Fumarase From Cyanidioschyzon merolae Stably Shows High Catalytic Activity for Fumarate Hydration Under High Temperature Conditions

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Fumarases (Fums) catalyze the reversible reaction converting fumarate to l-malate. There are two kinds of Fums: Class I and II. Thermostable Class II Fums, from mesophilic microorganisms, are utilized for industrial l-malate production. However, the low thermostability of these Fums is a limitation in industrial l-malate production. Therefore, an alternative Class II Fum that shows high activity and thermostability is required to overcome this drawback. Thermophilic microalgae and cyanobacteria can use carbon dioxide as a carbon source and are easy to cultivate. Among them, Cyanidioschyzon merolae and Thermosynechococcus elongatus are model organisms to study cell biology and structural biology, respectively. We biochemically analyzed Class II Fums from C. merolae (CmFUM) and T. elongatus (TeFum). Both CmFUM and TeFum preferentially catalyzed fumarate hydration. The catalytic activity of CmFUM for fumarate hydration in the optimum conditions (52°C and pH 7.5) is higher compared to those of Class II Fums from other organisms and TeFum. Thermostability tests of CmFUM revealed that CmFUM showed higher thermostability than those of Class II Fums from other microorganisms. The yield of l-malate obtained from fumarate hydration catalyzed by CmFUM was 75–81%. In summary, CmFUM has suitable properties for efficient l-malate production.

Keywords: fumarase, tricarboxylic acid cycle, l-malate, microalgae, cyanobacteria

INTRODUCTION

Fumarase, or fumarate hydratase (EC 4.2.1.2; hereafter referred to as Fum) is one of the enzymes of the tricarboxylic acid (TCA) cycle and catalyzes the reversible hydration/dehydration reaction of fumarate to l-malate. Based on biochemical analyses of isozymes of Fum from Escherichia coli, these enzymes are divided into two biochemically distinct classes named Class I and II (Woods et al., 1988). Amino acid sequence analysis revealed that there is no overall homology between Class I and II Fums (Woods et al., 1988). There is an approximately 40% difference between the amino acid sequences of the Class II Fums from eukaryotes and prokaryotes (Sacchetti et al., 1988). Class I Fums are thermolabile homo-dimeric enzymes, whereas Class II Fums are thermostable homo-tetrameric enzymes (Woods et al., 1988).

l-Malate is used in various industrial applications such as acidulants, flavor enhancers, color fixatives, medicines, and antimicrobial agents (Liu et al., 2017). Fum has been used as a biocatalyst for industrial l-malate production (Liu et al., 2017). Class II Fum from the mesophilic bacterium Corynebacterium glutamicum (Brevibacterium flavum) can be used for l-malate production.
Ito et al. CmFum Shows High Thermostability

V. Terasawa et al., 1990

TeFum requires Nakamura et al., 2002, °lT. Thus, natural Class ІІ Fums from culturable, °lLin et al., 2018 shows g°l). Thus, Fums used for |°lTeFum preparation of expression constructs of

MATERIALS AND METHODS

Preparation of Expression Constructs of CmFUM and TeFum

The genomic regions containing CmFUM (CMD058C) and TeFum (tll1534) with N-terminal BamHI and C-terminal XhoI sites were commercially synthesized by Eurofin Genomics Japan (Tokyo, Japan). Codon usage was optimized for E. coli. The synthesized DNA fragments were cloned into the BamHI-XhoI site of the pGEX6P-1 vector (GE Healthcare, Little Chalfont, United Kingdom).

Purification of CmFUM and TeFum

Glutathione-S-transferase (GST)-tagged CmFum and TeFum were purified using affinity chromatography as described with a few alterations (Takeya et al., 2017). The CmFUM and TeFum constructs were transformed individually into E. coli BL21 (DE3) competent cells (BioDynamics Laboratory Inc., Tokyo, Japan). BL21 (DE3) cells were cultivated overnight in 1.5 L LB media at 30°C with shaking (150 rpm). During the cultivation of BL21 (DE3) cells, the expression of GST-tagged Fums was induced by 0.01 mM isopropyl β-D-1-thiogalactopyranoside (Wako Chemicals, Osaka, Japan). The cells were collected by repeated centrifugation (5,800 g, 2 min, 25°C) and transferred to 50 mL tubes containing PBS-T (1.37 M NaCl, 27 mM KCl, 81 mM Na₂HPO₄, 12H₂O, 14.7 mM KH₂PO₄, and 0.05% Tween-20). To dissolve the GST-tagged Fums in PBS-T, the cells were sonicated for 200 s at 20% intensity (model VC-750, EYELA, Tokyo, Japan). After centrifugation (14,200 g, 15 min, 4°C), 800 μl of glutathione-Sepharose 4B resin (GE Healthcare Japan, Tokyo, Japan) was added to the supernatant containing the GST-tagged Fums. To bind the GST-tagged Fums to glutathione-Sepharose 4B resin, the mixture was shaken for 1 h on ice. After centrifugation (5,800 g, 2 min, 4°C) to remove the supernatant, the resin was washed three and five times using 3 ml and 700 μl of PBS-T, respectively, to remove non-specific proteins. Thereafter, the GST-tagged Fums were eluted five times using 500 μl of GST elution buffer (50 mM Tris-HCl pH 8.0 and 10 mM reduced glutathione) and concentrated using a VivaSpin 50 MWCO 50,000 device (Sartorius, Göttingen, Germany). The protein concentration was calculated using a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

Enzyme Assays for CmFUM and TeFum

The 1 ml assay solution of CmFUM contains 100 mM Tris-HCl [pH 7.5 (fumarate hydration) or 8.5 (l-malate dehydration)], 5 pmol CmFUM, and various concentrations of fumarate or l-malate. The 1 ml assay solution of TeFum contains 100 mM Tris-HCl [pH 7.0 (fumarate hydration) or 7.5 (l-malate dehydration)], 30 pmol TeFum, and various concentrations of fumarate or l-malate. The assay solution of CmFUM and TeFum before adding substrates was incubated for 5 min at 52°C and 50°C, respectively. Thereafter, various concentrations of fumarate or l-malate were added to the assay solution to start the reaction. The activities of CmFUM and TeFum were calculated by monitoring the changes in absorbance at A₂₄₀ using a Shimadzu UV-1850 (Shimadzu, Kyoto, Japan). One unit of Fum activity was defined as the amount of Fum that converts 1 μmol fumarate or l-malate per min. The maximum reaction velocity (v_max) and K_m (substrate concentration at 50% v_max) of CmFUM and TeFum were calculated by curve fitting of the Michaelis-Menten equation using the Kaleida.

1-l-Malate production using the C. glutamicum Fum requires heat treatment at 40-60°C for 10-300 min to repress by-product succinate formation because it is hard to separate succinate from l-malate (Terasawa et al., 1990). Thus, Fums used for l-malate production must maintain high activity after heat treatment. However, the stability of Class II Fums from mesophilic microorganisms, including C. glutamicum, is insufficient at these temperatures (Takata et al., 1983; Keruchenko et al., 1992; Lin et al., 2007, 2018; Song et al., 2011). Class II Fum from the highly thermophilic bacterium Thermus thermophilus shows high thermostability, with an optimum temperature of 85°C (Mizobata et al., 1998). However, this enzyme is not economically viable for l-malate production. The first reason is that maintaining the reaction temperature at 85°C is energy intensive. Second, the activity of this enzyme is lower than those of other Class II Fums from mesophilic bacteria such as C. glutamicum (Lin et al., 2018). Thus, natural Class II Fums from culturable microorganisms, which have suitable enzymatic properties for l-malate production, have not yet been identified. Previously, a Class II Fum from C. glutamicum was modified by introducing three mutations to enhance thermostability (Lin et al., 2018).

Eukaryotic microalgae and cyanobacteria are microorganisms that perform oxygenic photosynthesis and can use carbon dioxide as the sole carbon source. In recent years, biotechnological applications of thermophilic microalgae and cyanobacteria have been gaining attention because these organisms do not compete for food sources and their growth at high temperatures prevents contamination with other microorganisms (Patel et al., 2019). The entire genomic sequences of the hot-spring red alga Cyanidioschyzon merolae and the hot-spring cyanobacterium, Thermosynechococcus elongatus are known (Ohta et al., 1998, 2003; Nakamura et al., 2002; Matsuzaki et al., 2004; Nozaki et al., 2007) and they can be easily cultivated. C. merolae is a eukaryote that has the simplest cellular structure and has been primarily used for cell biological studies so far (Kuroiwa et al., 1998). T. elongatus is the simplest photosynthetic organism that displays thermostability and therefore has been used for structural analyses of the photosynthetic system (Murray et al., 2007; Laughlin et al., 2019; Schuller et al., 2019; Zhang et al., 2019). However, biochemical analyses of their enzymes of primary metabolic pathways, such as the TCA cycle, have not yet been performed. Additionally, Fums from thermophilic microalgae and cyanobacteria, have not been biochemically characterized. Genome sequencing revealed that both C. merolae and T. elongatus have a sole Class II Fum as a fumarase (Nakamura et al., 2002; Matsuzaki et al., 2004).

In this study, we biochemically characterized Fums from C. merolae (CmFUM) and T. elongatus (TeFum) and examined whether these Fums have suitable enzymatic properties for l-malate production.
Thermostability Measurements of CmFUM

Thermostability measurements of CmFUM were performed as described previously (Lin et al., 2018). The 1 ml assay solution of CmFUM contains 100 mM Tris-HCl (pH 7.5), 5 pmol CmFUM, and 0.5 mM fumarate. To measure the $T_{1/2}$ (temperature where the activity becomes 50% after heat treatment for 15 min), the assay solution before adding fumarate was pre-incubated at each temperature for 15 min. Thereafter, the enzyme assay described in the previous section was performed. To measure $T_{1/2}$ (time where the activity becomes 50% after heat treatment), the assay solution before adding fumarate was preincubated at 50°C for each time-point and then immediately cooled on ice for 1 min. Thereafter, the enzyme assay described in the previous section was performed.

Analysis of the Reaction Catalyzed by CmFum When Using 200 mM Fumarate as a Substrate

The 500 µl assay solution contains 100 mM Tris-HCl (pH 7.5), 500 pmol CmFUM, and 200 mM disodium fumarate. The assay solution before adding fumarate was pre-incubated for 5 min at 52°C. Thereafter, fumarate which was also pre-incubated for 5 min at 52°C was added to the assay solution and the reaction was started at 52°C. After the reaction for 5, 10, 20, 30, 40, 50, and 60 min, 50 µl of the assay solution was collected and the reaction was stopped by 100 mM HCl. The samples were analyzed by high-performance liquid chromatography (HPLC) using an LC-2000Plus System (JASCO, Tokyo, Japan). Organic acids were quantified using 0.2 mM bromothymol blue in 15 mM sodium phosphate buffer; peaks were detected at 445 nm, as described previously (Osanai et al., 2015).

Results

Biochemical Properties of CmFUM and TeFum

To characterize the biochemical properties of CmFUM and TeFum, we purified CmFUM and TeFum as GST-tagged proteins using affinity chromatography (Figure 1). We first measured their activities using fumarate as a substrate (hereafter “the activity for fumarate”) and l-malate as a substrate (hereafter “the activity for l-malate”) at different temperatures and pH values (Figure 2). CmFUM showed the highest activity for both substrates at 52°C (Figure 2A). CmFUM showed the highest activity for fumarate and l-malate at pH 7.5 and 8.5, respectively, (Figure 2B). We then set the measurement conditions of CmFUM activities for fumarate at 52°C and pH 7.5 and for l-malate at 52°C and pH 8.5. TeFum consistently showed high activity for both substrates at 45–55°C (Figure 2C) and showed the highest activity for fumarate and l-malate at pH 7.0 and 7.5, respectively (Figure 2D). We then set the measurement conditions of TeFum activities for fumarate at 50°C and pH 7.0, and for l-malate at 50°C and pH 7.5.

To calculate the kinetic parameters of CmFUM and TeFum, the enzymatic activities were measured at different substrate concentrations (Figure 3). The saturation curves of CmFUM and TeFum for both substrates were not sigmoidal but hyperbolic (Figure 3) and the kinetic parameters of CmFUM and TeFum were calculated using the Michaelis-Menten equation (Table 1). The $K_m$ (substrate concentration at 50% $V_{max}$) and $k_{cat}$ (turnover number) of CmFUM for fumarate were 0.27 ± 0.05 mM and 235 ± 22 s⁻¹, respectively (Table 1). The $K_m$ and $k_{cat}$ of CmFUM for l-malate were 1.49 ± 0.12 mM and 244 ± 6 s⁻¹, respectively (Table 1). The $k_{cat}/K_m$ (catalytic efficiency) of CmFUM for fumarate (872 ± 68 s⁻¹ mM⁻¹) was 5.3-fold higher than that for l-malate (164 ± 9 s⁻¹ mM⁻¹; Table 1). The $K_m$ and $k_{cat}$ of TeFum for fumarate were 0.14 ± 0.02 mM and 37 ± 2 s⁻¹, respectively (Table 1). The $K_m$ and $k_{cat}$ of TeFum for l-malate were 0.20 ± 0.01 mM and 15 ± 0.3 s⁻¹, respectively, (Table 1). The $k_{cat}/K_m$ of TeFum for fumarate (278 ± 23 s⁻¹ mM⁻¹) was 3.7-fold higher than that for l-malate (76 ± 1 s⁻¹ mM⁻¹). Succinate, citrate, and pyruvate act as effectors of Class ІІ Fums from higher plant Arabidopsis thaliana (mitochondrial Fum; Zubimendi et al., 2018) and mesophilic cyanobacterium...
Synechocystis sp. PCC 6803 (hereafter Synechocystis 6803; Katayama et al., 2019). We examined the effects of the three organic acids on CmFUM and TeFum activities (Figure 4). The three organic acids decreased the CmFUM activity for fumarate (Figure 4A). Succinate decreased the CmFUM activity for l-malate (Figure 4A). Succinate and citrate decreased the TeFum activity for fumarate (Figure 4B). In contrast, pyruvate increased the TeFum activity for fumarate (Figure 4B). The three organic acids decreased the TeFum activity for l-malate (Figure 4B).

**Further Biochemical Analyses of CmFUM for l-Malate Production**

Higher activity and specificity for fumarate were seen in CmFUM than in TeFum (Table 1). Therefore, we examined the important enzymatic property for l-malate production, thermostability of CmFUM (Figure 5). The residual activity of CmFUM for fumarate after heat treatment for 15 min decreased linearly depending on the heat treatment temperature within the range of 53–60°C (Figure 5A). The $T_{50}$ (temperature where the activity becomes 50% after heat treatment for 15 min) was calculated as 57.3°C using a linear equation (Figure 5A). In addition, the residual activity of CmFUM for fumarate after heat treatment at 50°C decreased linearly depending on the length of heat treatment (Figure 5B).

The $t_{1/2}$ (time where the activity becomes 50% after heat treatment) at 50°C was calculated as 507 min using a linear equation (Figure 5B).

Additionally, we examined the effects of metal cations and buffer solutions on CmFUM activity for fumarate and determined the condition where CmFUM shows the highest activity for fumarate (Figure 6). CmFUM activity for fumarate did not change in the presence of monovalent and divalent metal cations (Figure 6A). CmFUM activity for fumarate in HEPES-NaOH buffer was slightly lower than that in Tris-HCl buffer (Figure 6B). CmFUM activity for fumarate in each of the other three buffers tested was not significantly different from that in Tris-HCl buffer (Figure 6B).

Finally, in the optimum conditions of CmFUM, we analyzed the reaction catalyzed by CmFUM using high concentrations (industrial level) of fumarate as a substrate. CmFUM showed enzymatic activity in the presence of 200 mM and 1 M fumarate (Figure 7). When using 200 mM fumarate as a substrate of CmFUM, the yield of l-malate increased depending on reaction time, and the yield in an equilibrium state was 75% (Figure 7A). Also, the yield of l-malate when using 1 M fumarate as a substrate of CmFUM was 81% (Figure 7B). An unwanted by-product in l-malate production, succinate was not detected in all samples using high concentrations of fumarate as a substrate.
DISCUSSION

For the first time, Fums from a thermophilic microalga and cyanobacterium were purified and biochemically characterized. The optimum temperatures for the enzymatic activity of CmFUM (52°C) and TeFum (45–55°C) were higher than those of Class II Fums from mesophilic microorganisms (30–45°C, four species: *Streptomyces coelicolor*, *Rhizopus oryzae*, *Synechocystis* 6803, and *Streptomyces lividans*; Lin et al., 2007; Song et al., 2011; Su et al., 2014; Katayama et al., 2019), similar to that of Class II Fum from *Streptomyces thermovulgaris* (50°C; Lin et al., 2007), and lower than those of Class II Fums from *T. thermophilus* (85°C; Mizobata et al., 1998) and the thermophilic archaebacterium *Sulfobolus solfataricus* (85°C; Puchegger et al., 1990; Figures 2A,C). *C. merolae* grows optimally at 46°C (Moriyama et al., 2008), suggesting that CmFUM stably shows high activity at the optimum growth temperature. *T. elongatus* rapidly grows in the range of 50–60°C (Yamaoka et al., 1978). In *T. elongatus*, the activities of photosynthesis and entire electron transport were dependent on temperature and high in the range of 50–60°C (Yamaoka et al., 1978). It is suggested that TeFum also becomes active at these growth temperatures. The optimum pH for CmFUM (pH 7.5 for fumarate hydration; pH 8.5 for l-malate dehydration) and TeFum (pH 7.0 for fumarate hydration; pH 7.5 for l-malate dehydration) were approximately the same as those of Class II Fums from other organisms (pH 6.5–8.5, seven species: *R. oryzae*, *Synechocystis* 6803, *Saccharomyces cerevisiae*, *S. solfataricus*, *C. glutamicum*, *A. thaliana*, and *Homo sapiens*; Puchegger et al., 1990; Keruchenko et al., 1992; Genda et al., 2006; Song et al., 2011; Zubimendi et al., 2018; Ajalla Aleixo et al., 2019; Katayama et al., 2019; Figures 2B,D). Intercellular pH of *C. merolae* and cyanobacteria is maintained near neutral where CmFUM and TeFum show enzymatic activities (Coleman and Colman, 1981; Zenvirth et al., 1985; Mangan et al., 2016). Unlike Class II
FIGURE 4 | Effects of three organic acids on CmFUM and TeFum activity. (A) CmFUM activity using fumarate (left) and l-malate (right) as a substrate in the presence of organic acids. The measurements using fumarate and l-malate as substrates were performed at 52°C and pH 7.5, and 52°C and pH 8.5, respectively. The concentrations of fumarate and l-malate were the $K_m$ values of CmFUM, 0.27 mM and 1.49 mM, respectively. (B) TeFum activity using fumarate (left) and l-malate (right) as a substrate in the presence of organic acids. The measurements using fumarate and l-malate as substrates were performed at 50°C and pH 7.0, and 50°C and pH 7.5, respectively. The concentrations of fumarate and l-malate were the $K_m$ values of TeFum, 0.14 mM and 0.20 mM, respectively. All organic acids used as effectors were sodium salts. All the enzymatic activities in Figure 4 are represented by relative activities and the activity in the absence of effectors (gray bar) was set at 100%. All data in Figure 4 indicate the mean ± SD obtained from three independent experiments. All asterisks in Figure 4 indicate statistically significant differences between the absence and presence of the effector (Welch's $t$-test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$). All $p$-values obtained from Welch's $t$-test in (A,B) are listed in Supplementary Tables S1 and S2, respectively.

FIGURE 5 | Thermostability of CmFUM. (A) CmFUM activity after heat treatment at each temperature for 15 min. The measurements were performed at pH 7.5. The concentration of fumarate was 0.5 mM. The $T_{50}^{15}$ was calculated using a linear equation obtained from six values (53–60°C). (B) CmFUM activity after heat treatment at 50°C for each time-point. The measurements were performed at pH 7.5. The concentration of fumarate was 0.5 mM. The $t_{1/2}$ was calculated using a linear equation obtained from all the values. All the enzymatic activities in Figure 5 are represented by residual activities, and the activity without heat-treatment was set at 100%. All the data in Figure 5 show the mean ± SD obtained from three independent experiments.
Fums from *A. thaliana* (mitochondrial Fum; Zubimendi et al., 2018) and *Synechocystis* 6803 (Katayama et al., 2019), there was a significant difference between the optimum pH for fumarate hydration and l-malate dehydration in CmFUM and TeFum (particularly CmFUM; Figures 2B,D). Therefore, we can regulate the equilibrium of the reaction catalyzed by CmFUM by adjusting the pH. This property would be beneficial for l-malate production using fumarase.

Like Class II Fums from other organisms (*A. thaliana*, *Synechocystis* 6803, *C. glutamicum*, and *H. sapiens*; Genda et al., 2006; Zubimendi et al., 2018; Ajalla Aleixo et al., 2019; Katayama et al., 2019), CmFUM and TeFum preferentially catalyze fumarate hydration rather than l-malate dehydration (Table 1). The $K_m$ of CmFUM (0.27 mM) and TeFum (0.14 mM) for fumarate were within the range of most Class II enzymes (0.03–3.07 mM, nine species: *E. coli*, *C. glutamicum*, *R. oryzae*, *Synechocystis* 6803, *S. cerevisiae*, *S. solfataricus*, *A. thaliana*, *T. thermophilus*, and *H. sapiens*; Woods et al., 1988; Puchegger et al., 1990; Keruchenko et al., 1992; Mizobata et al., 1998; Song et al., 2011; Lin et al., 2018; Zubimendi et al., 2018; Ajalla Aleixo et al., 2019;
Katayama et al., 2019; Table 1). The $k_{cat}$ of CmFUM (235 s$^{-1}$) and TeFum (37 s$^{-1}$) for fumarate were within the range of Class II Fums (21–513 s$^{-1}$, four species: C. glutamicum, Synechocystis 6803, A. thaliana, and H. sapiens, Lin et al., 2018; Zubimendi et al., 2018; Ajalla Aleixo et al., 2019; Katayama et al., 2019; Table 1). The $k_{cat}/K_m$ of CmFUM (872 s$^{-1}$ mM$^{-1}$) for fumarate was similar to that of Class II Fum from H. sapiens (850 s$^{-1}$ mM$^{-1}$; Ajalla Aleixo et al., 2019), and higher than those of cyanobacterial Class II Fums (Synechocystis 6803: 415 s$^{-1}$ mM$^{-1}$; TeFum: 278 s$^{-1}$ mM$^{-1}$; Katayama et al., 2019) and Class II Fums from C. glutamicum (247 s$^{-1}$ mM$^{-1}$; Lin et al., 2018) and A. thaliana (30 s$^{-1}$ mM$^{-1}$; Zubimendi et al., 2018; Table 1). Thus, CmFUM shows high catalytic activity for fumarate hydration. Phylogenetic analysis of biochemically characterized Class II Fums revealed that the catalytic activities of closely related enzymes are not necessarily conserved (Figure 8). This suggests that some amino acid residues and motifs affect the activities of Class II Fums. A SS loop, a motif contributing to substrate binding and catalytic activity (Puthan Veetil et al., 2012) was highly conserved in Class II Fums (Figure 9). In contrast, a combination of a total of three amino acid residues that contribute to the activities of Class II Fums from S. coelicolor (equivalent to position 257 and 441 of CmFUM; Lin et al., 2007) and Synechocystis 6803 (equivalent to position 401 of CmFUM; Katayama et al., 2019) was different for each Class II Fum (Figure 9). This combination of the three amino acid residues might bring diversity to the catalytic activities of Class II Fums and contribute to the high catalytic activity of CmFUM. Unlike higher plants and algae, C. merolae do not possess an NAD$^+$-dependent malic enzyme in mitochondria, so that the pyruvate transport to mitochondria is essential to perform aerobic respiration (Kuroiwa et al., 2017). The respiratory oxygen consumption of C. merolae drastically increases when not organic acids in the TCA cycle such as l-malate but pyruvate is added to the cells as an exogenous substrate (Moriyama et al., 2015). This suggests that the pyruvate generation in glycolysis is a rate-limiting step of the aerobic respiration and the TCA cycle in C. merolae actively works for energy production unlike that in cyanobacteria (Wan et al., 2017). The high catalytic activity of CmFUM supports this hypothesis.

Similar to Class II Fums from higher plants (Zubimendi et al., 2018) and Synechocystis 6803 (Katayama et al., 2019), both CmFUM and TeFum were inhibited by succinate and citrate (Figure 4), suggesting a common mechanism of inhibition of Class II Fums from photosynthetic organisms. In contrast, Class II Fum from C. glutamicum is not inhibited by succinate and citrate (Genda et al., 2006). Analyses of the effects of pyruvate on the activities for fumarate and l-malate revealed that pyruvate moves the equilibrium of the reaction catalyzed by CmFUM and TeFum to l-malate dehydration and fumarate hydration, respectively (Figure 4). Pyruvate also moves the.

**FIGURE 8** | Phylogenetic analysis of biochemically characterized Class II Fums. Sequence alignment of 14 Class II Fums was performed using CLC Sequence Viewer ver. 8.0. A phylogenetic tree was generated by maximum likelihood method based on 423 conserved amino acid residues using PhyML online (http://www.atgc-montpellier.fr/phyml/). Bootstrap value obtained by 500 replications indicates the reliability of each branch.
equilibrium of the reaction catalyzed by Class II Fums from *A. thaliana* (mitochondrial Fum) and *Synechocystis* 6803 to l-malate dehydration and fumarate hydration, respectively (Zubimendi et al., 2018; Katayama et al., 2019). Phylogenetic analysis of biochemically characterized Class II Fums revealed that eukaryotic Class II Fums form an independent cluster, not including cyanobacterial Class II Fums (Figure 8). These suggest that pyruvate affects the equilibrium of the reaction catalyzed by Class II Fums from photosynthetic organisms and the effects are different between Class II Fums from photosynthetic eukaryotes and cyanobacteria. The difference in metabolism and physiological characteristics between photosynthetic eukaryotes and cyanobacteria might be associated with the effects of pyruvate on their Class II Fums.

Additional biochemical analyses of CmFUM clarified whether this enzyme has suitable enzymatic properties for efficient 1-malate production. The $T_{1/2}$ of CmFUM (57.3°C) was higher than both the Class II Fum from *C. glutamicum* (44.8°C) as well as its thermostable mutant (54.6°C; Lin et al., 2018; Figure 5A). Moreover, the $t_{1/2}$ at 50°C of the Class II Fum from *C. glutamicum* is 1 min (Lin et al., 2018), and the Class II Fums from *S. cerevisiae* and *A. coelocolor* are immediately denatured at 50°C (Keruchenko et al., 1992; Lin et al., 2007). Class II Fum from *S. thermovulgaris* shows higher thermostability than these mesophilic Class II Fums and its $t_{1/2}$ at 50°C is 300 min (Lin et al., 2007). CmFum showed higher thermostability than the Class II Fum from *S. thermovulgaris* and its $t_{1/2}$ at 50°C of CmFum was 507 min (Figure 5B). These suggest that CmFUM can show high activity after heat treatment in 1-malate production. Phylogenetic analysis of biochemically characterized Class II Fums revealed that as well as the catalytic activities, and the thermostability of closely related enzymes is not necessarily conserved (Figure 8). In the Class II Fum from *C. glutamicum*, three amino acid residues equivalent to position 268, 320, and 503 of CmFUM which contribute to the thermostability of Class II Fum from *C. glutamicum* (Lin et al., 2018). The yellow square represents a loop region containing the sequence GSSxxPxKxN (called a SS loop) which contributes to substrate binding and catalytic activity (Putman Veeti et al., 2012).

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in the presence of Na\(^+\) (Katayama et al., 2019). However, CmFUM activity for fumarate did not change in the presence of monovalent and divalent metal cations (Figure 6A). In industrial l-malate production using fumarase, fumarate salts exist as sodium and calcium salts, which are easy to dissolve in water and do not affect the pH of the reaction and thus, can be used as fumarase substrates (Terasawa et al., 1990). CmFUM, which is insensitive to metal cations, can use these fumarate salts as substrates. CmFUM consistently showed enzymatic activity in four buffer solutions except for the HEPES-NaOH buffer (Figure 6B). Considering the costs of the buffer solutions, we believe that Tris-HCl is a suitable buffer for CmFUM. The yield of l-malate when using CmFUM (75–81%) was higher than the yields when using Class II Fums from C. glutamicum (Chibata et al., 1987) and T. thermophilus (Ninh et al., 2013; Both are 70%; Figure 7). The yield of l-malate when using Class II Fum from R. oryzae is expected to be 75–80% (Naudé and Nicol, 2018).

In this study, we characterized the biochemical properties of Class II Fums from a thermophilic microalga and cyanobacterium. We demonstrated that CmFUM has suitable enzymatic properties for efficient l-malate production such as high activity and thermostability. The optimizations of l-malate production using CmFUM such as the utilization of a whole-cell biocatalyst and reactor will be future developments.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

**AUTHOR CONTRIBUTIONS**

SI designed the study, analyzed the data, and wrote the manuscript. KI and HS performed the experiments and analyzed the data. TO designed the study and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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The language of the manuscript has been corrected by Editage Co. Ltd.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.560894/full#supplementary-material

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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