Flavodoxin hydroquinone provides electrons for the ATP-dependent reactivation of protein-bound corrinoid cofactors

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Corrinoid-dependent enzyme systems rely on the super-reduced state of the protein-bound corrinoid cofactor to be functional, for example, in methyl transfer reactions. Due to the low redox potential of the [CoII]/[CoI] couple, autoxidation of the corrinoid cofactor occurs and leads to the formation of the inactive [CoII]-state. For the reactivation, which is an energy-demanding process, electrons have to be transferred from a physiological donor to the corrinoid cofactor by the help of a reductive activator protein. In this study, we identified reduced flavodoxin as electron donor for the ATP-dependent reduction of protein-bound corrinoid cofactors of bacterial O-demethylase enzyme systems. Reduced flavodoxin was generated enzymatically using pyruvate:ferredoxin/flavodoxin oxidoreductase rather than hydrogenase. Two of the four flavodoxins identified in Acetobacterium dehalogenans and Desulfotobacterium hafniense DCB-2 were functional in supplying electrons for corrinoid reduction. They exhibited a midpoint potential of about −400 mV (E_{SHE}, pH 7.5) for the semiquinone/hydroquinone transition. Reduced flavodoxin could be replaced by reduced clostridial ferredoxin. It was shown that the low-potential electrons of reduced flavodoxin are first transferred to the iron-sulfur cluster of the reductive activator and finally to the protein-bound corrinoid cofactor. This study further highlights the importance of reduced flavodoxin, which allows maintaining a variety of enzymatic reaction cycles by delivering low-potential electrons.

Introduction

Protein-bound corrinoid cofactors play an essential role as methyl group carriers in the C₁ metabolism of anaerobes [1]. To bind methyl groups, the corrinoid cofactor has to adopt the super-reduced [CoI]-state, which is highly sensitive to autoxidation. To maintain the catalytic reaction cycle, inadvertently oxidized [CoII]-corrinoid cofactors have to be rereduced to the [CoI]-state. For this reaction, low-potential electrons are required. The electron transfer reaction is challenging since the redox potential of the most negative physiological electron donors ferredoxin and flavodoxin is about 100 mV more positive [2] than the midpoint potential of the [CoII]/[CoI] couple (E_{SHE} ≈ −500 mV) [3-5]. To overcome this redox barrier, energy has to be invested [3,6,7]. So far, the electron transfer from a physiological donor to a protein-bound corrinoid cofactor was studied in detail only for MetH, the cobalamin-dependent methionine synthase of Escherichia coli [see, for

Abbreviations
AdoMet, S-adenosylmethionine; AE, activating enzyme; CoA, coenzyme A; CoFeSP, corrinoid-iron/sulfur protein; CP, corrinoid protein; Fd, ferredoxin;Fld, flavodoxin; FMN, flavin mononucleotide; MT, methyltransferase; PFOR, pyruvate:ferredoxin/flavodoxin oxidoreductase; RACE, reductive activator of corrinoid-dependent enzymes; TPP, thiamine pyrophosphate.
example, 8-10. The electrons for the reduction of \([\text{Co}^{II}]\) of MetH are provided by flavodoxin in the semiquinone or hydroquinone (reduced) form, depending on the oxygen availability during growth [9]. Further, this reaction depends on S-adenosylmethionine (AdoMet). AdoMet acts as methyl group donor to generate methylcobalamin, which re-enters the catalytic reaction cycle of MetH [11]. Only limited knowledge is available on the physiological electron donor(s) of corrinoid-dependent methyltransferase systems, which rely on ATP for their reactivation. These enzyme systems were found in archaea and bacteria and allow for the utilization of various methylated compounds as growth and energy substrate, for example, methanol, methylamines, or phenyl methyl ethers [12-14]. In contrast to MetH, which is a single protein (1227 aa) consisting of four modules [15], four single proteins are involved in the methyl transfer reaction: two methyltransferases (MT I, MT II), a corrinoid protein (CP), and a reductive activator [13-14,16-18]. MT I catalyzes the cleavage and transfer of the substrate methyl group to the super-reduced corrinoid cofactor (bound to CP) yielding methylcobalamin. MT II is responsible for the transfer of the methyl group from the corrinoid cofactor to an acceptor molecule, which is coenzyme M in archaea and tetrahydrofolate in bacteria. Finally, the methyl group is converted to methane (archaea) or acetate (acetogenic bacteria) [19,20]. In methanogenic archaea, MT I and CP can be tightly bound to each other [14]. Whereas MT I, MT II, and CP are important for the catalytic reaction cycle, the reductive activator is crucial for the reactivation of inadvertently oxidized corrinoid cofactors in the [CoI]-state; it catalyzes the ATP-dependent electron transfer from a physiological donor to the [CoI]-corrinoid cofactor yielding super-reduced active [CoI], thus maintaining the catalytic reaction cycle. The same mode of activation was observed for the corrinoid-iron/sulfur protein (CoFeSP) of *Carboxythermus hydrogenoformans* [21], which plays a crucial role as methyl group carrier in the synthesis of acetyl-CoA [22]. The activators, although quite different in amino acid sequence, belong to the same protein family and were summarized as RACEs, the reductive activators of corrinoid-dependent enzymes. They harbor two [4Fe-4S] clusters in archaea or one [2Fe-2S] cluster in bacteria [23]. So far, a few RACE proteins were isolated and characterized: (a) MAP and RamA as activators of the methanogenic methanol and methylamine methyltransferases of *Methanosarcina barkeri*, respectively [6,16,24], (b) RACo, the reductive activator of CoFeSP of *C. hydrogenoformans* [21,25,26], and (c) the reductive activators of the ether-cleaving *O*-demethylase enzyme systems of *Acetobacterium dehalogenans* [5,7,23,27,28], *Desulfitobacterium hafniense* DCB-2 [17] and *Eubacterium limosum* [18], which were termed activating enzymes (AEs). Previous works reported that ferredoxin coupled to hydrogenase can provide electrons for the reactivation of the corrinoid cofactors [6,29]; however, the electron transfer reaction was never a primary focus of former studies. The need of ATP to drive thermodynamically unfavorable electron transfer reactions is not limited to corrinoid-dependent enzyme reactions. Also nitrogenases, benzoyl-CoA reductases, and 2-hydroxyacyl-CoA dehydratases couple electron transfer processes to the hydrolysis of ATP [30,31]. Reduced flavodoxin [32-34] or ferredoxin [35,36] was reported as the physiological electron donors involved.

In this study, reduced flavodoxin was identified as electron donor for the ATP-dependent activation of *O*-demethylase corrinoid proteins of *A. dehalogenans* and *D. hafniense*. Reduction of flavodoxin was achieved enzymatically by pyruvate:ferredoxin/flavodoxin oxidoreductase (PFOR). Flavin mononucleotide (FMN) as well as flavodoxin semiquinone failed to deliver electrons for corrinoid reduction. The results of this study indicate that the low-potential electrons stored in reduced flavodoxin \((E_{\text{SHE}} \approx -400 \text{ mV})\) are first transferred to the [2Fe-2S]-cluster of the reductive activator \((E_{\text{SHE}} \approx -330 \text{ mV})\) and subsequently to the [CoI]-corrinoid cofactor leading to the formation of the super-reduced [CoI]-state. This article further highlights the importance of reduced flavodoxin, which allows maintaining a variety of enzymatic reaction cycles by delivering low-potential electrons.

**Results**

**Flavodoxins of *Acetobacterium dehalogenans* and *Desulfitobacterium hafniense* DCB-2**

In the genome of each microorganism, two putative flavodoxin-encoding genes were identified. According to their locus tags, they were designated as **Ad_Fld0540**, **Ad_Fld0918**, **Dh_Fld0519**, and **Dh_Fld0741**, respectively. They consist of 143–159 amino acid residues with a predicted molecular mass of 15–17 kDa. The maximum sequence identity on protein level is 60% (for **Ad_Fld0918** and **Dh_Fld0741**). The flavodoxins were produced as cofactor-free apoproteins in *E. coli* with the exception of **Dh_Fld0519**, which is the only long-chain flavodoxin among the four [37]. Successful constitution of the apoproteins with FMN was verified by UV/vis spectroscopy (shown for **Dh_Fld0741** in Fig. 1A, spectrum 1). The oxidized form of flavodoxin showed an absorption maximum at 445 nm and a
shoulder at ~470 nm, which is absent in free FMN. To test the functionality of the flavodoxins, they were applied as possible electron acceptors in the PFOR reaction. PFOR was obtained as recombinant protein from E. coli. The encoding gene originates from D. hafniense DCB-2 (see below). All flavodoxins were reduced when applied as electron acceptors in the PFOR assay. During PFOR-catalyzed reduction of Dh_Fld0741, Fldox was first converted to Fldsemi, and subsequently, Fldsemi was reduced to Fldred (Fig. 1A, B). The same behavior was observed for Ad_Fld0918. In the presence of Ad_Fld0540 or Dh_Fld0519, no semiquinone form was detected as intermediate. A direct transition of Fldox to Fldred was recorded (data not shown). Accordingly, midpoint redox potentials for both couples, Fldox/Fldsemi and Fldsemi/Fldred, could only be determined for Ad_Fld0918 and Dh_Fld0741, respectively, and were about −200 and −400 mV (E SHE, pH 7.5; Table 1). The midpoint redox potential obtained for the direct transition of Fldox to Fldred (Ad_Fld0540, Dh_Fld0519) was determined to be approximately −200 mV, which is similar to the value of the Fldox/Fldsemi couple of Ad_Fld0918 and Dh_Fld0741, respectively.

**Pyruvate:ferredoxin/flavodoxin oxidoreductase of A. dehalogenans and D. hafniense DCB-2**

Two PFORs are putatively encoded in the genome of each microorganism. In A. dehalogenans, a single-subunit (Ad_PFOR2453)- and a four-subunit-containing PFOR (Ad_PFOR1052–1055) were identified. In D. hafniense DCB-2, both PFORs are encoded by a single gene (Dh_PFOR0054, Dh_PFOR4766). Production of soluble recombinant protein in E. coli was achieved for Dh_PFOR4766 (Fig. 2A). The molecular mass of the protein was about 120 kDa on SDS/PAGE, which matches the prediction on the basis of the amino acid sequence (~130 kDa). The oxidized enzyme showed a shoulder near 400 nm, which disappeared after reduction with titanium(III) citrate indicating the presence of one or more [4Fe-4S] clusters (Fig. 2B). This finding is in accordance with the two [4Fe-4S] cluster-binding motifs detected in the amino acid sequence of Dh_PFOR4766 (aa 689–700, aa 745–756). The activity of the purified enzyme reached up to 300 nkat/mg with 5 mM methyl viologen and up to 200 nkat/mg with 50 µM flavodoxin as electron acceptor. For so far unknown reasons, the activity of purified PFOR decreased to 50% or lower within 2–3 h.

**Electron transfer to the protein-bound corrinoid cofactor**

Corrinoid reduction is catalyzed by AE in an ATP-dependent reaction. In vitro, titanium(III) citrate

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**Table 1.** Midpoint redox potentials of recombinant flavodoxins at pH 7.5 determined by potentiometry coupled to UV/vis spectroscopy. Ad, Acetobacterium dehalogenans; Dh, Desulfitobacterium hafniense DCB-2; n.d., not detectable.

|          | Ad_Fld0540 | Ad_Fld0918 | Dh_Fld0519 | Dh_Fld0741 |
|----------|------------|------------|------------|------------|
| E SHE (mV) |            |            |            |
| Fldox/Fldsemi | n.d.       | −201 ± 10  | n.d.       | −165 ± 5   |
| Fldsemi/Fldred | n.d.      | −420 ± 10  | n.d.       | −370 ± 20  |
| Fldox/Fldred  | −240 ± 25  |            | −170 ± 20  |            |

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**Fig. 1.** Reduction of flavodoxin Dh_Fld0741 (45 µM) by pyruvate:ferredoxin/flavodoxin oxidoreductase Dh_PFOR4766 (0.06 µM). (A) UV/vis spectra of flavodoxin were recorded between 350 and 700 nm every 55 s. (B) Kinetics of flavodoxin reduction deduced from panel A. Oxidized flavodoxin (Fldox) and flavodoxin semiquinone (Fldsemi) were detected at 445 and 578 nm, respectively.
($E_{\text{SHE}} = -480 \text{ mV}, \text{pH 7}; [38])$ is routinely applied as electron donor (Fig. 3A). 

In vivo, the low-potential electron carriers flavodoxin or ferredoxin might be involved in the supply of electrons for corrinoid reduction. To prove this assumption, titanium(III) citrate was replaced by flavodoxin (Fld_Dh0741) and a flavodoxin-reducing system, namely PFOR (Dh_PFOR4766), pyruvate, and coenzyme A (CoA). The reductive activator AE and the corrinoid protein applied in the following assays originate from A. dehalogenans [23,27,28]. In principle, these two proteins are exchangeable by the homologs found in D. hafniense DCB-2 as described previously [17].

In the presence of reduced flavodoxin, the corrinoid reduction activity of AE was similar to the control with titanium(III) citrate and reached up to $7 \text{nkat mg}^{-1}$ (Fig. 3A, B). In the absence of an electron donor (Fig. 3C), a background activity of AE of equal or below $0.3 \text{nkat mg}^{-1}$ was determined. By addition of flavodoxin, the rate of $[\text{Co}^\text{II}]$-formation considerably increased (approx. 20-fold) (Fig. 3C, +Fld), demonstrating the effectiveness of flavodoxin as electron donor for corrinoid reduction. Free FMN, although reduced by PFOR, was not able to replace the flavodoxin Dh_Fld0741 in this reaction (Fig. 4A). Also Ad_Fld0540 and Dh_Fld0519 could not serve as electron donors (data not shown). Besides Dh_Fld0741, only Ad_Fld0918 was functional. This result indicates that only low-potential electrons ($\approx -400 \text{ mV}$), stored in the reduced form of the flavodoxins Dh_Fld0741 and Ad_Fld0918, can be transferred to the corrinoid cofactor in the $[\text{Co}^{\text{III}}]$-state. To prove this assumption, the assay conditions of PFOR were adjusted to produce either Dh_Fld0741 semiquinone with electrons at a level of about $-200 \text{ mV}$ or reduced Dh_Fld0741 with electrons at a level of approximately $-400 \text{ mV}$. This was achieved by varying the ratio of CoA to flavodoxin in the assay mixture. In reaction mixture one, the concentration of CoA was lowered to 15 µM in the presence of

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**Fig. 2.** Purification of the pyruvate:ferredoxin/flavodoxin oxidoreductase Dh_PFOR4766 via affinity chromatography on Strep-Tactin®. (A) SDS/PAGE of soluble extract (SE, 5 µg) and eluates (E, 20 µL per lane) after Coomassie stain. (B) UV/vis spectrum of purified enzyme ($6 \text{ µg}$). Black line, oxidized enzyme; red line, reduced enzyme.

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**Fig. 3.** Determination of the corrinoid reduction activity of the activating enzyme of Acetobacterium dehalogenans with (A) titanium (III) citrate or (B, C) reduced flavodoxin Dh_Fld0741 as electron donor. The reaction was started by addition of 0.9 (A) or 0.7 µM (B, C) of AE. UV/vis spectra were recorded between 350 and 600 nm at the time points indicated. The concentration of $[\text{Co}^\text{II}]$ was calculated from the absorption maximum at 386 nm. In panel (C), flavodoxin Dh_Fld0741 was added to the reaction mixture after a lag time of 3 min. The concentration of flavodoxin, if applied, was 30 µM.
60 µM flavodoxin. Under these conditions, only flavodoxin semiquinone was produced. No reduction of the [Co⁹⁺]-corrinoid cofactor was observed (Fig. 5A). [Co⁹⁺]-formation started only after addition of an excess of CoA (180 µM), which enabled the formation of reduced flavodoxin in the assay. The second reaction mixture contained 10 µM flavodoxin and 25 µM CoA. This allowed a fast and complete reduction of flavodoxin (reduced form). As shown in Fig. 5B, [Co⁹⁺]-formation started. About 20% of the corrinoid cofactor, which corresponds to ~10 µM of corrinoid, were reduced. This concentration matches the initial amount of reduced flavodoxin present in the cuvette. Due to the kinetic properties of PFOR, the remaining CoA (~15 µM) was not sufficient to enable another fast production of reduced flavodoxin. Thus, the concentration of [Co⁹⁺] only slightly increased between 5- and 8-min reaction time (Fig. 5B). By further addition of CoA (180 µM), reduced flavodoxin was again produced and the reduction of the corrinoid cofactor was completed (Fig. 5B).

So far, attempts failed to isolate ferredoxin from A. dehalogenans or D. hafniense DCB-2. In addition, production of ferredoxin as recombinant protein in E. coli was not successful. Either the recombinant protein was not soluble or the incorporation of the Fe/S clusters into apo-ferredoxin failed. However, in order to assess the potential role of ferredoxin as physiological electron donor for corrinoid reduction, we applied ferredoxin purified from Clostridium pasteurianum in the enzyme assays. First, ferredoxin was applied as potential electron acceptor in the PFOR assay. In this assay, a decrease in the absorption of ferredoxin was detected between 350 and 600 nm, indicating the reduction of the Fe/S clusters of ferredoxin by Dh_PFOR4766. The reaction velocity was about one-fourth when compared with flavodoxin (data not shown). In the corrinoid reduction assay, clostridial
ferredoxin was as efficient as Fld_Dh0741 or Ad_Fld0918 (Fig. 4B).

The role of the Fe/S cluster of the activating enzyme in the electron transfer process

In previous experiments, it was shown that flavodoxin and ferredoxin can provide electrons for corrinoid reduction. It is suggested that the electrons from flavodoxin or ferredoxin are first transferred to the [2Fe-2S]-cluster of AE. Subsequently, the electrons are transferred from the reduced Fe/S cluster of AE to the corrinoid cofactor in the [CoII]-state, resulting in the formation of super-reduced [CoI]. To prove this assumption, the reducibility of AE by reduced Fld_Dh0741 was tested. As control, titanium(III) citrate was applied as artificial reducing agent. The oxidized [2Fe-2S] cluster of AE was characterized by absorption maxima at 415 and 460 nm as well as a shoulder at ~540 nm (Fig. 6A, black line). By addition of titanium (III) citrate, the Fe/S cluster was immediately reduced. This was obvious from the decrease in the absorption (‘bleaching’) and the disappearance of the two absorption maxima (Fig. 6A, red line). To test if the Fe/S cluster of AE is also reduced by flavodoxin, titanium(III) citrate was replaced by Fld_Dh0741 and the flavodoxin-reducing system (CoA, pyruvate, PFOR). First, the spectrum of the oxidized Fe/S cluster of AE was recorded (Fig. 6B, black line). Then, flavodoxin was added, which caused an increase in the absorption between 400 and 500 nm. The reduction of flavodoxin was initiated by the addition of PFOR. Subsequently, the reduction of the Fe/S cluster of AE started. After three scans, reduction of the cluster was complete (Fig. 6B, red line).

Discussion

Reduced flavodoxin was identified as electron donor for the ATP-dependent reduction of protein-bound corrinoid cofactors. This finding is in accordance with the results obtained for nitrogenases [32,33], 2-hydroxyacyl dehydratases [34], and MetH of E. coli (grown in the absence of oxygen) [8,9]. From the two flavodoxins encoded in the genome of A. dehalogenans and D. hafniense DCB-2, respectively, only one was functional in the corrinoid reduction assay. An explanation was found by analyzing the midpoint potentials of the flavodoxins. Usually, flavodoxin accepts two electrons, each at a different redox level [2]. This was true for Ad_Fld0981 and Dh_Fld0741, both functional as electron donor for corrinoid reduction when completely reduced. In contrast, the flavodoxins Ad_Fld0540 and Dh_Fld0519 reacted like free FMN without showing a semiquinone form as intermediate during reduction and a midpoint potential of around −200 mV [39,40]. Possibly, FMN, although bound to Ad_Fld0540 and Dh_Fld0519, was not correctly incorporated into the protein. This seems to be true for Ad_Fld0540, which was the only flavodoxin exhibiting a strong fluorescence signal at 520 nm after excitation at 450 nm (data not shown). A correct incorporation of FMN would lead to a fully quenched fluorescence signal [see, for example, 41-43].

The enzyme PFOR efficiently reduced the flavodoxins applied in this study. By adapting the assay conditions, it was possible to generate either flavodoxin semiquinone or reduced flavodoxin. PFOR also recognized ferredoxin of C. pasteurianum as electron acceptor. The reduced ferredoxin was able to replace reduced flavodoxin as electron donor in the corrinoid

![Fig. 6. Reduction of the [2Fe-2S] cluster of the activating enzyme of Acetobacterium dehalogenans by (A) titanium(III) citrate (0.5 mM) and (B) reduced flavodoxin Dh_Fld0741. UV/vis spectra were recorded between 350 and 600 nm every 45 s. The concentration of AE was 60 µM. The concentration of flavodoxin, if applied, was 10 µM. Black line, oxidized Fe/S cluster; red line, reduced Fe/S cluster; dotted lines, reduction (‘bleaching’) of the Fe/S cluster of AE after addition of flavodoxin.](image-url)
reduction assay. The substitution of one low-potential electron carrier by the other was also previously described for several enzymatic reactions including PFOR [2]. Whether flavodoxin or ferredoxin acts as electron carrier in vivo seems to depend on its availability in the cell, which is regulated by iron. Low concentrations of iron favor the production of flavodoxin, whereas high concentrations lead to an increase in the concentration of ferredoxin [36,44,45]. By contrast, a specific interaction of only one of the two electron carriers with its target protein is also feasible [46-48].

So far, it is not known if the pyruvate-dependent reduction of flavodoxin and/or ferredoxin also takes place in vivo, since pyruvate is not a direct intermediate in the methylothetic metabolism of A. dehalogena
ts and D. hafniense DCB-2. However, pyruvate, which is an important precursor for the biosynthesis of carbohydrates and amino acids [49], can be produced from acetyl-CoA, a central intermediate of the pathway, or from alanine. Recently, alanine was identified as growth substrate of Acetobacterium woodii [50] and also supported growth of A. dehalogenan
ts (data not shown). Interestingly, the gene encoding the single-subunit-containing PFOR is part of the operon responsible for alanine degradation [50]. A further indication for the in vivo participation of PFOR in flavodoxin and/or ferredoxin reduction is its activity detected in cell extracts of A. dehalogenan
ts and D. hafniense DCB-2 grown with phenyl methyl ethers. The activity of PFOR reached up to 20 nkat per mg protein. This value was up to 100-fold higher than the activity of hydrogenase or formate dehydrogenase measured in the same cell extracts (data not shown). All these aspects favor an in vitro-coupling of flavodoxin and/or ferredoxin reduction to PFOR instead of to hydrogenase, which was described previously [29].

With this study, we added another important piece of the puzzle of how protein-bound corrinoid cofactors are reactivated. Low-potential electrons were generated in the PFOR reaction and ‘stored’ in flavodoxin or ferredoxin. Subsequently, the electrons were trans
ferred to the [2Fe-2S] cluster of the activating enzyme. Flavodoxin as electron donor for corrinoid reduction will aim to elucidate how the binding and hydrolysis of ATP cause the shift in the midpoint potential of the corrinoid cofactor, which is still an enigmatic process.

Materials and methods

Construction of plasmids

DNA fragments harboring the flavodoxin-encoding genes Ad_Fld0540 (NCBI Ref. Seq. WP_035354890.1), Ad_Fld0918 (NCBI Ref. Seq. WP_026393714.1), Dh_Fld0519 (GenBank: ACL18586.1), and Dh_Fld0741 (GenBank: ACL18805.1) as well as a DNA fragment containing the pyruvate:ferredoxin/flavodoxin oxidoreductase-encoding gene Dh_PFOR4766 (GenBank: ACL22761.1) were amplified from genomic DNA of A. dehalogenan
ts (Ad) and D. hafniense DCB-2 (Dh), respectively. The Strep-tag DNA sequence (at the 3’ end) and the restriction sites for cloning into pET11a (Agilent Technologies, Waldbronn, Germany) were inserted by two following PCRs. In reaction 1, the gene of interest, the 5’ restriction site (NdeI), and a part of the Strep-tag sequence were amplified. In reaction 2, the Strep-tag sequence was completed and the 3’ restriction site (BamHI) for Ad_Fld0540 (SacII for Dh_Fld0741) was attached. PCRs were performed using Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific, Brunswick, Germany) according to the manufacturer’s protocol. The primer sequences are listed in Table 2. The insert was ligated into pET11a using the Ndel and BamHI restriction sites of the plasmid. Ligation of insert and vector, transformation of the plasmids into E. coli and selection of positive clones were performed according to Studenik et al. [17].

Heterologous expression of genes encoding flavodoxin and purification of the recombinant proteins

Escherichia coli BL21 (DE3) was used as expression strain. The production of recombinant proteins was performed in LB medium (10 g NaCl, 10 g tryptone, 5 g yeast extract per liter) supplemented with the required antibiotics at 18 °C. Gene expression was induced by addition of 0.25 mM IPTG. Cells were harvested by centrifugation (10 min; 10 000 g) 16 h after induction and were stored at −21 °C. Recombi
nant Strep-tagged flavodoxin was purified from cell extracts by affinity chromatography on Strep-Tactin® according to the manufacturer’s protocol (IBA GmbH, Göttingen, Germany). Sodium phosphate buffer (65 mM, pH 8.5) containing 150 mM NaCl was used as washing buffer and as basis for the preparation of elution and regeneration buffer. Soluble protein was obtained by disruption of E. coli cells in a French Pressure cell (working pressure 30 000 psi) followed by centrifugation (15 min; 16 000 g) at 10 °C. With the exception of Dh_Fld0519, recombinant flavodoxins were produced as cofactor-free apoproteins in E. coli.
incorporation of FMN, a twofold molar excess of FMN was added to oxygen-free, apo-flavodoxin-containing samples. After 48–72 h of incubation at 4 °C, the excess of FMN was removed by the use of centrifugal ultrafiltration devices. The concentration of FMN bound to recombinant flavodoxin was determined from absorption measurements at 445 nm (ε445 nm = 10.2 mM−1 cm−1) [51]. Protein samples were stored at −21 °C in 65 mM sodium phosphate buffer pH 7.5 containing 150 mM NaCl.

**Heterologous expression of the gene encoding pyruvate:ferredoxin/flavodoxin oxidoreductase and purification of the recombinant protein**

*Escherichia coli* BL21 (DE3) was used as expression strain. The production of the recombinant protein was performed in the absence of oxygen in medium containing (per liter) 0.09 g NaCl, 0.18 g NH4Cl, 0.18 g MgSO4 × 7 H2O, 0.01 g ammonium iron(III) citrate, 0.01 g CaCl2 × 2 H2O, 1.4 g KH2PO4, 14 g K2HPO4 × 3 H2O, 2 g yeast extract, and 10 mL trace element solution SL-6 [52]. After autoclaving, ampicillin (100 µg·mL−1) and glucose (15 mM) were added.

Cultivation was initially performed at 28 °C. After entering the exponential growth phase, the cultures were transferred to 18 °C and IPTG (0.25 mM) was added. Cells were harvested by centrifugation (10 min; 10 000 g) 5 h after induction and were stored at −21 °C. Recombinant Strep-tagged PFOR was purified under anaerobic conditions by affinity chromatography on Strep-Tactin as described above. The buffers were amended with 1 mM DTT and 1 mM thiamine pyrophosphate (TPP). Cell disruption and purification were performed in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA) with an atmosphere of 95% N2 and 5% H2. Protein samples were stored on ice and immediately applied in the assays.

**Heterologous expression of the O-demethylase genes encoding AE and CP of Acetobacterium dehalogenans and purification of the recombinant proteins**

The production and purification of AE were performed according to Sperfeld et al. [28]. CP production and purification as well as the incorporation of hydroxocobalamin as Table 2. Oligonucleotides used in this study. Genomic DNA of *Acetobacterium dehalogenans* (Ad) or *Desulfitobacterium hafniense* DCB-2 (Dh) was used as template for the first PCR. Restriction sites are underlined. FW, forward; RV, reverse.

| Gene            | Primer sequence 5’-3’                  | PCR step |
|-----------------|----------------------------------------|----------|
| Ad_Fld0540      | FW: GAT AAA ATT GGA ACC TCA ATT GC     | 1        |
|                 | RV: CTT GAC GTT CAC GAA CAT GG         |          |
|                 | FW: GAT ATA CAT ATG GAA TTG GTA GAT AAA ATT GG | 2 |
|                 | RV: CTG CGG GTG GCT CCA AGC GCT AAA CTC GGC AAA GG | 3 |
|                 | FW: GAT ATA CAT ATG GAA TTG GTA GAT AAA ATT GG | 3 |
|                 | RV: CAC CCA GAT CTT TAT TTT TCG AAC TGC GGG TGG C | 1 |
|                 | FW: ACC GTG TGG TGA TGG AG              |          |
|                 | RV: CAG CCG GTT ACC AGT GG              |          |
|                 | FW: GGA GAT ATA CAT ATG GAA AAA GAA GTA TCA ATT G | 2 |
|                 | RV: CTG CGG GTG GCT CCA AGC GCT ATT C    |          |
|                 | FW: GGA GAT ATA CAT ATG GAA AAA GAA GTA TCA ATT G | 3 |
|                 | RV: CAG CCG CAT CCT TAT TTT TCG AAC TGC GGG TGG C | 1 |
|                 | FW: CAG CCG CAT CCT TAT TTT TCG AAC TGC GGG TGG C | 3 |
| Dh_Fld0519      | FW: GCG ATT CGA TGG GAC AC              | 1        |
|                 | RV: CAA GAC ATA GAG TTG CTG G           |          |
|                 | FW: GGA GAT ATA CAT ATG AGG AAA AAA GTA TCA ATT G | 2 |
|                 | RV: CTG CGG GTG GCT CCA AGC GCT TAA CTG CTT C | 3 |
|                 | FW: GGA GAT ATA CAT ATG AGG AAA AAA GTA TCA ATT G | 3 |
|                 | RV: CAG CCG CAT CCT TAT TTT TCG AAC TGC GGG TGG C | 1 |
| Dh_Fld0714      | FW: GAC CCT GAA GGA TTT TCC            | 1        |
|                 | RV: GTG TCT ATG GGC GGA TTT TCC         |          |
|                 | FW: GGA GAT ATA CAT ATG AGG AAA ATT GTC GTC | 2 |
|                 | RV: CTG CGG GTG GCT CCA AGC GCT TGG TCC GGC C | 3 |
|                 | FW: GGA GAT ATA CAT ATG AGG AAA ATT GTC GTC | 3 |
|                 | RV: CAG CCG CAT CCT TAT TTT TCG AAC TGC GGG TGG C | 1 |
| Dh_PFOR4766     | FW: CTC ATG TCC AAC AGA TAA CAC G       | 1        |
|                 | RV: CTT GAA TCG GGA TTT CAA TCC         |          |
|                 | FW: GAT ATA CAT ATG GCA AAG AAA ATG AAA ACG | 2 |
|                 | RV: CTG CGG GTG GCT CCA AGC GCT ATG GGG CAT TCC CGC | 3 |
|                 | FW: GAT ATA CAT ATG GCA AAG AAA ATG AAA ACG | 3 |
|                 | RV: CAG CCG CAT CCT TAT TTT TCG AAC TGC GGG TGG C | 1 |
corrinoid cofactor were carried out according to Dürichen et al. [5]. In the last purification step of CP (column chromatography on MonoQ), KCl was replaced by NaCl.

**Growth of *C. pasteurianum* and purification of ferredoxin**

*Clostridium pasteurianum* was grown on glucose medium as described previously [53]. The purification of ferredoxin was performed according to Schönheit et al. [54]. The concentration of ferredoxin was determined spectrophotometrically at 390 nm ($\varepsilon_{390} = 30 \text{ mM}^{-1} \text{ cm}^{-1}$) [55].

**Determination of the midpoint potential of flavodoxin**

The midpoint potential of the flavodoxin oxidized/semiquinone and the flavodoxin semiquinone/hydroquinone couple was determined by UV/visible potentiometry at pH 7.5 under anoxic conditions as described previously [23]. Titanium(III) citrate was used as electron donor [38]. A redox standard solution at +220 mV, purchased from Mettler Toledo GmbH (Giessen, Germany) as well as a methyl viologen solution (50 mM in 100 mM Tris/HCl pH 7; $E'\theta = -449$ mV) titrated with titanium(III) citrate served as controls.

**Determination of enzyme activities**

The enzyme activities were measured photometrically under anoxic conditions in rubber-stoppered cuvettes with N$_2$ as the gas phase. The formation of flavodoxin semiquinone to hydroquinone were recorded at 445 nm ($\varepsilon_{445} = 10.2 \text{ mM}^{-1} \text{ cm}^{-1}$) [51] and 578 nm ($\varepsilon_{578} = 4.4 \text{ mM}^{-1} \text{ cm}^{-1}$) [56], respectively. The reduction of methyl viologen was followed at 578 nm ($\varepsilon_{578} = 9.7 \text{ mM}^{-1} \text{ cm}^{-1}$) [57]. PFOR activity was routinely measured in 100 mM Tris/HCl pH 8 containing 1 mM MgCl$_2$, 10 mM DTT, 2.5 mM TPP, 10 mM pyruvate, and 0.2 mM coenzyme A. Methyl viologen (5 mM) or flavodoxin (30–50 µM) was used as electron acceptor. The reaction was started by addition of PFOR-containing fractions. Corrinoid reduction activity was followed at 386 nm ($\Delta\varepsilon_{386} = 21 \text{ mM}^{-1} \text{ cm}^{-1}$) according to the method described in Schilhabel et al. [23]. The assay mixture contained 2 mM ATP, 10 mM MgCl$_2$, 250 mM potassium acetate, 0.5 mM titanium(III) citrate, and ~50 µM of CP in 50 mM Tris/HCl pH 7.5. For the measurement of the flavodoxin-dependent reduction of CP, titanium(III) citrate was replaced by flavodoxin (final concentration indicated in the figures or figure legends) and a flavodoxin-reducing system (DTT, 10 mM; TPP, 2.5 mM; pyruvate, 10 mM; coenzyme A, 0.2 mM; PFOR, nonlimiting conditions). The reaction was started by the addition of AE of *A. dehalogenans*.

All reactions were run in at least two biological and two technical replicates.

**Analytical methods**

The protein content was determined according to the method of Bradford [58] using bovine serum albumin as a standard protein. As dye, Roti$^\text{R}$-Nanoquant (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was applied.

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**Conflict of interest**

The authors declare no conflict of interest.

**Author contributions**

SS conceived the idea and designed the experiments. LK, HD, SS, and YG performed the experiments. LK, HD, and SS analyzed the data. SS wrote the manuscript with support from LK and HD. All authors read and approved the final version of the manuscript.

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