The molybdenum active site of formate dehydrogenase is capable to catalyze C-H bond cleavage and oxygen atom transfer reactions

Tobias Hartmann¹, Peer Schrapers², Tillmann Utesch³, Manfred Nimtz⁴, Yvonne Rippers³, Holger Dau², Maria Andrea Mroginski³, Michael Haumann², and Silke Leimkühler^{1*}

¹Department of Molecular Enzymology, Institute of Biochemistry and Biology, University of Potsdam, Karl-Liebknecht Str. 24-25, 14476 Potsdam, Germany;

²Institute of Experimental Physics, Arnimallee 14, Freie Universität Berlin, 14195 Berlin, Germany

³Institute of Chemistry, Technical University of Berlin, Straße des 17. Juni 135, 10623 Berlin, Germany;

⁴Helmholtz Center for Infection Research, Inhoffenstraße 7, 38124 Braunschweig, Germany;

***Corresponding Author**

Silke Leimkühler; Department of Molecular Enzymology, Institute of Biochemistry and Biology, University of Potsdam, Karl-Liebknecht-Str. 24-25, 14476 Potsdam, Germany; Tel.: +49-331-977-5603; Fax: +49-331-977-5128; E-mail: sleim@uni-potsdam.de

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Abbreviations

IAA, iodoacetamide, FDH, formate dehydrogenase, Moco, molybdenum cofactor, MGD, molybdopterin guanine dinucleotide, MV, methyl viologen, SeCys, selenocysteine, XAS, X-ray absorption spectroscopy

Abstract

Formate dehydrogenases (FDHs) are capable to perform the reversible oxidation of formate and are enzymes of high interest for fuel cell applications and for the production of reduced carbon compounds as energy source from CO₂. Metal-containing FDHs in general contain a highly conserved active site, comprising a molybdenum (or tungsten) center coordinated by two molybdopterin guanine dinucleotide molecules, a sulfido and a (seleno-)cysteine ligand, in addition to a histidine and arginine residue in the second coordination sphere. So far, the role of these amino acids for catalysis has not been studied in detail, due to the lack of suitable expression systems and the lability or oxygen sensitivity of the enzymes. Here, the roles of these active-site residues is revealed using the Mo-containing FDH from *Rhodobacter capsulatus*. Our results show that the cysteine ligand at the Mo ion is displaced by the formate substrate during the reaction, the arginine has a direct role in substrate binding and stabilization and the histidine elevates the pK_a of the active-site cysteine. We further found that in addition to reversible formate oxidation, the enzyme is further capable to reduce nitrate to nitrite. We propose a mechanistic scheme, which combines both functionalities and provides important insights into the distinct mechanisms of C-H bond cleavage and oxygen atom transfer catalyzed by formate dehydrogenase.

A specific enzyme system of increasing interest is formate dehydrogenase (FDH), being involved in the reversible conversion of CO₂ in biological systems (1,2). Formate in general is found in all forms of life from bacteria to animals. The diverse FDH enzyme systems catalyze the reversible oxidation of formate to CO₂, two electrons and one proton according to the reaction (3):

The equilibrium of the reaction usually favors formate oxidation. A particularly interesting FDH is the molybdenum cofactor (Moco) containing enzyme from *Rhodobacter capsulatus*, which consists of three different subunits, FdsGBA, assembled as an ($\alpha\beta\gamma$)₂ hexamer (4). Seven FeS clusters are bound to the enzyme, bridging electron transfer from the Moco center to a FMN cofactor, where NAD⁺-reduction occurs (4). One major advantage of the enzyme is its high level of oxygen-tolerance in its purified state. For *R. capsulatus* FDH a k_{cat} of 2189 min⁻¹ for formate oxidation was determined, however, CO₂ reduction was also catalyzed by this enzyme with a k_{cat} of 89 min⁻¹ (4). The enzyme, thus, is suitable for potential fuel cell applications, and provides a suitable tool for detailed investigations on the substrate conversion mechanism.

FDHs are highly diverse in terms of their structural composition and subcellular localization, as revealed by their roles in different metabolic pathways (C1-metabolism or energy conservation), however, their active sites are highly conserved (1,2). The crystal structures of the Mo-containing enzymes from *Escherichia coli*, namely FdhF (component of the formate hydrogenlyase complex) and FdnGHI (nitrate:formate respiratory enzyme), and the W-containing enzyme from *Desulfovibrio gigas* (FdhAB) have shown that the metal ion in the oxidized enzyme is coordinated by four sulfur ligands of two dithiolene groups of the bis-molybdopterin guanine dinucleotide (bis-MGD) cofactor, a selenocysteine (SeCys), and by a sixth ligand, which is now established as a sulfido group (5-9) (Fig. 1). After reduction with formate, the structure of *E. coli* FdhF showed that the SeCys ligand was displaced from the Mo ion (5). In the second coordination sphere, a highly conserved histidine and arginine are present in all FDH enzymes described so far. Experimental evidence in which these residues are replaced by other amino acids is lacking, due

to the high oxygen sensitivity of the *E. coli* and *D. gigas* enzymes and the lack of suitable overexpression systems.

Closely related to FDHs are periplasmic nitrate reductases (e.g. *Cupriavidus necator* NapA), which similarly belong to the DMSO reductase family of molybdoenzymes. Periplasmic nitrate reductases comprise an identical Mo coordination sphere containing six sulfur ligands in the oxidized state (including a Cys) and show a remarkable structural homology to FDHs (10-12) (Fig. 1). A conserved threonine and methionine are found close to the Mo ion in periplasmic nitrate reductases (Fig. 1 and S1) (11,12).

However, while FDHs have been shown to be the only example of mononuclear molybdoenzymes catalyzing naturally the C-H bond cleavage of formate by forming a single C=O bond on the product without the involvement of water (5,13-15), nitrate reductases, in contrast, generally catalyze a classical oxygen atom transfer reaction, reducing nitrate to nitrite, by releasing the substrate-derived oxygen as water (3):

The correlation between the identical active site metal configuration of formate dehydrogenases and nitrate reductases (six sulfur ligands in the Cys-containing enzymes) in relation to their distinct formate and nitrate conversion reactions is examined in this study. Site-directed mutagenesis was performed on the conserved active site residues Cys386, His387 and Arg587 in *R. capsulatus* FDH and their impact on the catalytic mechanism of formate oxidation was studied. We thereby define a role for the Arg587 in substrate binding and for His387 in increasing the proton affinity of the Cys386. During turnover, Cys386 is displaced from the Mo-active site and is proposed to play a significant role in the proton abstraction mechanism from the substrate. In addition, we observed a so far uncharacterized nitrate reductase activity of *R. capsulatus* FDH, which is likely not of physiological importance. The mechanism of nitrate reduction was shown to be independent of the sulfido ligand at the Mo atom. A conclusive mechanism combining formate oxidation and nitrate reduction is presented.

Experimental Procedures

Site-Directed Mutagenesis, Expression and Purification and Cofactor Analysis

Amino acid exchanges were introduced into the *fdsA* gene by PCR mutagenesis. *R. capsulatus* FDH was expressed, purified and the cofactor content was determined as described previously (4).

Stopped-Flow

Absorption spectra were recorded (SX20 instrument, Applied Photophysics) on samples containing 20 μ M FDH in 100 mM buffer with 20 mM Na-formate (final pH 6 to 10). Absorbance changes at 444 nm were fitted using single exponential functions.

Enzyme Assays

R. capsulatus FDH activity was detected using a UV-2401PC spectrophotometer (Shimadzu Europa GmbH, Duisburg, Germany) recording the change in NADH absorption at 340 nm ($\epsilon_{\text{NADH}} = 6,220 \text{ M}^{-1} \text{ cm}^{-1}$). 100 mM of either Britton-Robinson (pH 5-6), KPi (pH 6-8), CHES (pH 8.5-10) or CAPS (pH 10-11) was used as buffer.

Steady-state kinetics were performed at RT using formate ranging from 0.05 to 500 mM (dependent on the variant used) and 5 mM NAD^+ . Measurements started by addition of enzyme (100 nM FDH^{WT} ; 300 nM for variants $\text{FDH}^{\text{H387M}}$, $\text{FDH}^{\text{H387K}}$, $\text{FDH}^{\text{R587K}}$; 600 nM for variants $\text{FDH}^{\text{C386S}}$, $\text{FDH}^{\text{H387R}}$, $\text{FDH}^{\text{H387F}}$, $\text{FDH}^{\text{R587T}}$). Formate:NAD activities of the $\text{FDH}^{\text{R597+}}$ and $\text{FDH}^{\text{R587T/R597+}}$ variants (600 nM) were determined using 100 mM formate and 5 mM NAD^+ in 100 mM Tris/HCl (pH 9.0). For determination of the pH-dependent stability, 5 μ l of 1 μ M FDH^{WT} was mixed with 45 μ l of buffer containing either no, 1 mM or 10 mM potassium nitrate. Samples were incubated for 20 minutes at RT and the remaining activity was determined in the presence of 5 mM of formate and NAD^+ as substrates. Initial formate dehydrogenase activities were set to 100% and used as a reference.

The inhibition of FDH^{WT} by nitrate was determined using 100 nM FDH^{WT} and a concentration range of 0 – 1 mM of KNO_3 and 0.05-1 mM of formate and 5 mM of NAD^+ . Activities were plotted according to Lineweaver-Burk to determine the mechanism of nitrate inhibition and for calculation of the apparent $K_{\text{m}}^{\text{formate}}$ values. The competitive inhibition constant $K_{\text{i}}^{\text{nitrate}}$ was determined using a reciprocal plot of the apparent K_{m} values against the inhibitor concentration.

All measurements were performed at least three times from independent protein purifications. All activities were calculated as turnover numbers (k_{cat}) in respect to one catalytically active protomer $\alpha\beta\gamma$ (MW = 172 kDa).

Iodoacetamide Inactivation

FDH (3 μM) was incubated at pH 8.5 (CHES buffer) or in buffers with pH values ranging between 6 and 10 (using 100 mM of KPi or CHES buffer) in the presence of 10 mM KNO_3 in the presence or absence of 10 mM IAA at RT. Formate oxidation activities were determined every 5 minutes and after incubation for 20 min at RT, after the addition of one-tenth of the sample to either 100 mM Tris/HCl (pH 9.0) (for FDH^{WT}) or 100 mM KPi (pH 7.5) (for $\text{FDH}^{\text{H387M}}$) buffer containing formate (FDH^{WT} = 5 mM, $\text{FDH}^{\text{H387M}}$ = 50 mM) and 5 mM NAD^+ . As negative controls, FDH^{WT} and $\text{FDH}^{\text{H387M}}$ (both derived from anaerobic enzyme preparations) were incubated in the absence of KNO_3 and either in the presence or absence of 10 mM IAA for 20 min at RT inside a glove box. Formate oxidation activities of samples containing IAA (+/- KNO_3) were related to those obtained for enzyme incubated in absence of IAA (+/- KNO_3), respectively.

LC-MS/MS

Carboxamidomethylation of Cys386 was achieved by incubation of FDH (10 μM) with 10 mM KNO_3 and IAA at pH 9.0 for 2 h at RT. After SDS-PAGE gel pieces containing the FdsA subunit were digested with trypsin and peptides were extracted and purified with reversed-phase C18 ZipTips (Millipore, USA). LC-MS/MS analyses of desalted peptides were performed on a Dionex UltiMate 3000 n-RSLC system connected to an Orbitrap Fusion^{TM} Tribrid^{TM} MS (Thermo Scientific). For further details see SI.

X-Ray Absorption Spectroscopy

XANES and EXAFS spectra were collected for the following samples: as-isolated FDH^{WT} , nitrate-treated FDH^{WT} , as-isolated $\text{FDH}^{\text{desulfo}}$, and nitrate-treated $\text{FDH}^{\text{desulfo}}$. Enzymes were expressed as described above and purified in the absence or presence of 10 mM potassium nitrate. Samples (in 75 mM KPi buffer, pH 7.5) were transferred to ultracentrifugation devices and concentrated to ~1 mM of enzyme-

bound Mo. Samples were finally transferred to XAS sample holders and stored in liquid nitrogen. For further information see SI.

Quantification of Nitrite

FDH (6 μ M) was incubated with 50 mM KNO₃ and 5 mM reduced MV in 100 mM KPi (pH 6.8) buffer at RT inside a glove box. For extracted cofactor samples, FDH^{WT} was incubated at 95°C for 10 min followed by a centrifugation step. The supernatant (from 6 μ M enzyme) was used in the assays. For azide treated FDH^{WT} samples, 850 μ M of sodium azide was added to the assays. Aliquots (200 μ l) were removed from the chamber every 20 min, exposed to air and vortexed to reoxidize the remaining MV. 80 μ l detection solution (2:1 mix of 1% (w/v) sulfanilamide (solved in 25% HCl) and 0.08% (w/v) N-1-naphtyl-1-ethylendiamin-dihydrochloride) was added, the samples immediately vortexed and incubated for 5 min. Denatured proteins were removed by centrifugation (11.000 x g) for 5 min. Finally 200 μ l of the sample supernatant was transferred to 96-well plates and the absorbance of the formed azo-dye determined at 540 nm using a Varioskan Flash plate reader (Thermo Fisher Scientific, Waltham, MA, USA) (16). For quantification of produced nitrite, a standard curve of sodium nitrite (1-100 μ M) was used. Each experiment was performed in triplicates. Apparent turnover numbers ($k_{cat\ app.}$) derived from at least three independent experiments were calculated using the linear plots of the time-dependent nitrite production and correlated to the amount of enzyme used in the assays and to the respective enzyme bound Mo saturation.

Results

Characterization of Active-Site Variants of FDH

Our recently developed heterologous expression system in *E. coli* was used to study the role of the conserved amino acids in the active site of *R. capsulatus* FDH (4). Base-pair exchanges were introduced into the *fdsA* gene by site-directed mutagenesis. The generated variants C386S, H387K, H387F, H387M, H387R, R587T, R587K, H387M/R58T, R597⁺ and R587T/R597⁺ were purified (~90%) as ($\alpha\beta\gamma$)₂ heterodimers (Fig. S2). All variants displayed a similar elution profile after size-exclusion chromatography as the wild-type enzyme (FDH^{WT}), showing no changes with respect to the overall oligomeric state (data not shown). The iron content of all produced variants was comparable to FDH^{WT} (Table S1). Further analyses showed that the bis-MGD cofactor content was not affected in most variants (Table S1). Only FDH^{H387K}, FDH^{H387F}, and variants containing an additional Arg (FDH^{R597+} and FDH^{R587T/R597+}), exhibited decreased bis-MGD cofactor saturations, consistent with the lower Mo level (Table S1). The addition of an Arg residue (R597⁺) likely resulted in a slight structural change of the active site, which diminished the insertion of the bis-MGD cofactor.

Steady-State Kinetics and pH Optimum of FDH variants for Formate Oxidation

To test the influence of amino acid changes at the active site on the catalytic activity, steady-state kinetics were performed at pH values ranging from 6 to 10, following the reduction of NAD⁺. The resulting K_m^{formate} and k_{cat} values were determined and related to the overall Mo saturation (Fig. 2, Table 1).

For FDH^{WT}, the k_{cat} values showed a pH optimum of 9.0 for the formate:NAD⁺ activity ($k_{\text{cat}}^{\text{WT}} = 2124 \pm 36 \text{ min}^{-1}$) (Fig. 2). The reductive half reaction (k_{red}), as determined by the reduction of FMN at 444 nm using stopped-flow measurements, showed a similar pH optimum of 9.0 (Fig. 3). The maximal k_{red} of $1790 \pm 59 \text{ min}^{-1}$ at the pH optimum is consistent with the k_{cat} values for the formate:NAD⁺ reaction. This shows that electron transfer is not the rate limiting step of the reaction.

As expected, the FDH^{C386S} variant was inactive over the whole pH range (6-10), underlining the importance of Cys386 for the reaction. The substitution of His387 to a Phe or Arg also resulted in an inactive enzyme. Classical all-atoms molecular

dynamics simulations of both variants favored conformations of these residues, which blocked or drastically reduced substrate access to the active site (Fig. S4). In contrast, in FDH^{H387M} the k_{cat} remained similar to that of FDH^{WT} ($2022 \pm 164 \text{ min}^{-1}$), in agreement with the MD simulations showing a similar orientation of Met387 as found in the FDH^{WT} structure (Fig. S4). However, in this variant the pH optimum was lowered to 7.5 with a 19-fold increase of $K_{\text{m}}^{\text{formate}}$ to $3.6 \pm 0.1 \text{ mM}$. The replacement of His387 by a Lys reduced the activity to 3% of that of FDH^{WT} ($k_{\text{cat}} = 56 \pm 13 \text{ min}^{-1}$), accompanied by an increase of $K_{\text{m}}^{\text{formate}}$ to $27.5 \pm 2.7 \text{ mM}$ and a shift of the pH optimum to 8.0. The FDH^{R587T} and the FDH^{H387M/R587T} variants were inactive. When Arg587 was replaced by a Lys, retaining the positive charge of the side chain, one third of FDH^{WT} activity ($k_{\text{cat}} = 674 \pm 39 \text{ min}^{-1}$) was obtained, however, accompanied by an increase in $K_{\text{m}}^{\text{formate}}$ to $362.5 \pm 27.1 \text{ mM}$, and a shift of the pH-optimum to 8.0. These results show that His387 influences the pH optimum of the reaction, while the drastic increase in K_{m} reveals a role of Arg587 in substrate binding at the active site.

The Effect of Nitrate on FDH^{WT} Stability and Activity

In general, FDH enzymes were shown to be stabilized by the addition of nitrate during purification (4,17). We therefore investigated the effect of nitrate on the catalytic activity of FDH^{WT}. In the absence of nitrate, FDH^{WT} was rapidly inactivated during an incubation time of 20 min, particularly at higher pH values (Fig. 4A). The addition of 1 mM nitrate during the incubation time resulted in a much slower inactivation over time and ~83% of the initial activity was retained after 20 min at pH 6.0 (Fig. 4A). A higher nitrate concentration of 10 mM resulted in further stabilization and ~90% of the initial activity was retained after 20 min incubation in a pH range of 6 to 8 (Fig. 4A).

Steady-state kinetics with varying formate concentrations in the presence or absence of nitrate were performed to analyze the effect of nitrate on the formate reaction. The enzyme kinetics showed that nitrate acts as a competitive inhibitor for FDH (Fig. 4B) with a $K_{\text{i}}^{\text{nitrate}}$ value of $1.6 \pm 0.1 \text{ mM}$ (Fig. 4B, inset).

Carboxamidomethylation of Cys386 by Iodoacetamide

Further insights into the roles of the Cys386 and His387 residues were obtained by determining the effect of iodoacetamide (IAA) as an alkylating agent on FDH activity.

While in the absence of nitrate, the formate:NAD⁺ activity of FDH was not affected by IAA treatment, the addition of IAA to nitrate-incubated FDH^{WT} resulted in a linear decrease of activity over time (Fig. S5). When analyzing FDH^{H387M}, a stronger inactivation by IAA was observed compared to FDH^{WT} (Fig. S5). Further, FDH^{WT} and FDH^{H387M} were incubated at pH values ranging from 6 to 10 with nitrate in the presence of IAA. The resulting plots of relative activities against pH showed a linear decrease of enzyme activity with increasing pH for both FDH^{WT} and FDH^{H387M}, while in absence of nitrate no change of relative activities was observed (Fig. 5A). However, a direct comparison displayed a shift in the activity profile of FDH^{H387M} by one pH unit to the acidic region (Fig. 5A), which is consistent with the results shown above further underlining that His387 influences the pH optimum of the reaction. Mass spectrometry analysis of both enzymes, treated with IAA and nitrate, clearly revealed a carboxamidomethylation of Cys386 in both FDH^{WT} and in FDH^{H387M} (Fig. 5B). These results conclusively demonstrate that Cys386 is displaced by nitrate during the reaction, suggesting that nitrate binds similarly as formate to the Mo atom (consistent with the competitive inhibition mode).

XAS on Nitrate-Treated FDH

We used X-ray absorption spectroscopy (XAS) at the Mo K-edge to determine the Mo coordination in FDH^{WT} and in a variant, FDH^{desulfo}, which was expressed in the absence of the maturation protein FdsC and contains a Mo-oxo ligand instead of the sulfido ligand (4,6,18) (Fig. S6). In nitrate-treated FDH^{WT}, a short Mo=S bond was observed as in the absence of nitrate, but a long Mo-S distance (~2.6 Å), attributed to Cys386 at Mo^{VI} in untreated FDH^{WT}, was absent and a new Mo-O distance (~2.3 Å) was detected instead (Table S2). The structural features in nitrate- and formate-treated FDH^{WT}, thus, are similar (6), likely revealing replacement of the Cys386 ligand (and not of the sulfido ligand) by the oxygen of a nitrate or formate molecule. However, the elongated Mo=S bond may suggest protonation upon reduction to form a SH ligand at Mo^{IV}, which resembled the situation with formate (6). For FDH^{desulfo}, the increased number of short Mo=O bonds suggested replacement of the sulfido by an oxo ligand at Mo^{VI} and a doubled number of distances >2.8 Å, the lack of the ~2.6 Å Mo-S bond, and detection of a Mo-O bond (~2.1 Å) were compatible with Cys386

being replaced by a water species (Table S2) (6). For nitrate-treated $\text{FDH}^{\text{desulfo}}$, the observed XAS changes may suggest replacement of the water species by a nitrate molecule (see Fig. S6 for further details). These results suggest that the sulfido ligand is retained upon formate binding, but it is not important for nitrate binding at the Mo center. Additionally, the results confirm the observations above, that the Cys386-ligand is displaced by nitrate during the reaction.

Nitrate Reductase Activity of FDH

We further analyzed the catalytic activity of *R. capsulatus* FDH for the reduction of nitrate to nitrite. Incubation of FDH with nitrate and methyl viologen as an electron donor resulted in the detection of nitrite formation, which was quantified using a colorimetric assay (Fig. 6) to calculate the apparent turnover numbers (Table 2). While for FDH^{WT} an apparent k_{cat} value of $0.21 \pm 0.03 \text{ min}^{-1}$ was obtained, nitrate reductase activities were not observed when using $\text{FDH}^{\text{deMoco}}$ lacking the bis-MGD cofactor. Controls with just the cofactor fraction of heat-denatured FDH^{WT} or azide-inhibited FDH^{WT} (17,19,20) showed no nitrite production even over longer incubation times (Fig. 6). Surprisingly, turnover numbers of $0.31 \pm 0.04 \text{ min}^{-1}$ were obtained for $\text{FDH}^{\text{desulfo}}$, showing that the sulfido ligand is not essential for nitrate reduction in FDH. When analyzing $\text{FDH}^{\text{C386S}}$, activities were comparable to FDH^{WT} (Table 2). Further, FDH variants with substitutions of the conserved residues found in periplasmic nitrate reductases were analyzed. For the $\text{FDH}^{\text{H387M}}$, $\text{FDH}^{\text{H387M/R587T}}$, and $\text{FDH}^{\text{R587T}}$ reduced activities in comparison to the FDH^{WT} were determined (Table 2). Introducing an additional Arg at position 597, which is highly conserved in periplasmic nitrate reductase, but not present in FDH (see Fig. 1 and S1), resulted in 6-fold ($\text{FDH}^{\text{R597+}}$, $k_{\text{cat}} = 1.20 \pm 0.45$) and 11-fold ($\text{FDH}^{\text{R587T/R597+}}$, $k_{\text{cat}} = 2.23 \pm 0.43$) increased turnover numbers. Attempts to insert an Arg at position 597 in $\text{FDH}^{\text{H387M/R587T}}$ were not successful, since the amino acid exchange resulted in an unstable enzyme.

Discussion

Molybdoenzymes catalyze very diverse but specific reactions at the Mo active site, including oxygen atom transfer reactions and substrate hydroxylations using a variety of organic (e.g. DMSO, dimethylsulfide (DMS), formate, xanthine, ethylbenzene) or inorganic (e.g. nitrate, selenate, chlorate, sulfur, arsenic) substrates. However, formate dehydrogenase was shown to be strikingly different and, thus, a unique mononuclear molybdoenzyme, since formate oxidation occurs via C-H bond cleavage of the substrate resulting in a single C=O bond in the product, without the involvement of water. For metal-containing FDH enzymes no other substrates than formate and CO₂ were so far identified. In this study, we show that FDH from *R. capsulatus* can catalyze both C-H bond cleavage reaction accompanied by proton abstraction from formate and the classical oxygen atom transfer reaction by reduction of nitrate to nitrite and releasing the substrate-derived oxygen as water.

We substituted the conserved Cys386, His387 and Arg587 residues in the active site of *R. capsulatus* FDH and performed a detailed functional and structural analysis. The exchange of Cys386 to Ser completely abolished catalytic activity. Previous studies on FDH^{C386S} showed that Ser386 does not ligate to the Mo atom of bis-MGD (6). A pH-dependent alkylation of Cys386 by IAA was only observed in the presence of nitrate. Our results show that (a) both formate and nitrate bind similarly to the Mo atom, resulting in the displacement of the Cys386 ligand, which then is prone to alkylation by IAA, and (b) FDH likely catalyzes the oxidation of formate by a proton abstraction reaction mediated by Cys386 (Fig. 7), a reaction that can not be catalyzed by Ser386. The exchange of the Cys386 ligand in *R. capsulatus* FDH during the reaction by formate (6) or nitrate (this study) was further suggested by XAS studies and are consistent with the *E. coli* FdhF enzyme in which SeCys140 was displaced from the formate reduced Mo-center (5). In our results, the substrate is directly bound to the Mo atom, transiently replacing the Cys ligand, but retaining the Mo=S bond. In comparison to the *E. coli* FdhF enzyme, substitution of SeCys by Cys resulted in a more basic pH optimum of activity (21). The higher pK_a of Cys (pK_a = 8.1) vs. SeCys (pK_a = 5.4), thus, may explain the basic pH optimum (9.0) of *R. capsulatus* FDH, which ensures that Cys386 is deprotonated at this pH and can act

as a proton acceptor during the reaction.

Our results of Arg587 FDH variants further support previous crystallographic and theoretical data on the *E. coli* enzyme (5,13,14). The Arg587 likely interacts with the formate molecule to facilitate its correct orientation and stabilization throughout the whole catalytic cycle. This conclusion is supported by the observation that substitution to an uncharged Thr residue completely abolished catalytic activity. A Lys at this position retained 30% of FDH^{WT} activity, but the much increased K_m^{formate} suggests less effective substrate stabilization, possibly related to the lower pK_a of the Lys vs. Arg side chain and in agreement with the observed shift of the catalytic pH-optimum to the more acidic region.

Exchange of His387 by Arg or Phe completely abolished the catalytic activity of the enzyme. In contrast, the exchange to a Lys caused a strongly reduced activity (3% of FDH^{WT}) while a Met at this position did not influence the catalytic activity. In the latter substitutions, K_m^{formate} values were increased and rather dependent on pH. These findings imply that His387 stabilizes the formate molecule by hydrogen bonding in the initial state of substrate binding (Fig. 7). Since the His387 to methionine variant retained FDH^{WT} activity, His387 seems not to play a direct role in the formate oxidation reaction. A direct interaction of His387 with the Cys386 residue during catalysis can be excluded, since a Met at this position could not perform the interaction. Our results rather suggest that His387 modulates the strength of the Mo-S(Cys386) bond and the correct orientation of the Cys386 side chain when it is detached from the Mo center. This is supported by the previously observed elongation of the Mo-S(Cys386) bond in the FDH^{H387M} relative to the FDH^{WT} (6). Furthermore, the observed shift of the formate oxidation optimum to more acidic pH and sensitivity to IAA inactivation when analyzing the H387M variant suggests that His387 further raises the pK_a of the Cys386 side chain.

The addition of nitrate significantly increased *R. capsulatus* FDH stability as described for other FDH enzyme purifications reported previously (17,20). It has been suggested that nitrate likely protects the active site against inactivation by oxygen (19). We showed that in the presence of a strong electron donor, *R. capsulatus* FDH was able to reduce nitrate to nitrite at the Mo-center. The obtained nitrate reductase activities were four orders of magnitude lower in comparison to the

activities obtained for formate oxidation, so that the physiological relevance of nitrate reduction catalyzed by this enzyme can be excluded. Our data, however, show that the active site of FDH is capable to perform an oxygen atom transfer reaction. Since the active sites of FDHs and periplasmic nitrate reductases share a highly similar Mo coordination sphere both reactivities are possible in these enzymes, in spite of the differences in the substrate binding pockets. We found that the sulfido ligand, which is essential for formate oxidation (4,6), was not required for nitrate reduction. Further, substitution of Cys386 to a serine, which prevented proton abstraction of the formate molecule (4,6), did not alter the nitrate reductase activity of the enzyme. Since our previous results suggested that the serine at position 386 is not a ligand to the Mo-atom, nitrate can be bound to the vacant binding site. Instead, for formate oxidation the cysteine at this position is essential by acting as proton acceptor during the reaction.

The modification of active site residues in *R. capsulatus* FDH to mimic the substrate-binding pocket present in periplasmic nitrate reductase resulted in an increased nitrate reductase activity after the introduction of an Arg at position 597. This further underlines the importance of the positively charged Arg in the active sites of FDH and periplasmic NR to ensure the correct orientation of the negatively charged substrates as an essential step to initiate the catalytic process after substrate binding (22).

In summary, a reaction scheme for formate oxidation (C-H bond cleavage) and nitrate reduction (oxygen atom transfer reaction) of *R. capsulatus* FDH is presented in Figure 7:

A) Nitrate reduction: In the initial state, nitrate binds to the Mo center forming a Mo-O-nitrate bond and displacing Cys386 from the Mo. Likely, binding of nitrate to the active site is facilitated when Arg597⁺ and a Thr at position 587 in the active site, mimicking the situation found in periplasmic nitrate reductase. Nitrate reduction occurs by an oxygen atom transfer to the Mo-atom with concomitant release of nitrite (3). Finally, the oxo-group might be released as water after reduction of the Mo-center with help of an external electron donor similar to what is proposed for periplasmic nitrate reductase (22).

B) Formate oxidation: In the initial state, His387 and especially Arg587 are essential for formate binding to the Mo center establishing a H-bond network. Concomitant with formate binding, Cys386 is released from the Mo center and orients towards the proton of the bound formate molecule. The Arg587 adopts a position close to the thiol of Cys386, stabilizing its negative charge and in addition to the formate molecule. Formate oxidation occurs with abstraction of the α -proton by Cys386, followed by CO₂ release and two-electron transfer via the Mo-center to the FMN site. Finally the Cys386 ligand is reformed at the reoxidized Mo atom.

Conclusively, our results show that formate dehydrogenase has the capabilities to reduce both nitrate and oxidize formate at the same molybdenum site. This suggests common principles in the catalytic mechanism of both nitrate reductase and formate dehydrogenase. In both cases, the substrate displaces the amino acid ligand to the molybdenum atom at the active site. Since the nitrate reductase activity of FDH shown here is quite low, the reaction is likely not of physiological relevance. The results shown here are supported by previous results obtained in the crystal structure of *E. coli* FdhF, which showed that in the reduced state with formate the cysteine ligand was displaced from the active site (5). Further, the mechanism is consistent with the ones proposed by Cerqueira and coworkers by theoretical studies (14,23-25), but is inconsistent with a recent report by Nicks et al. (26), which proposed formate binding only in the second Mo coordination sphere by studies on *Cupriavidus necator* FDH. In future studies we aim to investigate the mechanism of substrate oxidation further, to reveal the mechanism of proton-transfer during the course of the reaction in detail.

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Supporting Information Available

- Table S1: Determination of the cofactors bound in FDH^{WT} and variants
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Figure Legends

Figure 1. Comparison of the Mo active sites of FDH and periplasmic nitrate reductase. Superimposed are structures of FDH-H (FdhF) from *Escherichia coli* (black; PDB entry: 1FDO) and periplasmic nitrate reductase (NapA) from *Cupriavidus necator* (white; PDB entry: 3ML1). The conserved residues found in FDH and their homologs found in nitrate reductase are labeled in addition to the Arg only found in nitrate reductase (*R. capsulatus* FDH residue numbering is shown in parentheses).

Figure 2. pH dependence of kinetic constants for formate oxidation. The kinetic parameters, k_{cat} (circles) and $K_{\text{m}}^{\text{formate}}$ (open squares), were derived from steady-state kinetics of the FDH^{WT} (A) and the variants FDH^{H387M} (B), FDH^{H387K} (C), and FDH^{R587K} (D). The formation of NADH was detected using saturating concentrations of NAD⁺ and a formate concentration range of 0.05-500 mM.

Figure 3. pH-optimum of the reductive half-reaction of FDH^{WT}. Pseudo-first order rate constants k_{red} (sec⁻¹) were obtained by stopped-flow measurements. FMN reduction was monitored at 444 nm in presence of formate and using either KPi (black squares), Tris/HCl (open diamonds) or CHES (gray circles) as buffers. The solid line represents a Gaussian fit to the averaged data.

Figure 4. Influence of nitrate on FDH^{WT} stability and formate oxidation. (A) Relative activities of FDH^{WT} after incubation for 20 minutes at RT in the absence (black circles) or presence of either 1 mM (gray circles) or 10 mM (open circles) nitrate. Activities were determined using formate as substrate and detecting the formation of NADH. (B) Reciprocal plots of activities in the absence (open squares) or presence of 0.05 mM (open circles), 0.1 mM (black diamonds), 0.25 mM (black triangles), 0.5 mM (black squares), and 1 mM (black circles) nitrate. Kinetics were performed using saturating NAD⁺ concentrations and a formate concentration range of 0.05 to 1 mM. Apparent k_{cat} values were fitted (solid lines) using Lineweaver-Burk equation. Inset: apparent $K_{\text{m}}^{\text{formate}}$ values vs. nitrate concentration.

Figure 5. Carboxamidomethylation of FDH by IAA in the presence of nitrate. (A) Relative formate oxidation activities for FDH^{WT} (black circles) or FDH^{H387M} (white circles) after incubation with 10 mM nitrate and 10 mM IAA for 20 minutes at RT. As negative controls, FDH^{WT} (black squares) and FDH^{H387M} (white squares) was incubated with 10 mM IAA in the absence of nitrate for 20 min at RT. (B) HPLC-ESI-MS comparison of the tryptic peptides containing the active site Cys386 of the FDH^{WT} and FDH^{H387M} variant. Depicted are the ion traces of the doubly charged molecular ions of the carboxamidomethylated and thiomethylated peptides allowing an approximate determination of their relative abundances. The amino acid sequences of the respective peptides were verified by additional MS/MS spectra of all relevant ions (data not shown).

Figure 6. Quantification of nitrite produced by FDH^{WT} and variants. Concentration of nitrite (μM) produced by FDH catalyzed nitrate reduction (enzyme-extracted cofactors = open diamonds, FDH^{WT} = black circles, FDH^{desulfo} = gray circles, FDH^{deMoco} = open circles, azide inhibited FDH^{WT} = gray diamonds, FDH^{C386S} = black diamonds, FDH^{H387M} = open triangles, FDH^{R587T} = black triangles, FDH^{H387M/R587T} = gray squares, FDH^{R597+} = open squares, FDH^{R587T/R597+} = black squares). Samples were incubated under anaerobic conditions using 50 mM nitrate as substrate and 5 mM methyl viologen as an electron donor. The formation of nitrite was determined every 20 min using a colorimetric assay as described in the experimental procedures section. Samples containing no enzyme were used as a negative control (gray triangles).

Figure 7. Reaction schemes for formate oxidation and nitrate reduction catalyzed by *R. capsulatus* FDH. (A) Proposed oxygen atom transfer mechanism with nitrate as substrate. (B) Proposed C-H bond cleavage mechanism with formate as substrate. Further details are given in the text.

Tables

Table 1: Kinetic Constants for Formate Oxidation at the pH Optimum

FDH Variant	NAD ⁺ :Formate reaction ^a		
	K_m^{formate} (mM)	k_{cat} (min ⁻¹)	k_{cat}/K_m (1/min*mM)
WT	0.19 ± 0.01	2124 ± 36	11181 ± 620
C386S	-	< 0.001	-
H387M	3.6 ± 0.1	2022 ± 164	563 ± 49
H387R	n.d.	n.d.	n.d.
H387K	27.5 ± 2.7	56 ± 13	2.0 ± 0.5
H387F	-	< 0.001	-
H387M/R587T	-	< 0.001	-
R587T	-	< 0.001	-
R587K	362 ± 27	674 ± 39	1.9 ± 0.2
R587T/R597 ⁺	-	37 ± 10 ^b	-
R597 ⁺	-	64 ± 20 ^b	-

^aParameters (±SD) relative to the Mo saturation of the FDH^{WT}

^bApparent k_{cat} values

-, Not determined

Table 2: Apparent Turnover Numbers for Nitrate Reduction

	Nitrate:MV Activities ^a
FDH Variant	Apparent k_{cat} (min ⁻¹)
Extracted Cofactors from WT	-
WT	0.21 ± 0.03
WT (deMoco)	-
WT (desulfo)	0.31 ± 0.04
WT (azide inhibited)	-
C386S	0.22 ± 0.05
H387M	0.06 ± 0.01
H387M/R587T	0.08 ± 0.01
R587T	0.13 ± 0.01
R597 ⁺	1.20 ± 0.45
R587T/R597 ⁺	2.23 ± 0.43

^aTurnover Numbers (± SD) calculated to a 100% Mo saturation
 -, No activity determined

Figures

Figure 1:

Figure 2:

Figure 3:

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Graphic for the Table of Contents

Title

The molybdenum active site of formate dehydrogenase is capable to catalyze C-H bond cleavage and oxygen atom transfer reactions

Authors

Tobias Hartmann¹, Peer Schrapers², Tillmann Utesch³, Manfred Nimtz⁴, Yvonne Rippers³, Holger Dau², Maria Andrea Mroginski³, Michael Haumann², and Silke Leimkühler^{1*}

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