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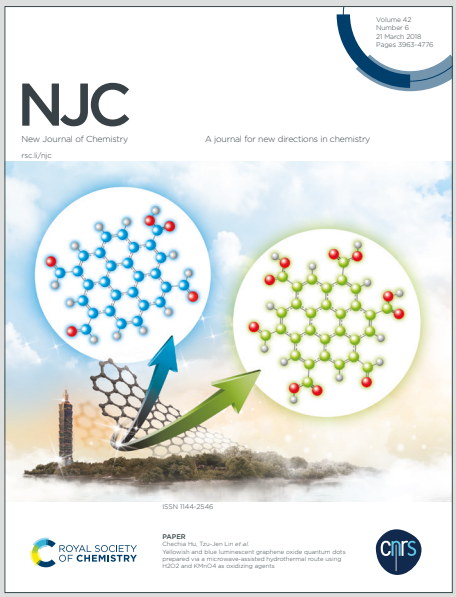

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COMMUNICATION

Can formate dehydrogenase from *Candida boidinii* catalytically reduce carbon dioxide, bicarbonate, or carbonate to formate?

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Formate dehydrogenase from *Candida boidinii* (CbFDH) reversibly catalyzes the formate to carbon dioxide with the redox coupling NAD^+/NADH. There have been many studies on CbFDH-catalyzed oxidation of formate to carbon dioxide in the presence of NAD^+. On the other hand, there are few studies on the detailed mechanism of the carbon dioxide reduction to formate catalyzed with CbFDH in the presence of NADH. In addition, it is not clear whether CbFDH can reduce carbon dioxide, bicarbonate or carbonate to formate. In this study, formate production with CbFDH in the presence of NADH was investigated in solutions with different ratios of carbon dioxide, bicarbonate and carbonate. The reaction rate of formate production with CbFDH increased in proportion to the concentration of carbon dioxide in the reaction solution. On the other hand, the formate production with CbFDH was suppressed in proportion to the concentration of bicarbonate or carbonate in the reaction solution. Thus, CbFDH was found to catalytically reduce only carbon dioxide to formate among the three types of carbonate species.

Formate dehydrogenases (FDHs) are a set of biocatalysts that catalyze the oxidation of formate to carbon dioxide, donating the electrons to a second substrate (S_{ox}) and the reverse reaction of carbon dioxide to formate, donating the electrons to a second substrate (S_{red}).[1-6] Second substrates of FDH are classified into (1) nicotinamide type (NAD^+ or $NADP^+$), (2) cytochrome type (cytochrome b_1 or c_{553}), (3) quinone type and (4) NAD^+-ferredoxin type. Among these FDHs, FDH from *Candida boidinii* (EC.1.2.1.2; CbFDH) is commercially available and can be easily handled as a catalyst for the CO_2 reduction to formate. The relationship between the $NAD(P)^+$-depend dehydrogenases and natural co-enzyme $NAD(P)^+$ or NAD(P)H is considered. For example, the kinetic parameters for the Michaelis constants (K_m) of NAD^+ and NADH for CbFDH in the formate oxidation to carbon dioxide and the reverse reaction are determined. The K_m values for NAD^+ in the formate oxidation and NADH in the carbon dioxide reduction to CbFDH are estimated to be 50 and 2087 mM, respectively.[6-8] Thus, CbFDH can be activated by the lower concentration of NAD^+, compared with that of NADH (1/400), and the affinity of NAD^+ for CbFDH is higher than that of NADH. There have been many studies on CbFDH-catalyzed oxidation of formate to carbon dioxide in the presence of NAD^+.[9-12] CdFDH catalyzes the stable oxidation of formate to carbon dioxide in the pH range of 5 to 10.[11] Figure 1 shows the molar fraction of formic acid and formate under various pH range.[13]

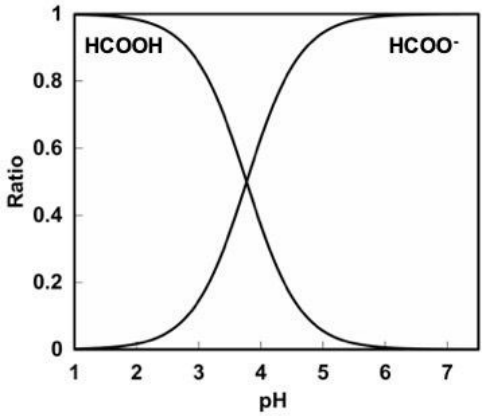

Fig. 1. The molar fraction of formic acid and formate under various pH.

This means that the catalytic function of CbFDH for formate oxidation is exhibited within the pH range in the stable presence of formate ion. Moreover, the binding sites of NAD^+ and formate in CbFDH are clarified using crystal structure analysis and docking simulation.[11] The detailed kinetic parameters for the catalytic formate oxidation with CbFDH in the presence of NAD^+ also are determined by the enzymatic reaction analysis.

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DOI: 10.1039/D0NJ01183E

In contrast, there are few studies on the detailed mechanism of the carbon dioxide reduction to formate catalyzed with CbFDH in the presence of NADH. One of the reasons is that the reaction rate with CbFDH in the presence of NADH is low compared with that of NAD^+ because of its low carbon dioxide reduction activity. The catalytic efficiency, k_{cat} / K_m value for conversion of carbon dioxide to formate (170×10^3 M^{-1} min^{-1}) was approximately 900 times lower than that of the reverse reaction, i.e., the conversion of formate to carbon dioxide (0.19×10^3 M^{-1} min^{-1}), catalyzed with CbFDH using a natural co-enzyme NAD^+/NADH.[6] We also found that the single-electron reduced of MV ($MV^•$) only acts as a co-enzyme for CbFDH, and oxidized form of MV is not recognized for a co-enzyme of CbFDH. Thus, the oxidation process with CbFDH suppressed using oxidized form of MV as an electron carrier molecule. Given that $MV^•$ effectively activates CbFDH, an enzymatic kinetic analysis of carbon dioxide reduction to formate with CbFDH in the presence of various reduced forms of 4,4'- and 2,2'-bipyridinium salts are determined.[14,15] Moreover, it is not clear whether carbon dioxide, bicarbonate or carbonate is reduced in the formate production process catalyzed by CbFDH. This is because, as shown in Figure 2, carbon dioxide, bicarbonate or carbonate are mixed in the reaction solution at a wide range of pH.[16]

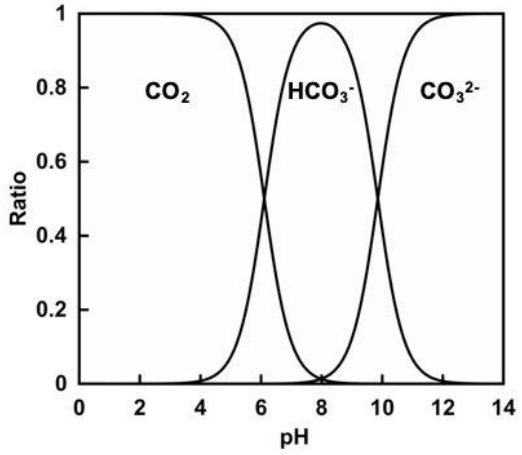

Fig. 2. The molar fraction of carbon dioxide, bicarbonate and carbonate under various pH.

Gaseous carbon dioxide can dissolve in aqueous media.[17,18] The hydrated carbon dioxide molecule reacts with water to produce carbonic acid (H_2CO_3). Dissociation of H_2CO_3 produces bicarbonate and carbonate depending on the pH value as shown in Fig. 2. From the low hydration equilibrium constant of H_2CO_3 in aqueous media, the dissolved carbon dioxide consists of mostly hydrated carbon dioxide together with a small amount of H_2CO_3. Thus, CO_2* is used to represent the two species of hydrated carbon dioxide and H_2CO_3 in the aqueous chemical equilibrium equation. The C_{total} is defined as the total concentration of all carbonate species of CO_2*, bicarbonate and carbonate. The first (K_1) and second (K_2) dissociation constants of H_2CO_3 can be denoted by the following equations, respectively. The K_1 and K_2 depend on the solution temperature according to Plummer and Busenberg.[18] From these equations, the concentration of CO_2*, bicarbonate and carbonate are represented as follows:

$$CO_2 + H_2O \underset{}{\overset{}{\leftrightarrows}} H_2CO_3 \overset{K_1}{\leftrightarrows} HCO_3^- + H^+ \overset{K_2}{\leftrightarrows} CO_3^{2-} + H^+$$

CO_2* = carbon dioxide in the solution

$$C_{total} = [CO_2^*] + [HCO_3^-] + [CO_3^{2-}]$$

$$K_1 = \frac{[H^+][HCO_3^-]}{[CO_2^*]} \qquad K_2 = \frac{[H^+][CO_3^{2-}]}{[HCO_3^-]}$$

$$[CO_2^*] = \frac{[H^+]^2}{[H^+]^2 + K_1[H^+] + K_1K_2} C_{total}$$

$$[HCO_3^-] = \frac{K_1[H^+]}{[H^+]^2 + K_1[H^+] + K_1K_2} C_{total}$$

$$[CO_3^{2-}] = \frac{K_1K_2}{[H^+]^2 + K_1[H^+] + K_1K_2} C_{total}$$

In this study, to clarify the carbonate species that CbFDH can reduce, the formate production with CbFDH in the presence of NADH was investigated in solutions with different ratios of carbon dioxide.

In the carbon dioxide reduction to formate with CbFDH and NADH(200 μM) and CbFDH (0 - 40 μM) obtained from Sigma-Aldrich in carbon dioxide saturated 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 6.3), 50 mM sodium bicarbonate containing MES buffer (pH 6.9) or 0.1 M carbonate-bicarbonate buffer (pH 9.3) was reacted for 60 min at 30.5 °C. The estimated formate concentration was calculated using the absorbance change at 340 nm with a molar absorption coefficient of NADH (ε_{340} = 6300 M^{-1} cm^{-1}) [19] using UV-visible absorption spectroscopy (SHIMADZU, MultiSpec-1500) as equivalent to the reduced concentration of NADH during reaction. The total carbonate species C_{total} in the solution can be measured as bicarbonate ions by adjusting the pH of the eluent to 8.0 using an ion chromatography (Dionex ICS-1100; electrical conductivity detector) with an ion exclusion column (Thermo ICE AS1; column length: 9 x 150 mm; composed of a 7.5 μm cross-linked styrene/divinylbenzene resin with functionalized sulfonate groups).

The concentrations of carbon dioxide, bicarbonate and carbonate in the solution were determined from the C_{total} and the molar fraction of carbon dioxide, bicarbonate or carbonate in the pH value of solution from Fig. 2. The initial rate for the estimated formate production (v_0) was determined from the gradient of the NADH consumption up to 60 min incubation. Figure 3 shows the time dependence of the absorbance change at 340 nm in the NADH(200 μM) and CbFDH (40 μM) in carbon dioxide saturated MES buffer (pH 6.3)(1), in 50 mM sodium bicarbonate containing MES buffer (pH 6.9) (2) and in 0.1 M carbonate-bicarbonate buffer (pH 9.3) (3), respectively.

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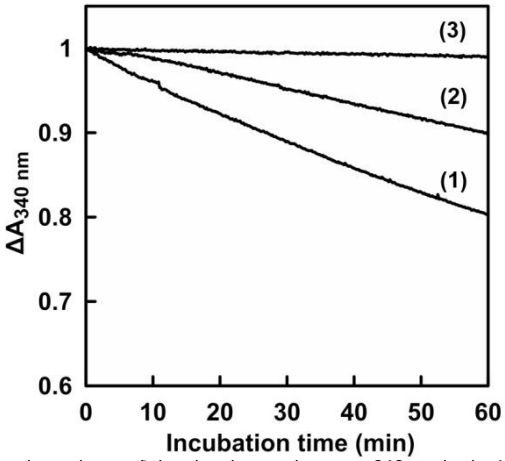

Fig. 3. Time dependence of the absorbance change at 340 nm in the NADH(200 μM) and CbFDH (40 μM) in carbon dioxide saturated MES buffer (pH 6.3)(1), in 50 mM sodium bicarbonate containing MES buffer (pH 6.9) (2) and in 0.1 M carbonate-bicarbonate buffer (pH 9.3) (3), respectively.

From the results of Figure 3, the absorbance change at 340 nm was decreased with increasing incubation time in all cases. The concentration of carbon dioxide in the carbon dioxide saturated MES buffer (pH 6.3), in 50 mM sodium bicarbonate containing MES buffer (pH 6.9) and in 0.1 M carbonate-bicarbonate buffer (pH 9.3) were estimated to be 30.7, 11.0 and 0 mM, respectively. Figure 4 shows the relationship between initial rate for the estimated formate production (v_0) and the concentration of CbFDH in the carbon dioxide saturated MES buffer (pH 6.3), in 50 mM sodium bicarbonate containing MES buffer (pH 6.9) and in 0.1 M carbonate-bicarbonate buffer (pH 9.3).

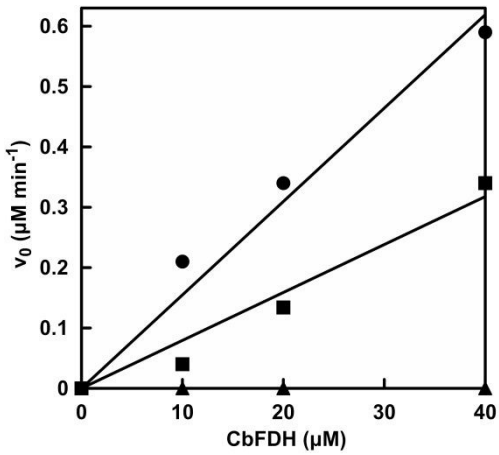

Fig. 4. The relationship between the estimated formate production (v_0) and the concentration of CbFDH in the carbon dioxide saturated MES buffer (pH 6.3) (circle), in 50 mM sodium bicarbonate containing MES buffer (pH 6.9) (square) and in 0.1 M carbonate-bicarbonate buffer (pH 9.3) (triangle)

From Figs 3 and 4, the lower pH in the solution, the more the formate production reaction with CbFDH proceeded. In these experimental conditions, however, the formate production enhancement by carbon dioxide and suppression by bicarbonate and carbonate is trade-off because the concentrations of each carbonate species are determined from the mol fraction of carbonate species. Therefore, it is necessary to investigate the effects of the concentrations and ratios of the three carbonate species on formate production with CbFDH.

Next, a GTA buffer consisting of consisting of 3,3-dimethyl-glutaric acid, tris(hydroxymethyl)aminomethane and 2-amino-2-methyl-1,3-propanediol available at a wide range of pH (3.5 – 10) was applied to the formate production with CbFDH to eliminate the effect of the buffer on the reaction. The fractions of carbon dioxide and bicarbonate in the solution were adjusted by the bubbling time of carbon dioxide gas and the pH of the GTA buffer. The initial rate for the estimated formate production (v_0) was determined from the gradient of the NADH consumption up to 60 min incubation in the reaction system of NADH(200 μM) and CbFDH (40 μM). Figure 5 shows the relationship among the initial rate for the estimated formate production (v_0), concentration of carbon dioxide (circle), of bicarbonate (square) , and of carbonate (triangle) with CbFDH.

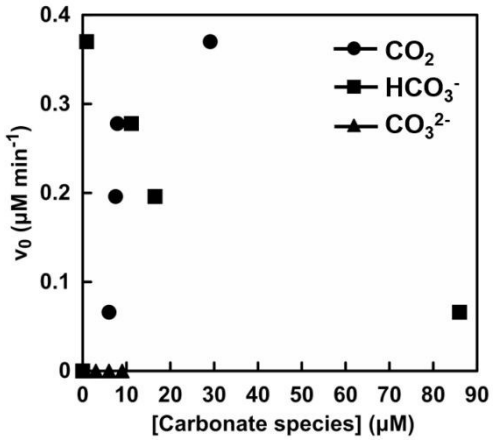

Fig. 5. The relationship between the estimated formate production (v_0), concentration of carbonate species with CbFDH. ●: Carbon dioxide, ■: bicarbonate, ▲: carbonate.

As the concentration of carbon dioxide increased, the formate production was tended to accelerate, while the increase in the concentration of bicarbonate tended to suppress the formate production. Moreover, no formate production was observed regardless of the change in carbonate concentration. However, there was no regular trend in these relationships. However, the concentration of total carbonate species in each sample solution was not uniform in various pH of GTA buffer.

Next, the relationship between the abundance ratio of carbon dioxide, bicarbonate or carbonate to the total carbonate species in the solution and the initial rate for the estimated formate production (v_0) was examined.

Figure 6 shows the relationship between the abundance ratio of carbon dioxide (circle), of bicarbonate (square), and of carbonate (triangle) to the total carbonate species and the initial rate for the estimated formate production (v_0) in the various pH of GTA buffer containing NADH (200 μM) and CbFDH (40 μM).

As the abundance ratio of carbon dioxide to the total carbonate species increased, the initial rate for the estimated formate production (v_0) was accelerated almost linearly. As the abundance ratio of bicarbonate to the total carbonate species increased, in contrast, the initial rate for the estimated formate production (v_0) was decelerated almost linearly. Moreover, no formate was produced with increasing carbonate abundance to total carbonate species. All these results suggest that CbFDH catalytically reduces only carbon dioxide to formate, whereas bicarbonate or carbonate

function as competitive inhibitors of carbon dioxide for the formate production with CbFDH.

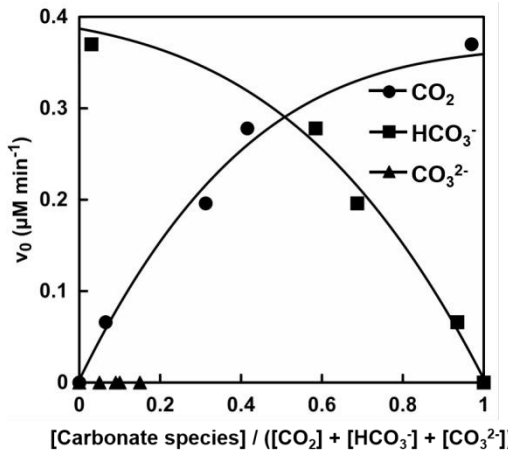

Fig. 6. Relationship between the abundance ratio of carbon dioxide (●), bicarbonate(■) or carbonate (▲) to the total carbonate species and the initial rate for the estimated formate production (v_0).

Figure 7 shows the binding model of formate (a),[20-22] bicarbonate (b), carbonate (c) and carbon dioxide (d) in the active site of CbFDH. It has been reported that formate are trapped at three amino acid residues (Asn119, Ile175 and Arg258) within the active site in CbFDH. It is predicted that bicarbonate, carbonate or carbon dioxide is also captured by the same three amino acid residues within the active site in CbFDH as shown in Figure 7. Thus, bicarbonate and carbonate act as competitive inhibitors of carbon dioxide for the formate production with CbFDH.

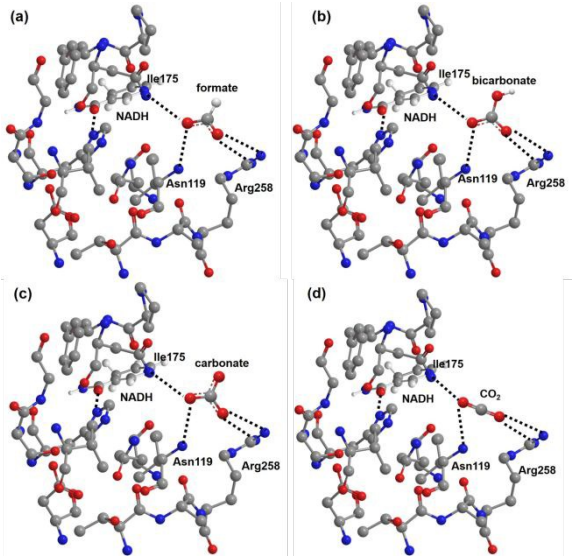

Fig. 7. The binding model of formate (a), bicarbonate (b), carbonate (c) and carbon dioxide (d) in the active site of CbFDH. The 3D structure of CbFDH used Protein Data Bank entry-5DN9(DOI: 10.2210/pdb5dn9/pdb.

From these results, CbFDH catalytically reduced only carbon dioxide to formate. In molybdenum containing NAD^+-dependent FDH from *Ralstonia eutropha* with catalytic activity of carbon dioxide reduction like CbFDH, the optimum pH was reported around 6.5. Also, carbon dioxide reduction is progressing in a pH region between 7.5 and 8.5, thus, it is possible that bicarbonate or carbonate can also be reduced to formate with molybdenum containing NAD^+-dependent FDH.[23,24] As far as we have surveyed, there are no reports of the detailed mechanism of the carbon dioxide reduction to formate catalyzed with CbFDH in the presence of NADH. In addition, it is not clear whether CbFDH can reduce carbon dioxide, bicarbonate or carbonate to formate. In this study, we found that CbFDH catalytically reduced only carbon dioxide to formate, and that other carbonate species acted as inhibitors for CbFDH for the first time.

Acknowledgement

This work was partially supported by Grant-in-Aid for Scientific Research on Innovative Areas “Artificial Photosynthesis (2406)”, “Innovations for Light-Energy Conversion (4906)” and Fund for the Promotion of Joint International Research (Fostering Joint International Research (B))(19KK0144).

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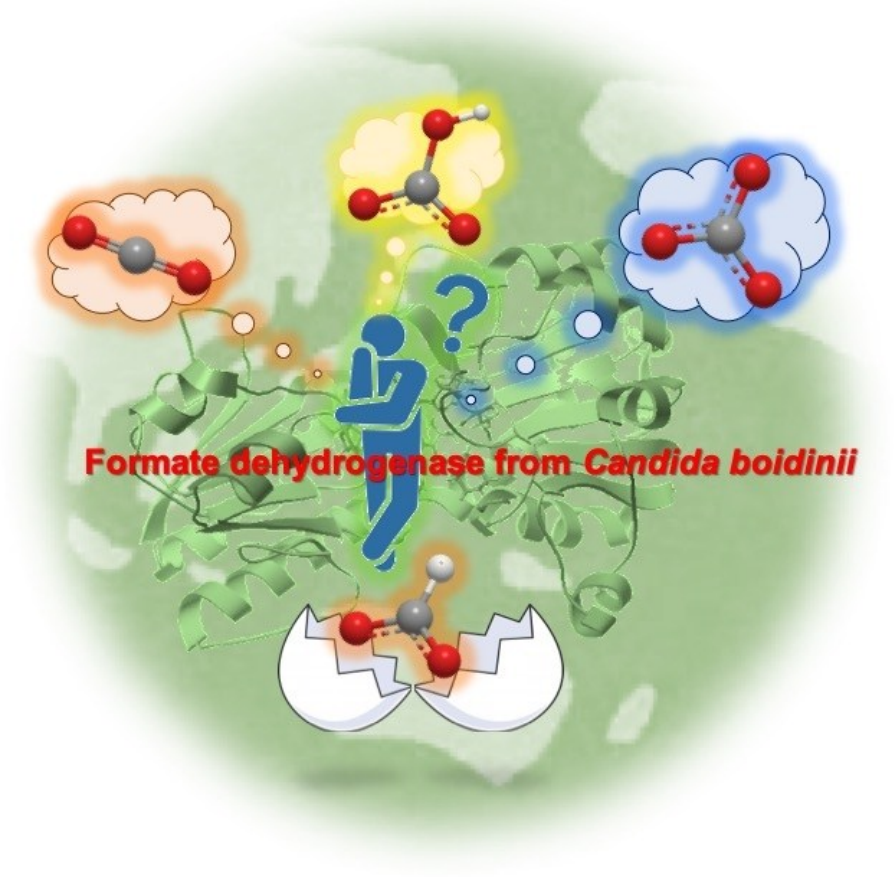

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