Stepwise assembly of the [NiFe]-hydrogenase active site

G. Caserta,[a]* S. Hartmann,[a] C. V. Stappen,[b] C. K. Retsou,[a] C. Lorent,[a] S. Yelin,[c] M. Keck,[c] J. Schoknecht,[a] I. Sergeev,[d] Y. Yoda,[e] C. Limberg,[c] P. Hildebrandt,[a] S. DeBeer,[b] I. Zebger,[a] S. Frielingsdorf,[a] and O. Lenz[a]*

[a] Institut für Chemie, Technische Universität Berlin, Straße des 17. Juni 135, 10623 Berlin, Germany

[b] Department of Inorganic Spectroscopy, Max Planck Institute for Chemical Energy Conversion, Stiftstraße 34-36, 45470 Mülheim an der Ruhr, Germany

[c] Department of Chemistry, Humboldt-Universität zu Berlin, Brook-Taylor-Straße 2, 12489 Berlin, Germany

[d] Deutsches Elektronen-Synchrotron, Notkestraße 85, 22607 Hamburg, Germany

[e] Japan Synchrotron Radiation Research Institute, 1-1-1 Kouto, Sayo-gun, Hyogo 679-5198, Japan

giorgio.caserta@tu-berlin.de

[NiFe]-hydrogenases catalyze the reversible reaction $H_2 \rightleftarrows 2H^+ + 2e^-$ under ambient conditions. Their basic module consists of a large subunit, hosting the heterobimetallic $NiFe(CN)_2(CO)$ cofactor, and a small subunit that carries electron-transferring Fe-S clusters. We have recently shown that the large subunits of the O_2-tolerant regulatory (RH) and membrane-bound (MBH) hydrogenases of *R. eutropha* (HoxC and HoxG, respectively), when separated from the corresponding small subunits (HoxB and HoxK), exhibit catalytic and spectroscopic properties that differ markedly from those of native enzymes.[1,2,3] The de-assembly process is reversible, as the RH subunits can be reassembled *in vitro* to yield a fully active enzyme. This provides a new tool for the isolation of native-like [NiFe]-hydrogenases equipped with ^{57}Fe exclusively at their catalytic site.[4]

Here, we have gone a step further and elucidated the stepwise assembly of the $NiFe(CN)_2(CO)$ cofactor in the large subunit prior to small subunit attachment by isolating key maturation intermediates of HoxG. These included the cofactor-free apo-HoxG, a nickel-free version carrying only the $Fe(CN)_2(CO)$ fragment, a precursor containing all cofactor components but redox-inactive, and the fully mature HoxG. Using biochemical analyses in combination with comprehensive spectroscopic studies, we present detailed insights into the sophisticated maturation process of [NiFe]-hydrogenase.

[1] G. Caserta, C. Lorent, A. Ciaccafava, M. Keck, R. Breglia, C. Greco, C. Limberg, P. Hildebrandt, S.P. Cramer, I. Zebger, O. Lenz, *Chem. Sci.*, **2020**, *11*, 5453–5465.

[2] G. Caserta, V. Pelmenschikov, C. Lorent, A.F. Tadjoung Waffo, S. Katz, L. Lauterbach, J. Schoknecht, H. Wang, Y. Yoda, K. Tamasaku, M. Kaupp, P. Hildebrandt, O. Lenz, S.P. Cramer, I. Zebger *Chem. Sci.*, **2021**, *12*, 2189–2197.

[3] S. Hartmann, S. Frielingsdorf, A. Ciaccafava, C. Lorent, J. Fritsch, E. Siebert, J. Priebe, M. Haumann, I. Zebger, O. Lenz *Biochemistry*, **2018**, *57*, 5339–5349.

[4] G. Caserta, C. Lorent, V. Pelmenschikov, J. Schoknecht, Y. Yoda, P. Hildebrandt, S.P. Cramer, I. Zebger, O. Lenz *ACS Catal.*, **2020**, *10*, 13890–13894.