CHAPTER 7

Conclusions and Outlook

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The metal cofactors of the three O_2-resistant Ni-Fe hydrogenases of *R. eutropha* are the subject of this thesis. In close cooperation with several workgroups (e.g. in the Sonderforschungsbereich 498) a spectroscopic approach, combining X-ray absorption spectroscopy (XAS) and complementary techniques (e. g. EPR, FTIR), has been applied for their investigation. The XAS experiments were performed at two synchrotron radiation sources (EMBL at DESY, BESSY). The experimental results, in combination with XANES and EXAFS simulations, provided structural features of the metal cofactors in various intermediates of the catalytic pathway of the enzymes. We have achieved a basic characterization of the metal sites. Here, a comprehensive overview of key results on the hydrogenases from *R. eutropha* as obtained in this work is provided.

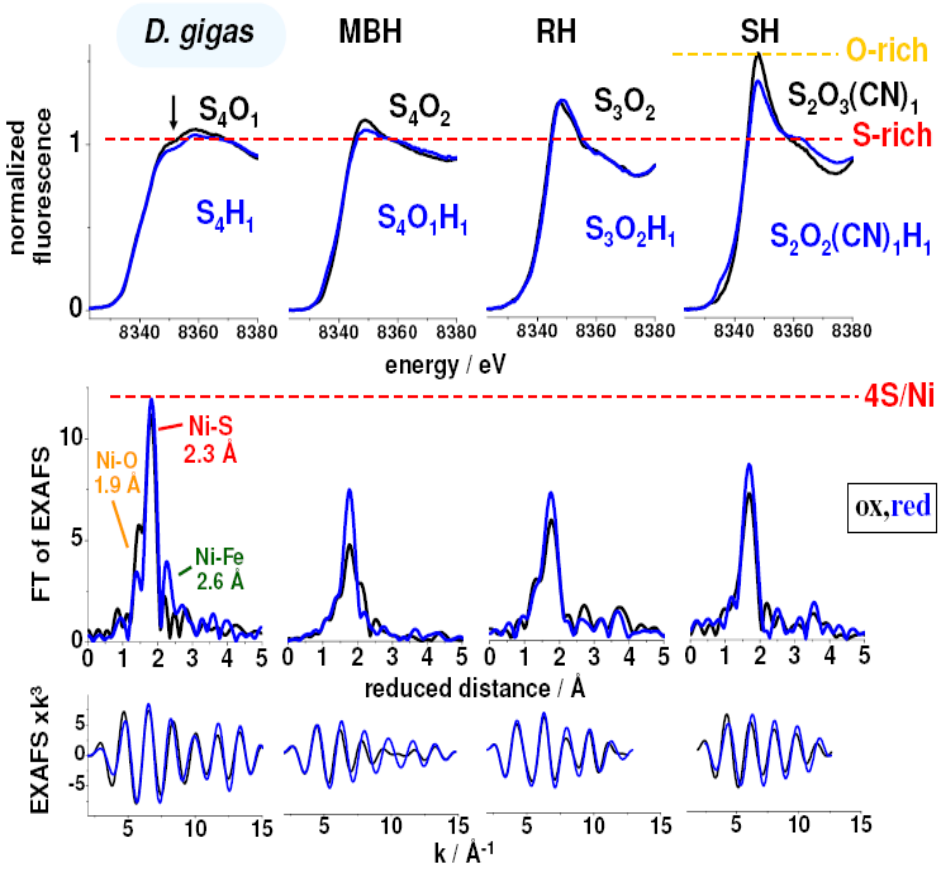

Figure 1: Overview of the Ni XANES and EXAFS spectra of the four hydrogenases obtained in this work. Note the pronounced differences between the hydrogenases from *R. eutropha* and *D. gigas.*

(1) XAS at the Ni K-edge has yielded quantitative information on the Ni site structures (Figs. 1,2). The structural features obtained for the Ni site of the *D. gigas* hydrogenase are in line with the crystallographic data. The structure of the Ni site in all three hydrogenases from *R. eutropha* differs from that in *D. gigas*. A general feature of the Ni site in the *R. eutropha* hydrogenases seems to be the presence of additional oxygen ligands bound to the Ni. In *D. gigas* hydrogenase, the Ni is coordinated by four thiols from cysteines plus one oxygen species in a Ni-Fe bridging position in the inactive state. In the hydrogenases from *R. eutropha*, in both inactive and active states Ni-O interactions are observed. The respective O-ligands may stem from sulfenates (Cys-SO) formed under aerobic conditions. The presence of sulfenates in *R. eutropha* may destabilize bridging oxygen species and thus, O_2-inhibited states. This may be one reason why the hydrogenases from *R. eutropha* are not inhibited by O_2.

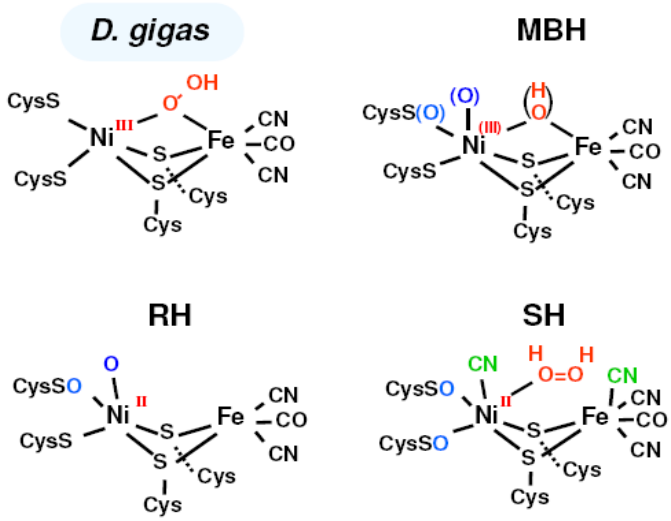

Figure 2: Tentative structural models of the Ni-Fe sites (as-isolated states). The additional O-ligands to Ni are apparent in the XAS data, but their locations are tentative.

On basis of the spectroscopic results, tentative structural models of the Ni-Fe sites have been formulated (Fig. 2). It has to be noted that the true three dimensional structure of the Ni-Fe site cannot be directly obtained by the applied spectroscopic approach. The structural models of the Ni-Fe site in *R. eutropha* hydrogenases represent working models which have been designed in analogy to the crystal structure of the standard hydrogenases. In particular it is unclear, which ones of the four cysteines are modified to sulfenates. Whether the proposed models are compatible with EPR data (e.g. on protein crystals) remains to be shown. At least in recent crystal structures of standard hydrogenases, sulfenates have been resolved. Further investigations are required to clarify this issue. In any event, the presence of sulfenates may favor the active-catalyst conformation of the Ni-Fe site of the H_2ases of *R. eutropha*.

(2) Investigations on the nature of the Fe-S clusters revealed an uncommon Fe-S cluster complement in all three O_2-tolerant hydrogenases of *R. eutropha* (Fig. 3). The XAS data excludes an Fe-S complement such as the one found in the *D. gigas* hydrogenase (two [4Fe-4S] and one [3Fe-4S] clusters). The results on the hydrogenases of *R. eutropha* indicate the presence of [2Fe-2S] cluster(s). In the RH additionally a [4Fe-3O-2S] cluster may be present. The presence of unusual Fe-S clusters in the hydrogenases of *R. eutropha* may be related to their function under aerobic conditions. One option is that the redox potentials of the Fe-S clusters are adjusted to the more positive potential at ambient O_2 partial pressure so that efficient electron transfer out of and towards the Ni-Fe active site also functions under such conditions. Future experiments in particular will address this question.

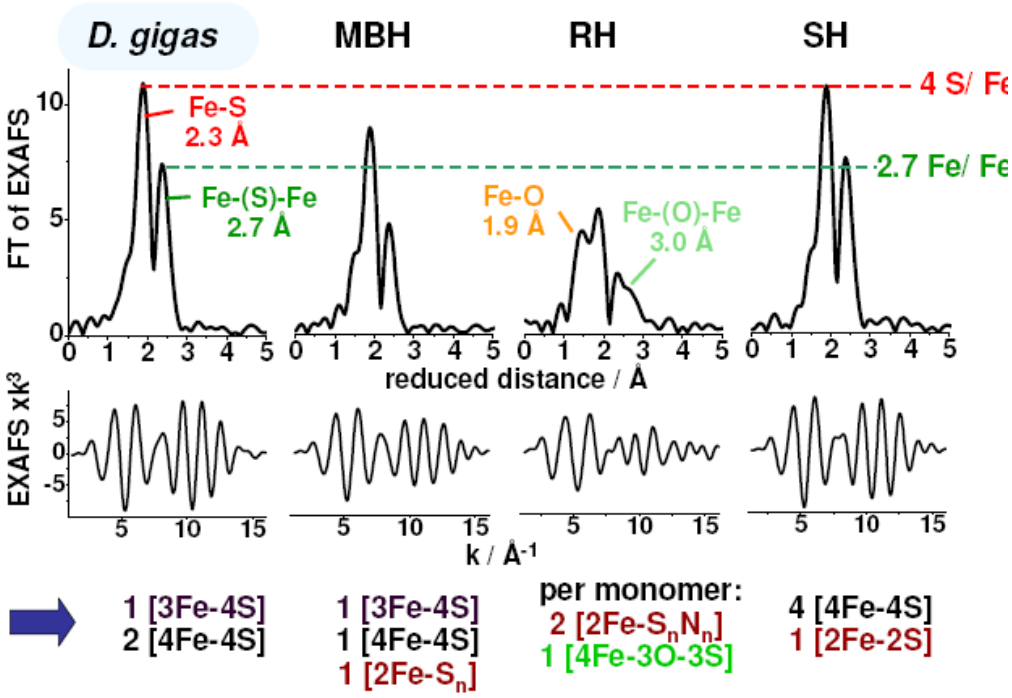

Figure 3: Comparison of the Fe XAS spectra of the four hydrogenases, pointing to a different Fe-S cluster complement in the hydrogenases of *R. eutropha* compared to the *D. gigas* enzyme. Lower row: Tentative assignment of the Fe-S cluster complements.

(3) EPR spectroscopy provides information on both, the oxidation state of the Ni and of the Fe-S clusters. The EPR data reveals pronounced differences between the hydrogenases of *R. eutropha* and the *D. gigas* enzyme (Fig. 4). A common feature of the *R. eutropha* hydrogenases is that inactive states (Ni-A, Ni-B) are not at all populated (RH, SH) or present only in a minor fraction of protein (MBH) at ambient pO_2. Notably most of the intermediates in the catalytic cycle seem to be EPR-silent. The absence of

the Ni-A state ensures rapid activation of the enzymes in the presence of H_2. This feature is favourable for efficient use of H_2 as an energy source in the presence of O_2. In the MBH and the RH, the active catalyst may be the prevailing form of the Ni-Fe site at ambient pO_2, in line with the XAS data. In the SH, O_2-tolerant H_2 catalysis may proceed via a pathway, that does not involve Ni(III) species at all (no Ni-A, B, C). The absence or presence of certain intermediates in the catalytic cycle may be a prerequisite for oxygen-tolerant H_2 catalysis. This idea has to be scrutinized in further experiments. EPR data point to a non-standard Fe-S cluster complement in the three hydrogenases of *R. eutropha*, in line with Fe XAS data. That the magnetic interactions between the Fe-S clusters differ from the *D. gigas* case may suggest an altered overall arrangement of the Fe-S clusters in the protein and/or different relaxation properties of the Fe-S clusters. Further information is required to finally assign the chemical nature and relative positioning of the Fe-S clusters in the *R. eutropha* hydrogenases.

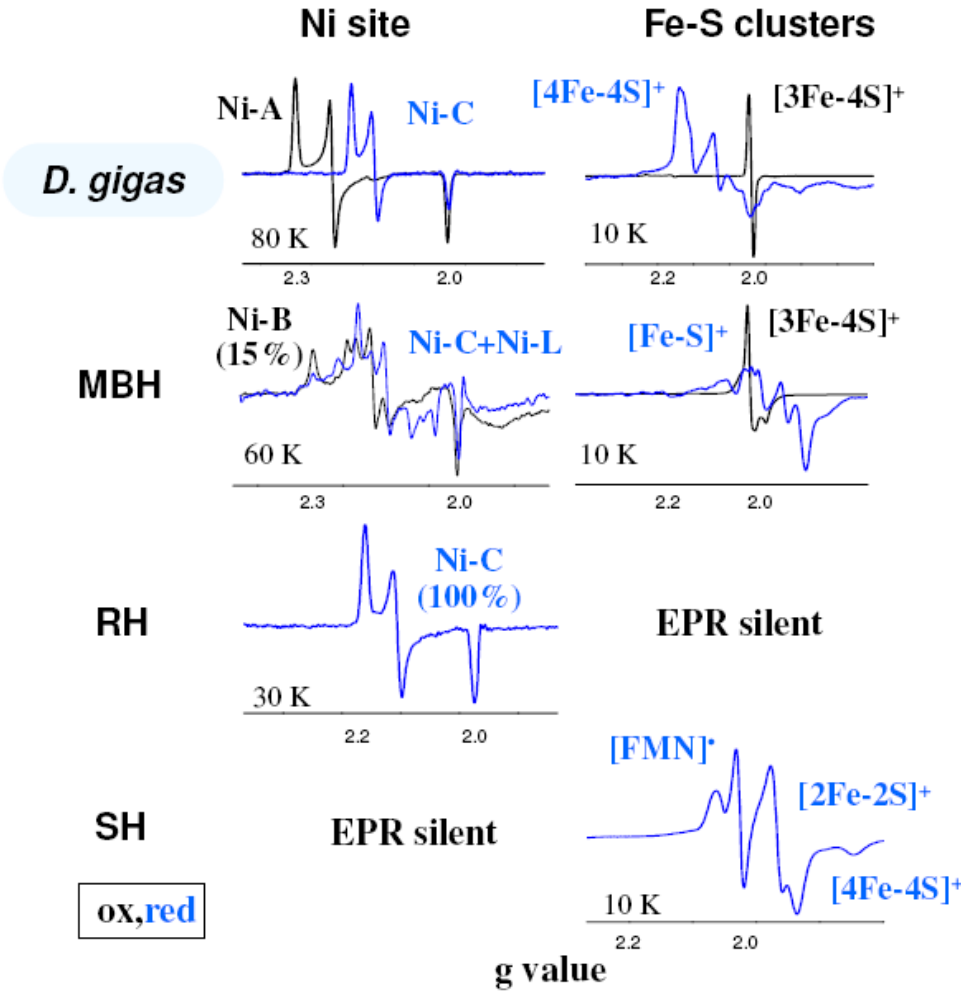

Figure 4: Overview of EPR spectra to characterize the Ni-Fe and the Fe-S cofactors in the four enzymes. Ni-A is not observed in *R. eutropha*.

The structural information on the metal cofactors derived in this work cumulates in general ideas how the Ni-Fe site and the Fe-S clusters in the hydrogenases from *R. eutropha* are arranged. The exceptional oxygen-tolerance of these enzymes may be mirrored by these structural features. The "hard" information that can be gained by the applied spectroscopic approach critically depends on the quality of the protein samples. In this respect, further improvements by the development of new purification procedures may be necessary.

In summary, a basal characterization of the metal cofactors of the hydrogenases from *R. eutropha* has been achieved in this work. The specific design of the Ni-Fe site and of the Fe-S clusters may be one reason for their O_2-tolerance.

The information on the overall protein organization, on the location and identity of metal cofactors, and on redox and structural changes in the intermediates of the catalytic cycle tentatively is summarized in Figure 5. It has to be emphasized that the shown models represent working hypotheses, summarizing the available information gained from spectroscopy and molecular biology. The depicted models are compatible with XAS, EPR, FTIR results. However, certain features may have to be revised if further information becomes available. In any event, the models in Fig. 5 are considered as a useful basis to design future experiments, addressing the sequence of events in the O_2-tolerant hydrogen catalysis of the hydrogenases from *R. eutropha.*

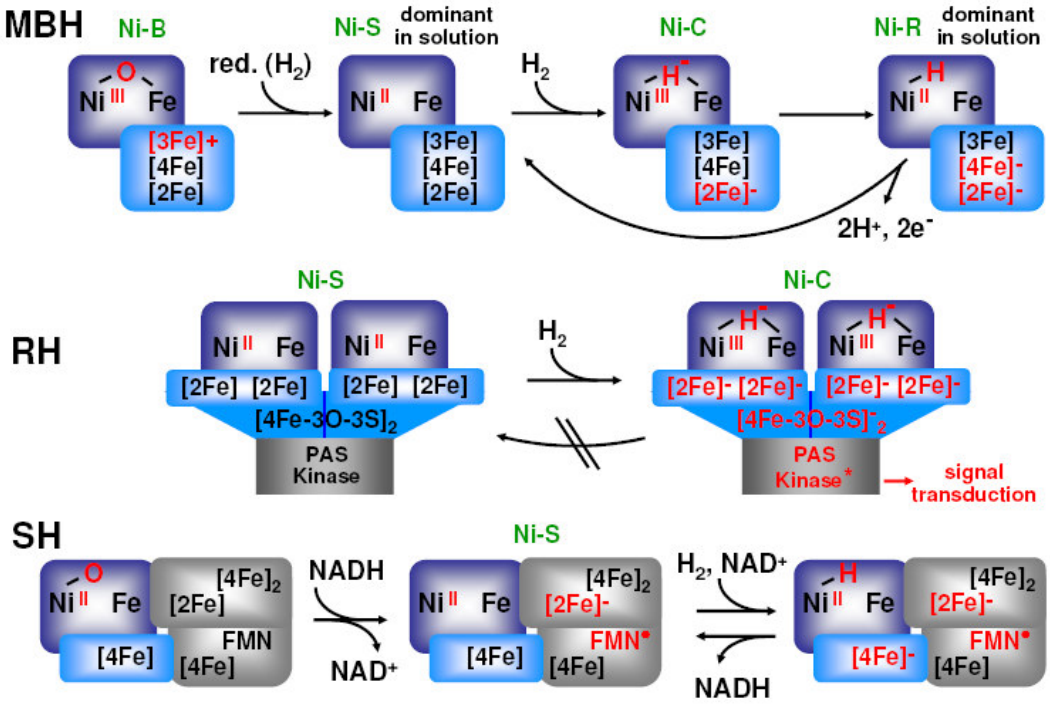

Figure 5: Tentative assignment of redox-reactions and cofactor locations in the hydrogenases of *R. eutropha* on the basis of spectroscopic and molecular biology results.