Iron Hydrogenases

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Dioxygen Sensitivity of [Fe]-Hydrogenase in the Presence of Reducing Substrates

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Abstract: *Mono-iron hydrogenase ([Fe]-hydrogenase) reversibly catalyzes the transfer of a hydride ion from H_2 to methenyltetrahydromethanopterin (methenyl-H_4MPT$^+$) to form methylene-H_4MPT. Its iron guanylylpyridinol (FeGP) cofactor plays a key role in H_2 activation. Evidence is presented for O_2 sensitivity of [Fe]-hydrogenase under turnover conditions in the presence of reducing substrates, methylene-H_4MPT or methenyl-H_4MPT$^+$/H_2. Only then, H_2O_2 is generated, which decomposes the FeGP cofactor; as demonstrated by spectroscopic analyses and the crystal structure of the deactivated enzyme. O_2 reduction to H_2O_2 requires a reductant, which can be a catalytic intermediate transiently formed during the [Fe]-hydrogenase reaction. The most probable candidate is an iron hydride species; its presence has already been predicted by theoretical studies of the catalytic reaction. The findings support predictions because the same type of reduction reaction is described for ruthenium hydride complexes that hydrogenate polar compounds.*

Mono-iron hydrogenase ([Fe]-hydrogenase) reversibly catalyzes the activation and heterolytic cleavage of H_2[1] and transfers the hydride anion to the substrate, methenyltetrahydromethanopterin (methenyl-H_4MPT$^+$), to form methylene-H_4MPT (Figure 1a).[2] The prosthetic group of [Fe]-hydrogenase is the iron guanylylpyridinol (FeGP) cofactor (Figure 1b; Supporting Information, Figure S1).[3] The iron center of the FeGP cofactor consists of a low-spin Fe^{II} ligated with two CO, cysteine-S, pyridinol-N, acyl-C, and a solvent molecule (Figure 1b).[4] The structural and catalytic features of [Fe]-hydrogenases are particularly useful for designing new hydrogenation catalysts.[1,5] Numerous model catalysts that mimic the active site of [Fe]-hydrogenase have been reported.[8]

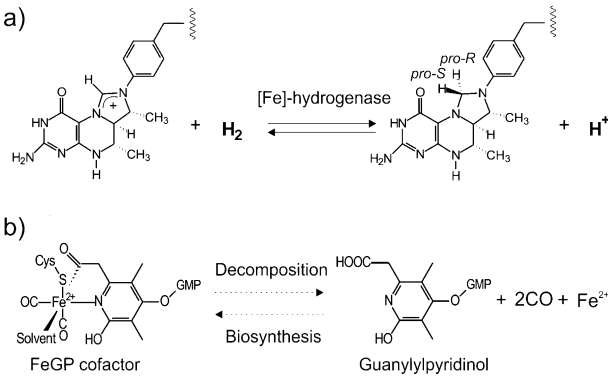

Figure 1. [Fe]-hydrogenase reaction and the FeGP cofactor structure. a) The *pro-R* stereospecific hydride-transfer reaction of [Fe]-hydrogenase.[6] b) Chemical structure of the FeGP cofactor and the light-decomposed product, guanylylpyridinol.[7]

The stability of hydrogenases and corresponding model compounds[9] is a critical factor for their applications. The dinuclear metal sites of [NiFe]- and [FeFe]-hydrogenases are redox active, and most of them react with O_2 and are quickly deactivated.[9,10] Nevertheless, several [NiFe]-hydrogenases from aerobic bacteria are O_2 tolerant to some degree and are able to reduce O_2 to H_2O_2, and further to water.[11] The iron center of the FeGP cofactor in [Fe]-hydrogenase is redox inactive[4a] and insensitive to O_2 under the conditions tested in previous studies.[12] However, reactive oxygen species, such as H_2O_2, and probably superoxide anion (O_2^-), deactivate [Fe]-hydrogenase.[12a] Herein, we report that the FeGP cofactor of [Fe]-hydrogenase is decomposed under aerobic conditions in the presence of reducing substrates. [Fe]-hydrogenase catalyzes reduction of O_2 to H_2O_2 and this reactive oxygen species decomposes the iron complex of the FeGP cofactor.

According to previous reports, [Fe]-hydrogenase is not deactivated in the absence of substrate under 100% O_2 (Supporting Information, Figure S2).[12] First information about a deactivating effect of O_2 on this enzyme was gained by the enzyme assays following the oxidation of methylene-H_4MPT and reduction of methenyl-H_4MPT$^+$. The rate of methylene-H_4MPT oxidation to methenyl-H_4MPT$^+$ decreases progressively under an N_2/O_2 (80%/20%) gas phase compared to 100% N_2 (Figure 2a). Notably, the initial rate of the reaction did not change and the exchange of the gas phase of the O_2-damaged [Fe]-hydrogenase sample to 100% N_2 did not reactivate the enzyme. Likewise, the enzyme activity of the reverse reaction, the reduction of methenyl-H_4MPT$^+$ with H_2,

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Supporting information, including technical details, procedures, sequences, structures, PDB codes, and the ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/anie.201712293.

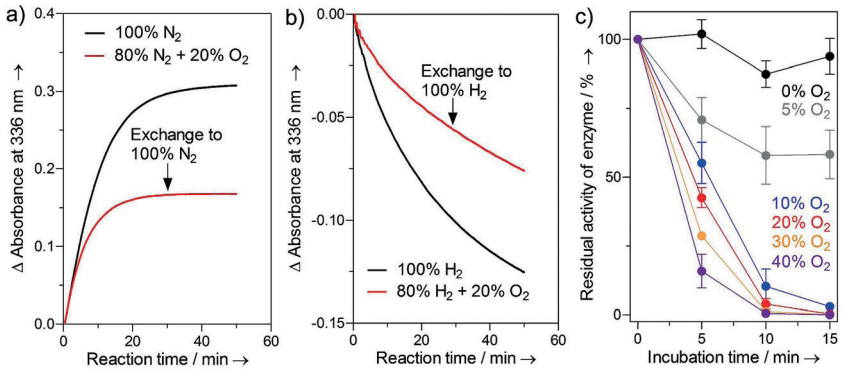

Figure 2. Deactivation of [Fe]-hydrogenase by O_2. a) Oxidation of methylene-H_4MPT to methenyl-H_4MPT$^+$. b) Reduction of methenyl-H_4MPT$^+$ to methylene-H_4MPT. The gas phases were exchanged at the time indicated by arrows. c) Effect of O_2 on [Fe]-hydrogenase activity in the presence of methylene-H_4MPT under N_2 containing 10% H_2 and different concentrations of O_2, as indicated.

also decreases with time under H_2/O_2 (80%/20%) compared to 100% H_2 (Figure 2b). Again, no reactivation has been achieved by exchanging the gas phase to 100% H_2. The initial rate of the reaction did not appear to be changed but the O_2-deactivation rate was quicker than in the case of methylene-H_4MPT oxidation. These results indicated that O_2 does not inhibit but slowly deactivates [Fe]-hydrogenase under the enzyme assay conditions.

Based on these observations, the effect of O_2 on the deactivation of [Fe]-hydrogenase was tested in the presence of variable O_2, H_2, methenyl-H_4MPT$^+$, and methylene-H_4MPT concentrations. We incubated [Fe]-hydrogenase with methylene-H_4MPT under a gas phase containing 10% H_2 and variable concentrations of O_2 (0–40%) prior to testing the activity (Figure 2c). [Fe]-hydrogenase is deactivated progressively and the deactivation rate increases with increasing O_2 concentrations.

The influence of the H_2 concentration on O_2-deactivation of [Fe]-hydrogenase was tested in the presence of the substrates methylene-H_4MPT (Supporting Information, Figure S3a) and methenyl-H_4MPT$^+$ (Supporting Information, Figure S3b). Increasing H_2 concentrations stimulate the deactivation of [Fe]-hydrogenase; the effect of H_2 is more obvious in the case of methenyl-H_4MPT$^+$ than that of methylene-H_4MPT. To study the impact of the substrate concentration, [Fe]-hydrogenase was incubated with various concentrations of methenyl-H_4MPT$^+$ and methylene-H_4MPT under an $N_2/O_2/H_2$ (85%/5%/10%) gas phase. Again, higher substrate concentrations and, concomitantly, a higher turnover rate increased the deactivation rate (Supporting Information, Figure S4).

The irreversibility of O_2 deactivation is presumably a result of the destruction of the FeGP cofactor of [Fe]-hydrogenase, which was investigated by UV/Vis and IR spectroscopies, and X-ray crystallography. UV/Vis absorbance peaks at 300 and 360 nm correspond to the absorbance of the pyridinol ring and iron complex of the intact FeGP cofactor, respectively. Upon deactivation with O_2, the absorbance at 360 nm decreases and the absorbance at 300 nm increases (Supporting Information, Figure S5). These changes indicate a decomposition of the FeGP cofactor because the same UV/Vis spectral behavior was observed when the iron site of [Fe]-hydrogenase was degraded by light.[12b,13] Initially, IR spectroscopic analysis confirmed the presence of the bands at 2008 and 1938 cm^{-1} of the intrinsic CO ligands of the enzyme-bound FeGP cofactor, which were previously reported (Supporting Information, Figure S6).[4b,d,12a] In the absence of methylene-H_4MPT and methenyl-H_4MPT$^+$, the bands did not change after incubating the enzyme under $O_2/N_2/H_2$ (20%/80%/0%) and $O_2/N_2/H_2$ (20%/70%/10%) gas phases (Supporting Information, Figure S6a). In their presence, however, the IR peaks decrease and almost disappear after long-term (1 h) incubation at 40°C indicating the loss of the CO ligands (Supporting Information, Figure S6b).

The 1.29 Å structure of O_2-deactivated enzyme (Supporting Information, Table S1) clearly revealed that the FeGP cofactor is decomposed into guanylylpyridinol (Figure 3b). Guanylylpyridinol was identified as a light-deactivated product of the isolated FeGP cofactor (Figure 1b).[7] The iron in the O_2-deactivated enzyme has lost pyridinol-N, acyl-C, and two CO ligands, but is still coordinated with Cys172-S, and

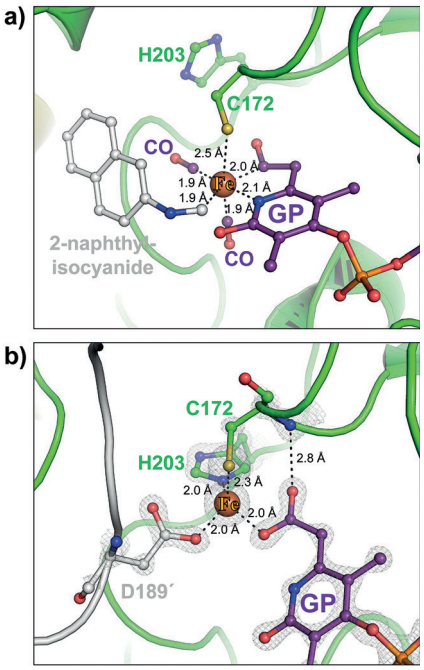

Figure 3. Crystal structure of O_2-deactivated [Fe]-hydrogenase at 1.29 Å resolution. a) The active-site structure of the intact [Fe]-hydrogenase inhibited with 2-naphthylisocyanide,[14] which is the only available crystal structure of [Fe]-hydrogenase derived from *Methanothermobacter marburgensis*. b) The active-site structure of the O_2-deactivated [Fe]-hydrogenase, in which the $2F_o-F_c$ electron density contoured at 3.0 σ is represented (Supporting Information, Table S1). GP: guanylylpyridinol.

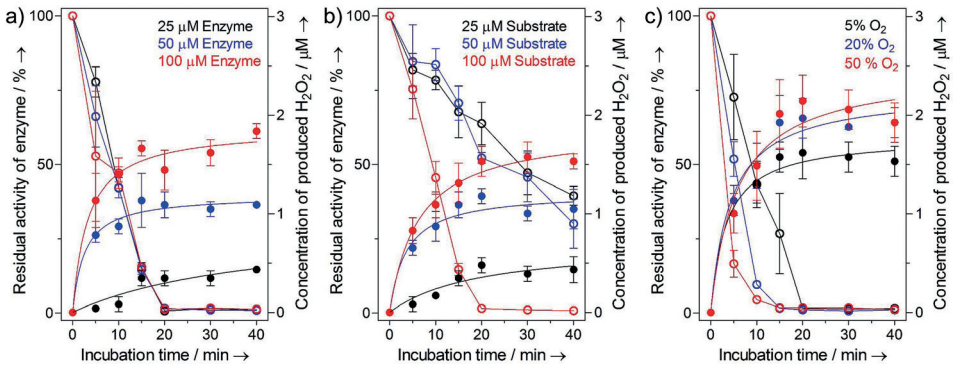

Figure 4. Kinetics of deactivation of [Fe]-hydrogenase exposed to O_2 in the presence of methylene-H_4MPT and H_2O_2 formation. a) Methylene-H_4MPT (100 μM) and variable concentrations of [Fe]-hydrogenase (enzyme) under N_2/O_2 (95%/5%). b) [Fe]-hydrogenase (100 μM) and variable concentrations of methylene-H_4MPT (substrate) under N_2/O_2 (95%/5%). c) [Fe]-hydrogenase (100 μM) and methylene-H_4MPT (100 μM) under N_2 with variable concentrations of O_2. The residual activity (○) and the respective H_2O_2 concentrations (●) were determined. The standard error of three measurements was calculated.

forms new bonds with the carboxymethyl-O of the guanylylpyridinol, His203, and Asp189 from the partner monomer. Thus, the number of ligands of iron is decreased from six to four by decomposition (Figure 3).

Based on the presented findings and previous data regarding decomposition of [Fe]-hydrogenase by reactive oxygen species,[12a] we hypothesized that O_2 is reduced by this enzyme to H_2O_2 or O_2^- and that the reactive oxygen species destroys the FeGP cofactor of [Fe]-hydrogenase. To test this hypothesis, [Fe]-hydrogenase was decomposed in an assay solution composed of [Fe]-hydrogenase (500 μM) and methylene-H_4MPT (500 μM) under 100% O_2. We found production of sub-stoichiometric amounts of H_2O_2 (ca. 3 μM), only in the presence of both enzyme and substrate (Supporting Information, Table S2). On the other hand, O_2^- was not detected. The relationship between the enzymatic activity of [Fe]-hydrogenase and H_2O_2 production under the O_2 gas phase was studied kinetically (Figure 4). Higher concentrations of the enzyme, methylene-H_4MPT or O_2 in the gas phase increased the amount of H_2O_2 produced, which directly correlates with the decrease in enzyme activity. When the enzyme became fully deactivated, the increase of H_2O_2 stopped.

To explore the dependency of the H_2O_2 concentration on the deactivation of [Fe]-hydrogenase, 26, 2.6, and 0.26 μM of H_2O_2 were added to a solution of 26 μM [Fe]-hydrogenase (final concentrations) and incubated (Supporting Information, Figure S7). In the presence of stoichiometric concentrations (26 μM) of H_2O_2, [Fe]-hydrogenase was fully deactivated within 5 min, but less than 10% of H_2O_2 was lost in the deactivation assay. In the case of sub-stoichiometric concentrations of H_2O_2 (2.6 μM and 0.26 μM H_2O_2), substantial amounts of [Fe]-hydrogenase were decomposed; the residual [Fe]-hydrogenase activity was only 30% and 60% within 10 min (Supporting Information, Figure S7). Altogether, these results demonstrated that [Fe]-hydrogenase is catalytically deactivated by sub-stoichiometric amounts of H_2O_2.

To determine whether the interaction of O_2 with the iron center and the reduction of O_2 to H_2O_2 are correlated with Fe^{II} oxidation, electron paramagnetic resonance (EPR) spectroscopy was used. The low-spin Fe^{II} active site of [Fe]-hydrogenase is EPR silent. Upon deactivation by O_2 in the presence of 0.1 mM methylene-H_4MPT under the gas phase ($N_2/O_2/H_2$ (70%/20%/10%)), only a minor EPR signal at $g=4.3$ from high-spin Fe^{III} emerged, with a spin concentration of less than 7% of the total enzyme (Supporting Information, Figure S8). An even weaker fraction of low-spin Fe^{III} products appeared around $g=2$. Thus, no significant oxidation of Fe^{II} to Fe^{III} occurs and most likely O_2 is not reduced in an iron-bound state.

The presented study indicates that the deactivation of [Fe]-hydrogenase upon O_2 exposure does not occur directly in response to O_2 but via a reduction to H_2O_2, which subsequently decomposes the FeGP cofactor without loss of H_2O_2. On the basis of the proposed catalytic mechanism of [Fe]-hydrogenase (Supporting Information, Figure S9), the most probable reductant is an iron hydride. The proposed type of metal-hydride-driven hydrogenation reaction has precedent in inorganic ruthenium complex chemistry;[15] thereby increasing the plausibility of the mechanism. Metal-hydride formation in the [Fe]-hydrogenase reaction was already postulated on the basis of calculations[8c,16] and model-complex studies[5,17] but experimental evidence was, thus far, not provided. If our conclusions are correct, [Fe]-hydrogenase stabilizes a hydride on its iron center in the catalytic cycle. This finding may open a perspective to apply [Fe]-hydrogenase for the reduction of non-polar compounds (which might be polarized in the enzyme) and as a template for developing new hydrogenating model catalysts (for example, a selective H_2O_2-forming catalyst[18]).

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Conflict of interest

The authors declare no conflict of interest.

Keywords: [Fe]-hydrogenase · enzyme catalysis · H_2 · hydrogenation · O_2 sensitivity

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