Bioconversion of Carbon Monoxide to Formate Using Artificially Designed Carbon Monoxide:Formate Oxidoreductase in Hyperthermophilic Archaea

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Bioconversion of carbon monoxide to formate using artificially designed carbon monoxide:formate oxidoreductase in hyperthermophilic archaea

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Running title: Direct electron transfer by a FeS-FeS fusion protein

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Abstract

Ferredoxin-dependent metabolic engineering of electron transfer circuits has been developed to enhance redox efficiency in the field of synthetic biology, e.g., for hydrogen production and for reduction of flavoproteins or NAD(P)⁺. Here, we present the bioconversion of carbon monoxide (CO) gas to formate via a synthetic CO:formate oxidoreductase (CFOR), designed as an enzyme complex for direct electron transfer between noninteracting CO dehydrogenase and formate dehydrogenase using an electron-transferring Fe-S fusion protein. The CFOR-introduced *Thermococcus onnurineus* mutant strains showed CO-dependent formate production *in vivo* and *in vitro*. The formate production rate from purified CFOR complex and specific formate productivity from the bioreactor were 348 ± 34 μmol/mg/min and 90.2 ± 20.4 mmol/g-cells/h, respectively. The CO-dependent CO₂ reduction/formate production activity of synthetic CFOR was confirmed, indicating that direct electron transfer between two unrelated dehydrogenases was feasible via mediation of the FeS-FeS fusion protein.
Electron transfer is central to various essential metabolic pathways. For example, the electron transport chain and Fe-S proteins are essential constituents of the respiratory complex in all life forms on Earth. Fe-S proteins, which are involved in enzyme catalysis, regulation, maintenance of protein structure, and biological electron transfer\textsuperscript{1,2}, include [2Fe-2S], [3Fe-4S], and [4Fe-4S] types\textsuperscript{1,3–5}. Quantum-mechanical electron transfer between two Fe-S clusters could explain the maximum distance of 14 Å for physiologically relevant electron tunneling\textsuperscript{6}. Ferredoxins are small (~11 kDa) soluble electron carriers that bind to proteins that contain electrons held by reduced Fe-S clusters, providing unique opportunities for engineering and synthetic biology applications\textsuperscript{7–13}. Artificial fusion of Fe-S proteins, such as ferredoxin and thioredoxin-like proteins, has been performed to improve electron transfer between redox partners\textsuperscript{7–10,14–16}. Despite the reliable efficiency of protein fusion for facilitating electron transfer, target enzymes of fusion constructs are restricted to specific redox pairs that naturally interact with each other, such as ferredoxin and ferredoxin-dependent hydrogenase. Thus, a novel synthetic electron transferring path between two noninteracting redox enzymes has not yet been reported.

Hyperfine magnetic field generation at a reduced Fe-S cluster was first observed by Johnson \textit{et al.},\textsuperscript{17} and it was applied to the synthesis of a novel electron transfer path in this study as a significant concept described in the ‘Discussion’ section. Subsequently, we attempted direct electron transfer between two different oxidoreductases, carbon monoxide dehydrogenase (CODH) and formate dehydrogenase (FDH), as a model system. CODH and FDH catalyze oxidoreduction of CO/CO\textsubscript{2} and formate/CO\textsubscript{2}, respectively\textsuperscript{18–24}. Theoretically, the overall reaction of the oxidation of CO to CO\textsubscript{2} with the reduction of CO\textsubscript{2} to formate is thermodynamically exergonic (ΔG\textsuperscript{o} = -16.5 kJ/mol). CO oxidation coupled with CO\textsubscript{2} reduction to formate by connecting CODH and FDH is an ideal system for monitoring electron flow between the two redox enzymes. The electric current can be easily read-out as formate, and the overall reaction does not require an additional substrate other than only CO. We chose the carboxydrotrophic and formatotrophic euryarchaeota \textit{Thermococcus onnurineus} NA1, which harbors genes encoding both CODH and FDH (\textit{codhB} and \textit{fdhA}, respectively) and shows high cell resistance against both CO and formate\textsuperscript{25–27}, as a model organism. Notably, the hydrogen-dependent CO\textsubscript{2} reductase (HDCR) enzyme complex catalyzes CO\textsubscript{2} reduction to formate in \textit{Acetobacterium woodii} using
hydrogen directly or coupled CODH-ferredoxin indirectly as an electron donor. However, no natural enzymes have been shown to catalyze direct CO oxidation coupled with formate production.

Here, we constructed synthetic carbon monoxide:formate oxidoreductase (CFOR) for direct electron transfer between CODH and FDH using an electron-transferring Fe-S fusion protein in *T. onnurineus*. The synthetic CFOR complex was purified and assayed to assess electron transfer ability *in vivo* and *in vitro*. 
Results

Construction of CO/formate bioconversion mutants via molecular fusion of two Fe-S proteins

The redox proteins CODH and FDH from *T. onnirineus* NA1 were engineered systematically through molecular fusion of electron-transferring Fe-S proteins to construct a single redox complex. The *codh* and *fdh3* gene clusters included the *codhABCD* and *focA-fdh3ABC* genes, respectively (Fig. 1a and Supplementary Table 3). CodhA and Fdh3B are homologous to the FDH-β subunit, FdnH (PDB 1FDI), in *E. coli*. FdnH has electron-transferring 4[4Fe-4S] clusters, and its amino acid sequence repeats the common motif for [4Fe-4S] cluster binding (CxxCxxCxxCP) or its slight variants. Sequence alignment of FdnH showed that the [4Fe-4S] cluster binding motifs were identical to Fdh3B and CodhA (Supplementary Fig. 1a). However, Fdh3C showed high similarity with the 2[4Fe-4S] cluster binding motif in ferredoxins (Supplementary Fig. 1b). The results suggested that Fdh3B and CodhA or Fdh3C had an extrinsic domain with 4[4Fe-4S] or 2[4Fe-4S] clusters, respectively. Protein structure and *in silico* analyses suggest that FDH small subunits (Fdh3B homolog) directly interact with the FDH catalytic subunit (Fdh3A homolog) and another Fe-S protein (Fdh3C homolog). Therefore, Fdh3B was predicted to transfer electrons from Fdh3A to Fdh3C by connecting them in the complex. The amino acid sequences of CodhAB were also homologous to the CooFS proteins (41.7% and 50.3% identity, respectively) in *Rhodospirillum rubrum*. CooF mediates electron transfer from the CODH catalytic subunit CooS to hydrogenase and interacts directly with CooS; hence, spontaneous enzyme complex formation of CodhA and CodhB is easily predictable. Accordingly, Fdh3C-CodhA and Fdh3B-CodhA were designed and constructed.

Fusion of Fe-S proteins led to the formation of novel protein complexes associated with CODH and FDH, termed synthetic CFOR. Therefore, the *fdh3B* or *fdh3C* genes were fused directly to the *codhA* gene using Gibson Assembly in two possible arrangements, *fdh3BC:codhA* and *fdh3B:codhA* (Fig. 1b and 1c). Structurally, the N- and C-termini of FdnH are located on the distal-end [4Fe-4S] cluster; thus, the distal-end [4Fe-4S] cluster at each Fe-S protein was expected to be aligned face-to-face in every possible fusion combinations. Predicted models of the synthetic CFORs are presented in Supplementary Fig. 2. Next, the two flexible linkers (GGGGG) and (GGGGGS) were designed with the
$fdh3BC:codhA$ fusion arrangement. However, $(GGGGS)_3$ insertion was obtained during the homologous recombination of the $(GGGGS)_2$, resulting in three different lengths of linkers, which was confirmed by sequencing analysis. The $fdh3BC:codhA$ fusion constructs pFd3CoL1C1118, pFd3CoL2C1119, and pFd3CoL3C1120 carried three different lengths of flexible linkers, i.e., $(GGGGS)_1$, $(GGGGS)_2$, and $(GGGGS)_3$, respectively (Supplementary Table 1). Notably, the shortest linker, $(GGGGS)_1$, showed the highest formate productivity (Supplementary Fig. 6b and 6c) and was selected for $fdh3B:codhA$ fusion.

A fosmid vector was used to facilitate cloning for chromosomal insertion of the synthetic CFOR. Insertion of the expression construct was targeted to a region of the chromosome between convergent genes TON_1126 and TON_1127, as previously described. A 9-kbp DNA fragment containing the $fdh3$ and $codh$ region and the $P_{0157}$ promotor-HMG cassette was inserted into the chromosome of $T. onnurineus$ D02 by transformation of pFd3CoL1C1118, pFd3CoL2C1119, and pFd3CoL3C1120, generating the mutant strains BCF01, BCF02, and BCF03, respectively (Fig. 1b). Strain BCF13 was then constructed by transformation of the pFd3NStrepCoL1C1149 fosmid, which contains the $fdh3B:codhA$ fusion with $(GGGGS)_1$, into $T. onnurineus$ D04 strain with additional deletion of the $fdh3$ whole gene cluster (Fig. 1c and Supplementary Fig. 3a). An affinity-purification Strep-tag was also inserted within the operon at the N-terminus of $fdh3A$ to allow easy purification of the synthetic CFOR enzyme at the strain BCF13 (Fig. 1c). Strain D05 was also constructed as a negative control for BCF13, which has no fusion linker between $fdh3B$ and $codhA$ (Supplementary Fig. 3b).

**Determination of CO-dependent cell growth and formate production**

Growth and metabolic profiles of the mutant strains were then compared. Initially, cell growth, formate production, and pH change were determined from $T. onnurineus$ BCF01, which contained the fusion of $fdh3C:codhA$ with the shortest length of $(GGGGS)_1$ linker. The growth rate of this strain was similar to that of the parental strain, whereas maximum cell growth was slightly reduced (Supplementary Fig. 4a). Cumulative formate production was detected during cell growth with a final concentration of $3.2 \pm 0.2$ mmol/L after 60 h of incubation for the BCF01 strain, whereas formate levels were below the detection
limit for the parental strain (Supplementary Fig. 4b). Based on the overall reaction, equivalent amounts of formate and H⁺ are produced, thereby decreasing the pH during formate production. Indeed, the pH of the cell supernatant was significantly decreased from pH 6.9 to 5.0 only in the BCF01 strain (Supplementary Fig. 4c). This may affect sustainable formate production and cell growth, which are optimal at pH 6.5 under CO supplementation conditions²⁷. Thus, 0.1 M bis-Tris propane (pH 6.5) buffer was added to the cell growth medium to prevent the abrupt pH change due to bioconversion of CO to formate, thus stabilizing the pH without growth inhibition (Supplementary Fig. 5).

Next, we compared the formate production ability of the remaining mutants. Cell growth was identical, and formate production was detected (Supplementary Fig. 6). BCF01 showed the highest formate production (7.5 mmol/L) after 60 h of incubation (Supplementary Fig. 6b). The relative formate productivities of BCF02 and BCF03 at the final time point were determined to be 78% and 84%, respectively, compared with BCF01 (Supplementary Fig. 6c). The (GGGGS)₁ linker showed the highest formate productivity; however, the effect on the electron transfer efficiency was insignificant. BCF13, carrying the fdh3B:codhA fusion with the (GGGGS)₁ linker, showed 6.6 ± 2.1 mmol/L formate production under CO-supplemented cell growth within 24 h (Fig. 2a), which was higher than that of BCF01 (4.3 ± 0.4 mmol/L formate) at the same time. Cell growth was similar to the other strains (Fig. 2a). Therefore, all subsequent experiments were conducted using the BCF13 strain. Formate production from the cell suspension was also investigated in serum vials using the strain D05 (without fusion linker) and BCF13 (with fusion linker) at an OD₆₀₀ of about 0.5, incubated at 80°C in the presence of CO gas with 2 bar (gauge pressure) CO/CO₂ (50:50 v/v) or CO/N₂ (50:50 v/v) mix gas. Activity and stability of the cell suspensions were confirmed by H₂ productivity that showed similar values among strains and gas conditions (Fig. 2c). In the BCF13, 3.0 ± 0.27 mmol/L formate was produced after 60 min incubation under the CO/CO₂ mix gas (Fig. 2b). In contrast, when the headspace was filled with CO/N₂, only 0.3 ± 0.04 mmol/L formate was produced due to the low CO₂ partial pressure (Supplementary Fig. 7). Although the CO oxidation reaction provides the equivalent CO₂ requirement for the formate production, the additionally supplemented CO₂ enhances the CO₂ reduction/formate production reaction. In the D05, formate production was detected under CO/CO₂ mix gas as a concentration of 0.1
± 0.007 mmol/L at 60 min, which is 30-fold lower than the BCF13. The results indicate that electron transfer between CODH and FDH modules is achievable just by overexpression of the enzymes but extremely enhanced by a flexible fusion of FeS-FeS in the synthetic CFOR complex.

Purification of the synthetic CFOR complex

We then purified the CFOR enzyme complex isolated from strain BCF13 grown in a fed-batch bioreactor with CO-supplemented MM1 medium to activate the expression of genes controlled by the strong promoter $P_{0157}$, which induces robust transcription and translation under CO-supplemented growth conditions. The CFOR complex was purified using a strep-tag fused to the N-terminus of the FDH catalytic subunit, Fdh3A, and then analyzed by SDS-PAGE. Fdh3A, CodhB, and Fdh3B-CodhA fusion subunits, were present, with apparent molecular masses of 76, 67, and 42 kDa, indicating that the FdhB-CodhA fusion protein spontaneously bound to CodhB and FdhA to form the CFOR complex (Fig. 3a). Protein bands consistent with the calculated molecular weights from deduced amino acid sequences were observed for all three subunits. The calculated size of Fdh3B-CodhA was 43,722 Da. The CFOR complex was further purified using size-exclusion chromatography, and the CFOR complex from gel filtration was eluted as a single major peak with an apparent mass of around 488 kDa (Fig. 3b and 3c). The three major bands of purified protein were identified by LC-MS/MS analysis by bands cut from the SDS-PAGE gel (Fig. 3a and Supplementary Table 4). The 76-kDa and 67-kDa protein bands were identified as FdhA and CodhB, respectively. The 42-kDa protein band was thought to be the FdhB-CodhA fusion protein, which was identified as two different proteins, i.e., Fdh3B and CodhA. The additionally inserted formate transporter FocA could enhance the secretion of intracellular formate synthesized by the CFOR complex. CodhC (29,274 Da) and CodhD (7,678 Da) are hypothetical proteins predicted to be involved in the maturation of the catalytic subunit CodhB. However, FocA, CodhC, and CodhD subunits were not detected in both the SDS-PAGE gel and by LC-MS/MS analyses. Thus, the entire CFOR complex was composed of the CO dehydrogenase catalytic subunit (CodhB),
FDH catalytic subunit (Fdh3A), and Fe-S fusion proteins (Fdh3B-CodhA) connecting the two catalytic subunits.

**Catalytic properties of the CFOR enzyme complex**

The activities of CODH and FDH from purified CFOR complex were determined individually. The isolated CFOR catalyzed CO oxidation with a specific activity of $2,209 \pm 159 \mu$mol/mg/min and formate oxidation with a specific activity of $369 \pm 181 \mu$mol/mg/min at $80^\circ$C (Supplementary Table 5). The specific activity of FDH was lower than that of CODH; therefore, the reaction of FDH was expected to be a limiting factor for the rate of formate production in the overall CFOR reaction. To confirm direct electron transfer by the Fe-S fusion protein, we investigated whether the purified CFOR could catalyze CO oxidation/formate production without any other additional electron carriers. The enzyme indeed catalyzed formate production from CO/CO$_2$ (50:50 v/v) mix gas with a specific activity of $348 \pm 34 \mu$mol/mg/min (Fig. 4 and Supplementary Table 5). However, formate was below the detection limit under conditions with 100% CO or without CFOR enzyme (Fig. 4). The maximum activity of the purified CFOR was determined at pH 7.5 in 50 mM sodium phosphate buffer (Supplementary Fig. 8). The results demonstrated that the simple, flexible fusion of two Fe-S proteins enabled electron transfer between them, leading to the formation of novel enzymes by assembly of noninteracting redox enzymes. Another formate production enzyme, HDCR in *A. woodii*, which reduces CO$_2$ to formate using hydrogen, has a specific activity of $10 \mu$mol/mg/min$^{28}$; therefore, synthetic CFOR showed a 35-fold higher formate production rate than native HDCR.

**Bioconversion of CO to formate in a bioreactor**

The formate production potential of the strain was tested in a bioreactor where 100% CO was continuously fed with a flowrate of 0.02–0.122 vvm (CO volume/working volume/min). Supplementary Table 6 summarizes the bioreactor parameters for the *T. onnurineus* BCF13 strain. Formate production was detected at a concentration of $56.4 \pm 6.4$ mmol/L after fermentation for 6 h (Fig. 5b). The formate production rate and specific formate productivity were calculated as $13.1 \pm 0.9$ mmol/L/h and $90.2 \pm$
20.4 mmol/g-cells/h, respectively. Recently CO-dependent formate production by coupling of CODH, ferredoxin, and HDCR was reported using *A. woodii* as a whole-cell biocatalyst; the formate production rate was 0.28 mmol/L/h\(^{39}\). *T. onnurineus* NA1 and its derivatives mutants used in this study are a basic hydrogenogenic carboxydotroph that can grow on CO as an energy source via the CO-dependent respiratory gene cluster *codh-mch-mnh3*\(^{25,27,29}\). Thus, the BCF13 strain showed carboxydotrophic properties, such as H\(_2\) and CO\(_2\) production (Fig. 2c and 5c), and could grow under 100% CO conditions with the maximum specific growth rate (\(\mu_{\text{max}}\)) of 0.621 ± 0.051 h\(^{-1}\) (Fig. 5a). This spontaneous CO\(_2\) production by the CO-dependent respiration enhances the formate production in the bioreactor. Therefore, *T. onnurineus* BCF13 could be used as an industrial microorganism for the production of H\(_2\) and formate simultaneously from CO.
**Discussion**

Fe-S proteins involved in the electron transfer chain have been well characterized, both structurally and functionally. Fe-S proteins in many oxidoreductase complexes are typically associated with a large subunit that has catalytic redox activity and forms an enzyme complex, such as respiratory complex I, FDH-N and formate hydrogenlyase in *E. coli*\[^{22,30,40}\]. However, direct electron transfer between CODH and FDH has not yet been found in nature. Thus, if one could construct an electron transfer system between these two oxidoreductases, it could serve as a universal electron transfer system. Accordingly, we generated fusion proteins between CodhA and Fdh3B to create a novel electron transfer path. The small subunits CodhA and Fdh3B specifically interacted with their catalytic large subunits. Therefore, the molecular fusion of Fe-S proteins may spontaneously mediate the formation of a novel CODH-FDH protein complex. However, a simple fusion between Fe-S proteins may not allow electron transfer. According to the electron tunneling theory, the maximum distance between the distal end [Fe-S] clusters at each protein must provide a distance of at least 14 Å for electron transfer, indicating that tight-binding and a sophisticated rearrangement of the two proteins are essential.

We showed that electron transfer was possible by simple fusion of the flexible (GGGGS) linker between electron-transferring Fe-S proteins. Based on the result, we suggest that the hyperfine magnetic field may affect this phenomenon. Indeed, Johnson *et al.* reported that the iron nuclei in the [Fe-S] cluster showed a magnetic hyperfine interaction with an electron spin S of \(\frac{1}{2}\), producing an effective field of about 180 kG in the reduced state\[^{17}\]. Therefore, we hypothesized that the [4Fe-4S] clusters located at both distal ends of the Fe-S fusion protein would combine tightly by physical magnetic interaction, providing the electron tunneling condition. Accordingly, we propose the following model for the CO oxidation and formate production-coupled bioactivity of the artificially constructed CFOR complex by simple, flexible fusion of two Fe-S proteins (Fig. 6). First, there was no CO oxidation reaction in the initial; CodhA and FdhB were connected by a flexible linker but could not bind tightly, and hence, there was no electron transfer occurring based on the electron tunneling theory. Additionally, when the CO oxidation reaction began in CodhB, the distal-end [4Fe-S4] cluster at CodhA was reduced by electron transfer, leading to generation of a hyperfine magnetic field on the [4Fe-4S] cluster.
Moreover, another fused Fe-S protein, Fdh3B, was forced into the magnetic field by the flexible linker. Next, the ferromagnetic Fe atoms in the distal-end [4Fe-4S] cluster at Fdh3B were magnetized based on the influence of the magnetic field, thus binding tightly and rearranging with the [4Fe-4S] cluster of CodhA to provide the electron tunneling distance. Finally, electrons could be transferred from CODH to FDH through CodhA-Fdh3B fusion protein. Therefore, the synthetic CFOR complex catalyzes the CO$_2$ reduction/formate production reaction. However, no previous reports have described an attempt to transfer electrons directly between noninteracting proteins. Here, we developed a FeS-FeS fusion protein to provide a novel electron transfer path for the production of formate from CO by the synthetic CFOR enzyme complex in hyperthermophilic archaea. The electron transfer strategy endeavored here is based on an elementary natural phenomenon that ‘Iron is attracted to a magnet.’ This approach also exploited the self-assembly and self-regenerating abilities of live cells to create a novel formate production species. Overall, our results provide important insights into the synthesis of a new electron path using synthetic biology-based metabolic engineering. We focused on the possibility of electron transfer between noninteracting Fe-S proteins in this study. However, the effect of introducing various fusion combinations among Fe-S proteins and oxidoreductases remains to be determined for the broad application of this approach.
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Author’s contribution

J.K.L. conceptualized the study and study design. The experimental investigation was carried out by J.K.L., J.I.Y., Y.J.K., and Y.J.P. Data analysis was performed by J.K.L. and Y.J.K. Writing of the original draft and editing were carried out by J.K.L. Reviewing was carried out by Y.J.K. and Y.H.K. Supervision was done by J.K.L.

Competing interests

Patent applications describing the development and applications of CFOR and mutant strains to the KIOST and J.K.L., J.I.Y., Y.J.K., is accepted (no. 10-2129279 and 10-2129282) or pending (no. 10-2018-0138475 and PCT/KR 2018/014807).
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Figure 1. Construction of the CO-formate bioconversion mutants by cloning of the synthetic CFOR. a, Gene arrangement of the fdh3 and codh gene clusters on the genome of T. onnurineus NA1. The name of genes and locus tags are indicated on the upper and lower side of the genes, respectively. b, Construction of T. onnurineus BCF01, BCF02, and BCF03 mutants by transformation and homologous recombination of the fdh3C:codhA fusion containing fosmids pFd3Col1C1118 (GGGGS)1, pFd3Col1C1119 (GGGGS)2, and pFd3Col1C1120 (GGGGS)3, respectively. c, Construction of T. onnurineus BCF13 mutant by the transformation of the fosmid
pFd3NStrepCoL1C1149, which contains a fusion of \textit{fdh3B:codhA} with linker (GGGS)\textsubscript{6} and strep-tag at the N-terminus of Fdh3A.
Figure 2. CO-dependent formate production. a, Formate production (●) and cell growth (▼) of T. onnurineus BCF13. Cells were cultured in MM1 medium supplemented with 0.1 M bis-Tris propane (pH 6.5) and 2 bar of CO gas at 80°C. b and c, Formate (b) and H₂ (c) production from cell suspensions of T. onnurineus BCF13 and T. onnurineus D05. Cells (OD₆₀₀ = 0.5) were incubated with CO/CO₂ (50:50 v/v) mix gas or CO/N₂ (50:50 v/v) mix gas in modified PBS buffer at 80°C. Symbols: ●, BCF13 with CO/CO₂ mix gas; ○, D05 with CO/CO₂ mix gas; ▼, BCF13 with CO/N₂ mix gas; △, D05 with CO/N₂ mix gas. Error bars represent the standard deviations (n = 3).
Figure 3. Purification and identification of the synthetic CFOR complex from *T. onnurineus* BCF13. 

a, Strep-tag purified CFOR complex (5 μg) was loaded to a 12% acrylamide gel and stained with Coomassie Brilliant Blue after gel running. CFOR subunits identified by LC-MS/MS are indicated with a black arrow. 
b, The affinity column purified CFOR complex (blue line), and standard marker (black line) were separated on a Superdex 200 10/300 GL column. A number on a standard peak indicates: 1. Thiroglobulin, 669 kDa; 2. Apoferritin, 443 kDa; 3. Beta-amylase, 200 kDa; 4. Albumin, 66 kDa; 5. Carbonic anhydrase, 29 kDa. 
c, The retention time versus the logarithm of the molecular weight of the CFOR complex (□) and standard marker (●) was plotted on the regression.
Figure 4. *In vitro* formate production by purified CFOR complex. The formate production was determined at the absence (●) or presence of 50 ug of purified CFOR complex that incubated with 2 bar (gauge pressure) of CO/CO$_2$ (50:50 v/v) (■) or 0.5 bar (gauge pressure) of 100% CO (▼) in 50 mM sodium phosphate buffer (pH 7.5). Formate production was determined by HPLC. Error bars represent the standard deviations (n = 3).
Figure 5. CO-dependent fed-batch culture in bioreactor. a, Cell growth was monitored by measuring OD\textsubscript{600}. b, Formate production was determined by HPLC. c, Partial pressure of CO (●), CO\textsubscript{2} (▼) and H\textsubscript{2} (■) in headspace was determined by GC. CO (100%) was supplied with initial flow rate of 0.02 vvm and was raised to 0.122 vvm during the fed-batch culture. Error bars represent the standard deviations (n = 4).
Figure 6. Proposed mechanism of electron transfer within Fe-S fusion protein by a hyperfine magnetic field.
## Supplementary Table and Figure

### Supplementary Table 1. Strains, plasmids and fosmids used this study.

| Strains/Plasmids/Fosmids | Description | Reference |
|--------------------------|-------------|-----------|
| **Strains**              |             |           |
| *E. coli*                |             |           |
| EPI300<sup>Tm</sup>-T1<sup>R</sup> | Fosmid cloning host | EPICENTRE |
| **T. onnurineus**        |             |           |
| NA1                      | Wild-type strain | 41        |
| D02                      | NA1 derivative, *Afđh1* gene cluster (TON_0266-TON_0282) *Afđh2* gene cluster (TON_1563-TON_1580) *Afđh3A* (TON_0539) | Previous construction (not reported) |
| D04                      | D02 derivative, *Afđh1* gene cluster (TON_0266-TON_0282) *Afđh2* gene cluster (TON_1563-TON_1580) *Afđh3* gene cluster (TON_0539-TON_0541) | This study |
| D05                      | D04 derivative *pFd3StrepCodhC2008* transformation, P<sub>0157hmgpfu</sub>::*focA-fđh3ABC-codhABCD*; Strep-tag inserted in N-terminus of Fdh3A; control strain for the strain BCF13 | This study |
| BCF01                    | D02 derivative *pFd3CoL1C1118* transformation, P<sub>0157hmgpfu</sub>::*focA-fđh3ABC-codhABCD*; fusion of fđh3C and *codhA* with linker (GGGGS)<sup>1</sup> | This study |
| BCF02                    | D02 derivative *pFd3CoL2C1119* transformation, P<sub>0157hmgpfu</sub>::*focA-fđh3ABC-codhABCD*; fusion of fđh3C and *codhA* with linker (GGGGS)<sup>2</sup> | This study |

(Continued)
| Strains/Plasmids/Fosmids | Description | Reference |
|-------------------------|-------------|-----------|
| BCF03                   | D02 derivative pFd3CoL3C1120 transformation, \( P_{0157\text{hmg}}::\text{focA-fdh3ABC:codhABCD} \); fusion of \( \text{fdh3C} \) and \( \text{codhA} \) with linker (GGGGS)\textsubscript{3} | This study |
| BCF12                   | D04 derivative pFd3NHisCoL1C1132 transformation, \( P_{0157\text{hmgpfu}}::\text{focA-fdh3AB:codhABCD} \); fusion of \( \text{fdh3B} \) and \( \text{codhA} \) with linker (GGGGS)\textsubscript{1}; His-tag inserted in N-terminus of Fdh3A | This study |
| BCF13                   | D04 derivative pFd3NStrepHisCoL1C1149 transformation, \( P_{0157\text{hmgpfu}}::\text{focA-fdh3AB:codhABCD} \); fusion of \( \text{fdh3B} \) and \( \text{codhA} \) with linker (GGGGS)\textsubscript{1}; Strep-tag inserted in N-terminus of Fdh3A | This study |

**Plasmids**

- **pUC vector**
  - **pUC118** Backbone plasmid; Amp\textsuperscript{r} | TAKARA |
  - **pFd1,2,3clusterA1135** pUC118 carrying \( P_{\text{gdh}} \) promoter, HMG cassette, and 1kbp Left-arm (LA) and Right-arm (RA) for deletion of \( \text{fdh3} \) gene cluster (TON_0539-TON_0541) | This study |

**Fosmids**

- **pCC1FOS** Backbone fosmid; Cm\textsuperscript{r} | EPICENTRE |
- **pNA1comFosC1096** pCC1FOS carrying \( P_{0157} \) promoter, HMG cassette, and 1kbp Left-arm (LA) and Right-arm (RA) for homologous recombination of \( T. \text{onnurineus} \) NA1 genome; backbone fosmid for mutant construction; Sim\textsuperscript{r} | This study |
- **pFd3CoL1C1118** pNA1comFosC1096 carrying \( \text{fdh3} \) region (\( \text{focA-fdh3ABC} \)) and \( \text{codh} \) region (\( \text{codhABCD} \)) from \( T. \text{onnurineus} \) NA1; fusion of \( \text{fdh3C} \) and \( \text{codhA} \) with linker (GGGGS)\textsubscript{1} | This study |

(Continued)
| Strains/Plasmids/Fosmids | Description | Reference |
|-------------------------|-------------|-----------|
| pFd3CoL2C1119           | pFd3CoL1C1118 carrying fusion of *fdh3C* and *codhA* with linker (GGGGS); | This study |
| pFd3CoL3C1120           | pFd3CoL1C1118 carrying fusion of *fdh3C* and *codhA* with linker (GGGGS); | This study |
| pFd3NHisCoL1C1132       | pNA1comFosC1096 carrying *fdh3* region (*focA-fdh3AB*) and *codh* region (*codhABCD*) from *T. onnurineus* NA1; fusion of *fdh3B* and *codhA* with linker (GGGGS); His tag inserted in N-terminus of Fdh3A | This study |
| pFd3NHisStrepCoL1C1149   | pNA1comFosC1096 carrying *fdh3* region (*focA-fdh3AB*) and *codh* region (*codhABCD*) from *T. onnurineus* NA1; fusion of *fdh3B* and *codhA* with linker (GGGGS); pFd3NHisCoL1C1132 carrying Strep-tag inserted in N-terminus of Fdh3A (TON_0539) | This study |
| pFd3NStrepCodhC2008      | pNA1comFosC1096 carrying *fdh3* region (*focA-fdh3AB*) and *codh* region (*codhABCD*) from *T. onnurineus* NA1 without fusion linker; Strep-tag inserted in N-terminus of Fdh3A (TON_0539) | This study |
## Supplementary Table 2. PCR primers used in this study.

Bold and underlined nucleotides indicate (GGGGS)n linker and tagging sequences, respectively.

| Construction/PCR      | Nucleotide sequence (5' to 3')                                      | Reverse                                           |
|-----------------------|---------------------------------------------------------------------|--------------------------------------------------|
|                        | **Forward**                                                        | **Reverse**                                      |
| pNA1comFosC1096        |                                                                     |                                                  |
| Left-arm-P<sub>o57-flm</sub> | AGCTCGGTACCCGGGATCCCCACAGCCTCTCGAGACGA                               | TGACGCCACGATCGGCTAGTCATCTCCCAAGCATTTATGAG        |
| Right-arm             | TGCTTGGGGATGACCTAGCGGTGGCTGAGCTAGCCTCAACGGATG                         | AGGTCGACCTAGAGGATCCAAATGGAAATACCAATCTACGG        |
| pFdh1,2,3clusterA1135 |                                                                     |                                                  |
|                         | Left-arm                                                           |                                                  |
|                         | AATTCGAGCTCGGTACCCGGGATCCACAGAATAATTCTCG                             | GGCCATCCTTAACACACACCCCGCTATCTTC                  |
|                         | Right-arm                                                         |                                                  |
|                         | GATAGCCGCGGTTGCTGTTAAGGGATGCGCTGTATG                               | CCAGTGCCAAGCCTGTCCAGAATGACCTTTGTTGATGCG         |
| pFd3CoL1C1118          |                                                                     |                                                  |
| focA-fdh3C:codhA(GGG  | TAAAATGCTTTGGGAGATGACCTAGGATGGCAGAATAATTCTACTCG                      | GGCACTGCTGCCCTCCGCCGCCCGCCCGCCAGGTAAGGCTCATATTTG |
| GS<sub>1</sub>)        |                                                                     |                                                  |
| fdh3C:codhA(GGG GS<sub>2</sub>) | TGGGGGGGGGGGAGGCGGGCGGAGGCAGCAGTCGCCAAGCTTTTCCGGTTCC                | TGGCCATCGGTGAGCAGCAGCAGGCTACCTCACTCCCTGAGTTTA   |
| pFd3CoL2C1119          |                                                                     |                                                  |
| focA-fdh3C:codhA(GGG GS<sub>1</sub>) | TAAAATGCTTTGGGAGATGACCTAGGATGGCAGAATAATTCTACTCG                      | GGCACTGCTGCCCTCCGCCGCCCGCCAGGTAAGGCTCATATTTG   |
| fdh3C:codhA(GGG GS<sub>2</sub>) | TGGGGGGGGGGGAGGCGGGCGGAGGCAGCAGTCGCCAAGCTTTTCCGGTTCC                | TGGCCATCGGTGAGCAGCAGCAGGCTACCTCACTCCCTGAGTTTA  |
| pFd3NHisCoL1C1132      |                                                                     |                                                  |
| focA-His:fdh3A         | TAAAATGCTTTGGGAGATGACCTAGGATGGCAGAATAATTCTACTCG                      | TCCTCGTGATGGTGGATGGTGCATCCGACCACCGCCCT          |
| His-fdh3A:fdh3B:codhA(GGG GS<sub>1</sub>) | GGATGCACCATCACCACCATCAAGAGGATTTAAGATGGCCGTG                         | GCTGGGGCTGCTGCCCGGCCGCCCGAGTAGAAGGCG           |
| pFd3NStrepCoL1C1149    |                                                                     |                                                  |
| focA-Strep:fdh3A       | TAAAATGCTTTGGGAGATGACCTAGGATGGCAGAATAATTCTACTCG                      | TCCTCGTGATGGTGGATGGTGCATCCGACCACCGCCCT          |
|               | Strep:fdh3A-codhD | pFd3NStrepCodhC200 8 | focA-fdh3B | codhA-codhD |
|---------------|------------------|----------------------|------------|-------------|
| **Strep:fdh3A-codhD** | GGATGCTTCTCGAAGCTGGGACCAGAGTAAAA TTGAGATTG GCCTG | TGGCCATCGGGCCAGCCACGAT CGACGTCTCATCCCTCTGAGTTTA AACCTCAT | TAAAATGGATGAGATGACCTAGGATGGCACAGAATAATTCACT CG | GCCTACTTTCGGTGAAACGAGGTGATCAAATGCC | TGGCCATCGGGCCACGATCGACGTCTCACCTCTGAGTTTA AACCTCAT |
| **pFd3NStrepCodhC200 8** | | | TGTTGATCAACCTTCGTTAACCCGAAAGTAGGCGAGCG | | |
### Supplementary Table 3. Information of the proteins used in this study.

| Proteins | Locug tag | M. W. (Da) | NCBI annotation                           |
|----------|-----------|------------|--------------------------------------------|
| Fdh3     |           |            |                                            |
| FocA     | TON_0538  | 35,570     | Hypothetical formate transporter            |
| Fdh3A    | TON_0539  | 75,626     | Hypothetical formate dehydrogenase, alpha subunit |
| Fdh3B    | TON_0540  | 18,342     | Oxidoreductase iron-sulfur protein          |
| Fdh3C    | TON_0541  | 13,925     | 4Fe-4S cluster-binding protein              |
| Codh     |           |            |                                            |
| CodhA    | TON_1017  | 23,581     | 4Fe-4S ferredoxin, iron-sulfur binding domain protein |
| CodhB    | TON_1018  | 67,671     | carbon-monoxide dehydrogenase, catalytic subunit |
| CodhC    | TON_1019  | 29,274     | Hypothetical ATP-binding protein            |
| CodhD    | TON_1020  | 7,678      | Hypothetical RNA-binding protein            |
Supplementary Table 4. LC-MS/MS identification of purified CFOR subunits showing number of peptides matched and peptide sequence coverage.

| Protein band | Subunit | Accession Number | NCBI Annotation                                      | Predicted M.W. (Da) | Sequence coverage (%) | MASCOT Score |
|--------------|---------|------------------|------------------------------------------------------|---------------------|-----------------------|--------------|
| 1            | Fdh3A   | gi|212008644        | Hypothetical formate dehydrogenase, alpha subunit    | 76324               | 74                    | 2602         |
| 2            | CodhB   | gi|212009124        | Carbon-monoxide dehydrogenase, catalytic subunit      | 68715               | 79                    | 2704         |
| 3            | Fdh3B   | gi|212008645        | Oxidoreductase iron-sulfur protein                    | 19243               | 81                    | 844          |
| 3            | CodhA   | gi|212009123        | 4Fe-4S ferredoxin, iron-sulfur binding domain protein | 24479               | 77                    | 1141         |
**Supplementary Table 5. Specific activities of CO oxidation, formate oxidation, and formate production by the purified CFOR.**

| Assay                  | Specific activities (μmol/mg/min) | Description                                      |
|------------------------|-----------------------------------|--------------------------------------------------|
| CO oxidation           | 2,209 ± 159                       | Methyl viologen as an electron acceptor          |
| Formate oxidation      | 369 ± 181                         | Methyl viologen as an electron acceptor          |
| Formate production     | 348 ± 34                          | CO dependent formate production                 |

**Supplementary Table 6. Bioreactor parameters of T. onnurineus BCF13.**

| Parameter                             | Value                      |
|---------------------------------------|----------------------------|
| Maximum specific growth rate, $\mu_{max}$ (h$^{-1}$)$^a$ | 0.621 ± 0.051             |
| Formate production rate (mmol/L/h)$^b$   | 13.1 ± 0.9                 |
| Specific formate productivity (mmol/g-cells/h)$^b$ | 90.2 ± 20.4               |

$^a$ The $\mu_{max}$ was determined using the values of the linear regression slope in time windows of 1 to 4.5 hours.

$^b$ Values were determined by dividing total yield by time difference from 2 to 6 hours.
### Supplementary Figure 1. Alignment of the amino acid sequence of Fe-S proteins within the CFOR in *T. onnurienus* (*To*) and other Fe-S proteins in which crystal structure has been determined.

The conserved [4Fe-4S] cluster is boxed, and the position of conserved Cys residues are indicated as bold. **a**, Amino acid sequences of Fdh3B and CodhA subunits are aligned with 4[4Fe-4S] clusters.
containing FdnH (PDB 1FDI) in *E. coli* (*Ec*). b, Multiple alignments of Fdh3C subunit was achieved with other 2[4Fe-4S] clusters containing ferredoxins (Fd) in *Clostridium acidurici* (*Ca*, PDB 1FDN), *Gottschalkia acidurici* (*Ga*, PDB 1FCA), *Clostridium pasteurianum* (*Cp*, PDB 1CLF), *Thauera aromatic* *a* K172 (*Ta*, PDB 1RGV), *Allochromatium vinosum* (*Av*, PDB 1BLU), and *Pseudomonas aeruginosa* (*Pa*, PDB 2FGO). The multiple alignments were obtained using Clustal Omega program on the webserver (https://www.ebi.ac.uk/Tools/msa/clustalo/).
Supplementary Figure 2. Proposed models of synthetic carbon monoxide:formate oxidoreductase (CFOR). a, The fusion constructions of Fdh3C-CodhA with linker (GGGGS)$_{1-3}$. b, The fusion constructions of Fdh3B-CodhA with linker (GGGGS)$_{1}$. 
Supplementary Figure 3. Schematic diagram of mutant construction. a, Additional deletion of fdh3 gene cluster for the construction of *T. onnurineus* D04 strain. Strain D02 was transformed with the pFdh1,2,3clusterA1135 vector containing a fdh3 gene cluster flanked by two ~1 kb DNA fragments, LA (left-arm) and RA (right-arm). Target gene cluster deletion was achieved by double-crossover event. b, Construction of *T. onnurineus* D05 mutant by the transformation of pFdNStrpCdhC2008 which has not including flexible linker fusion.
Supplementary Figure 4. Cell growth (a), formate production (b), and pH changes (c) of strain D02 (●) and BCF01 (▼) in 100% CO supplemented serum vials. Error bars represent the standard deviations (n = 3).
Supplementary Figure 5. Cell growth (a) and pH changes (b) of *T. onnurineus* parental strain D02 (●) and *T. onnurineus* BCF01 (▼), with 0.1 M bis-Tris propane buffer (pH 6.5). Error bars represent the standard deviations (n = 3).
Supplementary Figure 6. CO dependent formate production during serum vial cell growth. a, Cells were incubated at 80°C with 2 bar of CO gas in serum vial. Cell growth was monitored by measuring OD\textsubscript{600}. Symbols indicate \textit{T. onnurinues} D02 parental strain (●), BCF01 (▼), BCF02 (■), and BCF03 (◆). b, Formate production was measured using HPLC. Symbols are identical to cell growth. c, The relative formate productivity at 24 h has compared between BCF01 set to 100% and the other strains. Error bars represent the standard deviations (n = 3).
Supplementary Figure 7. Partial pressure changes of *T. onnurineus* BCF 13 (a) and *T. onnurineus* D05 (b) during cell suspension experiments. The headspace was filled with 2 bar (gauge pressure) of CO/CO\(_2\) (50:50 v/v) mix gas (closed symbols) or CO/N\(_2\) (50:50 v/v) mix gas (open symbols). The partial pressure was determined by gas chromatography. Symbols: ● and ○, with P(CO); ▼ and ▽, with P(CO\(_2\)); ■ and □, with P(H\(_2\)). Error bars represent the standard deviations (n = 3).
Supplementary Figure 8. Effect of pH on the formate production by the CFOR complex. The activity was assayed at 80°C with CO/CO$_2$ (50:50 v/v) mix gas. Symbols indicate 50 mM 4-Morpholineethanesulfonic acid (MES) (●), 50 mM sodium phosphate (▼), 50 mM bis-Tris propane (■), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (◆), and Tris-HCl (▲). Data obtained from one experiment.
Methods

Strains and cell culture conditions

_T. onnurineus_ NA1 wild-type and mutant strains were routinely cultured in modified medium 1 (MM1) containing 4g/L of yeast extract (BD Bioscience, San Jose, USA) and 4x Holden’s trace element/Fe-EDTA solution at 80°C. All procedures for the cultivation of _T. onnurineus_ strains were carried out in an anaerobic chamber (Coy Laboratory Products, Grass Lake, USA). For the cultures in serum bottles, bis-Tris propane (pH 6.5) buffer was additionally supplemented in the medium to a final concentration of 0.1 M, and the headspaces were filled with 100% CO (MMC) was provided to support the growth of wild-type and mutant strains. The serum bottles were sealed with bromobutyl rubber stops and aluminum crimp caps. All procedures were carried out under strictly anaerobic conditions.

For the pH-stat batch culture, _T. onnurineus_ strain BCF13 was serially cultured in a 150-ml serum bottle and 7 L bioreactors (Fermentec, Cheongwon, South Korea); the working volumes of which were 80 ml and 5 L of MM1 medium, respectively, at 80°C. The bioreactors were sparged with pure argon gas (99.999%) through a microsparger. The agitation speed was 500 rpm, and the pH was controlled at 6.2 using 2 M KOH in 3.5% NaCl. The inlet gas of 100% CO was supplied by using a mass flow controller (MKPrecision, Seoul, South Korea) at feeding rates of 100 - 610 ml/min.

_E. coli_ EPI300™-T1R (Epicentre Biotechnologies, Madison, USA) strain was used for fosmid based molecular cloning purposes. Fosmid containing _E. coli_ clones were cultured in LB medium containing 12.5 μg/ml chloramphenicol. General molecular biology manipulations and microbiological experiments were carried out by standard methods.

Cloning and construction of the CFOR expression vector

The cloning strains, plasmids and fosmids used in this study are listed in Supplementary Table 1. The _fdh3_ gene cluster deletion mutant (strain D04) was constructed by the previously used gene disruption system in _T. onnurineus_ NA1 (Supplementary Fig. 3a). To construct fosmid vector backbone, previously constructed complementary insertion site (Left_arm (TON1128-TON_1127) region-P0157 promotor-HMG cassette-Right_arm (TON_1126) region) was amplified by PCR using the primers
listed in Supplementary Table 2. The amplicon and fosmid vector pCC1FOS (Epicentre Biotechnologies, Madison, USA) were assembled into a single vector, pNA1comFosC1096 (Supplementary Table 1), using a SLIC method45. PCR products of the *T. onnurineus* NA1 Fdh3 encoding gene region (*focA-fdh3ABC* or *focA-fdh3AB*), Cdh encoding gene region (*codhABCD*), and fosmid vector backbone (pNA1comFosC1096 with *Avr*I enzyme digestion) were assembled into a single vector using Gibson Assembly Master Mix (New England Biolabs, Ipswich, USA). The fusion targeting Fe-S protein-encoding genes, *fdh3B* or *fdh3C*, and *codhA* from *T. onnurineus* NA1 were fused using a homologous recombination event with 26 bp complementary PCR primers to generate (GGGGS)$_{1-2}$ flexible linker sequence during the gene assembly reaction by the Gibson Assembly method. The 3'-end of *fdh3B* or *fdh3C* gene lacking its stop codon was fused to the 5'-end of *codhA* gene lacking its start codon mediated by (GGGGS)$_{1-2}$ linker (Fig. 1). The sequences of the fusion genes were verified by DNA sequencing. Transformations of *T. onnurineus* strains with the constructed fosmid and the confirmation of transformants were performed as previously described27. The primers used to create the fusion genes are listed in Supplementary Table 2.

**Analytical methods**

Cell growth was monitored by measuring optical density at 600 nm (OD$_{600}$) with a spectrophotometer (Eppendorf, Hamburg, Germany). The unit value of OD$_{600}$ corresponded to 0.361 g/L (dry cell weight) as previously determined35. The amounts of CO, CO$_2$, and H$_2$ gas were measured using a gas chromatograph (GC) as described previously35. Formate was measured by YL9100 high-performance liquid chromatography (HPLC; YL Instrument Co., Anyang, South Korea) with a Shodex RSpak KC-811 column (Showa Denko, Kanagawa, Japan). Ultrapure water containing 0.1% (v/v) phosphoric acid was used as the mobile phase at a flow rate of 1.0 ml/min. All samples were prepared with 1 ml of culture broth and centrifuged to remove cell debris at 4°C, 13,480 × g for 5 min. The supernatants were purified with a syringe filter and analyzed by HPLC.

**Cell suspension experiment**
To prepare cell suspensions, *T. onnurineus* strain BCF13 was anaerobically cultured in a 7-L fermentor with a working volume of 3 L as described above. At the end of the culture, the cells were harvested by centrifugation at 5,523 × g for 30 min at 20°C and then washed two times with an anaerobic modified PBS (600 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, and 2 mM DTT). Finally, cells were resuspended in the same buffer at cell densities of OD₆₀₀ = 0.5. Four milliliters of cell suspensions were transferred to a 20-ml serum vial under a headspace of CO/CO₂ (50:50 v/v) mix gas at about 2 bar (gauge pressure), or CO/N₂ (50:50 v/v) mix gas at about 2 bar (gauge pressure), respectively. The cell suspensions were incubated at 80°C, and then the formate concentration and headspace gas composition were determined by HPLC and GC, respectively.

**Purification of enzymes**

Purification of CFOR enzyme complex was carried out as described previously. Typically 2 to 4 g (wet weight) of fed-batch cultured *T. onnurineus* strain BCF13 cell pellets were harvested and washed with the modified PBS and then resuspended in buffer W (100 mM Tris–HCl, 150 mM NaCl, pH 8.0). Cells were then disrupted by sonication on ice, and cell debris was removed by centrifugation (15,000 × g for 40 min at 4°C). Affinity column purification was carried out following the manufacturer’s protocols with a Strep-Tactin system (IBA-Lifesciences, Göttingen, Germany). The molecular weight and additional purification of CFOR complex were determined by analyzing the purified protein on a calibrated Superdex 200 10/300 GL column equilibrated buffer W using fast protein liquid chromatography (Äkta FPLC System, Amersham Biosciences). The column was calibrated by using these standards: thyroglobulin (669 kDa), apoferritin (443 kDa), beta-amylase (200 kDa), albumin (66 kDa) and carbonic anhydrase (29 kDa). Strep-tag purified protein was loaded and eluted at a flow rate of 0.5 ml/min, then the fractions were selectively collected at about 488 kDa of a single peak. The above procedures were carried out under anaerobic conditions. The purified proteins by size exclusion chromatography were used for enzyme assay, and examined via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the standard methods. The major bands were identified by LC-MS/MS analysis service (Yonsei Proteome Research Center, Seoul, Korea).
Enzyme assays

CODH activity was assayed at 80°C by a colorimetric assay with methyl viologen (MV) as an electron acceptor \( (\varepsilon_{578} = 9.7 \text{ mM/cm at 578 nm}) \), and CO as an electron donor. The assay was conducted with 4.4 ng of purified CFOR complex in 2 ml volume of sodium phosphate buffer (50 mM sodium phosphate, and 2 mM DTT, pH 7.5) containing 5 mM MV in a 4 ml serum-stoppered glass cuvette. 100% CO gas was purged in the headspace at about 2 bar (gauge pressure), and then the reaction was initiated by incubation of the reaction mixture at 80°C. Formate dehydrogenase activity was assayed using 360 ng of purified CFOR complex under the same conditions except that CO was replaced by 10 mM sodium formate. The formate production assay was carried out in 2 ml volume of 50 mM sodium phosphate buffer (pH 7.5) containing 2 mM DTT and 50 ug of purified CFOR in a 20 ml serum-stoppered vial. CO gas was purged in the headspace at about 2 bar (gauge pressure) of CO/CO\(_2\) (50:50 v/v) mix gas, or at about 0.5 bar of 100% CO gas. The reaction was initiated by incubation at 80°C. Formate in reaction mixture was determined by HPLC.

Thermodynamic calculation

The biological standard Gibbs energy value \((\Delta G^\circ)\) was calculated by the Nernst equation using values of the standard Gibbs energy \((\Delta G^\circ)\) reported by Amend and Shock\(^4\)