A Sulfurtransferase Is Essential for Activity of Formate Dehydrogenases in *Escherichia coli* 1,2

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Background: The L-cysteine desulfurase IscS interacts with FdhD, a protein essential for the activity of formate dehydrogenases.

Results: IscS transfers sulfur to FdhD, and this is required to yield an active enzyme.

Conclusion: FdhD is a sulfurtransferase between IscS and formate dehydrogenases.

Significance: This helps us to understand how sulfur transfer occurs in living cells.

L-Cysteine desulfurases provide sulfur to several metabolic pathways in the form of persulfides on specific cysteine residues of an acceptor protein for the eventual incorporation of sulfur into an end product. IscS is one of the three *Escherichia coli* L-cysteine desulfurases. It interacts with FdhD, a protein essential for the activity of formate dehydrogenases (FDHs), which are iron/molybdenum/selenium-containing enzymes. Here, we address the role played by this interaction in the activity of FDH-H (FdhF) in *E. coli*. The interaction of IscS with FdhD results in a sulfur transfer between IscS and FdhD in the form of persulfides. Substitution of the strictly conserved residue Cys-121 of FdhD impairs both sulfur transfer from IscS to FdhD and FdhF activity. Furthermore, inactive FdhF produced in the absence of FdhD contains both metal centers, albeit the molybdenum cofactor is at a reduced level. Finally, FdhF activity is sulfur-dependent, as it shows reversible sensitivity to cyanide treatment. Conclusively, FdhD is a sulfurtransferase between IscS and FdhF and is thereby essential to yield FDH activity.

L-Cysteine is the sulfur source for the biosynthesis of a variety of cofactors, such as iron-sulfur clusters, thiamine, molybdopterin, and biotin, and for the modification of tRNA (1). Incorporation of sulfur into biomolecules involves pyridoxal 5'-phosphate-dependent L-cysteine desulfurases. They catalyze the conversion of L-cysteine to L-alanine with the concomitant release of the sulfane sulfur atom that forms a protein-bound persulfide intermediate (R-S-SH) on a conserved cysteine residue. The persulfide sulfur atom is then transferred to conserved cysteine residues in acceptor proteins to form new persulfide groups for the eventual incorporation of sulfur into an end product (2, 3). Sulfur transfer requires interaction between the L-cysteine desulfurase and the acceptor protein, usually resulting in stimulation of the activity of the former (4, 5). *Escherichia coli* synthesizes three L-cysteine desulfurases: SufS, CsdA, and IscS. In contrast to SufS and CsdA, IscS appears to be the physiological sulfur source for the cell. IscS belongs to the Isc (iron-sulfur cluster) system, the housekeeping [Fe-S] cluster biosynthesis system. In addition to providing sulfur for [Fe-S] assembly, IscS is involved in thiamine, biotin, and molybdopterin synthesis, selenium incorporation, and tRNA modifications (1). To commit sulfur to all of these different metabolic pathways, IscS is able to interact with and to transfer sulfur to different acceptor proteins. They all bind close to the catalytic cysteine residue of IscS (6). As IscS is a dimer, acceptor proteins bind on each side of IscS, as was shown with the heterotetramers IscS-TusA and IscS-IscU (6). TusA participates in a pathway that modifies tRNA, and IscU is a so-called [Fe-S] scaffold protein. In addition to TusA and IscU, large-scale interactome studies identified FdhD as a binding partner of IscS (7, 8). FdhD has been shown to be required for the activity of formate dehydrogenases (FDHs) in *E. coli* through an unknown mechanism (9, 10). *E. coli* synthesizes three FDHs that decompose formate to carbon dioxide and protons. FdnGHI (the so-called FDH-N) and FdoGHI (FDH-O) are respiratory enzymes anchored to the periplasmic side of the inner membrane (11, 12). FdhF (FDH-H) is part of the fermentative formate-hydrogen lyase complex and is located at the cytoplasmic side of the membrane (13). All three FDHs share a common requirement for FdhD for their activity (9, 14, 15). Moreover, they all contain a [4Fe-4S] cluster and a molybdenum cofactor (Moco) in their catalytic subunit as revealed by x-ray structure analysis of FdhF and FdnGHI (12, 16). According to the structure of the Moco, they belong to the dimethyl sulfoxide reductase family, which is widespread in prokaryotes (17).

Here, we address the role played by the interaction of IscS with FdhD in the activity of FdhF in *E. coli*. We show that FdhD stimulates the L-cysteine desulfurase activity of IscS and recuits sulfur as persulfides from IscS. Substitution of the...
strictly conserved residue Cys-121 of FdhD prevents sulfur transfer from IscS and impairs FdhF activity. FdhF activity is sensitive to cyanide treatment, with the activity being restored with an exogenous sulfur source. Overall, our data support a model in which sulfur transfer occurs between IscS and FdhF, the sulfur being ultimately transferred to FdhF, a step indispensable for activity.

EXPERIMENTAL PROCEDURES

Bacterial Two-hybrid Assay—Protein interactions were detected using a bacterial two-hybrid approach as described previously (18, 19). Protein interactions were estimated by measurement of β-galactosidase activity in cells grown to mid-log phase in anaerobiosis at 30 °C in L-broth supplemented with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside, 12.5 mM NaHCO3, 2 μM Na2MoO4, and 2 μM Na2S2O5. As a control for protein expression, stability, and correct folding, the plasmids used to express the fusion proteins were used to complement mutant strains, and wild-type levels of FDH activities were used to express the fusion proteins were used to complement mutant strains, and wild-type levels of FDH activities were measured. Zip is a yeast transcriptional activator used as a negative control (19).

Protein Purification—Because the variant protein FdhF(SeC140C) is active (20) and easier to overproduce due to the absence of selenocysteine incorporation during translation, the UGA codon encoding selenocysteine 140 of FdhF was exchanged with a UGC codon as described under supplemental “Experimental Procedures.” Purified FdhF(SeC140C) was thus employed for in vitro experiments. The expression and purification of a His6-tagged version of IscS, FdhF(SeC140C), FdhD, and its variants were performed by nickel affinity chromatography as described supplemental “Experimental Procedures.”

Determination of l-Cysteine Desulfurase Activity—l-Cysteine desulfurase activity was measured as described previously (21) by quantification of the produced l-alanine generated per min/mol of IscS. 2.5 μM IscS protein with or without 5 μM FdhD, BSA, FdhD treated with 20 μM N-ethylmaleimide (Sigma), or 5 μM FdhD variant was used in the assay.

Analysis of Protein-bound Persulfides by N-(Iodoacetyl)-N’-(5-sulfo-1-naphthyl)ethylenediamine (1,5-I-AEDANS) Gel Assay—The basic principle of the identification of protein-bound persulfides by use of 1,5-I-AEDANS has been described by Zheng et al. (22). Proteins were first treated with DTT to reduce the thiol groups and remove any persulfides. In a typical experiment, 20 μg of the IscS protein and 40 μg of the FdhD protein were incubated in 13.5 μl of 0.1 M Tris-HCl (pH 9.3) in the presence of l-cysteine. The reaction was allowed to proceed for 1 h at room temperature for persulfide formation. The excess of l-cysteine was removed by dialysis before 0.5 μl of 0.5 mM 1,5-I-AEDANS solution was added to the reaction mixture, followed by incubated for 1 h at 4 °C. After incubation, unbound 1,5-I-AEDANS was allowed to react with l-cysteine (1 μl of 2 mM stock solution) during 30 min at room temperature to prevent reaction of 1,5-I-AEDANS in subsequent reaction steps. To release possibly bound persulfides, 1 μl of 100 mM DTT solution was added to the reaction mixture, resulting in the formation of N-(thiocysteine)-N’-(5-sulfo-1-naphthyl)ethylenediamine (i.e. 1,5-AEDANS-sulfide conjugate) issued from reductive cleavage of the disulfide adduct. Subsequently, the reaction was supplemented with 2 μl of native loading buffer (2 m sucrose and 1% (w/v) bromphenol blue) and electrophoresed for 45 min at 200 V on SDS-12% polyacrylamide gels (adjusted to pH 9.5 with 4 M NaOH to improve separation). Upon exposure to UV light, 1,5-I-AEDANS reaction products such as 1,5-AEDANS-labeled proteins, 1,5-AEDANS-sulfide conjugate, and excess 1,5-AEDANS bound by l-cysteine were visualized. After exposure to UV light, proteins in the same gel were stained with Coomassie Brilliant Blue to allow comparison of protein amounts in each lane.

Immunoanalysis—Equal amounts of total proteins from crude extracts of the WT strain, FdhF, or FdhD transformed with an empty plasmid, pFdhD, or pFdhD(C121A/C124A) were subjected to SDS-12% PAGE. Proteins were then electrotransferred to a sheet of nitrocellulose, and immunoanalysis was performed using the ECL procedure (Amersham Biosciences) with a rabbit anti-FdhF primary antibody, followed by a goat secondary antibody.

Metal Analysis—Metal analysis of purified FdhF(SeC140C) was performed using inductively coupled plasma mass spectrometry (ICP-MS). Protein samples were wet-ashed in a 1:5 mixture with 65% (v/v) nitric acid for 2 days. Samples were then diluted with 3 ml of ultrapure water (18 megohms). The solutions obtained were then analyzed in triplicate by ICP-MS using a Thermo Series II ICP-MS apparatus (Thermo Electron, Les Ulis, France). As a reference, a multi-element standard solution (SCP Science, Quebec, Canada) was used. Metal concentrations were finally determined using PlasmaLab software (Thermo Electron).

FdhF and Nitrate Reductase Activities—FDH (FdhF) activity was measured at 37 °C in an anaerobic glove box (100% N2) by following the reduction of benzyl viologen spectrophotometrically at 600 nm coupled to the oxidation of sodium formate (23). FDH activity is expressed in micromoles of formate reduced per min/mg of protein.

Nitrate reductase (NarGH) activity was measured anaerobically at 37 °C by following the oxidation of reduced benzyl viologen spectrophotometrically at 600 nm coupled to the reduction of potassium nitrate (24). Nitrate reductase-specific activity is expressed in micromoles of nitrate reduced per min/mg of protein.

Sodium Sulfide Treatment—Sodium sulfide solution at a concentration of 10 mM was prepared by dissolving crystals in deionized water. The treatment was carried out with crude extract (140 μg) or purified FdhF(SeC140C) enzyme (10 μg) in the presence of 1.5 mg/ml benzyl viologen and 720 μM sodium sulfide in 500 μl of 50 mM Tris-HCl (pH 7.5). Samples were incubated at 37 °C for 60 min, allowing them to reach a plateau. After 60 min of incubation, substrate (sodium formate or potassium nitrate) was added to the sample, and oxidation or reduction of benzyl viologen was measured spectrophotometrically at 600 nm. All steps were done in an anaerobic glove box.

Potassium Cyanide Inactivation—260 μg of sodium sulfide-treated FdhF(SeC140C) was incubated with 1.5 mg/ml benzyl viologen and 50 mM KCN in 200 μl of 50 mM MES (pH 6), 50 mM Na2SO3, 2 mM DTT, and 3 mM NaN3 overnight at 4 °C. Excess KCN was separated from the protein with a 10-kDa cut-
of IscS with FdhD, we next tested if the L-cysteine desulfurase activity of IscS is enhanced through interaction with physiological partners (4, 5, 26–29). On the basis of the tight interaction of IscS with FdhD reported by large-scale studies (7, 8), bacterial two-hybrid experiments were conducted (Fig. 1A). IscS was confirmed to interact with FdhD. It is noteworthy that no interaction was detected between IscS and FdhE, a protein also required for the activity of the two periplasmic respiratory FDHs (9, 15). Such specific interaction between IscS and FdhD was further confirmed by co-purification of IscS with His6-FdhD from a cell-free extract (supplemental Fig. S1). Together, these results show that IscS specifically interacts with FdhD.

FdhD Stimulates IscS L-Cysteine Desulfurase Activity—Previous studies have shown that the activity of L-cysteine desulfurases is usually enhanced through interaction with physiological partners (4, 5, 26–29). On the basis of the tight interaction of IscS with FdhD, we next tested if the L-cysteine desulfurase activity of IscS is enhanced by FdhD. The addition of a 2-fold molar excess of FdhD increased the desulfurase activity of IscS by nearly 2-fold (Fig. 1B). The enhancement of IscS activity was specific to FdhD, as BSA, used as a control, showed no effect. Furthermore, FdhD showed no L-cysteine desulfurase activity.

Enhancement of the activity of L-cysteine desulfurases by their physiological partners is usually seen as the result of transfer of the sulfane sulfur generated by the L-cysteine desulfurase to surface-exposed thiol groups of the protein partner. To determine whether any of the seven cysteine residues of FdhD are involved in the stimulation of IscS activity, FdhD was pre-treated with the thiol-specific alkylating reagent N-ethylmaleimide. No stimulation of L-cysteine desulfurase activity was found (Fig. 1B), indicating that surface-exposed thiol groups of FdhD are involved in this stimulation. Multiple sequence alignment analysis of FdhD showed that, of the seven cysteine residues, only Cys-121 is strictly conserved (supplemental Fig. S2). Note that this residue is part of a cysteine- and glycine-rich region. In the sequence of E. coli FdhD, a second cysteine residue, Cys-124, is found close to Cys-121. To assess the importance of these cysteine residues, single and double variants were thus produced and tested for their ability to stimulate IscS activity (Fig. 1B). Although the FdhD(C124A) variant showed an unaffected stimulatory activity, substitution of the conserved Cys-121 residue led to reduction in (FdhD(C121A)) or a complete loss of (FdhD(C121A/C124A)) stimulation of the IscS activity. Used as a control, two-hybrid experiments confirmed that all variants were able to interact with IscS (supplemental Fig. S3). Therefore, the integrity of the conserved Cys-121 residue of FdhD is required for stimulation of the IscS activity.

Sulfuration of FdhD by IscS—To evaluate whether sulfur atoms are transferred from IscS to FdhD, the presence of persulfides on FdhD was assessed using the fluorescent sulfhydryl-reactive alkylating reagent 1,5-I-AEDANS. Without L-cysteine (Fig. 2, first lane), IscS was labeled with 1,5-I-AEDANS, supporting the existence of surface-exposed cysteine residues. In the presence of L-cysteine (second lane), IscS was not labeled due to the resolution of the persulfides resulting from the L-cysteine desulfurase activity under reducing conditions. The 1,5-AEDANS-sulfide conjugates thus produced were observed at 4673
Function of the IscS-FdhD Interaction

The FDH activity of FdhF was measured in crude extracts of the WT strain or fdhD transformed with an empty vector, pFdhD, or pFdhD(C121A/124A). The results represent the average of three independent experiments.

| Strain          | FdhF activity (units/mg protein) |
|-----------------|----------------------------------|
| WT              | 0.32 ± 0.1                       |
| pFdhD           | 0                                |
| pFdhD(C121A)    | 0.24 ± 0.08                      |
| pFdhD(C121A/124A) | 0                        |
| pFdhD(C124A)    | 0                                |

As the strictly conserved Cys-121 residue is essential for efficient sulfur transfer between IscS and FdhD, we next tested the importance of this residue for FdhF activity (Table 1). The C121A substitution led to a total loss of activity, supporting an essential role of this residue in the ability of FdhD to yield active FDHs. As expected, the double cysteine variant gave the same result as the C121A variant. Unexpectedly, the C124A substitution led to a total loss of FdhF activity. Two-hybrid experiments confirmed that all FdhD variants interacted with FdhF.

In Vivo Reactivation of FdhF Is Sulfur-dependent—The importance of Cys-121 of FdhD in both catalyzing efficient sulfur transfer from IscS to FdhD and promoting FdhF activity prompted us to investigate whether FDH activity is sulfur-dependent. To this end, a cell-free extract issued from an fdhD strain was treated in vitro with sodium sulfide as an exogenous sulfur source and further tested for activity.

As shown in Table 2, sulfide treatment restored the activity of FdhF from an fdhD extract to a level equivalent to the one obtained with a WT extract similarly treated. Used as a control, no activity was measured with an fdhF extract. These results demonstrate that the loss of FdhF activity in the absence of FdhD can be fully restored by an exogenous sulfur source. Xanthine oxidoreductase (XOR) constitutes a family of molybdoenzymes (widespread in prokaryotes and eukaryotes) distinct from the dimethyl sulfoxide reductase family. Indeed, these enzymes are characterized by the presence of an additional sulfur ligand in the molybdenum atom present at the active site (30). Accordingly, XOR family members display a sulfur-dependent activity. The sulfur atom can easily be removed by cyanide treatment with the concomitant loss of activity. Such a process is reversible upon treatment with an exogenous sulfur source (31). To evaluate whether FdhF activity displays a comparable pattern as members of the XOR family, we tested the ability of FdhD to bind persulfides generated by IscS. When both proteins were mixed in the presence of L-cysteine, FdhD showed no 1,5-AEDANS-sulfide conjugate was observed in the bottom of the gel. In contrast, in the presence of L-cysteine and FdhD (third lane), no 1,5-AEDANS-sulfide conjugate was produced at the bottom of the gel, and FdhD was strongly labeled. This confirms that FdhD by itself is devoid of cysteine desul furylase activity and that FdhD has surface-exposed cysteine residues. When both proteins were mixed in the presence of L-cysteine (fourth lane), a 1,5-AEDANS-sulfide conjugate was produced, IscS was no longer labeled, and only a low level of labeling was observed on FdhD. Sulfane sulfur atoms were thus deposited by IscS on surface-exposed cysteine residues of FdhD and later released by DTT treatment. This experiment demonstrates that FdhD accepts sulfur from IscS in the form of persulfides and explains why FdhD is able to stimulate IscS-L-cysteine desul furylase activity.

Furthermore, when the FdhD(C121A) variant was used instead of FdhD together with IscS (Fig. 2, fifth lane), 1,5-AEDANS remained bound to both proteins, indicating a reduced ability of FdhD(C121A) to bind persulfides generated by IscS. The same result was obtained with the double-substituted mutant FdhD(C121A/124A) (seventh lane), whereas in the case of the Cys-124 substitution (sixth lane), IscS and FdhD(C124A) were no longer labeled, indicating that all of the sulfane sulfur atoms generated by IscS were transferred to FdhD(C124A) and further cleaved off by DTT treatment as 1,5-AEDANS-sulfide conjugates. This situation is comparable with the one obtained with FdhD (fourth lane). Altogether, these results show that Cys-121 is crucial for efficient sulfur transfer between IscS and FdhD, whereas Cys-124 is not required.

Cys-121 and C124 of FdhD Are Required for FDH Activity—To analyze the importance of the fdhD gene product for FDH activity, the production and metal content of FdhF were evaluated in the absence of fdhD. At first, immunoblot analysis of cell-free extracts issued from the WT or fdhD strain revealed no differences in terms of FdhF production (Fig. 3). Next, the iron and molybdenum content was quantified by ICP-MS and expressed in moles of metal ion/mole of purified FdhF(SeC140C) issued from WT and fdhD cells, respectively. The iron content was unchanged (4.1 versus 4.2) and is in accordance with the presence of a single [4Fe-4S] cluster/enzyme. The molybdenum content was lower in the absence of FdhD (1 versus 0.6). Thus, FdhD appears to be essential neither for metal center insertion within FdhF nor for its stability.
family, purified FdhF(SeC140C) enzyme was incubated with cyanide, followed by sodium sulfide treatment.

As shown in Table 3, FdhF activity was severely affected by cyanide treatment with a 10-fold reduction. After removal of excess cyanide, the nearly inactivated enzyme was incubated with sodium sulfide, resulting in recovery of activity by up to 60%. The activity of the respiratory nitrate reductase complex NarGH, which belongs to the dimethyl sulfoxide reductase family (FdhF), was subjected to the same procedure. Although cyanide treatment resulted in a diminution of activity (likely affecting the [Fe-S] clusters of NarGH), such an effect was not reversible upon treatment with sodium sulfide (supplementary Table S3). Altogether, these results firmly demonstrate that the activity of the FDH FdhF is sulfur-dependent, likely in the same way as members of the XOR family.

**DISCUSSION**

In this study, we addressed the role played by the interaction of IscS with FdhD in promoting the activity of FdhFs. We have shown that the interaction of IscS with FdhD results in a sulfur transfer between IscS and FdhD, as FdhD stimulates the L-cysteine desulfurase activity of IscS and receives persulfides from IscS. We found that substitution of the strictly conserved residue Cys-121 of FdhD impairs both sulfur transfer from IscS to FdhD and FdhF activity and that substitution of Cys-124 abolishes FdhF activity. Moreover, supporting evidence that sulfur transfer is essential to yield active FdhF is provided by the fact that the absence of fdhD can be reversed solely by an exogenous sulfur source. In addition, we found that the activity of FdhF shows reversible cyanide inactivation. On the basis of the findings of this study, we propose that FdhD functions as a sulfurtransferase between IscS and FdhF and that, to yield active FdhF, FdhD ultimately transfers sulfur to the Moco of the enzyme. A working model describing the function of FdhD in the activity of FdhF is depicted in Fig. 4.

Data are available concerning the binding sites of IscS-interacting partners. IscS is a dimeric protein with a large and contiguous area of evolutionarily conserved amino acids surrounding the catalytic residue Cys-328 (E. coli numbering) (6, 32). Structural and biochemical experiments have mapped the binding sites of IscS-interacting partners (IscU, Thil, TusA, IscX, and CyaY) close to Cys-328 on the surface of each IscS monomer (6). Although TusA, CyaY, IscX, and Thil have overlapping binding footprints, TusA and IscU occupy distinct zones on IscS. The binding site of FdhD is very likely localized close to Cys-328 and on each opposite side of the IscS dimer. However, to date, it is unknown whether it overlaps or whether it is distinct from binding footprints of other IscS-binding partners. The structures of soluble TusA and IscU are rearranged when both proteins are in complex with IscS (6). These data raise the question about the folding of FdhD in solution and in complex with IscS. The x-ray crystal structure of FdhD from Desulfofadia psychrophila has been resolved at 2.1 Å (Protein Data Bank code 2pw9). FdhD exists as a dimer with a wide surface contact of 1900 Å². Such a structure questions the oligomeric state of FdhD in receiving sulfur.

Interestingly, careful analysis of the x-ray structure of D. psychrophila FdhD revealed the absence of a disordered loop of 20 amino acids carrying the conserved cysteine residue. It is most likely that the conserved cysteine needs to be carried on a flexible loop to be able to contact IscS for sulfur cargo and to be able to contact FdhD for sulfur delivery. We have provided evidence that Cys-121 plays an essential role in the whole sulfur transfer between IscS and FdhD, whereas the role of Cys-124 is more intricate. Although Cys-124 is dispensable for sulfur transfer between IscS and FdhD (Fig. 1A and Fig. 2), it is essential to yield active FdhF (Table 1). Closer examination of Fig. 2 reveals that, in the presence of IscS, the C124A variant is unlabeled compared with wild-type FdhD. This results from a higher level of persulfides on FdhD(C124A) than on FdhD. A model that can reconcile these data is that Cys-124 promotes sulfur release from the catalytic cysteine residue (likely Cys-121) through nucleophilic attack. Interestingly, this model shares analogies with the one described for 4-thiouridine biosynthesis by Thil (33, 34). Although a transient persulfide is formed on the active site Cys-456 residue of Thil in the presence of IscS, participation of a second cysteine residue (Cys-344) of Thil is required for turnover, with the ultimate formation of a disulfide bridge between both residues. It is unknown whether Cys-121 and Cys-124 from FdhD form a disulfide bridge. This hypothesis is currently under investigation.

Three lines of evidence are highly suggestive of sulfuration of FdhD being required for enzymatic activity. First, the absence of FdhD can be fully compensated by sulfide treatment of cell-free extracts (Table 2). Second, disturbance of the flexible active site loop carrying Cys-121 and Cys-124 affects both sulfur transfer from IscS (Fig. 2) and FdhF activity (Table 1). Third, the activity of FdhF can be cyanide-inactivated, and this inactivation can be reversed by chemical sulfuration (Table 3). Such reversibility of
the activity has long been described for XOR family members as resulting from sulfur coordination of the molybdenum atom that can be sequestered with cyanide to form thiocyanate and reintroduced by chemical sulfuration. It is noteworthy that the x-ray crystal structure of the tungsten-containing FDH from Desulfovibrio gigas sharing high structural similarity with FDH reveals the presence of an additional sulfur atom at the tungsten coordination sphere (35). Furthermore, reinterpretation of the x-ray data of E. coli FdhF shows that sulfur refines better than oxygen at the apical position of the molybdenum coordination sphere (36). Thus, we postulate that FdhD ultimately transfers sulfur to the molybdenum atom present at the active site of FdhF.

In addition to FDHs, the dimethyl sulfoxide reductase family includes other members for which an additional sulfur ligand of the molybdenum atom is encountered at the catalytic site. The x-ray crystal structure of the periplasmic nitrate reductase (Nap) of Cupriavidus necator was resolved at 1.5 Å and shows the presence of a terminal sulfur ligand at the molybdenum coordination sphere (37). These results confirm x-ray data obtained on the homologous NapA from Desulfovibrio desulfuricans ATCC 27774, which revealed a unique coordination sphere of six sulfur ligands bound to the molybdenum atom (38). Although the resolution was not sufficient to ascertain the identity of the molybdenum ligands in two other Nap structures (39, 40), altogether, these observations raise the question of the sulfuration of the Moco being a general feature of this group of enzymes. The mechanism could be enzyme-specific, as described herein for FdhD or for the Rhodobacter capsulatus xanthine dehydrogenase that requires XdhC for sulfuration (41, 42). Alternatively, the sulfuration mechanism for Nap could involve a sulfurtransferase dedicated to several enzymes, as is the case for ABA3, the Arabidopsis thaliana Moco sulfurase of all members of the XOR family. ABA3 carries L-cysteine desulfurase activity and interacts with the Moco for sulfuration of both XOR and aldehyde oxidase (43–45). In this regard, further work is required for the identification of the sulfurtransferase that would be required for the sulfuration and thus enzymatic activity of periplasmic nitrate reductases.

The x-ray crystal structure of FDHs, like that of other molybdoenzymes, shows that the Moco is buried in the enzyme and, as such, is not accessible to the external environment except for a narrow crevice for substrate access. Given this observation, one may question how FdhD obtains access to Moco for sulfuration, which is still a matter of debate for members of the XOR family. Sulfuration of the Moco prior to its insertion, as known for the XdhC protein (42), through direct interaction with FdhD would constitute, as such, a satisfying hypothesis. In this context, we observed a decrease of molybdenum content in purified FdhF expressed in the absence of FdhD, with only half containing molybdenum. Obviously, a putative interaction between FdhD and the Moco is not essential for Moco insertion. A similar situation is encountered for ABA3 and XdhC, which have been shown to interact with the Moco (41, 45), albeit the Moco is inserted into their respective molybdoenzymes in their absence (43, 46). In this regard, careful analysis of the x-ray crystal structure of D. psychrophila FdhD shows the presence of a structural motif known to interact with nucleotides: a α/β-Rossmann fold. As the Moco present in FdhF is composed of four nucleotidic motifs, two GMPs, and two molybdopterin, one can hypothesize that, through its Rossmann fold, FdhD interacts with the Moco by one of these motifs. This hypothesis is currently under investigation.

Altogether, our results provide a comprehensive description of an active role for FdhD in sulfur transfer from IscS, a major player in sulfur metabolism, to the active site of FDHs. This special dependence may also hold true in other molybdoenzymes and be of paramount importance for their reactivity.

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Altogether, our results provide a comprehensive description of an active role for FdhD in sulfur transfer from IscS, a major player in sulfur metabolism, to the active site of FDHs. This special dependence may also hold true in other molybdoenzymes and be of paramount importance for their reactivity.

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