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Formate dehydrogenases for CO_2 utilization

Liliana Calzadiaz-Ramirez and Anne S Meyer

New measures for reducing atmospheric CO_2 are urgently needed. Formate dehydrogenases (FDHs, EC 1.17.1.9) catalyze conversion of CO_2 to formate ($HCOO^-$) via a reverse catalytic ability. This enzymatic conversion of CO_2 represents a novel first step approach for biocatalytic carbon capture and utilization targeting both CO_2 reduction and substitution of petrochemical-based production of important commodity chemicals. To achieve robust and efficient FDH catalyzed CO_2 conversion for sustainable large-scale implementation, it is critical to focus on the efficacy of the electron donor, enzyme stabilization, and on how the desired reverse FDH reactivity can be enhanced. Recent advances include the realization that NADH, the most common natural cofactor for reverse FDH catalysis, is an inefficient electron donor for FDH catalyzed CO_2 conversion. Improved understanding of the redox reaction details and structure-function relations of both metal-dependent and metal-independent FDHs provides the foundation for achieving rational technological advancements to promote enzymatic CO_2 utilization.

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Introduction

Although the rate of increase of global CO_2 emissions from fossil resource utilization has slowed recently, atmospheric CO_2 levels are currently higher than ever, close to 420 ppm, and continue to increase [1]. Clearly, natural photosynthesis processes are unable to capture the CO_2 emissions, and current strategies for CO_2 reduction are mainly emission mitigation strategies [2]. Utilizing CO_2 as a carbon source for production of high-in-demand chemicals represents a double gain by both replenishing fossil-fuel processes and simultaneously assisting in mitigating CO_2 accumulation and climate change.

Formic acid/formate ($HCOOH/HCOO^-$) is a consolidated commodity chemical having an annual value of about 780 million US$. Formic acid is moreover considered a potential liquid fuel compound and a feedstock for green synthesis of a range of 'petro'chemicals including methanol in the new bioeconomy [3,4,5••]. Formate dehydrogenases (FDHs, EC 1.17.1.9) naturally catalyze oxidation (dehydrogenation) of $HCOO^-$ to CO_2 ($HCOO^- \rightleftarrows CO_2 + H^+ + 2e^-$). The enzymatic reaction uses various natural cofactors as electron acceptors, notably nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$). FDHs also readily catalyze the reverse one-step CO_2 reduction to $HCOO^-$ under mild reaction conditions, that is, $CO_2 + H^+ + 2e^- \rightleftarrows HCOO^-$ using NADH (or NADPH) as electron donor. Whether the actual substrate for this reverse FDH catalyzed reaction is dissolved CO_2 or hydrated species such as HCO_3^- has been unclear, but recent electrochemical experiments have confirmed that CO_2 is the substrate in the reverse FDH catalysis [6]. FDHs are widespread in Nature and found in a broad range of anaerobic and aerobic bacteria as well as in some yeasts and plants. (For the sake of completion, the existence of another group of formate dehydrogenases, categorized as EC 1.2.2.1, that require cytochrome as acceptor, should be mentioned. This latter type is currently not considered suitable for technical CO_2 conversion).

CO_2 reduction with FDHs

FDHs are divided into two types: (i) metal-dependent enzymes that contain molybdenum (Mo) or tungsten (W) in their active site and (ii) metal-independent enzymes that function without relying on metal-driven redox catalysis [7–9]. In general, the metal-dependent FDHs favor catalyzing the reverse CO_2 reduction reaction relatively more than the metal-independent FDHs. The redox potential for the enzymatic reduction of CO_2 to COO^- is $E^{\circ\prime} = -420$ mV, but both types of FDHs mainly catalyze $HCOO^-$ oxidation, hence their dehydrogenase designation. The difference in catalytic reaction reversibility is due to differences in the catalytic reaction mechanism and the enzyme structure governing the energy reorganization during catalysis. However, the detailed understanding of how the reverse and the forward catalytic reactions are controlled is incomplete. NADH and NADPH are expensive and unstable [10••,11,12] and unfit for large-scale technical applications, despite being the natural and hence the most frequently applied electron donors (cofactors) in FDH catalysis studies. FDHs have been intensely studied for cofactor regeneration ($NAD(P)^+ \rightleftarrows NAD(P)H$) in various oxidoreductase-driven synthesis reactions [13].

Electrochemical reduction by alternative cofactors

During reaction, the metal-dependent FDHs transfer electrons within the enzyme through a FeS mediated electron transfer chain. Yet, for the actual FDH catalyzed conversion of CO_2 to $HCOO^-$ a balanced regeneration of reducing equivalents is required to sustain the continued enzymatic reaction. The metal-dependent FDHs accept a broader spectrum of electron donors than metal-independent FDHs, but appear to be more sensitive to oxygen than the metal-independent FDHs [10••].

Although the redox potential of $E^{\circ\prime} = -320$ mV for the reaction $NADH \rightleftarrows NAD^+ + 2e^-$ presents a thermodynamically favorable half-reaction to FDH catalyzed CO_2 reduction, NADH is both expensive and unstable, and exhibits poor kinetic rates in practical *in vitro* reactions. For this reason, a range of artificial cofactors as well as direct supply of electrons via electrodes have been explored for electron donation to promote fast and efficient FDH catalyzed CO_2 reduction for technical applications. Methylviologen (MV^{2+}), also known as 1,1-dimethyl-4,4′- bipyridinium dichloride [14,15] (and its various 2,2′-bipyridinium salt derivatives (1,1′-dimethyl (DM), trimethylene (TB), ethylene (DB), and tetramethylene (QB)) has been shown to be a particularly efficient electron vehicle for FDH catalyzed conversion of CO_2 to $HCOO^-$ [16,17]. Hence, the FDHs derived from *Desulfovibrio desulfuricans* and *Acetobacterium woodii* exhibit higher turnover rates (k_{cat}) and higher specificity constants (k_{cat}/K_M) than what is achieved with other electron donors in other systems, and notably higher than with NADH (Figure 1). However, the available data provide a comparison of the inherently more efficient metal-dependent FDHs with metal-independent FDHs from *Candida boidinii*, *Candida methylica*, *Chaetomium thermophilum*, *Myceliophthora thermophila*, and *Thiobacillus* sp. *KNK65MA* (Figure 1). An interesting comparison, however, is that the catalytic efficiency in *A. woodii* FDH is 13-fold higher with MV^{2+} than with H_2 (Figure 1), corroborating the significant impact of the electron donor selection for reverse FDH kinetics.

Stabilization by enzyme immobilization

In addition to high enzymatic turnover rates (k_{cat}) providing fast CO_2 conversion and high catalytic specificity constants (k_{cat}/K_M) supporting efficient CO_2 reduction rates at low CO_2 concentrations, enzyme robustness is a paramount prerequisite for large scale implementation of FDHs for CO_2 utilization. Insight into the structural features of FDHs are not yet mature enough to guide enzyme stabilization by rational protein engineering. Improved recombinant expression, especially of the metal-dependent FDHs will pave the way for production of high enzyme yields for further structural studies in turn allowing robustness improvements by rational enzyme engineering. Meanwhile experimental enzyme immobilization of metal-independent as well as metal-dependent FDHs has been deployed for stabilization, often combined with assessment of other electron donors than NADH or NADPH. From a kinetics point of view, immobilization of FDHs appears a particularly wise strategy because both the reactants and products of the FDH catalysis are small, supporting fast mass transfer, although efficient cofactor contact is an issue.

Covalent immobilization was used to immobilize *Desulfovibrio vulgaris* FdhAB FDH to chemically modified gold and low-density graphite electrodes. This technology allowed measuring high electrocatalytic currents by direct electron transfer for both $HCOO^-$ oxidation and CO_2 reduction, using electrostatic interactions to favor spatially advantageous immobilization of the enzyme [18]. Recent work has shown that FDH of *C. boidinii* immobilized covalently with mesoporous silica modified with glyoxyl groups, exhibited higher thermal stability than the free enzyme [19].

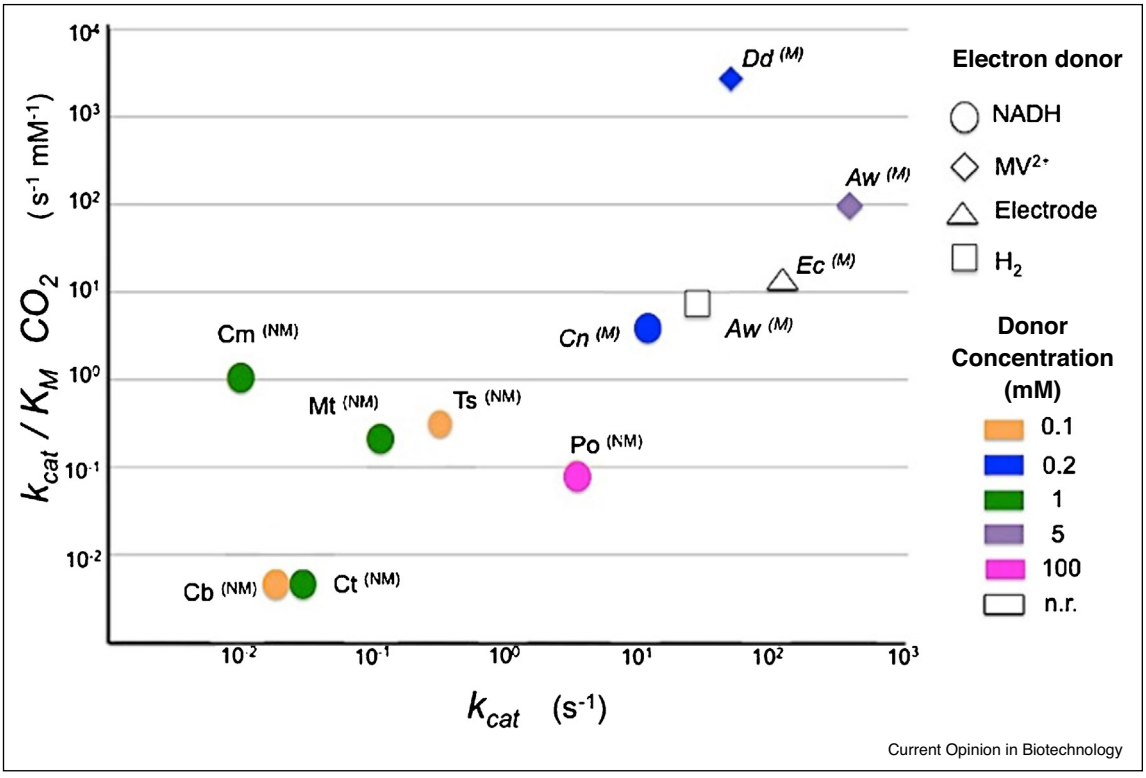
Immobilization of the FDH from *C. boidinii* by cross-linked enzyme aggregate (CLEA) technology using dextran polyaldehyde and glutaraldehyde as cross-linking reagents increased thermal stability of the enzyme 3.6 times compared to the free enzymes, but the activity of the CLEA-immobilized enzyme for CO_2 reduction was only 13% of that of the free FDH. Glutaraldehyde supports have generally been widely studied for enzyme immobilization, but despite producing stable, covalent immobilization, the activity of the immobilized FDH is often vastly reduced compared to free enzyme [20,21]. Recently, the use of polyethyleneimine, which is a common modifier in the field of CO_2 adsorption, with plenty of amine groups, low volatility and good chemical stability, has proven effective for FDH immobilization [22,23].

Direct electrocatalytic FDH mediated reduction of CO_2 to C_1 compounds by suppling electrons to enzymes immobilized directly on an electrode has given promising results, and may be particularly useful for designing *in vitro* cascade reactions, for example, catalyzing conversion of CO_2 to methanol via co-immobilization of several enzymes involving reverse FDH catalysis as the first step [24–27].

FDH protein engineering

Most studies have been focused on engineering of bacterial and yeast FDHs to improve catalytic activity and chemical and thermal stability [28,29]. The main FDHs subjected to protein engineering enhancements have been on metal-independent FDHs derived from for example, *Pseudomonas* sp. *101*, *Granulicella mallensis* MP5ACTX8, *Candida bodiini*, *Burkholderia stabilis* 15516, *Mycobacterium vaccae N10*, and *Saccharomyces cerevisiae*, but initial protein engineering has also been done on the more complex metal-dependent FDHs from

Figure 1



Kinetic properties of FDH catalyzed CO_2 reduction for different types of electron donors, discriminated by color and shape of markers. The compared FHDs are: (M) metal-dependent (i.e. metal-containing) FDHs from: (Ec) *Escherichia coli*, (Po) *Pseudomonas oxilatus*, (Cn) *Cupriavidus necator*, (Dd) *Desulfovibrio desulfuricans*, and (Aw) *Acetobacterium woodii*; (NM): Metal-independent FDHs from: (Mt) *Myceliophthora thermophila*, (Ts) *Thiobacillus* sp. KNK65MA, (Ct) *Chaetomium thermophilum*, (Cb) *Candida boidinii* and (Cm) *Candida methylica* (Figure adapted from Ref. [10••]).

Escherichia coli, *D. vulgaris*, *Rhodobacter capsulatus*, and *Cupriavidus necator*. Besides the main aim to improve the catalytic properties, engineering providing changed cofactor specificity from NAD^+ to $NADP^+$ for improved cofactor regeneration has been of huge research interest [30–33]. In this regard, *Pseudomonas* sp. *101* FDH appears to be the most promising NADPH regenerator and determination of the crystal structure of this enzyme has been used to establish the main residues governing the cofactor specificity and active site conformation [34,35]. The high sequence similarity of the metal-independent FDHs now permit their structural comparison via homology modeling. In contrast, the structural details of metal-dependent FDHs, including the structural features and residues responsible for cofactor specificity, catalytic activity, and reaction direction specificity, are not yet fully elucidated, although recently reported structural data for the Mo-containing *R. capsulatus* FDH provide important new insight into the active site structure and notably the electron transfer pathways in this enzyme [36••]. However, this new structural insight, achieved by cryo-electron microscopy, has in fact revealed an unexpected complexity by showing that the FdsD subunit is retained as a subunit in the active enzyme, and that the main electron pathway takes place through five of the seven Fe–S clusters, and that the functional enzyme appears to exist as a heterotetrameric dimer that may interconnect the electron pathway in the enzyme [36••]. Hence, despite this recent progress, targeted protein engineering of metal-dependent FDHs remains a huge challenge.

One of the few protein engineering attempts reported for FDHs aimed for CO_2 reduction, was to engineer the metal-free FDH from *C. thermophilum* (CtFDH) to enhance its CO_2 reduction activity through directed evolution. Three variants were identified (G93H/I94Y, G93H/I94R and R259C) which were characterized in presence of both aqueous $CO_{2(g)}$ and HCO_3^- [37,38•,39,40]. The three variants exhibited up to three-fold higher turnover rate (k_{cat}) than the wild-type (WT), but only the variant G93H/I94Y exhibited an improved specificity constant (k_{cat}/K_M) for CO_2 reduction (2.8-fold improvement) [37,38•,39]. Although it has recently been proven that a metal-dependent FDH (specifically a W-dependent FDH from *D. vulgaris*) indeed catalyzes reduction of CO_2 rather than HCO_3^- [6], it should be

mentioned that the metal-independent *C. thermophilum* FDH (CtFDH) has been claimed to catalyze conversion of HCO_3^- to $HCOO^-$, and models of this enzyme shows that HCO_3^- fits the active site [40]. Likewise, the engineered variant G93H/I94Y of the CtFDH enzyme was reported to show a 5.4-fold increase in k_{cat}/K_M for HCO_3^- reduction compared to the WT [37,38•,39,40]. However, due to the spontaneous interconversion between CO_2 and HCO_3^- in aqueous solution and the approximate *pKa* of 6.4 of the reaction $CO_2 + H_2O \rightleftarrows HCO_3^- + H^+$ it cannot be ruled out that the reaction kinetics reported for the enzymatic HCO_3^- conversion in reality concerned CtFDH catalyzed CO_2 conversion to $HCOO^-$.

FDHs for *in vivo* CO_2 utilization

Development of *in vivo* systems, meaning using of microorganisms to obtain specific commercial compounds such as methanol, certain organic acids (lactic and succinic) as well as L-alanine and L-serine from CO_2 is a hot research area. However, the low solubility of CO_2 in water and the lack of efficient CO_2 assimilation pathways are challenging. Therefore, $HCOO^-$ is favorably produced from CO_2 using FDHs, and the advantage is that $HCOO^-$ presents a more convenient C-storage compound than CO_2 because it is a liquid. In addition, as a carbon source $HCOO^-$ can be assimilated more efficiently than CO_2 by microorganisms [41].

In order to prepare an engineered whole-cell biocatalyst for producing $HCOO^-$, the FDHs from *Clostridium carboxidovorans* (CcFDH), *Pyrococcus furiosus* (PfFDH), or *Methanobacterium thermoformicicum* (MtFDH) have been attempted overexpressed in *E. coli* JM109. The strain overexpressing PfFDH was able to produce 44 mM of $HCOO^-$ from sodium bicarbonate and gaseous hydrogen [42].

Despite the existence of native formatotrophs such as *Methylobacterium extorquens* that can grow with $HCOO^-$ as sole carbon source, its growth rate is too slow for practical exploitation. Therefore, different metabolic pathways to support formatotrophic growth have been designed in different microorganisms through rational metabolic engineering, involving new enzymes and/or adaptive laboratory evolution. Notably, the reductive glycine pathway (rGlyP), which is a synthetic pathway for formate assimilation, has become promising after it was shown that an rGlyP engineered *E. coli* strain could grow under formatotrophic conditions [43••]. Furthermore the full rGlyP was implemented in the bioplastic producer *C. necator* and formate assimilation to glycine through the core module of rGlyP was shown to work in *S. cerevisiae* [44,45]. The expression of FDHs to catalyze reduction of CO_2 to $HCOO^-$ through synthetic formate assimilation pathways thus appears as a budding strategy for CO_2 reduction with simultaneous bioproduction of new products. In this regard, the production of methyl ketones, isoprenoids and terpenes, isobutanol, alkanes and alkenes from CO_2 using *C. necator* looks particularly promising [46].

Conclusion

The high atmospheric CO_2 levels are of crucial concern in relation to climate change and global warming and new strategies for CO_2 utilization have to be developed. In this review, we defined the application of FDHs for conversion of CO_2 to $HCOO^-$ as a potential and promising strategy for CO_2 utilization. By comparing the kinetic rates (k_{cat}) and specificity constants (k_{cat}/K_M) achieved for FDH catalyzed CO_2 conversion we highlighted the improvements in catalytic conversion efficiency achievable by alternative electron donors and illustrated that NADH is in fact a poor electron donor for FDH catalysis. Immobilization of FDHs to electrodes can also optimize the FDH catalyzed CO_2 conversion kinetics, and pave the way for using electricity as electron source for FDH catalyzed CO_2 utilization. Such use will allow very large scale reactions and provide an option for $HCOO^-$ production by use of excess electricity from for example, wind turbines. Although $HCOO^-$ is a valuable base chemical, the application of FDHs for multi-enzymatic cascades to convert CO_2 to various high-in-demand chemicals, including methanol, is of high interest; such reaction cascades support a dual gain, namely CO_2 mitigation and substitution of current petrochemical processes (methanol is considered a top 10 'petrochemical'). Improved recombinant expression, deeper understanding of structural features of FDHs, notably of metal-dependent FDHs, and provision of robust enzymes and fast reactions via proper electron donor development, will pave the way for practical use of FDHs in enzymatic CO_2 conversion processes.

Conflict of interest statement

Nothing declared.

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