Oxygen Tolerance of the H₂-sensing [NiFe] Hydrogenase from Ralstonia eutropha H16 Is Based on Limited Access of Oxygen to the Active Site*

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Hydrogenases, abundant proteins in the microbial world, catalyze cleavage of H₂ into protons and electrons or the evolution of H₂ by proton reduction. Hydrogen metabolism predominantly occurs in anoxic environments mediated by hydrogenases, which are sensitive to inhibition by oxygen. Those microorganisms, which thrive in oxic habitats, contain hydrogenases that operate in the presence of oxygen. We have selected the H₂-sensing regulatory [NiFe] hydrogenase of Ralstonia eutropha H16 to investigate the molecular background of its oxygen tolerance. Evidence is presented that the shape and size of the intramolecular hydrophobic cavities leading to the [NiFe] active site of the regulatory hydrogenase are crucial for oxygen insensitivity. Expansion of the putative gas channel by site-directed mutagenesis yielded mutant derivatives that are sensitive to inhibition by oxygen, presumably because the active site has become accessible for oxygen. The mutant proteins revealed characteristics typical of standard [NiFe] hydrogenases as described for Desulfovibrio gigas and Allochromatium vinosum. The data offer a new strategy how to engineer oxygen-tolerant hydrogenases for biotechnological application.

Hydrogen metabolism, catalyzed by hydrogenase, is widespread in the microbial world. According to the composition of the hydrogen-activating site, hydrogenases are classified as [NiFe], [FeFe], and [Fe] enzymes (1, 2). [NiFe] hydrogenases predominantly oxidize hydrogen to obtain reducing equivalents, whereas [FeFe] hydrogenases are mostly involved in the reduction of protons to dispose of reducing power. Crystal structures, available for both types of hydrogenase, uncovered a complex architecture of the hydrogen-activating site, showing in addition to the cysteine-bound metals diatomic ligands such as CO and CN⁻ (3).

Hydrogenases are usually sensitive to inhibition by oxygen. In particular [FeFe] hydrogenases are irreversibly destroyed by oxygen, whereas oxygen does not affect the structural integrity of [NiFe] hydrogenases but reversibly inactivates their catalytic function. It was shown by various spectroscopic techniques that an oxygen species is bound between nickel and iron (4). This bridging ligand occupies the position that is required for binding of a formal hydride under turnover conditions (5). The bridging oxygen ligand is removed reductively, hence giving hydrogen access to the catalytic site.

Some microorganisms that thrive in oxic environments harbor oxygen-tolerant [NiFe] hydrogenases capable of metabolizing hydrogen under aerobic conditions. Oxygen-insensitive hydrogenases are of increasing biotechnological interest, e.g. as catalysts in fuel cells or in biological hydrogen production (6, 7). The β-proteobacterium Ralstonia eutropha hosts three different oxygen-tolerant [NiFe] hydrogenases, which enable the organism to use hydrogen as the sole energy source in the presence of oxygen. The periplasmically oriented membrane-bound hydrogenase (MBH) is connected to the respiratory chain via a b-type cytochrome (8, 9). The soluble hydrogenase (SH) is a cytoplasmic enzyme that directly reduces NAD⁺ at the expense of hydrogen (10). The third hydrogenase (H₂-sensing regulatory hydrogenase (RH)) of R. eutropha is not involved in energy conservation but acts as a hydrogen sensor in a H₂-dependent signaling cascade triggering hydrogenase gene transcription (11). Different strategies seem to exist to acquire oxygen tolerance. In the case of SH, two additional cyanide ligands, attached to the [NiFe] site, one bound to the iron and one bound to the nickel, have been identified by Fourier-transformed infrared spectroscopy (12). The lack of the nickel-bound CN⁻ in a mutant protein correlated with the occurrence of oxygen sensitivity of the SH suggesting that the modification of the active site accounts for the oxygen tolerance of this specific enzyme (13). On the other hand, based on spectroscopic and chemical data, the RH of R. eutropha shows a standard [NiFe] site so far as the number of CO and CN⁻ ligands is concerned (14), raising the question as to what kind of mechanism may account for the oxygen tolerance of this hydrogenase.

Defined hydrophobic gas channels were identified in both [NiFe] and [FeFe] hydrogenases (15, 16). It seems as if molecular hydrogen does not randomly diffuse to the active site but is guided through distinct gas channels as demonstrated by xenon labeling experiments in combination with molecular modeling studies. In case of [NiFe] hydrogenases, the gas channel ends close to the nickel of the active site, gated by two conserved hydrophobic amino acids, valine and leucine (Fig. 1A). These residues are highly conserved in [NiFe] hydrogenase large subunits. In the subclass of H₂-sensing hydrogenases, represented by the RH of R. eutropha and the HupUV proteins from Rhodobacter capsulatus (17) and Bradyrhizobium japoni-

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1 The abbreviations used are: MBH, membrane-bound hydrogenase; SH, soluble hydrogenase; RH, H₂-sensing regulatory hydrogenase; ST, Strep-tag.
cum (18), valine and leucine are replaced by the more bulky residues isoleucine and phenylalanine, respectively (19). It was proposed that the presence of these residues might narrow the exit of the gas channel thereby limiting access of molecules larger than \( \text{H}_2 \), e.g. \( \text{O}_2 \), to the \([\text{NiFe}]\) site (20).

To investigate this theoretical consideration experimentally we replaced the corresponding isoleucine and phenylalanine in the RH large subunit HoxC of \( R. \text{eutropha} \) by valine and leucine. In a stepwise approach single and double mutants were constructed that finally yielded the conserved standard signature of \([\text{NiFe}]\) hydrogenases at this specific location of the protein. Analysis of the mutant derivatives clearly revealed oxygen sensitivity of the RH supporting the notion that the shape of the putative gas channel plays an important role in protecting the hydrogenase from inactivation by oxygen.

**MATERIALS AND METHODS**

**Strains and Plasmids**—The strains and plasmids used in this study are listed in Table I. Strains with the initials HF are derived from \( R. \text{eutropha} \) H16. \( E. \text{coli} \) strains and plasmids were used as a control. \( E. \text{coli} \) S17-1 (22) was employed for conjugative plasmid transfer. Mobilizable plasmids were transferred from \( E. \text{coli} \) to \( R. \text{eutropha} \) using a spot mating technique (22).

A plasmid for the overproduction of a \( \text{Strep-tagII} \) protein was constructed as follows. The 162-bp \( 3' \) modified 228-bp SacII fragment from pCH1123 resulting in pCH1124. The 360-bp SacII wild-type fragment from pCH594 was replaced by the synthetic oligonucleotides

\[
5' \text{ACGTTTGGTGGTGGGGCC} -3'
\]

SH promoter was removed from pCH1124 as a 2.68-kbp HindIII-SpeI plasmid transfer. Mobilizable plasmids were transferred from \( E. \text{coli} \) to \( R. \text{eutropha} \) using a spot mating technique (22).

**TABLE I**

**Strains and plasmids used in this study**

| Strain or plasmid | Relevant characteristics | Source or Ref. |
|-------------------|--------------------------|---------------|
| \( R. \text{eutropha} \) H16 | Wild-type, SH \^\text{+} MBH \^\text{+} RH \^\text{+} | \( \text{DSM}482, \text{ATCC} \text{17699} \) |
| HF574 | SH \(^{\text{+}}\) (hox\( \Delta \)) MBH \(^{\text{+}}\) (hox\( \Delta \)) RH \(^{\text{+}}\) (hox\( \Delta \)Box\( \Delta \)) | 24 |
| \( E. \text{coli} \) JM109 | \( F' \) traD36 lacZ, \( \Delta \)lacYZM15 3\text{proA} A^B' \( \text{fl}14^4 \) (\( \text{MerA} \)) \( \Delta \)lac-proAB \( \text{thi} \) gyr\( \text{A96} \), endA1, hsdR17, relA1 supE44 recA1 | 21 |
| S17-1 | Tra\(^{\text{+}}\) recA \text{pro} hsdR, chr-RP4-2 | 22 |

Plasmids

- \( \text{LITMUS 28/29} \): Ap\(^\text{+} \), lacZ, ColE1 ori
- \( \text{pGE574} \): 2.68-kbp HindIII-SpeI fragment from pCH1141 containing P\( \text{SH} \)-tagII-encoding region controlled by the strong \( \text{SH} \) promoter
- \( \text{pGE572} \): 2.68-kbp HindIII-SpeI fragment from pCH1139 containing P\( \text{SH} \)-tagII-encoding region controlled by the strong \( \text{SH} \) promoter
- \( \text{pGE573} \): 2.68-kbp HindIII-SpeI fragment from pCH1140 containing P\( \text{SH} \)-tagII-encoding region controlled by the strong \( \text{SH} \) promoter

**Strains and Plasmids**—The plasmids used in this study were constructed as follows. The 162-bp \( 3' \) modified 228-bp SacII fragment from pCH1123 resulting in pCH1124. The 360-bp SacII wild-type fragment from pCH594 was replaced by the synthetic oligonucleotides

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Media and Growth Conditions—E. coli strains were grown in Luria Broth (LB). R. eutropha strains were grown in modified LB medium containing 0.25% sodium chloride (LSLB) or in mineral salts medium containing 0.4% fructose (FN) or a mixture of 0.2% fructose and 0.2% glycerol (FGN) (25). Solid media contained 1.5% agar. Antibiotics were used at the following concentrations: 100 μg ml⁻¹ ampicillin and 15 μg ml⁻¹ tetracycline. Large scale cultivation of R. eutropha strains was done in FGN medium at a 10-liter scale using a Braun Biostat fermentor. Cells were harvested by low speed centrifugation after 48 h at an OD₅₇₀ of 11. The cell pellet was frozen in liquid nitrogen and stored at −80 °C.

Protein Purification—The wild-type RH and its derivatives were overproduced in R. eutropha as Strep-tag II fusion proteins. Protein purification was carried out either in the presence of air or in the absence of oxygen in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI) under an atmosphere containing 95% N₂ and 5% H₂. 18 g of cells of the respective R. eutropha strain were resuspended in 18 ml of Buffer A (100 mM Tri-HCl (pH 8.0), 150 mM NaCl) and disrupted by two passages through a chilled French pressure cell at 1100 p.s.i. (SLM Aminco). Cell debris and membranes were removed by ultracentrifugation (45 min, 90,000 × g, 4 °C), and the clear supernatant was subjected to a Strep-Tactin Superflow column (2 ml bed volume; IBA, Göttingen, Germany). The column was washed with 12 column volumes of Buffer A to remove unbound proteins. Subsequently, proteins were eluted with 6 column volumes of Buffer B (Buffer A, 5 mM desthiobiotin) and concentrated by ultrafiltration (Amicon Ultra-15 30,000 MWCO; Millipore, Schwalbach, Germany).

Assays of Enzymatic Activity—H₂ oxidizing activity was quantified by an amperometric H₂ uptake assay as described before (26) using a H₂-electrode with methylene blue as an electron acceptor. One unit of H₂-oxidizing blue oxidoreductase activity was the amount of enzyme that catalyzed the consumption of 1 μmol of H₂ per min. Protein concentrations were determined according to the protocol of Bradford (27).

Calculation of the Gas Channels—The crystal structure of the D. gigas [NiFe] hydrogenase (Protein Data Bank entry 2FRV) was used as the basis for calculating the putative gas channels in wild-type and mutant RH from R. eutropha. In D. gigas hydrogenase, the valine and the leucine residue relevant to this study were replaced by isoleucine and phenylalanine, respectively, using the program O (28). After selecting the side chain rotamers of these residues, which do not cause steric conflicts with their local environment, the geometry was optimized by energy minimization using DeepView/Swiss-PdbViewer (www.expasy.org/spdbv/). The program CAVEnv (29) of the CCP4 suite (30) was used to calculate the gas channel as the cavity accessible to a probe atom of a given radius.

RESULTS

Modeling of the Putative Gas Channel of the RH Based on the Structure of the D. gigas [NiFe] Hydrogenase—A crystal structure of an H₂-sensing hydrogenase is not available so far. Thus, the structure of the D. gigas [NiFe] hydrogenase was used to analyze the RH from R. eutropha for the presence of intramolecular hydrophobic cavities. A universal channel running from the protein surface to the vacant binding site of hydrogen at the [NiFe] active site was visualized by using a probe radius of 1.0 Å (Fig. 1A). A gas channel of that size would allow not only hydrogen but also oxygen to enter the active site. The two conserved hydrophobic residues highlighted in Fig. 1A were replaced in silico by the two more bulky residues as found in the sequence of HoxC, the RH large subunit. After geometry optimization, the internal cavities were also calculated for that derivative. Fig. 1B clearly shows that the hydrophobic channel was interrupted by the amino acid replacements, no longer allowing oxygen to enter the active site. This theoretical consideration could explain why the RH is not sensitive to inhibition by oxygen (14, 20).

To test this hypothesis experimentally, site-directed mutagenesis was employed to replace the bulky residues present in the sequence of HoxC (Fig. 1B) by the smaller residues that are present in standard [NiFe] hydrogenases, e.g., in the D. gigas enzyme (Fig. 1A). Therefore, isoleucine at position 62 of HoxC was replaced by valine (I62V). In analogy, phenylalanine at position 110 of HoxC was replaced by leucine (F110L). Finally, a double mutant was constructed harboring both replacements (V+L) to generate a situation as found in the oxygen-sensitive D. gigas hydrogenase (Fig. 1A). The mutations were introduced into a plasmid which provides the basis for overexpression and fast purification of the RH. The plasmid harbors a modified RH small subunit gene (hoxB), which leads to the production of a polypeptide in which the C-terminal 55 amino acids are replaced by a Strep-tag II sequence. The so-called RHstopST protein is beneficial for in vitro studies, because (i) it can easily be isolated by affinity chromatography, and (ii) unlike the wild type this RH derivative forms a monodimer (αβ) and not a double dimer ((αβ)₂) complexed with a histidine protein kinase (31). This monodimeric RH conformation resembles the standard [NiFe] hydrogenases. Recently it was dem-

![Fig. 1. Zoomed view of the gas channel, shown as a fine grid, of D. gigas [NiFe] hydrogenase.](image-url)
Table II

| RH derivative | Specific H₂ uptake activity | units of protein |
|---------------|-----------------------------|----------------|
|               | After aerobic purification | After anaerobic purification |
|               |  |  |
| Wild-type (RHstopST) | 1.87 | 1.78 |
| I62V          | 0.06 | 0.93 |
| F110L         | 0.30 | 1.27 |
| V+L           | <0.01 | 0.84 |

The values given are the mean of three independent experiments. Standard deviations were less than 10%.

FIG. 2. SDS-PAGE analysis of purified RH samples. Preparations of RHstopST and its derivatives were separated by SDS-PAGE (12% gels) and subsequently stained with Coomassie Brilliant Blue. 5 μg of protein of the respective preparation was subjected on each lane as indicated above the figure.

FIG. 3. Reductive reactivation of the V+L mutant protein. H₂ uptake activity of aerobically purified RH samples was recorded in an amperometric assay using a Clark-type electrode that directly detects the amount of hydrogen present in the reaction chamber (26). The addition of hydrogen-saturated water (H₂; 51 μM final concentration), dithionite (Dit; 1 mM) and protein (Prot; about 200 nM) is indicated by arrows. After 1 min of incubation the reaction was started by addition of methylene blue (MB; 1.5 mM).

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Standard deviations were less than 10%.

within 2 h, and the V was fairly stable during this treatment for at least 3 h.

After 30 min of air exposure (Fig. 4). The wild-type RH activity observed with the mutant proteins was not the result of protein instability.

Attempts were made to reductively activate the purified V+L mutant protein. Exposure to an atmosphere of 100% hydrogen for 24 h was not sufficient to restore H₂ uptake activity (data not shown). The mutant protein was, however, successfully reactivated upon treatment with dithionite in the presence of hydrogen (Fig. 3). Control experiments revealed that dithionite had no effect on the H₂ uptake activity of the oxygen-tolerant wild-type RH. Thus, these findings strongly indicate that the loss of activity of the mutant protein originates from reversible oxidative inactivation, a property that has been described for standard [NiFe] hydrogenases (33–35).

To analyze the effect of oxygen in more detail, protein purification was performed under exclusion of oxygen in a glovebox. The mutant proteins only displayed a moderate decrease of H₂ uptake activity maintaining a level of 50–70% compared with the wild-type level (Table II). This result contrasts the data obtained after aerobic protein isolation (Table II) and implies that during purification the mutant proteins are inactivated by oxygen. Moreover, a rapid inactivation of the anaerobically purified mutant proteins was observed when the anaerobic atmosphere was replaced by air. In case of the I62V and the F110L mutant protein, H₂ activity was decreased by 50% within 2 h, and the V+L mutant protein lost 50% of its activity after 30 min of air exposure (Fig. 4). The wild-type RH activity was fairly stable during this treatment for at least 3 h.

**DISCUSSION**

Molecular hydrogen is considered as an important renewable energy source for future technologies. At least two modes of application of hydrogen-metabolizing protein catalysts are being discussed. First, hydrogenases may be used as catalysts in hydrogen production. One approach focuses on coupling oxygenic photosynthesis with biological hydrogen production by using green algae that harbor hydrogen-evolving [FeFe] hydrogenases (7). Photosynthesis, however, produces oxygen, which severely inactivates the oxygen-sensitive algal hydrogenase. This dilemma has to be solved experimentally before the system can be applied successfully. The second approach is directed to the use of hydrogenase in biological fuel cells (6). The oxygen sensitivity of hydrogenases, again, turned out to be one of the major problems in maintaining a stable system.

The identification of defined hydrophobic channels within hydrogenases that guide gaseous molecules to the active site paved the way for the present study. On the basis of the x-ray structures of the [NiFe] hydrogenases of *D. gigas* and *D. fructosovorans*, molecular dynamics simulations of hydrogen diffusion in combination with xenon labeling experiments showed that defined channels serve as pathways for gas access to the active site (15, 20). Hydrophobic gas channels have also been described for the CO dehydrogenase/acyetyl-CoA synthase from *Moorella thermoacetica* (36) and for the [FeFe] hydrogenase from *Desulfovibrio desulfuricans* (16). Recently, hydrogen and
oxygen diffusion in the [FeFe] hydrogenase from Clostridium pasteurianum (CpI) was studied by molecular dynamics simulation. It was shown that hydrogen can randomly diffuse through the protein, although the majority of the molecules migrate through a number of conserved cavities. Oxygen, on the other hand, exclusively migrates through these hydrophobic channels (37). Thus, the physical properties of the gas itself seem to be crucial for the access to the active site of hydrogenase.

The concept of defined gas channels inspired us to examine the molecular background of oxygen tolerance using the RH from R. eutropha. We addressed the question as to whether the size or shape of the putative gas channel determines whether molecules larger than hydrogen can enter the active site. Previous biochemical and spectroscopic analysis of the RH had demonstrated that the protein is absolutely insensitive to inactivation or shape of the putative gas channel determines whether molecules larger than hydrogen can enter the active site. Pre-vious biochemical and spectroscopic analysis of the RH had demonstrated that the protein is absolutely insensitive to inhibition by oxygen (14, 26). Biochemical data of the present study clearly showed that modification of the putative gas channel by site-directed mutagenesis yielded mutant proteins that are no longer oxygen-resistant. Replacement of either of the two residues gating the gas channel by a smaller residue resulted in moderate oxygen sensitivity of the RH, whereas construction of the V+L double mutant led to a protein which was completely inactive in the presence of oxygen. Inactivation of the mutant proteins, however, was reversible. Thus, the mechanism of oxidative inactivation and reductive reactivation of the RH derivatives resembled that of standard [NiFe] hydrogenases such as the enzyme from D. gigas (38). This indicates that the active site of RH itself is sensitive to inhibition by oxygen as described for the standard hydrogenases.

The binding of either hydrogen or oxygen to the active site of standard [NiFe] hydrogenases is well characterized by using a number of spectroscopic techniques. In particular EPR spectroscopy had been employed to examine the various redox states of [NiFe] hydrogenases (39). In the presence of hydrogen a formal hydride binds to the [NiFe] as a bridging ligand between the two metals. Hydrogen binding is accompanied by the oxidation of the nickel ion from Ni2+ to Ni3+ resulting in the EPR-detectable Ni-C state of the reduced enzyme. The electron released by the nickel ion is transferred to an iron-sulfur cluster located in the hydrogenase small subunit. The reversible switch from the EPR-silent Ni-Si state with the vacant binding site for hydrogen to the EPR-active Ni-C state has been described not only for standard [NiFe] hydrogenases but also for the RH from R. eutropha (14, 26). In the case of standard enzymes, the reaction of the [NiFe] site with oxygen also results in the oxidation of the nickel ion from Ni2+ to Ni3+, which can be detected by EPR as the so-called Ni-A and Ni-B states of the oxidized, inactive enzyme (40). In contrast to standard [NiFe] hydrogenases, the RH shows neither a Ni-A nor a Ni-B state under oxidative conditions, being another argument for the assumption that oxygen cannot bind to the active site of the RH (14).

On the other hand, binding of oxygen to the active site may not automatically lead to the oxidation of the nickel ion and therefore remains EPR-silent. For example, the [NiFe] active site of the oxidized SH of R. eutropha is EPR-silent. X-ray absorption spectroscopy analysis, however, revealed that an oxygen species, presumably a peroxide, is bound to nickel in the oxidized state of the SH. Moreover it was shown that this ligand is removed upon reduction of the enzyme (41). In case of the SH, two different mechanisms of oxygen protection have been discussed. First, an additional CN− ligand bound to the nickel of the [NiFe] site contributes to the oxygen tolerance of the enzyme. Mutant proteins devoid of the nickel-bound CN− ligand turned out to be oxygen-sensitive (13, 41). Second, x-ray absorption spectroscopy revealed that in contrast to standard [NiFe] hydrogenases the active site of the SH is coordinated by more oxygen ligands and by less sulfur ligands. The different coordination of the [NiFe] active site may also contribute to the oxygen tolerance of the enzyme (41). Variations of the coordination of the [NiFe] site have also been observed for the RH of R. eutropha. X-ray absorption spectroscopy analysis of the oxidized protein revealed that nickel is coordinated by three sulfur and two oxygen ligands (42). On the basis of the biochemical data presented in this study, we propose that due to an enlarged gas channel oxygen directly binds to the [NiFe] site, thus leading to oxygen-sensitive RH derivatives. Currently, we cannot exclude that the amino acid replacements possibly alter the coordination of the [NiFe] active site, thus contributing to an increased oxygen sensitivity of the protein. A detailed spectroscopic analysis of the RH mutant proteins, including EPR and x-ray absorption spectroscopy, is under way.

In conclusion, our findings show a clear direction for how to cope with problems of oxygen-sensitive hydrogenases. The results give insights into variations of the reaction mechanism of [NiFe] hydrogenases and provide clues for engineering oxygen-tolerant enzymes in applied research.

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REFERENCES

1. Schwartz, E., and Friedrich, B. (2003) in The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community (Dworkin, M., Schleifer, K. H., and Stackebrandt, E., eds) 3rd Ed., Release 3.14, Springer, New York

2. Vignais, P. M., and Colbeau, A. (2004) Curr. Issues Mol. Biol. 6, 159–188

3. Nicolet, Y., Cavazza, C., and Fontecilla-Camps, J. C. (2002) J. Inorg. Biochem. 91, 1–8

4. Armstrong, F. A. (2004) Curr. Opin. Chem. Biol. 8, 133–140

5. Breech, M., van Gastel, M., Buhrze, T., Friedrich, B., and Lubitz, W. (2003) J. Am. Chem. Soc. 125, 13075–13083

6. Karyakin, A. A., Morezev, S. V., Karyakina, E. E., Zorin, N. A., Pereklyug, V. V., and Cosnier, S. (2005) Biochem. Soc. Trans. 33, 73–75

7. Melis, A., and Happe, T. (2001) Plant Physiol. 127, 740–748

8. Schink, B., and Schlegel, H. G. (1979) Biochim. Biophys. Acta 567, 315–324

9. Bernhard, M., Benelli, B., Hochkoeppler, A., Zannoni, D., and Friedrich, B. (1997) Eur. J. Biochem. 245, 179–186

10. Schneider, K., and Schlegel, H. G. (1976) Biochim. Biophys. Acta 452, 66–80

11. Lenz, O., and Friedrich, B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 12474–12479

12. Happe, R. P., Roseboom, W., Egert, G., Friedrich, C. G., Massanz, C., Fried, P., and Albracht, S. P. (2000) FEBS Lett. 466, 209–263

13. Bleilevans, B., Buhrze, T., van der Linden, E., Friedrich, B., and Albracht, S. P. (2004) J. Biol. Chem. 279, 46666–46691

14. Bernhard, M., Buhrze, T., Bleilevans, B., De Lacey, A., Fernandez, Y. M., Albracht, S. P., and Friedrich, B. (2001) J. Biol. Chem. 276, 15592–15597

15. Montet, Y., Amara, P., Volbeda, A., Vernede, X., Hatchikian, E. C., Field, M. J., Frey, M., and Fontecilla-Camps, J. C. (1997) Nat. Struct. Biol. 4, 523–526

16. Nicolet, Y., Piras, C., Legrand, P., Hatchikian, C. E., and Fontecilla-Camps, J. C. (1999) Structure Fold. Des. 7, 13–23

17. Elen, S., Colbeau, A., Chabert, J., and Vignais, P. M. (1996) J. Bacteriol. 178, 5174–5181
Oxygen Tolerance of the RH from R. eutropha

18. Black, L. K., Fu, C., and Maier, R. J. (1994) J. Bacteriol. 176, 7102–7106
19. Kleihues, L., Lenz, O., Bernhard, M., Buhrke, T., and Friedrich, B. (2000) J. Bacteriol. 182, 2716–2724
20. Volbeda, A., Montet, Y., Vernede, X., Hatchikian, C., and Fontecilla-Camps, J. C. (2002) Int. J. Hydrogen Energy 27, 1449–1461
21. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Gene (Amst.) 33, 103–119
22. Simon, R., Priefer, U., and Hülser, A. (1983) Bio/Technology 1, 717–743
23. Chen, B., and Przybyla, A. E. (1994) BioTechniques 17, 657–659
24. Buhrke, T., Brecht, M., Lübzyt, W., and Friedrich, B. (2002) J. Biol. Inorg. Chem. 7, 897–908
25. Schwartz, E., Gerischer, U., and Friedrich, B. (1998) J. Bacteriol. 180, 3197–3204
26. Pierik, A. J., Schmelz, M., Lenz, O., Friedrich, B., and Albracht, S. P. (1998) FEBS Lett. 438, 231–235
27. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
28. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kieldgaard, M. (1991) Acta Crystallogr. Sect. A 47, 110–119
29. Volbeda, A. (1999) Les Ecoles Physique et Chimie du Vivant, numero 1, pp. 47–52, CNRS Editions, Paris
30. Collaborative Computational Project, No. 4 (1994) Acta Crystallogr. Sect. D Biol. Crystallogr. 50, 760–763
31. Buhrke, T., Lenz, O., Porthun, A., and Friedrich, B. (2004) Mol. Microbiol. 51, 1677–1689
32. Buhrke, T., Lösch, S., Lenz, O., Schloßer, E., Zebger, I., Andersen, L. K., Hildebrandt, P., Meyer-Klaucke, W., Dau, H., Friedrich, B., and Haumann, M. (2005) J. Biol. Chem. 280, 19488–19495
33. Carepo, M., Tierney, D. L., Brondino, C. D., Yang, T. C., Pamplona, A., Telser, J., Moura, I., Moura, J. J., and Hoffinan, B. M. (2002) J. Am. Chem. Soc. 124, 281–286
34. Bleelevens, B., van Broekhuizen, F. A., De Larey, A. L., Roseboom, W., Fernandez, V. M., and Albracht, S. P. (2004) J. Biol. Inorg. Chem. 9, 743–752
35. Lamle, S. K., Albracht, S. P., and Armstrong, F. A. (2004) J. Am. Chem. Soc. 126, 14899–14909
36. Serva, J., and Ragsdale, S. W. (2000) Biochemistry 39, 1274–1277
37. Cohen, J., Kim, K., Posewitz, M., Gharardi, M. L., Schulten, K., Seibert, M., and King, P. (2005) Biochem. Soc. Trans. 33, 80–82
38. Cammack, R., Fernandez, V. M., and Schneider, K. (1998) Biochimie (Paris) 80, 85–91
39. Albracht, S. P. J. (2001) in Hydrogen as a Fuel-Learning from Nature (Cammack, R., Frey, M., and Robson, R., eds) pp. 116–125, Taylor & Francis, London
40. Bleelevens, B., Faber, B. W., and Albracht, S. P. (2001) J. Biol. Inorg. Chem. 6, 763–769
41. Burgdorf, T., Łoscher, S., Liebisch, P., Van der Linden, E., Galander, M., Lendzian, F., Meyer-Klaucke, W., Albracht, S. P., Friedrich, B., Dau, H., and Haumann, M. (2005) J. Am. Chem. Soc. 127, 576–592
42. Haumann, M., Porthun, A., Buhrke, T., Liebisch, P., Meyer-Klaucke, W., Friedrich, B., and Dau, H. (2003) Biochemistry 42, 11004–11015
43. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950
44. Lawrence, M. C., and Bourke, P. (2000) J. Appl. Crystallogr. 33, 990–991
45. Merritt, E. A., and Bacon, D. J. (1997) Methods Enzymol. 277, 505–524