) value determined for RHstop is in good agreement with the value that would be expected in the presence of two cysteinyl-coordinated [2Fe-2S] clusters (49). The ε(420 nm) of RHWT, on the other hand, is significantly higher, confirming the data from the metal analysis which indicate that RHWT contain additional iron species (see “Determination of Metal Contents”). The ε(390 nm) values estimated for RHWT as well as for RHstop were significantly larger than the value of ε(390 nm) that was expected if only one [4Fe-4S] cluster was present (Table II).

When the RH was reduced by flushing of samples with H2, increased absorption was observed in the whole spectral range (not shown); the curvature of the background (proportional to λ-4) suggested its origin from a scattering contribution. Because this background could be removed by centrifugation of
H₂-flushed samples, apparently a small portion of the RH protein precipitated, thereby light-scattering aggregates were formed. Aggregation was also observed after flushing with argon, which indicated that it was not specifically caused by H₂.

Reduction of RHWT by flushing with H₂ and subsequent removal of aggregates by centrifugation yielded a spectrum with clearly decreased absorption between 350 and 600 nm as observed from the UV-visible spectra (Fig. 4, A and B) showed two main minima (bleachings) around 410 and 550 nm, respectively. Whereas bleaching solely around 410 nm has been observed upon reduction of [4Fe-4S] clusters (24, 28), the absorption at ~280 nm was almost unchanged. Reduction of RHstop with H₂ yielded similar results (Fig. 4B, dotted line). The absorption at ~280 nm was almost unchanged. Reduction of RHstop with H₂ yielded similar results (Fig. 4B, dotted line). The absorption at ~280 nm was almost unchanged.

Characterization of Fe-S Clusters by XAS—By XAS (22, 28) at the iron K-edge, the iron sites in the RH preparations were selectively studied. Because the coordination of the iron of the Ni-Fe site was similar in RHWT and RHstop, both in the oxidized and H₂-reduced states and this iron remained in its divalent oxidation state, any changes in the iron XAS spectra were expected to be attributable to the putative Fe-S clusters.

The XANES spectra from both RH preparations (Fig. 5) were similar to typical spectra of Fe-S clusters (29–31). A pronounced shift of the iron K-edge to lower energies by 1.0–1.2 eV after reduction by H₂ was observed (dotted lines). The shift was by 0.2 eV larger in the RHWT (Table III). A shift by ~0.2 eV was also evident in the pre-edge peak of the XANES spectra (Fig. 5, insets). Such spectral shifts are typical for Fe-S cluster reduction (29, 31), implying that such clusters became reduced in RHWT and RHstop upon formation of the Ni-C state.

The maxima of the XANES spectra of the RHWT were larger, and the pre-edge peak magnitudes and areas were smaller than in the RHstop, pointing to an average coordination of iron in the RHWT by less sulfur and more O/N ligands (32). For one-electron reduction of single-Fe(III) compounds with mixed O/N/S ligation of iron, a shift of the K-edge by about ~2.5 eV may be expected (29, 31, 33). Hence, it was estimated that 3–4 and ~2 iron atoms (on basis of 8 and 5 iron atoms in total) became reduced in the RHWT-H₂ and RHstop-H₂, respectively (Table III). In both preparations obviously more than one electron was transferred to iron in the Ni-C state. Moreover, the overall iron coordination was different, and additional iron atoms seemed to become reduced in the RHWT.

The Fourier transforms (FTs) of iron EXAFS oscillations (Fig. 6) of oxidized RHWT and RHstop showed two prominent peaks immediately revealing the presence of at least two backscattering shells, likely due to Fe-O/N/S and Fe-Ni interactions around 2–2.2 and 2.7 Å (the true iron-backscatterer distances are by about 0.4 Å larger than the reduced distances indicated in Fig. 6). The FTs of RHWT-ox and RHstop-ox were not identical; FT peak I was larger in RHWT-ox, and an additional shoulder was present on FT peak II in RHWT-ox (Fig. 6, arrow). Precise structural information was obtained from simulations of the EXAFS oscillations (Fig. 6, inset). In both preparations the EXAFS was expected to be dominated by contributions from Fe-C/N/O/S and Fe-Ni/Fe-Ni vectors in the first and second ligand spheres. The broad shoulder at lower distances on FT peak I in RHWT (Fig. 6) suggested the presence of C/O/N atoms with significantly shorter distance from iron than the S-atoms at about 2.3 Å. A first simulation where one Fe-C/N/O/S

TABLE II
Extinction coefficients of preparations of RHWT and RHstop at 390 and 420 nm as obtained from the UV-visible spectra (Fig. 4).

| Preparation | 390 nm | 420 nm |
|-------------|--------|--------|
| RHWT-ox     | 31,500 | 28,300 |
| RHWT-ox     | 23,900 | 21,800 |
| [Fe-4S]     | 15,200 | 14,800 |
| 2 × [2Fe-2S] | 19,200 | 18,000 |

*The values for RHWT were corrected by a factor of 1.18 due to the fact that preparations of RHWT contained 16% impurities.

The values for RHWT were taken from Lippard et al. (49).
one Fe-S, and one Fe-Fe/Ni vector were included yielded an error factor $R_F$ of about 10% for both RHWT and RHstop (Table IV, A, fit I). The coordination number of the Fe-Fe vector $N_{Fe-Fe}$ was close to one in both cases. For the presence of two [4Fe-4S] clusters plus one [4Fe-4S] or [3Fe-4S] cluster as in standard hydrogenases, a value of 2.5 $< N_{Fe-Fe} < 3$ was expected because each iron in such clusters has three (in [4Fe-4S]) or two (in [3Fe-4S]) iron neighbors at about 2.7-Å distance (34–36). Thus, fit I immediately suggested that the iron EXAFS of the RH was not dominated by cubane clusters. Instead, a value of $N_{Fe-Fe}$ close to one was compatible with the predominant presence of iron clusters where each iron has only one iron neighbor in both RH preparations. Such a situation is realized in [2Fe-2S] clusters. That $N_S$ was much lower than four suggested the mixed coordination of several iron ions by O/N and S ligands and not predominantly by terminal Cys-S and bridging δ-S ligands as observed for [4/3Fe-4S] clusters. If [2Fe-2S] clusters were present, they could, therefore, be of the Rieske type ([(2Fe-2S)Cys-S]δ(His-N)δ).

The EXAFS of RHWTox showed a shoulder on FT peak II that was absent in RHstopox. Inclusion of a second Fe-Fe vector in the simulations to account for this feature almost halved the $R_F$ value for RHWTox (Table IV, A, fit II), whereas it did not significantly improve the fit for RHstopox. Furthermore, the Debye-Waller parameters (2$\sigma^2$) were unrealistically large for both Fe-Fe vectors, meaning that a distinct long Fe-Fe distance was likely not present in RHstop. The estimated longer Fe-Fe distance of $>3$ Å in RHWTox is not observed in normal [4Fe-4S], [3Fe-4S], or [2Fe-2S] clusters where typical Fe-Fe distances are $\sim 2.7$ Å (29, 31, 34–36). These results seemed to imply the presence of an additional more unusual iron cofactor in the RHWT.

In a third simulation a C-shell was included to account for the three CN/O ligands of the iron of the Ni-Fe site. The fit for RHWTox was not improved, supporting its lower (CN,CO)/Fe ratio. A significantly improved fit was obtained for RHstopox (Table IV, A, fit III) where the coordination number of the Fe-(CN,CO) vector was in agreement with its larger (CN,CO)/Fe ratio of $\sim 0.6$ according to the lower iron content.

To gain more support for the unexpected result that the Fe-Fe coordination number ($N_{Fe-Fe}$) was close to one, particularly stringent analysis (37) of the Fe-Fe vector in RHstop was performed. The values of $N_{Fe-Fe}$ and $2\sigma^2_{Fe-Fe}$ were varied, an EXAFS simulation was carried out for each parameter couple, and the resulting fit errors ($R_F$) were depicted in Fig. 7. Clearly, the absolute minimum ($R_F = 12\%$) in the fit function was obtained at a value of $N_{Fe-Fe}$ close to 1. The presence of only one [4Fe-4S] cluster was strongly disfavored because at the then expected $N_{Fe-Fe}$ of 2.6 (taking into account the iron from the Ni-Fe active site) $R_F$ was 3-fold increased. The presence of only 1 [3Fe-4S] or of 1 [2Fe-4S] plus 1 [3Fe-4S] in RHstop was also disfavored because at the then predicted $N_{Fe-Fe}$ values of 1.75 and 1.5 (including the single Fe-Ni vector) $R_F$ was already about doubled (Fig. 7). Thus, the presence of 2 [2Fe-2S] clusters appeared to be the most likely option in RHstop.

The Fe-Fe distances in [4/3Fe-4S] clusters in crystallized Ni-Fe hydrogenases range between 2.62 and 2.77 Å (Refs. 34–36; unusually long 38) and short (39) Fe-Fe distances were neglected); the range is similar in [2Fe-2S] clusters (29, 31). The Debye-Waller parameters of the $\sim 2.7$-Å Fe-Fe vectors correspond to a smaller distance spread of only $\sim 0.07$ Å (Table IV, A). Superimposition of EXAFS oscillations from Fe-Fe vec-

**Table IV**

**Simulation results of EXAFS spectra from RHWT and RHstop (oxidized state (ox); +H2, Ni-C state)**

|        | $N_1$ (per iron atom) | $R_F$ | $2\sigma^2$ | $R_F$ |
|--------|-----------------------|-------|-------------|-------|
|        | $A$ | $A^2$ | %   |
| **A**, RHWTox (fit I/fit II/fit III) | | | |
| Fe-C | -/0.11 | -/1.95 | -/0.002* | 9.2/5.4/5.6 |
| Fe-N, O | 2.52/2.40/2.15* | 2.06/2.05/2.05 | 0.021/0.022/0.023 |
| Fe-S | 2.60/2.60/2.74* | 2.26/2.26/2.26 | 0.012/0.012/0.012 |
| Fe-Fe, Ni | 0.85/1.16/1.16 | 2.70/2.74/2.74 | 0.004/0.005/0.005* |
| Fe-Fe | -0.46/0.46 | -0.02/0.02 | -0.008/0.009* |
| Fe-C | -/0.54* | -/1.90 | -/0.002* | 9.5/9.1/6.5 |
| Fe-N, O | 2.22/2.36/1.69* | 2.08/2.07/2.10 | 0.046/0.054/0.012 |
| Fe-S | 2.46/2.64/2.80* | 2.26/2.26/2.25 | 0.005/0.005/0.010 |
| Fe-Fe, Ni | 1.09/1.16/1.11 | 2.72/2.72/2.73 | 0.005/0.016/0.008 |
| Fe-Fe, Ni | -0.37 | -0.01 | -0.015* |
| **B**, RH+H2 (WT/stop) | | | |
| Fe-N, O | 2.28/1.69* | 2.12/2.21 | 0.036/0.015 | 8.5/10.7 |
| Fe-S | 2.72/3.31* | 2.28/2.27 | 0.009/0.010 |
| Fe-Fe, Ni | 1.01/1.14 | 2.72/2.71 | 0.005/0.008 |
| **C**, extra Fe in RHWT (4Fe-3S-3O)* | | | |
| Fe-N, O | 1.50* (1.50) | 1.19 (1.94) | 0.001 (0.001) | 9.8 |
| Fe-S | 2.50* (2.50) | 2.28 (2.26) | 0.008 (0.007) |
| Fe-Fe | 1.00* (1.00) | 2.72 (2.73) | 0.005 (0.006) |
| Fe-Fe | 1.00* (1.00) | 3.02 (3.11) | 0.007 (0.004) |

* Values in parenthesis refer to the [4Fe-3S-3O] cluster in D. desulfuricans (39).
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Fig. 7. Contour plot of \( R_F \) values (numbers in %) derived from two shell simulations (1 x Fe-S and 1 x Fe-Fe; \( N_{Fe,N} \) was allowed to vary in the simulations; \( 2\sigma^2 \), was restricted to positive values) of the RHstop EXAFS under variations of the coordination number \( N_{Fe,N} \) and Debye-Waller parameter \( 2\sigma^2 \) of the 2.7-Å Fe-Fe vector. Contour lines are spaced by \( \Delta R_F = 3\% \). A white color denotes the lowest \( R_F \)-values (best fit).

Debye-Waller parameter 2\( \sigma^2 \)

Fig. 8. FT of a \( k^3 \)-weighted EXAFS spectrum (solid line) tentatively attributed to the extra iron present in RHWT but not in RHstop. The FT was calculated from normalized EXAFS oscillations \( \chi(k) \) representing \( \langle R_F(k) - \langle R_F \rangle \rangle / \langle R_F \rangle \). Here, the presence of four extra iron ions in HoxB of RHWT was assumed (taking into account the error range of the iron content, Table I) to allow for comparison with the theoretical EXAFS spectrum (triangles) of the [4Fe-3S-3O] cluster (inset) in the D. desulfuricans crystal structure (39) calculated using parameters given in Table IV, C (in parenthesis), a.u., arbitrary units.

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In RHWT the fit minimum for the 2.7-Å Fe-Fe vector was also observed for \( N_{Fe,N} = 1 \) irrespective of the specific fit approach, e.g. inclusion of 1–3 O/N/C ligand shells and/or of an additional 2.7-Å Fe-Fe vector (not documented). Presumably, RHWT contained the same [2Fe-2S] clusters as RHstop and, furthermore, an additional Fe-S cluster.

In the H2-reduced state, the EXAFS spectra of both RH preparations were overall similar to the ones in Fig. 6; in particular, the shoulder on FT peak II due to long Fe-Fe distances was still present in RHWT\(^{+H2} \) (data not shown). Simulations revealed significantly elongated Fe-O/N and Fe-S distances; the Fe-Fe/Ni vectors of 2.7-Å length were slightly shortened (Table IV, B). Very similar effects have been observed upon reduction of the [2Fe-2S]Rieske cluster in Pseudomonas cepacia phthalate dioxygenase (29). Fe-S cluster reduction was clearly detectable both in the XANES and EXAFS of the RH in the Ni-C state.

Tentative Identification of the Additional Iron Cofactor in RHWT — The EXAFS of RHWT revealed Fe-Fe distances of \( -3 \) Å that were absent in RHstop. Similar Fe-Fe distances were found in the [4Fe-3S-3O] cluster in the proximal position of the small subunit of the Ni-Fe hydrogenase from Desulfovibrio desulfuricans (36). To test the hypothesis of whether the RHWT may contain a [4Fe-3S-3O] cluster, a tentative isolation of the contribution of the extra iron to the RHWT EXAFS was performed (Fig. 8, see legend). The resulting EXAFS difference spectrum (from oxidized enzymes) revealed pronounced splitting of both FT peaks (the difference from the H2-reduced enzymes was similar (not shown)). The best fit was achieved using a (O/N)\( _{1.25-1.75} \)S\( _{2.25-2.75} \) iron coordination and two Fe-Fe vectors with \( N_{Fe,N} = 1 \) and \( -2.7- \) and \( -3- \)Å length (Table IV, C). The [4Fe-3S-3O] cluster in D. desulfuricans reveals similar structural motifs (Fig. 8, inset) and also 4-coordinated iron ions (Table IV, C, in parentheses). The FT (Fig. 8, triangles) of an EXAFS spectrum calculated on basis of the crystal structure (36) was similar to the one tentatively attributed to the extra Fe-S species in RHWT. The binding of one extra [4Fe-3S-3O] to each HoxB subunit was thus, a conceivable option in RHWT.

DISCUSSION

The RH of R. eutropha belongs to a subclass of Ni-Fe hydrogenases acting as hydrogen sensors (3). H2-sensing proteins have also been examined in B. japonicum (7) and R. capsulatus (8). Moreover, genome sequencing projects of a number of microorganisms have uncovered sequences of additional potentially H2-sensing Ni-Fe hydrogenases (Fig. 9). These proteins may all be similarly organized, the large subunit carrying a Ni-Fe site and the small subunit harboring Fe-S clusters. However, due to the lack of crystal structures, only little information about the chemical nature of the putative Fe-S clusters in the H2-sensing hydrogenases was available. In this study, for the first time the structure and function of Fe-S clusters in the oxygen-insensitive hydrogen sensor of R. eutropha was investigated.

Alignment of the HoxB amino acid sequence with those of the small subunits of crystallized Ni-Fe hydrogenases and of potential H2 sensors (Fig. 9) shows that the conserved residues coordinating the iron ions of Fe-S clusters in standard hydrogenases are always present. Thus, on the level of amino acid primary sequences one might argue that the small subunits of H2-sensing hydrogenases possibly contain Fe-S clusters such as found in the standard hydrogenases. Crystal structure analyses (34–36) revealed that the standard hydrogenases harbor a proximal [4Fe-4S] cluster coordinated by four cysteine residues (\( P_1 \) to \( P_4 \) in Fig. 9). The medial cluster usually is a [3Fe-4S] cluster coordinated by three cysteines except for the Ni-Fe-Se enzyme from Desulfomicrobium baculatum (38), where a [4Fe-4S] cluster is found due to the replacement of one polyns residue by another cysteine (39). All four cysteines are present in the sequences of the small subunits of the H2 sensors (\( M_1 \) to \( M_4 \) in Fig. 9). The distal [4Fe-4S] cluster is coordinated by three cysteines and one histidine, which are conserved in all Ni-Fe hydrogenases including the H2 sensors (Fig. 9). The distal [4Fe-4S] cluster is coordinated by three cysteines and one histidine, which are conserved in all Ni-Fe hydrogenases including the H2 sensors (Fig. 9). One cysteine is shifted by one amino acid in the binding motif for the distal Fe-S cluster (\( D_1 \) to \( D_4 \) in Fig. 9). Despite these striking sequence similarities, our experimental data provided no evidence for [4Fe-4S] or [3Fe-4S] clusters in the RH but instead favored the presence of [2Fe-2S] clusters possibly of the Rieske type. These may be coordinated by two cysteines and possibly two histidines. Conserved histidine residues present only in the sequences of the H2 sensors that could
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be involved in Fe-S cluster binding are highlighted in Fig. 9 (H₁ to H₄). The coordination of iron ions by non-sulfur ligands could also be explained by the oxidative conversion of cysteine thiols to sulfenates (Cys-SOH) or sulfinates (Cys-SO₂H) observed in a variety of proteins (40, 41), which would impair direct coordination of iron ions by Cys-S. The presence of [2Fe-2S] clusters highlighted by a black background coordinate the iron ions of the proximal (F₁ to P₄), the medial (M₁ to M₃) and the distal (D₁ to D₄) Fe-S clusters in the standard hydrogenases. Amino acids highlighted by a gray background indicate the proline residue the codon of which has been replaced by a stop codon to generate the RHstop protein.

The range of the iron content in RHWT was also compatible with the binding of only one extra Fe-S cluster per HoxBC₁₂ by residues from the two HoxB proteins. Such a binding mode was observed in Rhodospirillum rubrum dimeric carbon monoxide dehydrogenase (CODH) where a [4Fe-4S] cluster is ligated by two cysteines from each of the two CODH molecules (44). The binding of a [4Fe-3S] cluster in between two HoxB units may be less likely for symmetry reasons since this cluster should be coordinated by three cysteines and by one glutamate residue.

In RHWT where the C-terminal extension of HoxB was truncated, XAS-detectable 3-Å Fe-Fe distances were absent. The Ni-Fe-S cluster in between two HoxB units may be less likely for symmetry reasons since this cluster should be coordinated by three cysteines and by one glutamate residue. In RHWT where the C-terminal extension of HoxB was truncated, XAS-detectable 3-Å Fe-Fe distances were absent. The Ni-Fe-S cluster in between two HoxB units may be less likely for symmetry reasons since this cluster should be coordinated by three cysteines and one glutamate residue.

In RHstop where the C-terminal extension of HoxB was truncated, XAS-detectable 3-Å Fe-Fe distances were absent. The Ni-Fe-S cluster in between two HoxB units may be less likely for symmetry reasons since this cluster should be coordinated by three cysteines and one glutamate residue.

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FIG. 9. Alignment of the amino acid sequences of Ni-Fe hydrogenase small subunits from known and potential H₂-sensing hydrogenases from R. eutropha H16 (R.eut; accession number AAB49364), Alcaligenes hydrogenophilus (A.hyd; AAB49360), Burkholderia cepacia R1808 (B.cepc; ZP00224364), Rubrivivax gelatinosus PM1 (R.gelm; ZP00243688), Azorhizobium caulinodans (A.cau; AAS91235), Dechloromonas aromatica RCB (D.aroc; ZP00203774), Magnetospirillum magnetotacticum MS-1 (M.mag; ZP00654235) R. capsulatus (R.cap; AAS91239), Rhodobacter sphaeroides (R.sph; ZP00243688), Rhodopseudomonas palustris (R.pal; ZP00243688), Desulfovibrio gigas (D.giga; ZP00298862), B. japonicum USDA110 (B.jap; AAA62627), Rhodopseudomonas palustris CGA009 (R.pal; CAE26403), and from the five crystallized standard Ni-Fe hydrogenases from D. gigas (D.giga; P12943), Desulfovibrio fructosovorans (D.fruc; P18187), Desulfovibrio vulgaris Miyazaki F (D.vulg; P21853), D. desulfuricans G20 (D.des; ZP00129917) and from the Ni-Fe-Se hydrogenase from D. baculatum (D.bac; AAA23376), Amino acids highlighted by a gray background coordinate the iron ions of the proximal (F₁ to P₄), the medial (M₁ to M₃) and the distal (D₁ to D₄) Fe-S clusters in the standard hydrogenases. Amino acids highlighted by a black background indicate the proline residue the codon of which has been replaced by a stop codon to generate the RHstop protein.
between RHWT and RHstop. However, the hydrogen cleavage activity at the Ni-Fe site was unchanged. Assuming a maximal distance between the Fe-S clusters of about 25 Å as in standard hydrogenases, electron transfer between these clusters may in any event proceed at least within seconds (45).

Compared with energy-generating hydrogenases, the RH displays a very low but clearly defined hydrogen cleavage turnover rate (3, 10). Consequently, electrons have to be released from the Ni-Fe active site. In standard Ni-Fe hydrogenases these electrons are transferred to the Fe-S clusters, which when reduced show typical EPR signals (1, 13). In the RH, splitting of H2 at the Ni-Fe site yields the EPR-detectable Ni-C cluster when reduced show typical EPR signals (1, 13). In the RH, these electrons are transferred to the Fe-S clusters, which when reduced show typical EPR signals (1, 13). In the RH, the distance between the Fe(II) ions, rendering them EPR-invisible by a small distance of about 12 Å may cause strong magnetic coupling between the Fe(II) ions, rendering them EPR-invisible by a small distance of about 12 Å may cause strong magnetic coupling. In standard Ni-Fe hydrogenases, electron transfer between these clusters may in any event proceed at least within seconds (45).

The sensing of H2 involves complex formation between double dimeric RH and a tetramer of the histidine protein kinase HoxJ (14). A similar arrangement has been described in the R. capsulatus regulatory hydrogenase (47). In the RH, reductive splitting of Fe-S clusters of the HoxJ subunit may cause a structural change of the RH-HoxJ complex, thereby modifying its phosphorylation activity. These events may represent the first step in the signal transduction chain leading to the expression of the energy converting Ni-Fe hydrogenases in R. eutropha.

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Reduction of Unusual Iron-Sulfur Clusters in the H₂-sensing Regulatory Ni-Fe Hydrogenase from Ralstonia eutropha H16
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