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Clostridium carboxidivorans Strain P7T Recombinant Formate Dehydrogenase Catalyzes Reduction of CO_2 to Formate

Apostolos Alissandratos, Hye-Kyung Kim, Hayden Matthews, James E. Hennessy, Amy Philbrook, Christopher J. Easton

CSIRO Biofuels Research Cluster, Research School of Chemistry, Australian National University, Canberra, Australia

Recombinant formate dehydrogenase from the acetogen *Clostridium carboxidivorans* strain P7^T, expressed in *Escherichia coli*, shows particular activity towards NADH-dependent carbon dioxide reduction to formate due to the relative binding affinities of the substrates and products. The enzyme retains activity over 2 days at 4°C under oxic conditions.

Formate dehydrogenases (FDHs) catalyze the interconversion of CO_2 and formic acid through an oxidoreductive process (Fig. 1) (1). Consequently, when catalyzing CO_2 reduction, they are of interest for the sequestration of CO_2 and for the production of formic acid as a stabilized form of hydrogen fuel and as a source of commodity chemicals. In many bacteria and eukaryotes, FDHs catalyze the final step of catabolic processes in which formate is oxidized to CO_2 (2). The ability of certain members of this class, such as FDH from *Candida boidinii* in particular, to efficiently regenerate NADH in conjunction with formate oxidation has been a research focus (3).

Acetogens are known to possess a number of pathways distinct from those found in the other species. FDHs present in acetogens are known to take part in a carbon fixation metabolic pathway producing acetate (the Eastern branch of the Wood-Ljungdahl pathway), in which the first step involves reduction of CO_2 to formate (4). Several FDHs are known to catalyze CO_2 reduction under appropriate conditions (5–9). Those enzymes from acetogenic and related anaerobes, such as *Moorella thermoacetica* and *Clostridium pasteurianum*, are better than other FDHs as reduction catalysts but also show similar catalytic efficiency toward formate oxidation and are considered highly oxygen labile, requiring anaerobic expression and purification as well as anoxic assay conditions (10, 11). *Clostridium carboxidivorans* strain P7^T (equivalent to ATCC BAA-624^T and DSM 15243^T) was isolated from the sediment of an agricultural settling lagoon after enrichment with CO as the substrate and is an obligate anaerobe that can grow autotrophically with H_2 and CO_2 or CO (fixing carbon via the Wood-Ljungdahl pathway) (12, 13). Therefore, when the gene of a selenocysteine-containing formate dehydrogenase H (FDH_H) from the acetogen *Clostridium carboxidivorans* strain P7^T was first identified, it was suggested that FDH_H would catalyze the conversion of CO_2 to formate (14, 15). Here we report the first production of FDH_H and its catalytic preference for CO_2 reduction, as well as its tolerance for oxic conditions.

Cloning, expression, and purification of FDHs. The overexpression and purification of recombinant FDH_H from the *Clostridium carboxidivorans* strain P7^T (FDH_H_*CloCa*) was carried out, along with that of NAD^+-dependent recombinant FDH from *Candida boidinii* (FDH_*CanBo*), in order to compare expression and activity of formate dehydrogenases that take part in distinct metabolic pathways. The DNA sequences for the FDH_H_*CloCa* (UniProt E2IQB0) and FDH_*CanBo* (UniProt O13437) genes were codon optimized for expression in *Escherichia coli* (commercially synthesized by GeneArt, Germany), with the exception of residue 139Sec, which was modified to 139Cys for FDH_H_*CloCa* to avoid the need for use of a different expression system with selenoprotein-expressing elements. Plasmid DNAs were then reconstructed with the respective FDH genes inserted into the T7 promoter vector pETMCSIII for subsequent expression with an N-terminal His_6 tag (16). The successful reconstruction of the plasmid DNAs was confirmed by PCR coding region amplification and DNA sequencing. The recombinant plasmid was transformed into the *E. coli* BL21(DE3) strain for expression. The transformed cells for both FDH_H_*CloCa* and FDH_*CanBo* were grown overnight in 50 ml of LBA medium (Luria-Bertani medium supplemented with 0.1 mg/ml ampicillin) at 37°C in a shaking incubator with full aeration. Overexpression of both FDH_H_*CloCa* and FDH_*CanBo* was efficient under the conditions tested, with no need for induction. The cells were harvested by centrifugation (4,000 × *g*, 15 min), and approximately 0.5 g of cells were obtained. Cell pellets were resuspended in binding buffer (20 mM sodium phosphate [pH 7.4], 500 mM NaCl, and 20 mM imidazole). Resuspended cells were lysed using a high-pressure homogenizer (Emulsiflex-B15; Avestin, Canada), and the soluble fraction of the cell lysate was collected by centrifugation (20,000 × *g*, 1 h, 4°C). Proteins were purified by metal ion affinity chromatography (His GraviTrap; GE Healthcare). The supernatant of the cell lysate was loaded onto the column and washed with 10× the column volume of binding buffer, followed by washing with 20× the column volume of washing buffer (20 mM sodium phosphate [pH 7.4], 500 mM NaCl, and 40 mM imidazole). Proteins were eluted with 4 ml of elution buffer (20 mM sodium phosphate [pH 7.4], 500 mM NaCl, and 500 mM imidazole) and concentrated with a YM-10 centrifugation filter (Millipore). Final concentrated proteins were stored in 50 mM phosphate buffer (pH 7.0) at 4°C. The yields of FDH_H_*CloCa* and FDH_*CanBo* were 0.4 mg and 3.3 mg per 50 ml of cell culture, respectively. The higher yield of the latter reflects its greater overexpression level and larger proportion in the soluble protein fraction. All purification procedures were carried out at 4°C under normal oxic conditions with no atmosphere control. The expression levels of the formate dehydrogenases were assessed by 20% SDS-PAGE (Fig. 2A).

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Address correspondence to Christopher J. Easton, easton@rsc.anu.edu.au.
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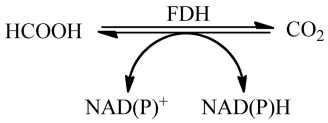

FIG 1 The reaction catalyzed by formate dehydrogenase, coupled to the redox of NAD.

The production of the formate dehydrogenases was also confirmed by liquid chromatography-mass spectrometry (LC/MS). Protein mass spectrometry was carried out by direct injection onto an Agilent 1100 series LC/MSD (mass spectrometer detector) time-of-flight (TOF) instrument running a mobile phase of 50:50 (vol/vol) 0.1% formic acid in MeCN–0.1% aqueous formic acid. The calculated molecular masses based on protein sequence for the N-terminally His_6-tagged FDH_*CanBo* and FDH_H_*CloCa* were 41,324 and 80,739 Da, respectively. Mass spectrometry gave molecular masses of 41,324 (data not shown) and 80,735 Da (Fig. 2B) for the expressed FDH_*CanBo* and FDH_H_*CloCa*, respectively.

Several formate dehydrogenases, as well as many oxidoreductases, are known to contain Mo-pterin cofactors (17, 18); however, there have been cases where W replaces Mo in the cofactor (19). Inductively coupled plasma-optical emission spectrometry (ICP-OES) (Perkin-Elmer) revealed the presence of W in an FDH_H_*CloCa* sample, which indicates that this enzyme, like the acetogenic *Moorella thermoacetica* formate dehydrogenase, contains a W cofactor.

Catalytic activities of FDHs. The purified FDHs were assessed for their catalytic activities. The initial velocities for enzyme catalysis in both directions were studied for these FDHs by monitoring NADH absorbance at 340 nm. It is impractical to define a reaction equilibrium position for the assay, since CO_2 is a substrate or product and the system is open. However, an excess of either formate or bicarbonate was present in order to ensure that the assays were initiated far from equilibrium. An initial assay was carried out with 1 μM enzyme in 0.1 M sodium phosphate buffer (pH 6.8) at 37°C with an excess (0.1 M) of sodium formate or sodium bicarbonate (depending on the direction monitored) and 0.2 mM NAD^+ or NADH, respectively. Formate dehydrogenase activity was assessed by measuring the change in absorbance at 340 nm due to the reduction of NAD^+ or oxidation of NADH (Shimadzu UV 2450 spectrophotometer equipped with a thermostated cell compartment).

The two FDHs displayed opposite catalytic behaviors under the conditions tested (Fig. 3A). The eluent from the purification of nontransformed *E. coli* BL21(DE3) cell lysate was used as a control and presented no conversion (data not shown). FDH_*CanBo* showed high activity in NAD^+ reduction in the presence of formate, as expected. On the contrary, when presented with NADH with an excess of bicarbonate, it was inactive. Conversely, FDH_H_*CloCa* showed minimal activity toward NAD^+ reduction but was active toward NADH oxidation. Under the conditions tested, the activities of these two enzymes seems to be consistent with what is known about their metabolic roles. The progress of the preferred reaction for each enzyme was more directly compared using the aforementioned assay conditions but with 0.1 μM FDH_*CanBo* and 5.0 μM FDH_H_*CloCa*, under which circumstances similar initial reaction rates were observed (Fig. 3B).

The FDHs were further characterized by monitoring their catalytic activities over a range of substrate concentrations. By maintaining an excess of one substrate and varying the concentration of the other, Michaelis-Menten kinetics were observed. Assays were performed for a range of NAD^+ (0.02 to 0.75 mM) or NADH (0.02 to 0.6 mM) concentrations in 0.1 M sodium phosphate (pH 6.8) at 37°C with 0.1 M sodium formate or sodium bicarbonate and 0.1 μM FDH_*CanBo* or 1 μM FDH_H_*CloCa*.

From the kinetic data presented in Table 1, it can be seen that both enzymes show similar binding affinities to their dinucleotide substrate; however, FDH_*CanBo* exhibits a much higher turnover. This translates into an approximately 50-fold-higher efficiency of FDH_*CanBo* than of FDH_H_*CloCa* in their respective "preferred" directions.

In order to gain a better understanding of the differences in this

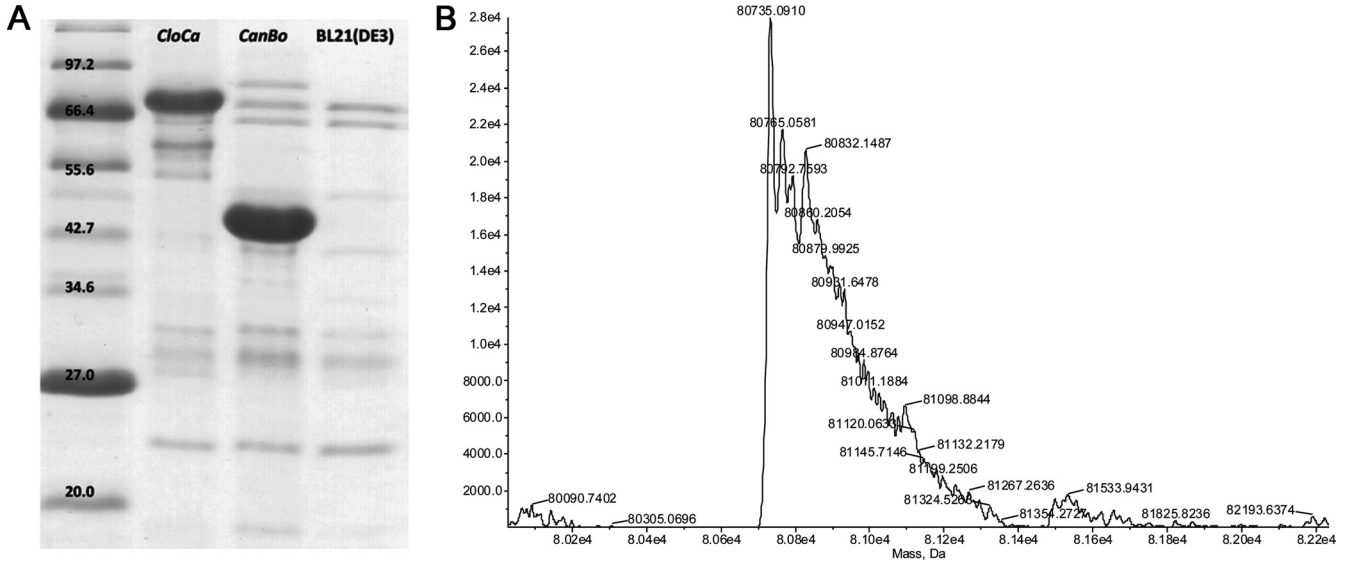

FIG 2 Twenty percent SDS-PAGE of purified FDH_H_*CloCa* (80.7 kDa) and FDH_*CanBo* (41.3 kDa), as well as the background from blank BL21(DE3) cells (A), and the mass spectrum of FDH_H_*CloCa* (B).

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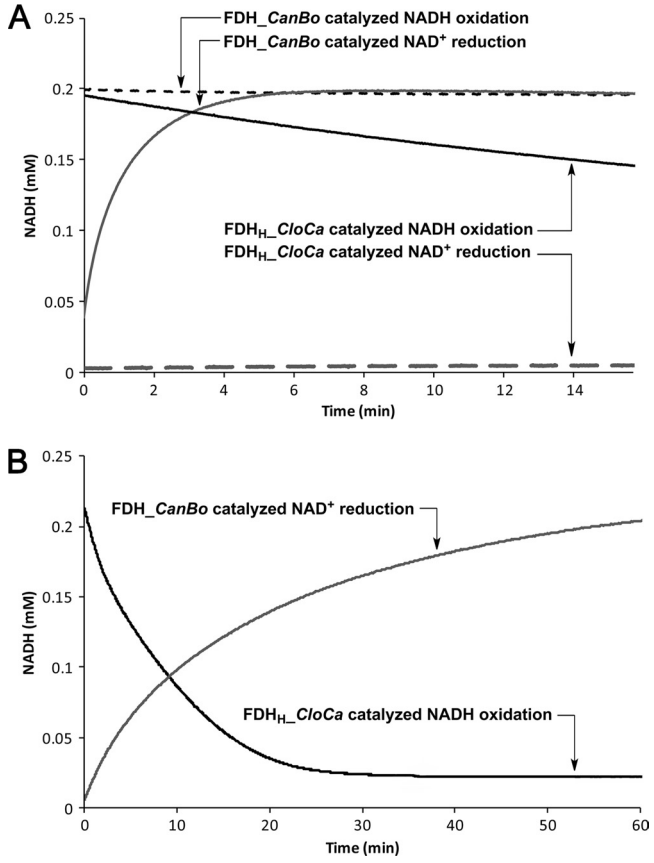

FIG 3 Catalytic activities of FDHs: all with 1 μM enzyme (A) or FDH_*CanBo*-catalyzed NAD^+ reduction with 0.1 μM enzyme and FDH_H_*CloCa*-catalyzed NADH oxidation with 5 μM enzyme (B).

preference of the two FDHs, the binding constants of the dinucleotide products were also calculated (Table 2). If a product is added to an assay mixture at initiation, it will compete with a substrate in binding to the enzyme. Since catalysis of the reverse reactions in each case is negligible, the Michaelis constant of the product reduces to the binding constant, which has the significance of an inhibition constant (K_P).

$$v_i = \frac{V_{max}[S]}{K_m\left(1 + \frac{[P]}{K_p}\right) + [S]}$$

[*S*] is the substrate concentration, [*P*] is the product concentration, v_i is the initial velocity, V_{max} is the maximum initial velocity for the substrate, K_m is the Michaelis constant for the substrate, and K_P is the Michaelis constant for the product.

TABLE 1 FDH kinetic parameters

Parameter	Value for indicated substrate	
	FDH_H_*CloCa* for NADH	FDH_*CanBo* for NAD^+
K_m (mM)	0.05	0.07
V_{max} (μM min^{-1})	5.0	38
k_{cat} (s^{-1})	0.08	6.3
k_{cat}/K_m (s^{-1} mM^{-1})	1.6	90

TABLE 2 FDH formate and nicotinamide binding affinities

Substrate/product	Binding affinity, mM	
	FDH_*CanBo*	FDH_H_*CloCa*
NAD^+	0.07 (K_m)	0.75 (K_P)
NADH	0.01 (K_P)	0.05 (K_m)
Formate	3.7 (K_m)	>100 (K_P)

For a fixed concentration of NADH (0.1 mM), the initial velocity of FDH_*CanBo*-catalyzed NAD^+ reduction was measured for a range of NAD^+ concentrations (0.1 M sodium phosphate buffer [pH 6.8] and 0.1 M sodium formate). The saturation kinetics observed allowed for calculation of an apparent K_m ($K_{m,app}$) (0.74, Eadie-Hofstee), which was used to calculate the binding constant of NADH: $K_{m,app} = K_m\{1 + ([P]/K_P)\}$.

Based on the above equation, the K_P of FDH_*CanBo* for NADH was calculated at 0.01 mM (Table 2). In the case of FDH_H_*CloCa*, it was found that a larger concentration of product (NAD^+) was required to significantly reduce the initial velocity, which suggested that the affinity of the enzyme for the product dinucleotide is very low. This was confirmed with saturation kinetics observed for various NADH concentrations when 1.5 mM NAD^+ was present in the assay (0.1 M sodium phosphate buffer [pH 6.8] and 0.1 M sodium bicarbonate). The K_P was calculated at 0.75 mM ($K_{m,app}$ = 0.15 mM, Eadie-Hofstee), which is approximately 15-fold higher than the K_m for NADH with this enzyme. The K_m of FDH_*CanBo* for formate was measured at 3.7 mM (0.6 to 100 mM sodium formate, 0.1 M sodium phosphate [pH 6.8], 37°C, and 0.5 mM NAD^+). In the case of FDH_H_*CloCa*, however, when formate at a concentration of 100 mM was added to the assay mixture (0.1 M sodium phosphate [pH 6.8], 37°C, 0.1 M sodium bicarbonate, and 0.2 mM NADH), the initial reaction velocity remained the same. This suggests that the binding constant of FDH_H_*CloCa* for formate ($K_{P,HCOO^-}$) has to be greater than 100 mM.

Despite the fact that both enzymes show a preference to bind NADH, it is noteworthy that for FDH_*CanBo*, the preference is less (K_{m,NAD^+} is 7-fold greater than $K_{P,NADH}$). This is combined with a relatively high binding affinity for formate, making the enzyme an efficient catalyst of formate oxidation. For FDH_H_*CloCa*, the preference for NADH is greater (K_{P,NAD^+} is 15-fold greater than $K_{m,NADH}$), which, combined with a low binding affinity for formate, makes this enzyme a more efficient catalyst for formate production through CO_2 reduction.

Concluding remarks. For the first time, recombinant formate dehydrogenase from the *Clostridium carboxidivorans* strain $P7^T$ was expressed and purified using an *E. coli* host cell. FDH_H_*CloCa* and FDH_*CanBo* prepared in the same way displayed different catalytic behaviors. While FDH_*CanBo* was more efficient in NAD^+ reduction, FDH_H_*CloCa* displayed a strong preference for NADH oxidation. Relative to FDH_*CanBo*, FDH_H_*CloCa* shows a 10-fold-lower binding affinity for NAD^+ and a binding affinity at least 30-fold lower for formate. These lower affinities for both products of NADH-dependent CO_2 reduction make it a much better catalyst for formate production. Expression was carried out aerobically, and the enzyme retained catalytic activity under normal oxic assay conditions, thus presenting relative robustness in comparison to related enzymes that are reported to be oxygen sensitive. Cur-

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rently, we are investigating the activity of FDH_H_*CloCa* in *E. coli* host cells, where formate production could serve in a useful role in alleviating fossil fuel dependence.

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