**The Catalytic Bias of 2-Oxoacid:ferredoxin Oxidoreductase in CO₂:
evolution and reduction through a ferredoxin-mediated electrocatalytic
assay**

Bin Li^a and Sean J. Elliott^{a,1,*}

^a Department of Chemistry, Boston University, 590 Commonwealth Ave., Boston, MA
02215, USA

* corresponding author contact information: elliott@bu.edu; Boston University, 590
Commonwealth Ave., Boston, MA, 02215 USA; phone 617-358-2816; FAX: 617-353-6466

¹ ISE Member

ABSTRACT:

Enzymes from the 2-oxoacid: ferredoxin oxidoreductase (OFOR) family engage in both CO₂ evolution and reduction in nature, depending on their physiological roles. Two enzymes and their redox partner ferredoxins (Fds) from *Hydrogenobacter thermophilus* and *Desulfovibrio africanus* were examined to investigate the basis of the catalytic bias. The Fd1 from *H. thermophilus* demonstrated a potential of ~ -485 mV at room temperature, the lowest for known single [4Fe-4S] cluster Fds. It suggests a low potential electron donor may be the key factor in overcoming the large thermodynamic barrier of CO₂ reduction. The Fd-mediated electrocatalytic experiments further demonstrated the impact of Fd's potential on the direction of the OFOR reaction: as OFOR enzymes could essentially catalyze both CO₂ evolution and reduction *in vitro*, the difference in their physiological roles is associated with the reduction potential of the redox partner Fd. The electrocatalytic assay could study both CO₂ evolution and reduction in one setup and is a good tool to probe Fds' reactivity that arise from their reduction potentials.

KEYWORDS: electron-transfer; voltammetry; CO₂; ferredoxin

1. Introduction

Fixation of CO₂ is one of the most important biological processes on Earth, providing the building blocks for all life forms. The most prominent example is the “dark” reaction of photosynthesis where the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) converts CO₂ and ribulose 1,5-bisphosphate to glycerate 3-phosphate. ATP and NADPH provide the driving force required for the endergonic process [1]. Alternatively, a few distinct CO₂ fixation pathways exist in the prokaryotes world [2-5]. These pathways offer a fascinating insight into the early evolution of autotrophy and provide many intriguing examples of how CO₂ is transformed to different organic molecules. Of our particular interest is the reductive tricarboxylic acid (rTCA) cycle in which two members of the 2-oxoacid:ferredoxin oxidoreductase (OFOR) enzyme family, pyruvate:ferredoxin oxidoreductase (PFOR) and 2-oxoglutarate:ferredoxin oxidoreductase (OGOR), catalyze the reductive carboxylation of acetyl-CoA and succinyl-CoA to pyruvate and 2-oxoglutarate [6, 7].

This reaction is an energetically challenging process, with an E°' on the order of -500 mV [2, 8-10]. Reduced ferredoxins (Fds) fulfill the energetic requirement of the reaction, serving as the electron donor. Fds are a group of small iron-sulfur proteins that serve as electron mediators in various biological redox processes. Analyses of the energetics of biological pathways usually denote Fds with a reduction potential around -400 mV [2, 8]. How could

Fds with a reduction potential at -400 mV compensate for the -500 mV thermodynamic gap of an OFOR reaction? It is proposed that Fds must be kept almost entirely in its reduced form *in vivo* so that the actual potential can reach -500 mV [2]. While this view is plausible, it means another energy source is needed to drive Fds into such disequilibrium. Also, it seems such a burden for the cell to express a high concentration of Fd that is essentially unused and must stay reduced, in order to catalyze one reaction. Another view suggests even with Fds at a reduction potential of -400 mV, if the ratio between substrates and products is kept very high, the energy barrier of the reaction could be lowered [3, 8]. The caveat of this hypothesis is that cells may be able to direct high flux of substrates for reactions, however, a low concentration of products may impact their downstream reactions, considering pyruvate and 2-oxoglutarate are key components of the rTCA cycle. While the two hypotheses are not mutually exclusive and together they could account for the strong driving force of CO₂ reduction, the impact of the reduction potential of Fd has usually not been considered a variable.

In this study of the OFOR-Fd system, we hypothesized that such a minimalized view of the role of Fd redox potentials may ignore the diversity of Fds in nature and their possible difference in reactivity. For example, as a redox enzyme family, OFORs are conserved in the three domains of life, yet demonstrate different biological roles in either oxidative or reductive chemistry [9, 11]. For example, OFOR enzymes identified from rTCA organisms reduce CO₂ using the physiological electron donor of an Fd, yet those from non-autotrophic organisms oxidize a 2-oxoacid to CO₂ with Fd as the electron acceptor. Remarkably, all OFOR enzymes share similar structural features and contain the same cofactors: a thiamine pyrophosphate (TPP) as the active site where the redox reaction occurs and one or three [4Fe-4S] clusters that engage in electron transfer [9, 11]. How do these structurally similar OFOR

enzymes operate at opposite directions *in vivo*? We suggest that the Fd redox relay itself may be essential for the directionality of OFOR reactions.

Here, we compare the electrocatalytic properties of an OGOR with its electron donor Fd from *Hydrogenobacter thermophilus*, a model system for the study of rTCA cycle, with a PFOR and its electron acceptor Fd from *Desulfovibrio africanus*, a heterotrophic organism that does not fix CO₂ [12-17]. Previous studies have established that at physiological conditions, HtOGOR reduces CO₂ to 2-oxoglutarate with HtFd1 as the electron donor while DaPFOR oxidizes pyruvate to CO₂ with DaFdI as the electron acceptor [12, 15]. In addition to the study of OFOR enzymes with their native redox partners, we also paired OFOR enzymes with a non-native Fd. Through these experiments with different OFOR-Fd combinations, we find that both enzymes are essentially capable of catalyzing both CO₂ evolution and reduction reactions regardless of their actual physiological roles. The difference in CO₂ evolution versus reduction is associated with the potential of Fd, regardless its source. HtFd1, serving as an electron donor, demonstrate a potential near -500 mV, 100 mV more negative than that of DaFdI, which serves as the electron acceptor. This finding underlines the importance of the reduction potential of the biological electron mediator, Fd.

To arrive at this comparison, we found that an electrochemical assay format was extremely advantageous (Fig.1a). Traditionally, a CO₂ reduction assay by an OFOR enzyme would consist of three key components: the reduction of CO₂, using reduced Fds as the electron donor, a upstream reaction to keep Fds reduced and a downstream reaction to measure product formation (Fig.1b). Both upstream and downstream reactions are indispensable in this coupled enzymatic assay. First, in order to provide the driving force for CO₂ reduction, the concentration of reduced Fds need to be maintained by a regeneration system. Methods developed separately in Ragsdale group and Igarashi group utilize carbon monoxide dehydrogenase (CODH) or an orthogonal OFOR enzyme in the upstream reaction [18-20].

The latter makes use of the substrate specificity of different OFOR enzymes: when measuring CO₂ reduction activity of an OGOR (OFOR2), a PFOR is used in the upstream reaction (OFOR1), vice versa. Second, the product of CO₂ reduction, either pyruvate or 2-oxoglutarate, is not measurable optically. By coupling a downstream reaction, a lactate dehydrogenase (LDH) or glutamate dehydrogenase (GDH) consumes pyruvate or 2-oxoglutarate with the concomitant oxidation of NADH, which is optically measured at 320 nm. The coupled assay is designed as such that the CO₂ reduction by OFOR is the rate-limiting step and thus the measured downstream rate could be considered the rate of CO₂ reduction. In the Fd-mediated electrochemical assay we presented here, the electrode replaces both the upstream (electron donor) and downstream reactions (product detection), simplifying the study of OFOR enzyme directionality. Details and benefits of this electrochemical assay are further discussed below.

2. Experimental

2.1 Instrumentation

All electrochemistry experiments were performed under anaerobic conditions in an MBraun LABmaster glovebox with a constant positive pressure at > 5 mbar and O₂ level <1 ppm. The electrochemical analyses were conducted with a Metrohm/Eco Chemie Autolab PGSTAT 12 potentiostat and operated by the GPES software. Electrochemical cells were water jacketed and connected to a circulator for temperature control. The electrode configuration includes a standard calomel reference electrode (Fisher Scientific), a platinum counter electrode (Fisher Scientific) and a pyrolytic graphite edge (PGE, Minerals Technologies) working electrode. The working electrode was sanded on 1500 grit waterproof silicone carbide sandpaper, polished with 1 µm alumina slurry (Beuhler) and sonicated for

>10 min in each experiment. The voltammograms were analyzed by SOAS and plotted with ProFit (QuantumSoft) [21].

2.2 Materials and reagents

Purification of all proteins in this study was based on procedures reported elsewhere with modifications [13, 14, 22]. More details regarding the recombinant expression and purification of these proteins can be found in Appendix A. Supplementary data. All chemicals were purchased from Sigma Aldrich. 2xYT broth, tryptone and yeast extract for cell culture was purchased from Fisher Scientific. All anaerobic buffers were made based on the procedure previously reported [23].

2.3 Protein characterization and enzyme activity

The concentration of DaPFOR and HtOGOR was determined using a Quick Start™ Bradford protein assay kit (Bio-Rad) and the concentration of DaFdI and HtFdI was determined with a RC DC™ protein assay (Bio-Rad), both with bovine serum albumin (BSA) as the standard. The iron content of the proteins was determined by a ferrozine colorimetric assay, while the activity of the enzymes was routinely determined based on procedures reported previously [15, 24]. Optically-coupled assays were performed on a Cary 50 or 100 Bio UV-vis spectrophotometer (Varian), by monitoring the reduction of methyl viologen at 604 nm ($\epsilon_{604} = 13.6 \text{ mM}^{-1} \text{ cm}^{-1}$). The reaction was carried out in a 3 mL serum-stopper cuvette containing 10 mM sodium pyruvate or disodium 2-oxoglutarate, 2 mM methyl viologen, 5 mM dithioerythritol, 0.2 mM sodium coenzyme A and 100 nM enzyme. A mixed buffer consisted of 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 3-(*N*-morpholino)propanesulfonic acid (MOPS), *N*-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS), *N*-Cyclohexyl-2-aminoethanesulfonic acid (CHES) and *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) was used. This mixed buffer allows buffering over a range of pH. In this study the experiments were performed at pH 7.0 to be

consistent with the electrochemical experiment. Reaction mixture was made anaerobic by purging with argon for 5 min. Concentrations of sodium pyruvate or disodium 2-oxoglutarate were varied to determine the K_M for the substrate. The assay temperature was maintained at 30 °C for DaPFOR and 60 °C for HtOGOR.

2.4 Cyclic voltammetry of Fds

The cyclic voltammetry experiment of Fds was carried out in solution at various temperatures. Typically, Fds were diluted to a final concentration of 10~20 μM in the electrochemical cell by a mixed buffer. The mixed buffer consisted of 5 mM MES, MOPS, TAPS, CHES and CAPS. All experiments were performed at pH 7.0. Neomycin sulfate was added in the cell at a final concentration of 1 mM to promote interaction between Fds and electrode surface.

2.5 Fd-mediated OFOR turnover cyclic voltammetry

Turnover experiments involve OFORs interacting with their native or non-native redox partner Fds in the presence of substrates for both 2-oxoacid oxidation and CO_2 reduction. These experiments were carried out over different ranges between -0.30 and -1.05 V (vs SCE), depending on which Fd was used and the direction of OFOR reaction. Cyclic voltammetry is set at a scan rate of 1 mV/s, with a 0.15 mV step potential. Typically, OFOR enzymes were added to a final concentration of 2-4 μM , with a 1:5 ratio to Fds. In 2-oxoacid oxidation experiments, 10 mM sodium pyruvate or disodium 2-oxoglutarate and 0.2 mM coenzyme A were added, similar to that of the activity assay. In the CO_2 reduction experiments, 10 mM sodium bicarbonate and 1 mM acetyl or succinyl coenzyme A were added. Each set of turnover experiment is done with a control where the enzyme is omitted from the assay. In all experiments, neomycin was added to a final concentration of 1 mM. The raw data were processed by SOAS and plotted by ProFit. The voltammograms of the

control experiment and its corresponding turnover experiment were overlaid after normalizing the current of the background capacitance.

3. Results and Discussion

3.1. Protein characterization and enzyme activity

All proteins in this study were purified to homogeneity (Appendix A. Supplementary data). Iron assays indicated that both enzymes and the Fds were purified with appropriate iron-sulfur cluster loading, indicating homogenous proteins could be produced recombinantly for the subsequent electrochemical analyses. As such, all proteins were used with no further chemical reconstitution. The oxidative decarboxylation activity of OFOR enzymes was found to be pH dependent, as reported previously, with an optimum at 9.0 for DaPFOR and 7.5~7.8 for HtOGOR, respectively [12, 15]. In this study, all activity assays were carried out at pH 7.0 for consistency. Additionally, temperature impacts upon activity were considered as HtOGOR is from a thermophilic organism, and its activity starts to increase at 60 °C drastically. Thus, the activity assay for HtOGOR was carried at 60 °C, compared to DaPFOR, which was examined at 30 °C. At these experiment conditions, both enzymes exhibit similar activity in terms of 2-oxoacid oxidation, with a k_{cat} of $\sim 350 \text{ min}^{-1}$ (Fig. 2).

3.2. Cyclic voltammetry reveals the difference of DaFdI and HtFd1 in reduction potential

Both DaFdI and HtFd1 belong to a type of single [4Fe-4S] cluster Fds that are presumed to be structurally similar, based upon overall homology. Despite the net negative charge on the protein surface of either Fd, they generate robust electrochemical responses on the PGE electrode in the presence of the positively charged aminoglycoside co-adsorbant, Neomycin. Fig. 3a shows typical voltammograms for DaFdI and HtFd1 at a scan rate of 50 mV/s. The

reduction potential of HtFd1 and DaFdI were determined at -485 mV and -385 mV at pH 7.0 at room temperature (23°C). The reduction potential value for DaFdI determined by electrochemistry is in agreement with previous reported value of -385 ± 15 mV, determined by redox titration monitored by Electron Paramagnetic Resonance (EPR) [25]. The reduction potential value of -485 mV for HtFd1 reported here is the lowest for proteins with a single [4Fe-4S] cluster in the literature.

There is very little variation for potential values measured at different scan rates. The potential values are, however, dependent on temperature and buffer pH (data not shown). As shown in Fig. 3b, reduction potential values for both DaFdI and HtFd1 decrease as the increase of temperature, with nearly identical slopes: -0.88 mV/ $^{\circ}$ for DaFdI and -0.87 mV/ $^{\circ}$ for HtFd1. The very similar slopes of temperature dependence indicate the similarity of protein fold of the two Fds, and the relative contribution of entropy to the thermodynamics associated with the redox reaction. Since *H. thermophilus* is a thermophilic organism with an optimal growth temperature between 70 and 75 °C, the reduction potential of HtFd1 could reach as least -533 mV at its physiological condition, again implicating its very low potential nature [12]. The determination of HtFd1 reduction potential provides an initial evidence that Fds with low potentials ($\sim$ -500 mV) are utilized in CO₂ reduction catalyzed by OFOR enzymes from the rTCA cycle. How the difference in reduction potentials of Fds may impact the reactivity of OFOR reactions is further addressed in the turnover experiment described below.

3.3. Both DaFdI and HtFd1 can serve as electron acceptors supporting 2-oxoacid oxidation

The cyclic voltammetry experiments have demonstrated DaFdI and HtFd1 could freely diffuse in solution and generate electrochemical response on the electrode. In the turnover electrochemical experiments, the two Fds are further coupled to an enzymatic reaction

catalyzed by an OFOR present in solution as well. Here, Fds serve as robust electron mediators, shuttling electrons between the enzymes and the electrode during catalysis. At the experiment condition, the direct interaction between OFOR enzymes and the electrode does not generate observable currents discernible from the charging capacitance of the electrode, largely due to the bulky nature of these enzymes. Pieulle and coworkers have used a similar method to investigate the kinetics of intermolecular electron transfer between DaPFOR and three Fds from the same organism [26]. In a general formulation of this experiment, electrocatalytic responses are anticipated if the following scheme is followed where a 2-oxoacid is oxidized, and Fds are cycled through the oxidized and reduced states:

In the control experiment, enzymes are omitted and thus only the electrochemical reaction is at work. Fds will be cycled between their oxidized and reduced states under cyclic voltammetry conditions and a voltammogram with reversible “peaks” centered at the reduction potential of the Fd will be observed. When enzymes are added, the enzymatic reaction can proceed and electrocatalytic redox cycling of the Fd’s results. Reduced Fds generated from the enzymatic step will be re-oxidized by the electrode at potential higher than the Fd’s reduction potential. The continuous re-oxidation of a constantly reduced Fds generates a catalytic anodic current. Here, we mixed the two enzymes and their respective redox partners, to see how reduction potential of the Fds impact the resultant electrocatalytic reactivity.

Considering the reactions mediated by DaPFOR and DaFdI (value based on reduction potential at 30°C, Fig.4a, dashed line), in the absence of the enzyme, simple, reversible DaFdI voltammetry is observed centered around -390 mV. However, when DaPFOR is added, the shape of the voltammogram changes into a sigmoidal wave and a significant

anodic current is detected (Fig.4a, solid line). This is in agreement with the previous observation, indicating DaFdI is able to accept electrons from DaPFOR, during enzymatic oxidation of pyruvate. In a similar fashion, when HtOGOR and HtFd1 are examined, in the presence of substrates for 2-oxoglutarate oxidation, HtFd1 can act as a redox intermediary for HtOGOR, and again a significant anodic current is detected (solid line, Fig. 4d). The reduction of HtFd1 by HtOGOR and its substrates has been also observed in spectrophotometric measurements.

With a good understanding on the interaction of OFOR enzymes and their native redox partners Fds in the direction of 2-oxoacid *oxidation*, we went on to check how these enzymes might interact with the *other* Fd, again comparing their ability to support oxidation of the native substrate. Fig. 4b illustrates that when DaPFOR is paired with HtFd1, and again a significant oxidative current results. Similarly, when HtOGOR is examined with DaFdI in a similar experiment setup (Fig. 4c) anodic electrocatalytic currents result. Thus, both Fds can accept electrons from a non-native redox partner OFOR.

There are two minor considerations among the four experiments described above. First, as HtFd1 has a reduction potential about 100 mV lower than that of DaFdI, the potential range of cyclic voltammetry experiments involving HtFd1 is shifted 100 mV lower than that of DaFdI, to ensure a similar, comparable shape of the voltammograms. At potential lower than -600 mV (vs SHE), there is a small reductive feature generated by 2-oxoacid itself. This has been observed when only 2-oxoacid is added to the buffer, presumably due to the direct reduction of the carboxyl group of 2-oxoacid to carbonyl, but it does not impact the results of enzymatic electrochemistry [27]. Second, the temperature is set at 60 °C (which is the highest stable temperature for our setup) whenever HtOGOR is involved. This is to ensure the thermophilic enzyme is active at the experiment condition. As shown in enzyme activity assays, HtOGOR in this study exhibit a similar activity at 60 °C to that of DaPFOR at 30 °C.

However, at this temperature, the diffusion rates of Fds are higher than those at 30 °C (experiments with DaPFOR), making the shapes of baselines slightly different (compare Fig. 4a and 4c, 4b and 4d, dashed line).

The fact that all Fds could serve as the electron acceptor for OFOR enzymes during 2-oxoacid oxidation, whether they are native redox partners or not, provides direct evidence to a common notion that the intermolecular electron transfer between Fd and its redox partners is largely driven by its reduction potential [16, 28-31]. At the experiment condition, DaFdI was poised at -390 and -415 mV and HtFd1 was at -490 and -520 mV (at 30°C and 60 °C, respectively). All of these values are lower than or within the range of the standard potential of 2-oxoacid oxidation ($E^0 \sim -500$ mV). Thus this energetically “downhill” reaction could always proceed.

3.4 Only HtFd1 serves as an electron donor supporting the reduction of CO₂

In the 2-oxoacid oxidation experiments, the detection of the anodic turnover current is the result of continuous re-oxidation of enzyme-reduced Fds on the electrode. In the same manner, when the substrates are CO₂ and acyl-CoA, a cathodic current would suggest a continuous re-reduction of enzyme-oxidized Fd on the electrode. Similarly, two steps need to proceed in this experiment for detection of a cathodic current:

In comparison with the oxidative experiments, the DaPFOR-DaFdI pair (Fig.5a) reveals a simple voltammetric response of DaFdI centered around -390 mV when the enzyme is absent. Further, no change was observed upon addition of bicarbonate (as a CO₂ source) and acetyl-CoA. However, when enzyme is present, the simple voltammogram becomes distorted, with separation between the oxidative and reductive currents, yet still centered

around -390 mV. This distortion is probably due to the intermolecular electron transfer between DaFdI and DaPFOR, yet clearly does not indicate electrocatalysis, either. We presume that DaPFOR can receive electrons from DaFdI, but the potential of the electrons is not sufficient to support substrate (CO₂) reduction. Thus once the equilibrium of electron transfer is established between DaFdI and DaPFOR, no more Fd could be continuously reduced to generate a cathodic turnover current. We can conclude a Fd within this potential regime cannot thermodynamically support the steady-state requirements of CO₂ reduction.

In contrast, the HtOGOR and HtFd1 pair yields electrocatalysis, in the presence of substrates. As determined in previous experiments, the reduction potential of HtFd1 is around -520 mV at 60 °C, which is also observed in the presence of substrates, but in the absence of HtOGOR (Fig. 5d, dashed line). When the enzyme was present, cathodic electrocatalytic current results, with an onset potential around -520 mV (Fig. 5d, solid line). The formation of cathodic current suggests that the Step (2) enzymatic reaction is at work, where the reduction of CO₂ is supported by continually, electrochemically reduced HtFd1.

Similarly, we investigated the non-native pairing of enzyme and redox mediator, and our observations were consistent with that of the native OFOR-Fd pairs. When DaFdI was used as an electron donor for HtOGOR, at 60 °C, it was unsuccessful in supporting CO₂ reduction (Fig. 5c). Interestingly, when HtFd1 is used as an electron donor for DaPFOR, at 30 °C, a cathodic current is generated with an onset potential at about -490 mV, similar to the reduction potential of the HtFd1 at this temperature (Fig. 5b). Finally, we note that the background response of Fds in Fig. 5c and 5d (and Fig. 4c and 4d) takes on a quasi-sigmoidal shape due to the higher diffusion rate of Fds at 60 °C compared to data collected at lower temperatures (*i.e.*, compare Fig. 5a and 5c, 5b and 5d, dashed line).

With the two sets of experiments, it is evident that CO₂ reduction catalyzed by OFOR enzymes from rTCA cycle requires an electron donor at “ultra” low reduction potential (~ -

500 mV). Another model system for the study of rTCA, *Chlorobium tepedium*, contains two double-cluster Fds that demonstrate potential at -514 and -584 mV at pH 7.5 [32, 33]. Our unpublished data also indicate a second Fd from *H. thermophilus*, and a Fd from a closely related species, *Aquifex aeolicus*, exhibit reduction potential approaching -500 mV [13]. The occurrence of low potential Fds (≤ -500 mV) may be a common feature for organisms that operate rTCA. Future studies on Fds from rTCA organism may help to establish the link.

Furthermore, the fact that DaPFOR, an enzyme at physiological condition predominantly engages in 2-oxoacid oxidation, could be “driven” to the opposite direction, suggests the reduction potential of Fd dictates the direction of OFOR reaction. Previous studies on OFOR enzymes and Fds from heterologous organisms support this observation. For example, OGOR from *Chlorobium limicola* demonstrated full activity in 2-oxoglutarate synthesis when supplied with Fds from *Chromatium* or *Rhodospirillum rubrum*, both are organisms that engage in N₂ fixation and contain Fds that exhibit potential ≤ 500 mV [34-37]. Fds from *Clostridium pasteurianum* (~ -420 mV) resulted only $\sim 5\%$ activity and Fds from spinach (~ -420 mV) or blue-green algae ($-390 \sim -425$ mV) resulted no activity [38-40]. The dependence on Fd potentials, regardless of their origin, could provide inspiration for bioengineering of CO₂ fixation pathways. Fds and OFORs from heterologous organism could be used together to build chimeric pathways to achieve maximum efficiency. Alternatively, as the reduction potential of Fds is largely modulated by the proteinaceous environment of the iron-sulfur cluster, engineering efforts could also be focused on modulating the potential of Fds by creating different molecular variants.

In the electrochemical experiment, Fds play a key role as the electron carrier, shuttling electrons between the electrode and the enzymatic reaction. Essentially, the electron flow is “wired” from substrates, enzyme, Fd to the electrode (Fig. 1a). Especially in CO₂ reduction, the electrode serves as the ultimate electron donor and at the same time, provides the readout

for CO₂ reduction. Its function is analogous to the upstream and downstream reactions of the coupled assays for CO₂ reduction by OFOR enzymes (Fig. 1b). The benefits of using this electrochemical CO₂ reduction assay are many. First, it eliminates the use of two additional enzymes in the system and thus could avoid possible cross-talks of the three separate redox systems. One case in point is that the two upstream reactions that could generate reduced Fds are either an orthogonal OFOR enzyme or a CODH [18-20]. Both produce CO₂ when they regenerate reduced Fds. This complicates the measurement of K_M on CO₂ of the OFOR enzyme in CO₂ reduction. Second, using an electrode as the ultimate electron donor allows precise, flexible control of the electron flow in the redox process. This could enable us to design further electrochemical experiments to address the dependence of Fd reactivity on its reduction potential in a more quantitative manner and to probe the kinetics of OFOR enzymes and Fds in CO₂ reduction.

4. Conclusions

The large difference in reduction potential (~100 mV) of two [4Fe-4S] Fds provided more insight in their diverse biological functions in CO₂ metabolism. The low reduction potential of HtFd1 (-485 mV at 23°C, pH 7.0, or <-530 mV at the organism's optimal growth temperature) is a key factor in overcoming the large thermodynamic barrier of CO₂ reduction and could be a common feature for organisms that operate rTCA cycle. The electrocatalytic experiments showed that essentially the OFOR enzymes could catalyze both CO₂ evolution and reduction. They also supported the previous notion that the reduction potential of Fd is the dominating factor in biological intermolecular electron transfer, and specifically, construes the catalytic bias of the OFOR reaction. The Fd-mediated electrochemical assay here provides a useful tool to evaluate the dependence of Fd reactivity on its reduction

potential, especially in the CO₂ reduction direction. Future efforts will be focused on more quantitative analysis of the potential dependence and probing the kinetics of OFOR enzymes in CO₂ reduction.

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Appendix A. Supplementary data

Protein expression and purification

The plasmid encoding PFOR from *Desulfovibrio africanus* was a gift from Professor Patrik Jones from University of Turku, Finland. This construct is based on a modified version of pCDFDuet-1 (Novagen/EMD Millipore), containing an N-terminal His₈-tag followed by a small linker (MGHHHHHHHHGTKL-). The genes for OGOR from *Hydrogenobacter thermophilus* (KorA and KorB) were synthesized by GenScript with an internal ribosomal binding site sequence (AAGGAGA) and inserted into pET52b(+) (Novagen/EMD Millipore) linearized with KpnI and SacI. The pET52b(+) plasmid contains an N-terminal Strep-tag followed by an HRV 3C cleavage site (MASWSHPQFEKGALEVLFGQP-). Genes for Fds from *Desulfovibrio africanus* and *Hydrogenobacter thermophilus* were synthesized by GenScript and inserted into pCDFDuet-1 (Novagen/EMD Millipore) linearized with NcoI and XhoI. The plasmid constructs for the two Fds result in expression of tag-free proteins. All plasmid constructs were confirmed by DNA sequencing by Genewiz.

The plasmids were separately transformed to *E.coli* BL21(DE3) ΔiscR cells (gift from Professor Patrik R. Jones, University of Turku, Finland) on LB-agar plates with 50 µg/mL streptomycin (DaPFOR and Fds) or 50 µg/mL ampicillin (HtOGOR). A single colony was inoculated into a 10 mL of LB starter culture, grown at 37 °C overnight until saturated. The 10 mL starter culture was then diluted to 1 L 2xYT media, grown at 37 °C with shaking at 200 rpm until OD₆₀₀ reaches between 0.4 and 0.6. To induce the expression of proteins, IPTG (GoldBio) was added to a final concentration of 100 µM and the growth temperature was decreased to 23 °C and the shaking down to 100 rpm. To ensure cofactor loading, all cultures were supplemented with ammonium ferrous sulfate at a final concentration of 1 mM. Cultures for DaPFOR and HtOGOR were additionally supplemented with thiamine pyrophosphate at a final concentration of 1 mM. Cells were harvested by centrifugation

(6000 g, 10 min, 4 °C) after 12 h of IPTG induction. Cell pellets were saved in -20 °C freezer until use.

DaPFOR was purified with affinity columns packed with Ni-Sepharose resin (GE healthcare). The cell pellets were suspended in 30 mL Buffer A (50 mM HEPES, 100 mM NaCl, 20 mM imidazole, 1 mM MgCl₂, 1 mM thiamine pyrophosphate, 10% glycerol (v/v) pH 8.0), supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mg/mL lysozyme and 1 µg/mL DNase. The suspension was incubated on ice for 30 min before sonication. Cell lysate was clarified by centrifugation (15000g, 30 min, 4 °C) and the clear fraction was loaded on Ni column. After the column was washed with 5 column volume of Buffer A, the protein was eluted with 3 column volume of Buffer A1 (same as Buffer A, 200 mM imidazole). The elution was concentrated and buffer-exchanged to Buffer B (20 mM HEPES, 1 mM MgCl₂, 1 mM thiamine pyrophosphate, 10% glycerol, pH 8.0) by PD-10 column (GE Healthcare). Protein purity was checked by SDS-PAGE on 12.5% Tris Gel.

The purification procedure of HtOGOR was similar to that of DaPFOR, but carried out anaerobically in the Coy chamber (Coy Laboratory) with Strep-Tactin resin (IBA). All buffer were made anaerobic based on the procedure reported by Lanz and coworkers [23]. The protein was eluted with Buffer A2 (same as Buffer A, imidazole is replaced with 2.5 mM desthiobiotin), concentrated and buffer-exchanged to Buffer B.

Fds were purified in two steps, similar to previously reported procedure [13]. Specifically, cell pellets were suspended in Buffer C (10 mM Tris-HCl, 1 mM MgCl₂, pH 8.0) supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mg/mL lysozyme and 1 µg/mL DNase. After sonication and centrifugation, the clear lysate was loaded on a DEAE column, washed with low salt buffer and subsequently eluted by Buffer C with a step gradient of NaCl (0-500 mM, 50 mM increase per step). The elution was concentrated and loaded on a gel filtration column (HiPrep 26/60 Sephacryl S100 or S200 HR, GE Healthcare) pre-

equilibrated with Buffer D (50 mM Tris-HCl, 100 mM NaCl, 1 mM MgCl₂, pH 8.0) on the ÄKTApurifier system (GE healthcare). Proteins were eluted with Buffer D at a flow rate of 0.7 ml/min. Protein purity was checked by SDS-PAGE on 16% Tricine Gel.

Figures

Fig 1. (a) The schematic representation of the Fd-mediated electrochemical assay in this study. OFOR enzymes, Fds and substrates are present in the electrochemical cell. CoA and acyl-CoA are omitted for the sake of simplicity. Both 2-oxoacid oxidation (red arrow) and CO₂ reduction (blue arrow) are depicted. (b) Commonly used coupled enzymatic assay to study OFOR in CO₂ reduction. OFOR1 and OFOR2 are orthogonal OFOR enzymes that use different substrates. Typically one is PFOR and the other is OGOR, and the acyl-CoA is acetyl-CoA and succinyl-CoA respectively. CODH: carbon monoxide dehydrogenase. LDH: lactate dehydrogenase. GDH: glutamate dehydrogenase.

Fig.2 Michaelis-Menten enzyme activity plots for DaPFOR (a) and HtOGOR (b) in this study.

Fig 3. (a) Typical cyclic voltammograms of DaFdl and HtFdl1 at pH 7.0. Scan rate: 50 mV/s. (b) Dependence of Fd reduction potential on temperature.

Fig.4. Typical voltammograms of Fd-mediated catalytic electrochemical experiment in 2-oxoacid oxidation. (a)-(d) represent different combinations of OFOR enzymes and Fds.

Fig.5. Typical voltammograms of Fd-mediated catalytic electrochemical experiment in CO_2 reduction. (a)-(d) represent different combinations of OFOR enzymes and Fds. Blue dashed lines indicate the reduction potential of the Fd at the experiment condition.

Figure for Appenix. A Supplementary Data. (a) Purified enzymes visualized by SDS-PAGE, 12.5% Tris-HCl gel. Lane 1, protein standard, Lane 2, HtOGOR, which is a heterodimer with an α subunit at 70 kD and β subunit at 33 kD (theoretical molecular weight based on protein sequence), Lane 3, DaPFOR, which is homodimer with a 135 kD molecular weight per protomer. (b) Purified Fds visualized by SDS-PAGE, 16% Tris-Tricine gel. Lane 1, protein standard, Lane 2, HtFd1, with a theoretical molecular weight at 7.9 kD, Lane 3, DaFdI, with a theoretical molecular weight at 7.3 kD. The size discrepancy in SDS-PAGE is probably due to the acidic surface and low molecular weight of Fds [13].

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