Clostridium acidurici Electron-Bifurcating Formate Dehydrogenase

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Cell extracts of uric acid-grown *Clostridium acidurici* catalyzed the coupled reduction of NAD^+ and ferredoxin with formate at a specific activity of 1.3 U/mg. The enzyme complex catalyzing the electron-bifurcating reaction was purified 130-fold and found to be composed of four subunits encoded by the gene cluster *hylCBA-fdhF2*.

*C*lostridium acidurici* ferments uric acid to acetate, CO_2, and ammonium ([1], [2]) in a process involving a selenium- and tungsten-containing formate dehydrogenase ([3], [4]). In cell extracts, formate dehydrogenase activity requires both NAD^+ and ferredoxin ([5]) but the purified enzyme is active with neither $NAD(P)^+$ nor ferredoxin (Fd) ([6]). We report here that the cytoplasmic enzyme catalyzes the coupled reduction of NAD^+ and ferredoxin with formate and propose that coupling proceeds via the mechanism of flavin-based electron bifurcation ([7]) in this reaction, as follows: $2\ HCOO^- + Fd_{ox} + NAD^+ \leftrightharpoons 2\ CO_2 + Fd_{red}^{2-} + NADH + H^+$.

Flavin-based electron-bifurcating enzymes were first discovered in 2008, when it was shown that the butyryl-coenzyme A (butyryl-CoA)/EtfAB complex in *Clostridium kluyveri* couples the exergonic reduction of crotonyl-CoA with NADH with the endergonic reduction of ferredoxin with NADH ([8], [9]). Since then, five other types of electron-bifurcating enzyme complexes have been reported, namely, the NAD^+-specific [FeFe]-hydrogenase complex HydABC(D) from *Thermotoga maritima* ([10]), *Acetobacterium woodii* ([11]), and *Moorella thermoacetica* ([12]), the NADH-dependent reduced ferredoxin:$NADP^+$ oxidoreductase complex NfnAB from *C. kluyveri* ([13]) and *M. thermoacetica* ([14]), the [NiFe]-hydrogenase/heterodisulfide reductase complex MvhADG/HdrABC from methanogenic archaea ([15], [16]), the caffeyl-CoA reductase complex CarCDE from *A. woodii* ([17]), and the $NADP^+$-specific [FeFe]-hydrogenase/formate dehydrogenase complex HytA-E/FdhA from *Clostridium autoethanogenum* ([18]). All these complexes have the following characteristics in common: they are present in the cytoplasm of anaerobic bacteria or archaea, they are flavoproteins (flavin mononucleotide [FMN] or flavin adenine dinucleotide [FAD]), they simultaneously use two different electron acceptors or donors, and one of the electron acceptors or donors is a ferredoxin. Formate dehydrogenase from *C. acidurici* is the seventh enzyme to share these properties and is the first identified electron-bifurcating formate dehydrogenase.

C. acidurici (strain 9a; DSM 604) ([19]) was grown at 37°C in three fermentors, each containing 10 liters of uric acid medium supplemented with tungstate and selenite ([3], [4]). The cells (15 g [wet mass]) from the 30-liter culture were harvested by centrifugation and stored under 100% N_2 at −80°C. Cell extracts containing 53 mg protein per ml were prepared by passing a suspension of 5 g cells (wet mass) in 10 ml 100 mM potassium phosphate (pH 7) through a French press three times, followed by removal of cell debris and membranes by centrifugation at 65,000 × *g* for 40 min. In all steps, strictly anoxic conditions were employed.

Enzymes were assayed at 37°C in 1.5-ml cuvettes (optical path = 1 cm) containing 0.8 ml assay mixture and 0.7 ml N_2 or H_2

as gas phase. The reduction of NAD^+ was monitored at 340 nm ($\varepsilon = 6.3\ mM^{-1}\ cm^{-1}$), that of ferredoxin (from *Clostridium pasteurianum*) ([12]) was monitored at 430 nm ($\Delta\varepsilon_{ox-red} \approx 13.1\ mM^{-1}\ cm^{-1}$), and that of methyl viologen was monitored at 578 nm ($\varepsilon = 9.8\ mM^{-1}\ cm^{-1}$).

Cell extracts catalyzed the coupled reduction of ferredoxin from *Clostridium pasteurianum* (25 μM) and NAD^+ (1 mM) with formate (20 mM) at a specific activity of 1.3 U/mg protein (in 100 mM potassium phosphate, pH 7.5) (Table 1). Ferredoxin was not reduced in the absence of NAD^+, and NAD^+ was not reduced in the absence of ferredoxin. $NADP^+$ could not substitute for NAD^+. The reduction of methyl viologen (10 mM) with formate (20 mM) was independent of $NAD(P)^+$. The proposed function of the ferredoxin- and NAD^+-dependent formate dehydrogenase in uric acid metabolism ([19], [20]) is shown in Fig. 1.

We also determined the specific activities of the other oxidoreductases involved in purine metabolism (Table 1; Fig. 1): xanthine dehydrogenase was tested in 100 mM Tris-HCl (pH 8.5) containing 2 mM xanthine and 25 μM ferredoxin from *C. pasteurianum* ([21]); 5,10-methylenetetrahydrofolate dehydrogenase was tested in 100 mM MOPS-KOH (pH 6.5) (where MOPS is mospholinepropanesulfonic acid) containing 50 mM 2-mercaptoethanol, 0.5 mM tetrahydrofolate, 10 mM formaldehyde, and 0.5 mM NAD^+ ([22]); the glycine cleavage system was tested in 100 mM potassium phosphate (pH 7.5) containing 2 mM glycine, 0.1 mM pyridoxal phosphate, 0.5 mM tetrahydrofolate, 0.1 mM D,L-alpha lipoic acid, and 1 mM NAD^+ ([23]); pyruvate:ferredoxin oxidoreductase was tested in 100 mM potassium phosphate (pH 7.5) containing 10 mM pyruvate, 25 μM ferredoxin from *C. pasteurianum*, 0.1 mM thiamine pyrophosphate, and 1 mM CoA ([18]); and reduced ferredoxin:NAD^+ oxidoreductase (RnfA-G) was tested in 100 mM potassium phosphate (pH 7.5) containing 25 μM ferredoxin, 1 mM NAD^+, 1 U monomeric [FeFe]-hydrogenase from *C. pasteurianum*, and 100% H_2 as gas phase ([18]). The genes for these oxidoreductases are found in the genome of *C. acidurici* (GenBank accession number CP003326; chromosome) ([19]).

To characterize the electron-bifurcating formate dehydrogenase, we purified the enzyme from 12 ml cell extract under anoxic conditions essentially as described by Kearny and Sagers ([6]). All

TABLE 1 Specific activities of oxidoreductases involved in acetic acid formation in uric acid-grown *C. acidurici*[a]

Enzyme activity in cell extracts	Substrates	Specific activity (U/mg)
Formate dehydrogenase (Fdh)	HCOO⁻ + NAD⁺ + Fd_ox	**1.3**
	HCOO⁻ + NADP⁺ + Fd_ox	<0.01
	HCOO⁻ + Fd_ox	<0.01
	HCOO⁻ + NADP⁺	<0.01
	HCOO⁻ + NAD⁺	<0.01
	HCOO⁻ + MV	5.9
Xanthine dehydrogenase (Xdh)	Xanthine + Fd_ox	**0.3**
	Xanthine + NAD⁺	<0.01
	Xanthine + NADP⁺	<0.01
	Xanthine + MV	2.3
Methylene-H₄F dehydrogenase[b]	Methylene-H₄F + NAD⁺	**4.3**
	Methylene-H₄F + NADP⁺	**1.0**
Pyruvate:Fd oxidoreductase (PFOR)	Pyruvate + CoA + Fd_ox	**0.6**
	Pyruvate + CoA + MV	0.9
Fd_red²⁻:NAD⁺ oxidoreductase[c] (RnfA-G)	Fd_red²⁻ + NAD⁺	**0.2**
	Fd_red²⁻ + NADP⁺	<0.01
Glycine-cleavage sytem (GCS)[d]	Glycine + H₄F + NAD⁺	**>0.02**
	Glycine + H₄F + NADP⁺	**>0.01**
Hydrogenase	H₂ + MV	<0.01

[a] Red lettering indicates substrates or products whose reductions were monitored; boldface indicates physiologically relevant specific activities. MV, methyl viologen; Fd, ferredoxin from *C. pasteurianum*; H₄F, tetrahydrofolate. One unit equals 2 μmol electrons transferred per min. For assay conditions, see the text.
[b] The genome harbors two genes for methylene-H₄F dehydrogenase. The NADP-specific enzyme has been purified (22).
[c] Reduced ferredoxin was regenerated by H₂ and [FeFe]-hydrogenase from *C. pasteurianum* (9).
[d] Specific activity increases exponentially with the protein concentration, indicating a multicomponent enzyme system (23). The specific activity was found at a protein concentration of 4.5 mg per 0.8 ml assay mixture.

the buffers were supplemented with 5 μM FAD, 5 μM FMN, and 2 mM dithiothreitol (DTT). The procedure involved centrifugation at 115,000 × *g* for 40 min, ammonium sulfate fractionation (the precipitate between 50% and 55% contained most of the activity), hydrophobic chromatography on a phenyl Sepharose

FIG 2 NAD⁺-dependent ferredoxin (Fd) reduction with formate catalyzed by electron-bifurcating ferredoxin- and NAD⁺-dependent formate dehydrogenase (Fdh/Hyl) from *C. acidurici*. (A) Kinetics of NAD⁺ and Fd reduction with formate monitored simultaneously at 340 nm (for NAD⁺ reduction) and 430 nm (for Fd reduction). (B) Stoichiometry of NAD⁺ and Fd reduction. The amounts of NADH ($\varepsilon_{340\ nm}$ = 6.3 mM⁻¹ cm⁻¹) and reduced ferredoxin ($\Delta\varepsilon_{ox\text{-}red,\ 430\ nm}$ = 13.1 mM⁻¹ cm⁻¹) were calculated based on their molar extinction coefficients. The absorbance change at 340 nm caused by ferredoxin reduction was very small and could therefore be disregarded. The data for panel B were taken from panel A.

high-performance column (2.6 cm by 12 cm), and size exclusion chromatography on a Superdex G200 column (26 cm by 60 cm). The enzyme was purified 130-fold to a specific activity of 168 U/mg. The enzyme preparation was found to contain FMN rather than FAD, which was identified by thin-layer chromatography on an RP-18 F254 aluminum sheet (Merck, Darmstadt, Germany) with FAD and FMN as the standards (12). It catalyzed the coupled reduction of NAD⁺ and ferredoxin from *C. pasteurianum* with formate (apparent K_m, 0.5 mM) in an almost 1:1 stoichiometry (Fig. 2). NADP⁺ could not substitute for NAD⁺. Methyl viologen reduction with formate was not dependent on the presence of NAD(P)⁺. Ferredoxin from *C. acidurici* was not tested.

SDS-PAGE of the preparation revealed the presence of mainly four proteins (Fig. 3), which were sequenced via matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF/MS) analysis after digestion with trypsin to iden-
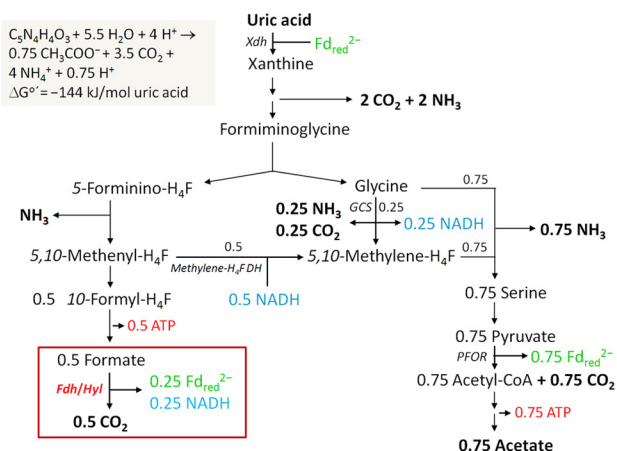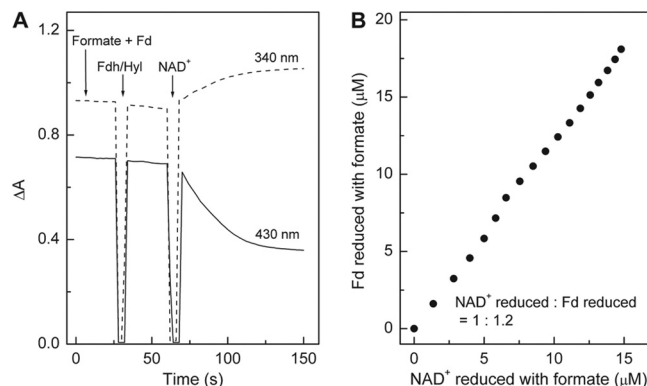

FIG 1 Scheme of the energy metabolism of *C. acidurici* growing on uric acid. The scheme highlights the proposed role of the electron-bifurcating formate dehydrogenase. Fd, ferredoxin; H₄F, tetrahydrofolate; Xdh, xanthine dehydrogenase; Fhd/Hyl, electron-bifurcating formate dehydrogenase; methylene-H₄F DH, 5,10-methylene-H₄F dehydrogenase; GCS, glycine cleavage system; PFOR, pyruvate:Fd oxidoreductase. The reduced ferredoxin required for uric acid reduction is regenerated in the Fdh/Hyl and PFOR reactions.
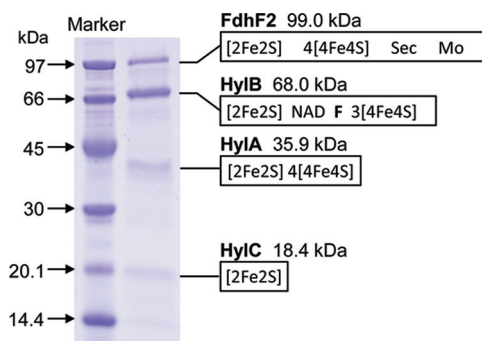

FIG 3 SDS-PAGE of purified electron-bifurcating ferredoxin- and NAD⁺-dependent formate dehydrogenase from *C. acidurici*. The molecular masses and cofactor binding sites of the four subunits were deduced from the amino acid sequences predicted from the DNA data (19) (see Fig. 4). Fdh, formate dehydrogenase; Hyl, [FeFe]-hydrogenase-like protein; Sec, selenocysteine; Mo, molybdopterin, to which either molybdate or tungstate can be bound; F, flavin. There is clearly more than one HylB per FdhF2.
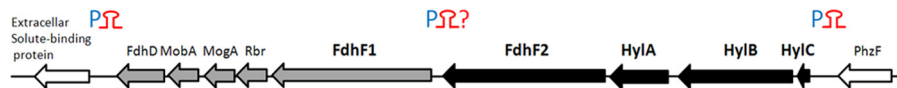
FIG 4 The *hylCBA-fdhF2-fdhF1-rbr-mogA-mobA-fdhD* gene cluster (gene numbers Curi_c29410 to Curi_c29330) in *C. acidurici*. Hyl, [FeFe]-hydrogenase-like protein; FdhF2, formate dehydrogenase with an active-site selenocysteine; P, promoter; Ω, terminator. Bioinformatics analysis was performed with Softberry software (Softberry, Inc., NY) and the software provided by the ARNold finding terminators at IGM-Web Server (http://rna.igmors.u-psud.fr/toolbox/arnold/index.php). The gene products Rbr (rubredoxin), MogA, MobA, FdhD, and FdhF1 are not found in the purified enzyme complex. MogA, MobA, and FdhD are involved in molybdopterin cofactor synthesis and formate dehydrogenase maturation. FdhF1 is a formate dehydrogenase with an active-site cysteine and is probably formed only under selenium-deficient growth conditions.

tify the encoding genes (12). The four encoding genes were found in a cluster that may form a transcription unit (Fig. 4). The four genes have already been annotated by Hartwich et al. (19) as *hydC*, *hydB*, *hydA*, and *fdhF2*. The genes *hydA*, *hydB*, and *hydC* were at that time predicted to encode a heterotrimeric electron-bifurcating [FeFe]-hydrogenase, and the gene *fdhF2* was predicted to encode a molybdopterin- and selenocysteine- containing formate dehydrogenase.

However, the purified formate dehydrogenase preparation, like the cell extracts (Table 1), did not show hydrogenase activity, neither with viologen dyes nor with $NAD(P)^+$ and/or ferredoxin as electron acceptors. Closer inspection of the sequences revealed the lack of typical amino acid residues involved in the H cluster (active site of [FeFe]-hydrogenase) formation in HydA. These residues are also not present in the homologues HydA1 and HydA2 encoded by *hydA1* and *hydA2* located elsewhere in the genome. Consistently, the genes *hydE*, *hydF*, and *hydG*, which are required for H cluster biosynthesis and assembly (24, 25), were not found in the genome of *C. acidurici*. Growing cultures of *C. acidurici* neither form nor consume H_2 (20). Because of all these findings, it is very unlikely that the *hydCBA* genes in *C. acidurici* code for an electron-bifurcating [FeFe]-hydrogenase (HydABC). We therefore renamed the *hydCBA* genes in *C. acidurici* the *hylCBA* genes (*hyl* for hydrogenase-like). HylB from *C. acidurici* shows high sequence identity (51% to 66%) to HydB and HytB from organisms with FMN-based electron-bifurcating [FeFe]-hydrogenases. Apparently, this FMN-containing, electron-bifurcating module can be combined with both formate dehydrogenases and [FeFe]-hydrogenases, just as the EtfAB module can be combined with butyryl-CoA dehydrogenase and caffeyl-CoA reductase (17).

The oxidation of protein-bound flavoquinol in two one-electron steps at different redox potentials is the mechanistic basis of flavin-based electron bifurcation (7). The two-electron reduction and twice-occurring one-electron oxidation at different potentials are inherent properties of flavins (26). It is therefore not surprising that several electron-bifurcating flavoproteins have evolved independently (27, 28), namely, HydB/HytB/HylB (10–12, 18), HdrA (15, 29), NfnB (13, 14), and EtfAB (9, 17). Genes encoding these flavoproteins—neighbors of genes with quite different annotated functions—are found in many anaerobic microorganisms, which indicates that the presently known flavin-based electron-bifurcating enzyme complexes are only the tip of an iceberg.

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