

# An Electron-bifurcating Caffeyl-CoA Reductase\*

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**Background:** Energy conservation by Na<sup>+</sup>-translocating ferredoxin oxidation requires a low potential ferredoxin.

**Results:** Endergonic reduction of ferredoxin with NADH is driven by exergonic caffeyl-CoA reduction catalyzed by a soluble caffeyl-CoA reductase-electron transfer flavoprotein complex.

**Conclusion:** Caffeyl-CoA reductase uses electron bifurcation for energy coupling.

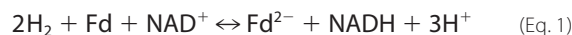
**Significance:** Ferredoxin reduction by electron bifurcation is essential for energy conservation in caffeate respiration.

A low potential electron carrier ferredoxin ( $E_0' \approx -500$  mV) is used to fuel the only bioenergetic coupling site, a sodium-motive ferredoxin:NAD<sup>+</sup> oxidoreductase (Rnf) in the acetogenic bacterium *Acetobacterium woodii*. Because ferredoxin reduction with physiological electron donors is highly endergonic, it must be coupled to an exergonic reaction. One candidate is NADH-dependent caffeyl-CoA reduction. We have purified a complex from *A. woodii* that contains a caffeyl-CoA reductase and an electron transfer flavoprotein. The enzyme contains three subunits encoded by the *carCDE* genes and is predicted to have, in addition to FAD, two [4Fe-4S] clusters as cofactor, which is consistent with the experimental determination of 4 mol of FAD, 9 mol of iron, and 9 mol of acid-labile sulfur. The enzyme complex catalyzed caffeyl-CoA-dependent oxidation of reduced methyl viologen. With NADH as donor, it catalyzed caffeyl-CoA reduction, but this reaction was highly stimulated by the addition of ferredoxin. Spectroscopic analyses revealed that ferredoxin and caffeyl-CoA were reduced simultaneously, and a stoichiometry of 1.3:1 was determined. Apparently, the caffeyl-CoA reductase-Etf complex of *A. woodii* uses the novel mechanism of flavin-dependent electron bifurcation to drive the endergonic ferredoxin reduction with NADH as reductant by coupling it to the exergonic NADH-dependent reduction of caffeyl-CoA.

Strictly anaerobic bacteria devoid of cytochromes are classically considered as fermenting bacteria without having the capability for ion gradient-driven ATP synthesis, as they lack ion-motive electron transfer systems (1, 2). This view has changed dramatically with the discovery of decarboxylation-driven (3) and methyltransfer-driven (4, 5) ATP synthesis. However, these types of reactions are limited to only a few species. Recently, an ion-motive electron transport system was dis-

covered in the acetogenic model bacterium *Acetobacterium woodii*: a membrane-integral, sodium-motive ferredoxin:NAD<sup>+</sup> oxidoreductase (Rnf complex) that couples the exergonic electron flow from reduced ferredoxin to NAD<sup>+</sup> with the export of Na<sup>+</sup> from the cytoplasm (6–11). The Rnf complex is the only coupling site in *A. woodii*, and the established transmembrane electrochemical Na<sup>+</sup> gradient then drives the synthesis of ATP via a well characterized Na<sup>+</sup> F<sub>1</sub>F<sub>0</sub>-ATP synthase (12–16). The Rnf complex is apparently widespread in the bacterial world and present also in a few archaea (17, 18).

A cellular bioenergetics based on just one coupling site that is fueled by reduced ferredoxin raises the question of how ferredoxin is reduced. The redox potential of the ferredoxin in *A. woodii* can be considered to be in the range of  $\approx -500$  mV, and thus, electron transfer from hydrogen ( $E_0' = -414$  mV) or NADH ( $E_0' = -320$  mV) to ferredoxin is highly endergonic and thermodynamically impossible. Recently, a soluble hydrogenase was purified from *A. woodii* that drives the endergonic ferredoxin (Fd) reduction with H<sub>2</sub> as reductant by coupling it to the exergonic electron transfer from hydrogen to NAD<sup>+</sup> by electron bifurcation (19) according to Equation 1.



Electrons derived from NADH are then channeled by soluble cytoplasmic enzymes to the terminal electron acceptor carbon dioxide (carbonate respiration or acetogenesis) or caffeate (caffeate respiration) (7, 20–23).

Caffeate respiration involves activation of caffeate to caffeyl-CoA (22, 24) and a subsequent reduction of caffeyl-CoA to hydrocaffeyl-CoA. After a CoA is transferred to caffeate, hydrocaffeate is released into the ecosystem. In addition to the genes encoding the CoA transferase and an ATP-dependent caffeyl-CoA synthetase required for the initial activation, the *car* operon encodes a potential acyl-CoA dehydrogenase (*CarC*) and the two subunits of an electron transfer flavoprotein (*CarDE*). The reduction of caffeyl-CoA with NADH as reductant is exergonic, and the energy gained from this reaction may be used to drive the endergonic ferredoxin reduction with NADH as reductant by flavin-based electron bifurcation (23, 25, 26). To address this question, we purified the caffeyl-CoA reductase-Etf complex and present evidence for ferredoxin

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reduction coupled to caffeyl-CoA reduction with NADH as reductant. The energetics of caffeate reduction is discussed.

## EXPERIMENTAL PROCEDURES

**Growth of Cells and Purification of Caffeyl-CoA Reductase**—*A. woodii* (DSM 1030) was grown at 30 °C under anaerobic conditions in 20-liter flasks (Glasgerätebau Ochs, Bovenden/Lengeln, Germany) using 20 mM fructose as the carbon and energy source as described previously (27, 28). At  $A_{600} \sim 0.3$ , caffeate was added to a concentration of 5 mM. The medium and all buffers were prepared using the anaerobic techniques described previously (29, 30). All buffers contained 2 mM dithioerythritol (DTE)<sup>3</sup> and 4  $\mu\text{M}$  resazurin. 5  $\mu\text{M}$  FAD was added to all purification buffers to avoid loss of activity. All purification steps were performed under strictly anaerobic conditions at room temperature in an anaerobic chamber (Coy Laboratory Products, Grass Lake Charter Township, MI) filled with 98–95%  $\text{N}_2$  and 2–5%  $\text{H}_2$  as described (27). Cells of *A. woodii* were harvested at  $A_{600} = 1.5$  and washed twice with 25 mM Tris-HCl (pH 7.0) and 420 mM sucrose and resuspended in 150 ml of 25 mM Tris-HCl (pH 8.0) and 420 mM sucrose. 500 mg of lysozyme was added, and the suspension was incubated for 1 h at 37 °C. After centrifugation, the protoplasts were resuspended in 25 mM Tris-HCl (pH 7.5), 20 mM  $\text{MgSO}_4$ , 20% glycerol, 0.5 mM PMSF, and 0.1 mg/ml DNase I and passed three times through a French pressure cell at 110 megapascals. Cell debris was removed by centrifugation at  $24,000 \times g$  for 40 min. Membranes were removed by centrifugation at  $130,000 \times g$  for 45 min. The supernatant containing the cytoplasmic fraction with  $\sim 1600$  mg of protein was applied to a Q Sepharose high performance column ( $2.6 \times 5$  cm) equilibrated with buffer A (50 mM Tris-HCl, 20 mM  $\text{MgSO}_4$ , 5  $\mu\text{M}$  FAD, and 20% glycerol (pH 7.5)). Protein was eluted with a 150-ml linear gradient of 0–0.35 M NaCl in buffer A. Methyl viologen-dependent caffeyl-CoA reductase activity eluted at  $\sim 0.3$  M NaCl. After the addition of ammonium sulfate (1.25 M), the pooled fractions were loaded onto a phenyl-Sepharose high performance column ( $1.6 \times 10$  cm) equilibrated with 1.25 M  $(\text{NH}_4)_2\text{SO}_4$  in buffer A. Protein was eluted with a 250-ml linear gradient of 1.25 to 0 M  $(\text{NH}_4)_2\text{SO}_4$ . Caffeyl-CoA reductase activity eluted at  $\sim 0.7$  M  $(\text{NH}_4)_2\text{SO}_4$ . Pooled fractions were concentrated by ultrafiltration in 50-kDa Vivaspin tubes (Sartorius Stedim Biotech GmbH). For the following purification step with a HiTrap Blue Sepharose high performance column (5 ml), the removal of the ammonium sulfate in the sample was crucial to ensure binding to the column; this was done by a 10-fold dilution with buffer A. The sample was loaded onto the column equilibrated with buffer A, and protein was eluted with a linear gradient of 0–500 mM NaCl. Caffeyl-CoA reductase activity eluted in a broad peak at  $\sim 170$  mM NaCl. Pooled fractions were concentrated by ultrafiltration in 50-kDa Vivaspin tubes. The sample was applied to a Sephacryl S-300 high resolution column ( $1.6 \times 60$  cm) equilibrated with buffer A and eluted at a flow rate of 0.5 ml/min. Caffeyl-CoA reductase activity eluted as a single peak. Pooled fractions were concentrated to a minimum of 5 mg/ml and stored at 4 °C.

<sup>3</sup> The abbreviation used is: DTE, dithioerythritol.

**Assays of Caffeyl-CoA Reductase Activity**—All measurements were performed at 30 °C in 1.8-ml anaerobic cuvettes (Glasgerätebau Ochs) sealed with rubber stoppers in a  $\text{N}_2$  atmosphere. A caffeyl-CoA regeneration system was used in all measurements (200 units of CoA transferase and 0.25 mM caffeate), and the CoA transferase was overproduced and purified as described (24). Measurements were done routinely using a methyl viologen oxidation assay with 5 mM methyl viologen in buffer B (50 mM Tris-HCl, 20 mM  $\text{MgSO}_4$ , and 2 mM DTE, pH 7.5). The addition of 1 mM sodium dithionite resulted in partial reduction of the methyl viologen. The reaction was started by the addition of 5  $\mu\text{M}$  caffeyl-CoA. Methyl viologen oxidation was monitored at 604 nm ( $\epsilon = 13.8 \text{ mM}^{-1} \text{ cm}^{-1}$ ), and 1 unit is defined as the reduction of 1  $\mu\text{mol}$  of caffeyl-CoA/min. Physiological caffeyl-CoA reductase activity was measured in buffer B with 0.5 mM NADH as electron donor. The reaction was started by the addition of at least one of the following electron acceptors: caffeyl-CoA (5  $\mu\text{M}$ ), ferredoxin (20  $\mu\text{M}$ ), FAD (0.25 mM), or FMN (0.25 mM). The reduction of caffeate was monitored at 312 nm ( $\epsilon = 13.72 \text{ mM}^{-1} \text{ cm}^{-1}$ ), and the reduction of ferredoxin was monitored at 430 nm ( $\epsilon = 13.1 \text{ mM}^{-1} \text{ cm}^{-1}$ ). Ferredoxin was purified from *Clostridium pasteurianum* as described (31). In initial experiments, caffeyl-CoA was synthesized chemically (24). For the experiments described here, caffeyl-CoA was synthesized enzymatically with the caffeyl-CoA synthetase from *A. woodii* (22). The synthesis of caffeyl-CoA in 50 mM Tris-HCl (pH 7.0), 20 mM  $\text{MgSO}_4$ , 50 mM KCl, 1 mM ATP, 1 mM CoA, 0.25 mM caffeate, and 2 mg of enzyme was followed by ultrafiltration in 30-kDa Vivaspin tubes to remove the enzyme. The yellow flow-through was concentrated in a centrifugal evaporator to a final concentration of 1 mM caffeyl-CoA ( $\epsilon_{346} = 18.0 \text{ mM}^{-1} \text{ cm}^{-1}$ ) and stored at 4 °C.

**Analytical Methods**—The concentration of proteins was measured according to Bradford (32). Proteins were separated on 12% polyacrylamide gels and stained with Coomassie Brilliant Blue G-250. The molecular mass of the purified caffeyl-CoA reductase was determined using a calibrated Sephacryl S-300 column equilibrated with buffer A. The iron and sulfur content of the purified enzyme was determined by colorimetric methods (33, 34). To determine the flavin content of the purified enzyme, 1 nmol of protein was precipitated with trichloroacetic acid to a final concentration of 500 mM. The nature of the flavin in the supernatant was identified by thin layer chromatography on Silica Gel 60 sheets (Macherey-Nagel GmbH & Co. KG) with 5%  $\text{Na}_2\text{HPO}_4$  in  $\text{H}_2\text{O}$  or 1-butanol/acetic acid/ $\text{H}_2\text{O}$  (12:3:5) as the mobile phase. The amount of bound flavin was calculated by comparing the absorbance of the yellow supernatant at 450 nm with a calibration line with FAD treated the same way (35).

## RESULTS

**Assay for Caffeyl-CoA Reduction**—The activity of the caffeyl-CoA reductase-Etf complex was measured using dithionite-reduced methyl viologen as electron donor and caffeyl-CoA as electron acceptor. Methyl viologen oxidation was followed spectroscopically at 604 nm. Because caffeyl-CoA is not commercially available, a caffeyl-CoA regeneration system was established. Caffeyl-CoA was reduced with the caffeyl-CoA

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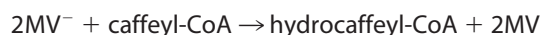
**TABLE 1**

Purification of the caffeyl-CoA reductase-Etf complex from *A. woodii*

Purification step	Protein	Specific activity <sup>a</sup>	Yield	Purification
	mg	units/mg	%	-fold
Cell-free extract	2579.2	0.57	100.0	1.0
Cytoplasm	2161.9	0.57	83.2	1.0
Q Sepharose	381.4	2.51	65.0	4.4
Phenyl-Sepharose	99.5	7.83	52.9	13.7
Blue Sepharose	46.5	12.93	40.8	22.6
Gel filtration Sephacryl S-300	14.5	13.61	13.4	23.8

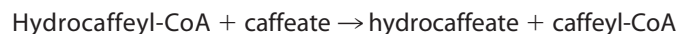
<sup>a</sup> The activity of the caffeyl-CoA reductase-Etf complex was determined by following the caffeyl-CoA-dependent oxidation of reduced methyl viologen at 604 nm. The assay buffer (buffer B) contained 5 mM reduced methyl viologen (reduced with 1 mM sodium dithionite) as electron donor, 5 μM caffeyl-CoA, and the caffeyl-CoA regeneration system (200 units of the CoA transferase CarA and 0.25 mM caffeate) under an atmosphere of 100% N<sub>2</sub>. Measurements were performed at 30 °C in anaerobic cuvettes.

reductase-Etf complex to hydrocaffeyl-CoA according to Equation 2,



(Eq. 2)

where MV is methyl viologen. Next, hydrocaffeyl-CoA was used as CoA donor by the hydrocaffeyl-CoA:caffeate-CoA transferase CarA from *A. woodii* (24) to activate caffeate to caffeyl-CoA (Equation 3).



(Eq. 3)

Thus, in sum, caffeate was reduced to hydrocaffeate according to Equation 4, and the rate of methyl viologen (MV) oxidation is proportional to caffeyl-CoA reduction (1 unit = 1 μmol of caffeyl-CoA/mg).

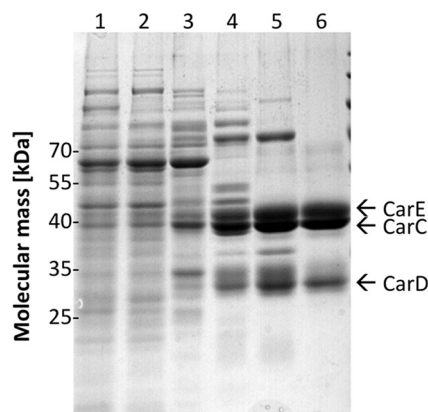


(Eq. 4)

The dependence of methyl viologen oxidation on the caffeyl-CoA concentration showed saturation at 5 μM caffeyl-CoA with  $K_m = 0.5 \mu\text{M}$ .

**Purification of the Caffeyl-CoA Reductase-Etf Complex from *A. woodii***—To purify the caffeyl-CoA reductase-Etf complex, fructose/caffeate-grown cells were grown to the late exponential growth phase, harvested, and disrupted with a French pressure cell. Membranes were removed by ultracentrifugation, and the caffeyl-CoA reductase-Etf complex was purified from the cytoplasm by ion exchange chromatography on Q Sepharose, phenyl-Sepharose, and Blue Sepharose, followed by gel filtration on Sephacryl S-300. The enzyme was purified by 24-fold to apparent homogeneity with an average specific activity of 14 units/mg in the methyl viologen oxidation assay (Table 1).

The purified enzyme apparently has three subunits of 41, 39, and 29 kDa (Fig. 1). This fits well with the predicted masses of CarE (42.5 kDa), CarC (41.4 kDa), and CarD (28.2 kDa) and indeed, the proteins were identified by peptide mass fingerprinting as CarE, CarC, and CarD. CarDE is identical to EtfAB identified previously by a proteomics approach to be up-regulated during growth on caffeate (7). Analytical gel filtration, as well as separation on a native gel, revealed that the caffeyl-CoA reductase and the electron transfer flavoprotein formed a stable complex. Because the masses of the subunits of the complex are rather small, an accurate estimate of the subunit composition could not be made, but the estimated molecular mass of 350



**FIGURE 1. Purification of the caffeyl-CoA reductase-Etf complex.** Samples from the different purification steps were separated by SDS-PAGE, and proteins were stained with Coomassie Brilliant Blue. Lane 1, cell extract; lane 2, cytoplasm; lane 3, pooled fractions from Q Sepharose; lane 4, pooled fractions from phenyl-Sepharose; lane 5, pooled fractions from Blue Sepharose; lane 6, pooled fractions from Sephacryl S-300. 10 μg of protein was applied to each lane.

kDa upon gel filtration is consistent with a homotrimer of CarCDE, which would have a mass of 336 kDa.

**Basic Biochemical Properties of the Caffeyl-CoA Reductase-Etf Complex of *A. woodii***—The spectrum of the yellow-brownish enzyme shows absorbance maxima at 372 and 445 nm (Fig. 2), which indicated oxidized flavin as the prosthetic group (35). The purified complex contained  $4 \pm 0.2$  mol of flavin/mol of CarCDE. This flavin was tightly bound because it could not be removed by gel filtration. Thin layer chromatography identified the flavin as FAD. Iron and acid-labile sulfur were determined colorimetrically according to Fish (33) and Beinert (34). The enzyme contained  $9 \pm 0.5$  mol of iron/mol of CarCDE and  $9 \pm 1.3$  mol of acid-labile sulfide/mol of CarCDE. The C terminus of CarE contains eight conserved cysteine residues, which are homologous to the cysteine residues in the ferredoxins from *C. pasteurianum* and *Clostridium acidi-urici* harboring two [4Fe-4S] clusters (36, 37). With the determined content of iron and sulfur, two [4Fe-4S] clusters/CarCDE can be predicted.

The basic biochemical properties of the enzyme were determined using the caffeyl-CoA-dependent methyl viologen oxidation assay. The caffeyl-CoA reductase activity had an optimum at pH 7.5, and activity decreased to ~30% at pH 6.0 or 9.0. The enzyme activity exhibited a rather broad temperature optimum, and maximum activity was detected at 30 °C, which is the optimum growth temperature of *A. woodii*. If caffeate was substituted with *p*-coumarate or ferulate, the corresponding CoA thioesters were reduced as well.

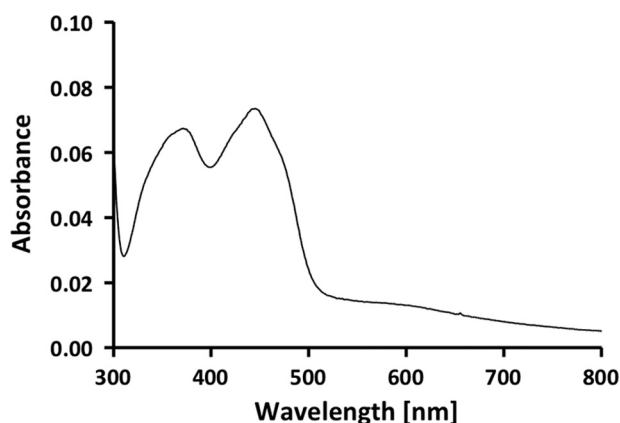


FIGURE 2. UV-visible spectrum of the purified caffeyl-CoA reductase-Etf complex. The spectrum of the enzyme (0.57 mg/ml) was recorded in buffer B.

*Caffeyl-CoA Reductase Uses NADH as Reductant and Catalyzes Flavin Reduction*—The redox potential of caffeyl-CoA/hydrocaffeyl-CoA is not known, but considering that the reduction of the acrylate side chain of caffeate is thermodynamically equivalent to the reduction of fumarate (21), it can be considered to be in the same range as fumarate/succinate with +33 mV (38). We first determined whether the enzyme can catalyze the energetic “downhill” transport of electrons from NADH ( $E_0' = -320$  mV) to caffeyl-CoA ( $E_0' = +33$  mV). Thus, in the enzyme assay, reduced methyl viologen (Equation 4) was replaced with NADH, which is the physiological electron donor of caffeate reduction in *A. woodii* (7), and the decrease in caffeate was measured spectroscopically at 312 nm, which is proportional to the reduction of caffeyl-CoA (1 unit = 1  $\mu$ mol of caffeyl-CoA/mg). With NADH (0.5 mM) as electron donor, caffeyl-CoA (5  $\mu$ M) was reduced with an activity of 1 unit/mg.

To prevent loss of activity, 5  $\mu$ M FAD had to be added to all buffers during purification. Thus, the CarCDE complex requires FAD for catalytic activity. Next, we tested whether flavins could be used as artificial electron acceptors. No activity could be detected when FAD or FMN (0.25 mM) was used alone as electron acceptor, but together with caffeyl-CoA, they were completely reduced with NADH as reductant. Caffeyl-CoA reduction was stimulated by 26- and 12-fold with FAD and FMN, respectively. The  $K_m$  value for both FAD and FMN was 100  $\mu$ M. Thus, caffeyl-CoA and flavins were reduced simultaneously.

*Coupling of Caffeyl-CoA and Ferredoxin Reduction*—Next, we tested whether the addition of ferredoxin also stimulated NADH-dependent caffeyl-CoA reduction, and indeed, the reduction of caffeyl-CoA increased by 10-fold to 10 units/mg after the addition of 20 nmol of ferredoxin. The observed stimulation of caffeyl-CoA reduction by ferredoxin raised the question of whether ferredoxin was reduced alongside caffeyl-CoA. To test this, spectral analyses were performed (Fig. 3). The spectrum of oxidized ferredoxin reveals the characteristic shoulder of oxidized ferredoxin between 370 and 450 nm (*trace a*). Reduction with CO dehydrogenase purified from *A. woodii* with 100% CO in the gas phase led to the disappearance of the shoulder (*trace b*). The addition of NADH, the caffeyl-CoA regeneration system (caffeate and the CoA transferase CarA, with no caffeyl-CoA present), and the purified caffeyl-CoA

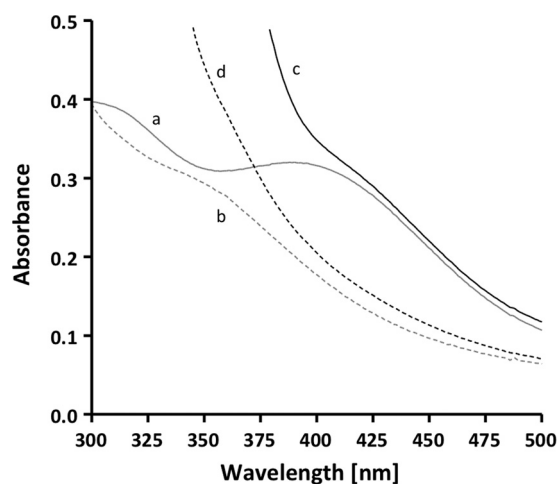


FIGURE 3. Caffeyl-CoA-dependent ferredoxin reduction. The assay buffer (buffer B) contained 0.2 mM NADH, 20  $\mu$ M ferredoxin, 5  $\mu$ M caffeyl-CoA, the caffeyl-CoA regeneration system (200 units of the CoA transferase CarA and 0.2 mM caffeate), and 8  $\mu$ g of the purified CarCDE complex. *Trace a*, ferredoxin; *trace b*, ferredoxin reduced with CO dehydrogenase purified from *A. woodii* and 100% CO in the gas phase; *trace c*, ferredoxin, NADH, CarCDE, and the caffeyl-CoA regeneration system (with no caffeyl-CoA present); *trace d*, same as trace c after the addition of caffeyl-CoA and incubation of 5 min at 30 °C.

reductase-Etf complex to oxidized ferredoxin led to the spectrum shown in *trace c*. The change in absorbance below 400 nm was due to NADH and caffeate, but above 400 nm, the spectrum was identical to that of oxidized ferredoxin. This spectrum did not change, indicating that ferredoxin alone was not reduced. The addition of caffeyl-CoA and incubation for 5 min gave rise to the spectrum shown in *trace d*. The change in the absorbance spectrum above 400 nm was similar to the change in the absorbance spectrum when ferredoxin was reduced with CO dehydrogenase and CO. This experiment clearly demonstrated that ferredoxin was reduced alongside caffeyl-CoA.

To determine the kinetic constants for this reaction, ferredoxin reduction was measured spectroscopically by following the decrease in absorbance at 430 nm. The caffeyl-CoA reductase-Etf complex reduced ferredoxin at a rate of 6 units/mg (1 unit = 1  $\mu$ mol of ferredoxin/min). The dependence of the ferredoxin reduction on NADH (Fig. 4A) and ferredoxin (Fig. 4B) was hyperbolic. Saturation with NADH was at 500  $\mu$ M with  $K_m = 100$   $\mu$ M. Values in the same range were obtained when caffeyl-CoA reduction was followed or when FAD ( $K_m$  for NADH = 125  $\mu$ M) or FMN ( $K_m$  for NADH = 150  $\mu$ M) was used as oxidant. Saturation with ferredoxin was at 15  $\mu$ M, but the  $K_m$  value was below 5  $\mu$ M and thus below the detection limit.

To determine the ratio for the reduction of the two electron acceptors, the reduction of ferredoxin and caffeyl-CoA was monitored simultaneously at 430 and 312 nm, respectively (Fig. 5). The decrease in absorbance at 430 nm was used to calculate the amount of ferredoxin reduced, and the decrease in absorbance at 312 nm was used to calculate the amount of caffeyl-CoA reduced. From a number of experiments, a stoichiometry of 1:1.8 (ferredoxin:caffeyl-CoA) was calculated. However, NADH oxidation also led to a decrease in absorbance at 312 nm ( $\epsilon = 3.6$  mM<sup>-1</sup> cm<sup>-1</sup>) (Fig. 6). All of the other substances involved did not have absorbance at 312 nm (hydrocaffeate and hydrocaffeyl-CoA), or the absorbance did not change after

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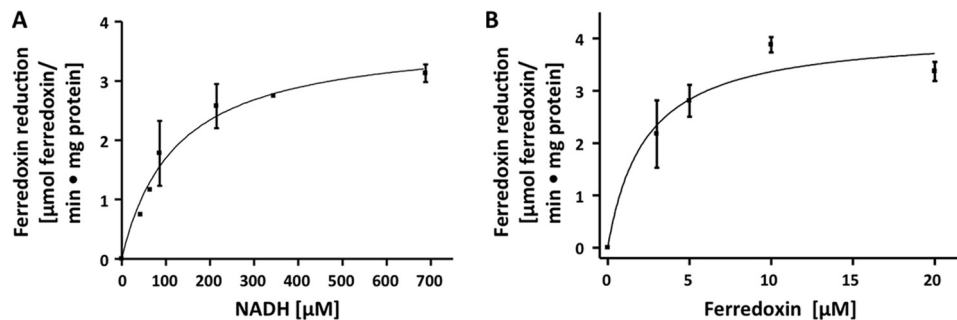


FIGURE 4. **Dependence of ferredoxin reduction on NADH (A) and ferredoxin (B).** Enzyme activity was measured in assay buffer (buffer B) containing the caffeyl-CoA regeneration system (200 units of the CoA transferase CarA and 0.2 mM caffeate), 8 μg of protein, and 5 μM caffeyl-CoA. All assays were done at 30 °C under an atmosphere of 100% nitrogen. For NADH dependence (A), NADH was added, and the reaction was started by the addition of 20 μM ferredoxin. For ferredoxin dependence (B), 0.5 mM NADH was added, and the reaction was started by the addition of ferredoxin.

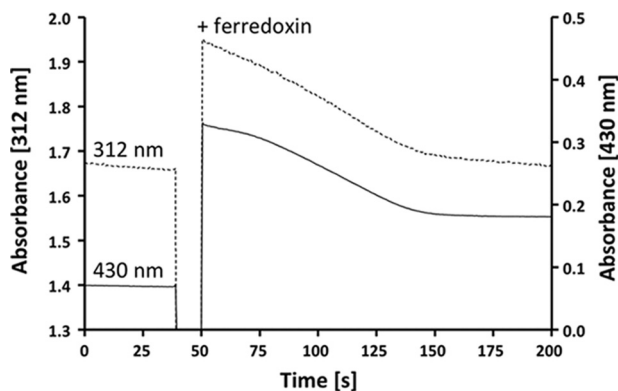


FIGURE 5. **The purified CarCDE complex catalyzes reduction of ferredoxin and caffeyl-CoA simultaneously.** The assay buffer (buffer B) contained 0.2 mM NADH, 5 μM caffeyl-CoA, 8 μg of the purified CarCDE complex, and the caffeyl-CoA regeneration system (200 units of the CoA transferase CarA and 0.2 mM caffeate). The reaction was started by the addition of 20 μM ferredoxin. Reduction of caffeyl-CoA (dashed line; monitored at 312 nm via decline of caffeate) and reduction of ferredoxin (solid line; monitored at 430 nm) were monitored simultaneously.

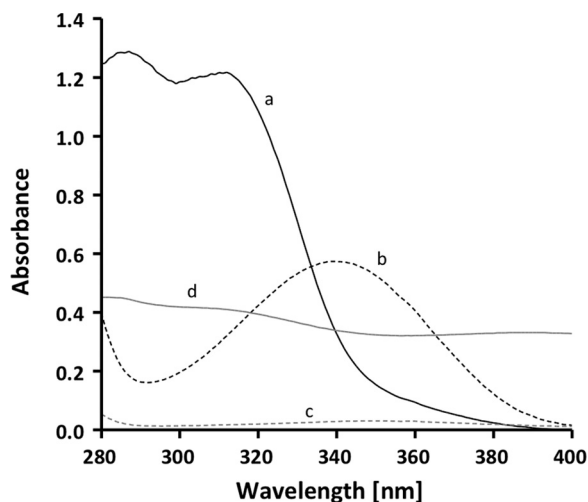


FIGURE 6. **Spectral properties of components used in the enzyme assay.** Trace a, 0.2 mM caffeate; trace b, 0.2 mM NADH; trace c, 5 μM caffeyl-CoA; trace d, 20 μM ferredoxin (measured in buffer B).

reduction (ferredoxin). Thus, the change in absorbance at 312 nm was the result of a combination of caffeyl-CoA reduction and NADH oxidation. Because one NADH is required to reduce one ferredoxin and the amount of ferredoxin reduced can be measured unequivocally at 430 nm, the amount of

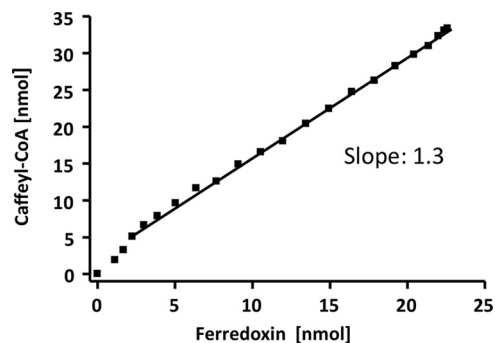


FIGURE 7. **The purified CarCDE complex reduces ferredoxin and caffeyl-CoA in stoichiometric amounts.** The reduction of caffeyl-CoA and ferredoxin was monitored simultaneously (see Fig. 5). Data points from 5-s intervals were taken to calculate the amount of reduced electron carrier from the absorbance difference and the molar extinction coefficient. The amount of reduced caffeyl-CoA is plotted against the amount of reduced ferredoxin.

NADH used to reduce ferredoxin was subtracted. This gave a stoichiometry of 1:1.3 (ferredoxin:caffeyl-CoA) (Fig. 7). Considering that this ratio still reflects the amount of NADH required to reduce caffeyl-CoA and that reduction of caffeyl-CoA is also catalyzed in the absence of ferredoxin, under fully coupled conditions, the ratio should come close to 1:1. If FAD or FMN was used instead of ferredoxin as an additional electron acceptor, the stoichiometry without any corrections was 1:1.2 (flavin:caffeyl-CoA).

### DISCUSSION

We have shown that the gene *carC* encodes a caffeyl-CoA reductase and that the genes *carD* and *carE* encode the two subunits of the electron transfer flavoprotein. The Etf protein forms a stable complex with the caffeyl-CoA reductase, a homolog of the acyl-CoA dehydrogenase-Etf complexes from *Clostridium propionicum* (35) and *Clostridium kluyveri* (25). Electron transfer flavoproteins are found in all three domains of life (39) and consist of a large and a small subunit building a heterodimer containing noncovalently bound FAD. In general, they mediate the electron transfer between different donors and acceptors, e.g. between acyl-CoA dehydrogenases and the Etf:quinone oxidoreductase in the mitochondrial matrix in Mammalia (40, 41). Etf proteins usually contain, in addition to FAD, an AMP molecule buried in the small subunit, which may play a role in facilitating the correct structure of the protein (42, 43). In the Etf protein of *Megasphaera elsdenii*, this second

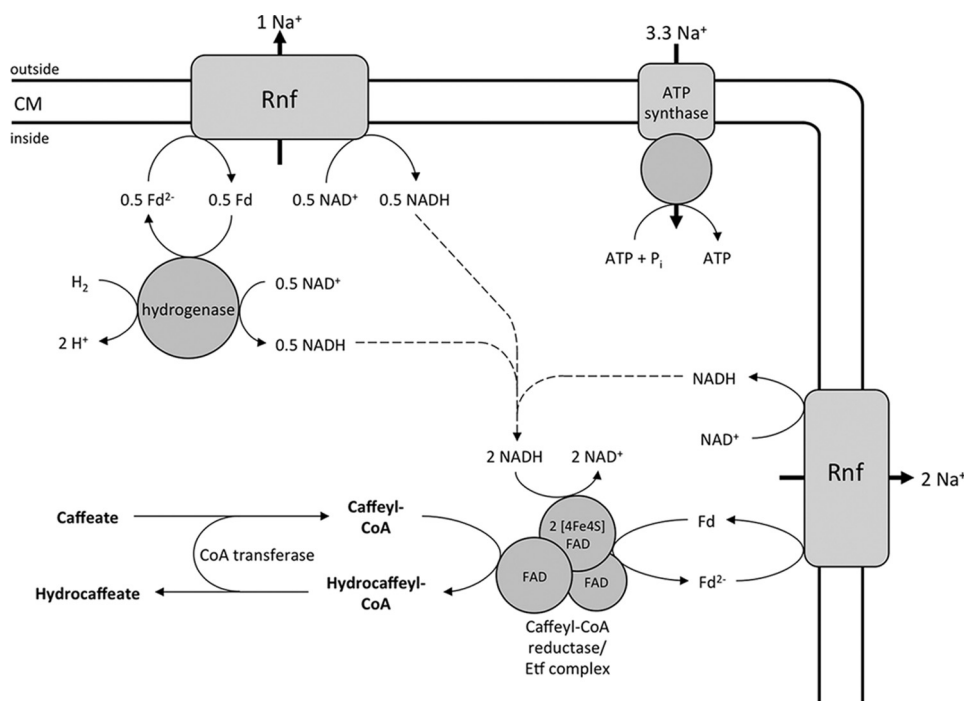


FIGURE 8. **Enzymology and bioenergetics of hydrogen-dependent caffeate respiration in *A. woodii*.** The electron-bifurcating hydrogenase reduces ferredoxin (*Fd*) and  $\text{NAD}^+$  simultaneously. The Rnf complex couples the exergonic electron transfer from reduced ferredoxin to  $\text{NAD}^+$  with the translocation of  $\text{Na}^+$  ions. The electron acceptor caffeyl-CoA is reduced by the caffeyl-CoA reductase-Etf complex with NADH, which is coupled to the endergonic reduction of ferredoxin with  $\text{NAD}^+$ . The reduced ferredoxin is re-oxidized at the Rnf complex. The electrochemical  $\text{Na}^+$  gradient drives ATP synthesis by the  $\text{Na}^+$   $\text{F}_1\text{F}_0$ -ATP synthase. The hydrocaffeyl-CoA:caffeate-CoA transferase CarA uses hydrocaffeyl-CoA to activate caffeate to caffeyl-CoA; this energy-saving CoA loop provides the regeneration of the acceptor under steady-state conditions. Initially, caffeate is activated by the caffeyl-CoA synthetase CarB at the expense of ATP (not shown). CM, cytoplasmic membrane.

nucleotide-binding site is occupied by a second FAD molecule (44), which seems to be the case also for the Etf proteins of other anaerobic bacteria characterized so far (25, 35). Besides these two FAD molecules bound to the Etf protein (CarDE), another one can be expected to bind to CarC (Fig. 8). This is in contrast to the four FAD molecules/CarCDE experimentally determined here. Because FAD can substitute ferredoxin and is also reduced in a stoichiometry close to 1:1 (FAD:caffeyl-CoA), a possible explanation is that an additional FAD molecule is tightly bound to the ferredoxin-binding site.

In the Etf protein of *A. woodii*, in addition to FAD, 9 mol of iron/CarCDE and 9 mol of sulfur/CarCDE were determined. The large subunit of the Etf protein of *A. woodii* contains an N-terminal extension that contains two copies of the conserved consensus sequence (CX<sub>2</sub>CX<sub>2</sub>CX<sub>3</sub>CP) responsible for the binding of the [4Fe-4S] cluster in several ferredoxins (36, 37). This extension can be found in the large Etf subunit of other anaerobic bacteria such as *Clostridium saccharolyticum* and was proposed for several other organisms by comparing genomic sequences (41). In the mitochondrial electron transfer flavoprotein:ubiquinone oxidoreductase, a [4Fe-4S] cluster is the immediate acceptor for electrons derived from the Etf protein (45, 46). Thus, the iron-sulfur cluster in EtfA from *A. woodii* may be involved in electron transport, possibly in the intramolecular electron transfer from the Etf protein subunit to the caffeyl-CoA reductase subunit inside the complex.

The caffeyl-CoA reductase-Etf complex of *A. woodii* apparently couples the endergonic ferredoxin reduction with NADH as reductant to the exergonic reduction of caffeyl-CoA with the

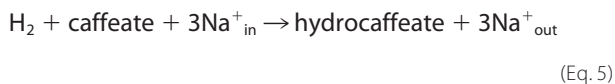
same reductant. A flavin is involved, and thus, the enzyme apparently uses the novel mechanism of electron bifurcation to overcome the steep energy barrier in ferredoxin reduction. The first electron-bifurcating enzyme discovered was the butyryl-CoA dehydrogenase-Etf complex from *C. kluyveri* (23, 25). This complex couples the exergonic reduction of crotonyl-CoA with NADH to the endergonic reduction of ferredoxin. For this complex, a similar stoichiometry of 1:1.4 (ferredoxin:butyryl-CoA) was determined because this reaction is also slightly uncoupled.

In *C. kluyveri*, the ferredoxin is used by the ferredoxin-dependent hydrogenase to reduce protons to molecular hydrogen. Part of the reduced ferredoxin can also be oxidized at the Rnf complex, improving the energy yield via electron transport phosphorylation. Additional electron-bifurcating enzymes have been described since then. The hydrogenase from *Thermotoga maritima* uses electron confurcation to drive hydrogen production from  $\text{NADH}$  by coupling to exergonic ferredoxin-dependent proton reduction (47). Transhydrogenases from *C. kluyveri* and *Moorella thermoacetica* couple the NADH-dependent reduction of  $\text{NADP}^+$  to the ferredoxin-dependent reduction of  $\text{NADP}^+$  (48, 49), and the heterodisulfide reductase complex from *Methanothermobacter marburgensis* couples the endergonic ferredoxin reduction with  $\text{H}_2$  to the exergonic reduction of a heterodisulfide with  $\text{H}_2$  (50). Recently, a hydrogen-activating, electron-bifurcating hydrogenase was purified and characterized from *A. woodii* (19). The enzyme oxidizes hydrogen only when  $\text{NAD}^+$  and ferredoxin are present, and both substrates are reduced in a stoichiometry of 1:1. This

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activity is strictly dependent on the presence of FMN. This hydrogenase couples the FMN-dependent exergonic reduction of NAD<sup>+</sup> with H<sub>2</sub> to the FMN-dependent endergonic reduction of ferredoxin with the same electron donor using flavin-based electron bifurcation. Here, we have described the caffeyl-CoA reductase-Etf complex, which couples the exergonic reduction of caffeyl-CoA with NADH to the endergonic reduction of ferredoxin with the same electron donor by flavin-based electron bifurcation.

The caffeyl-CoA reductase-Etf complex was the last missing link in our understanding of the enzymology and bioenergetics of caffeate respiration in *A. woodii* (Fig. 8). This remarkable example of microbial life under extreme energy limitations has only one coupling site, the sodium-motive ferredoxin:NAD<sup>+</sup> oxidoreductase (Rnf) and two soluble enzymes that conserve energy in the form of reduced ferredoxin that fuels the ferredoxin:NAD<sup>+</sup> oxidoreductase. Oxidation of 1 mol of hydrogen yields 0.5 mol of NADH and 0.5 mol of reduced ferredoxin. Oxidation of the latter via the ferredoxin:NAD<sup>+</sup> oxidoreductase may be coupled to the translocation of one Na<sup>+</sup> (Fig. 8) across the membrane. The NADH is then oxidized by the caffeyl-CoA reductase-Etf complex, and electrons are bifurcated to caffeyl-CoA and ferredoxin. The reduced ferredoxin is re-oxidized by the ferredoxin:NAD<sup>+</sup> oxidoreductase, which gives rise to the translocation of two more Na<sup>+</sup> ions. Thus, the internal Rnf loop accounts for up to 60% of the total amount of ions translocated during this respiration. In sum, 3 mol of Na<sup>+</sup> are translocated (Equation 5).



Considering that the Na<sup>+</sup> F<sub>1</sub>F<sub>0</sub>-ATP synthase of *A. woodii* has 10 ion-binding sites (14, 16, 51), this makes 3.3 ions/ATP, and thus, caffeate respiration yields 0.9 mol of ATP/mol of caffeate. This is in good agreement with the experimental data (21).

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