

Genetic engineering studies of
Ni-carbon monoxide dehydrogenase
from a thermophilic carboxydotrophic bacterium

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Chapter 1

General introduction

Carbon monoxide utilizing microbes and carbon monoxide dehydrogenases

Carbon monoxide (CO) is well known as a toxic gas, especially, CO is toxic for human being, binding strongly and almost irreversibly to hemoglobin (100). Despite the potent toxicity, many recent researches have suggested CO is key metabolic function and signal transduction in eukaryote, archaea and bacteria. For example, in human CO is important molecule in neural signaling and human circadian rhythms are regulated by CO-responsive transcriptional regulators (19, 36, 78). Notably, both cultivation and genome sequence studies of bacteria and archaea have demonstrated that numerous anaerobic microorganisms can utilize CO as a source of carbon and/or energy for their growth (29, 85, 92). These CO-utilizing anaerobic microbes use Ni-containing carbon monoxide dehydrogenases (CODHs). CODHs are the enzyme for reversible oxidation of CO to CO₂ ($\text{CO} + \text{H}_2\text{O} \rightleftharpoons \text{CO}_2 + 2\text{e}^- + 2\text{H}^+$). CODH genes are widely found in anaerobic bacteria and archaea including hydrogenogen, acetogen, methanogen and sulfate-reducing microbes. Specifically, about 6% of sequenced bacterial and archaeal genomes possessed at least one CODH catalytic subunit gene (*cooS*) (130). CODH genes were also found in several metagenomic data sets such as iron mountain acid mine drainage, mangrove site in the global ocean sampling and hindgut of a wood-feeding higher termite (129, 132, 133, 137, 143). Recent phylogenetic analyses of CODH genes demonstrated CODH showed extensive diversity, being divided into six distinct clades (Clade A to Clade F), in addition, among CODH encoding organisms, more than 40% organisms encode multiple CODHs (130). While the detailed metabolic role of CODH, except a few model enzymes, remains unknown, it is

suggested that these CODHs play significant role in their CO metabolism (63, 102).

Hydrogenogenic CO-utilizing microbes

Hydrogenogenic CO-utilizing microbes can grow utilizing CO and producing CO₂ and hydrogen, as waste products (115), which is analogous to the water-gas-shift reaction (43, 80, 152). CODH-hydrogenase enzyme complex, encoded by CODH/hydrogenase gene cluster, play a key role in these microbes. This gene cluster includes CODH catalytic subunit gene (*cooS*), a ferredoxin like gene (*cooF*), CODH Ni insertion maturation gene (*cooC*), CO-dependent transcriptional regulator gene (*cooA*), genes of a six-subunit of proton-pumping hydrogenase (Ech) and several hydrogenase accessory genes (97). As a result of 988 microbial genome analyses, the CODH/hydrogenase gene cluster are specific to these microbes (130). In addition, CODH-hydrogenase complex were isolated from *Rhodospirillum rubrum* and *Carboxydotherrmus hydrogenoformans* where CODH catalytic subunit, ferredoxin-like protein and several hydrogenase genes were identified (26, 110, 113). These results demonstrated CODH generates the electrons from CO and the electrons are subsequently conveyed via the ferredoxin-like protein CooF to the hydrogenase and finally hydrogenase reduces intracellular protons to H₂. This reaction would causes ion translocation through the cytoplasmic membrane.

The model organsims of hydrogenogenic CO-utilizing microbes are *R. rubrum* and *C. hydrogenoformans* (59, 123). Since the discovery of *C. hydrogenoformans*, which was isolated from a hot swamp of Kunashir Island, a lot of themophilic and strictly anaerobic hydrogenogenic CO-utilizing microbes have been isolated from hot environment such as fresh water hot spring and hydrothermal vents (83, 111, 112, 114, 116, 117, 151). Techtmann *et al.* proposed in thermophile microbial consortia, the hydrogenogenic CO-utilizing microbes played a key ecological role as a ‘safety valve’, i.e. not only reducing toxic level of CO which is derived from some microbial metabolism such as methanogenesis and sulfate reduction,

volcanic volatile emission and photo-degradation of organic substrates but also providing H_2 as substrates for this microbial consortia (24, 34, 44, 129, 135). From the view point of application, these microbes are potentially applied to the industrial H_2 production. Especially, considerable studies have focused on the H_2 production from synthesis gas (syngas) using these hydrogenogenic CO-utilizing microbes (21, 39, 153). Referred to syngas, through gasification, organic wastes are converted into a mixture of CO, CO_2 and H_2 gas (36, 42). CO-utilizing hydrogenogenic microbes can efficiently produce H_2 from this wastes.

CO-utilizing Acetogen

Some acetogen can grow utilizing CO and producing acetate (15, 104). Many acetogen produce acetate from CO_2 or organic substrate via reductive acetyl-CoA pathway (Wood-Ljungdahall pathway) (94, 103, 145, 146). In this pathway one CO_2 molecule is converted to methyl-group by several enzymes and one CO_2 molecule is reduced to CO by CODH-Acetyl-CoA synthase enzyme complex (CODH/ACS), respectively. Subsequently, they are combined with CoA by CODH/ACS to form acetyl-CoA. Enzymes which constitute acetyl-CoA pathway are encoded by CODH/ACS operon. Early study of acetogen showed CO can be directly metabolized through this pathway, mainly using CODH/ACS (18, 45, 96). In addition to CODH/ACS, several CODH catalytic subunit genes which do not constitute CODH/ACS and CODH/hydrogenase gene cluster (monofunctional CODH) were identified on the genomes of many acetogens (63), suggesting these monofunctional CODHs play an important role in CO-utilizing acetogen. However, the metabolic function of these CODHs is not well-known. The model organism is *Moorella thermoacetica*, and many *Clostridium* showed this CO-utilizing ability (2, 70). Recently, in hindgut microbial communities of lower termites and the wood roach, CODH genes including both CODH/ACS and monofunctional CODH were identified (76, 138). This suggested CODH play a significant role in the acetogen living in such

environment. CO-utilizing acetogen was potentially applied to industrial field, because some organisms such as *Clostridium carboxydovorans* produce significant amounts of ethanol, butyrate and butanol during growth on CO. Many studies have focused on production of these useful products from synthetic gas using genetic engineered CO-utilizing acetogen (12, 64, 91, 93).

CO-utilizing Methanogen

Several methanogenic archaea show the capacity to grow on CO (14, 16, 29, 84, 101). In CO-utilizing methanogenic archaea, CODH is involved in aceticlastic energy metabolism, carbon fixation and methanogenesis. On the genome of CO-utilizing methanogen, both CODH/ACS and monofunctional CODH were identified. CODH/ACS fixes CO₂ through an analogous route to acetyl-CoA pathway. In contrast, in aceticlastic methanogenesis metabolism, CODH/ACS split acetyl-CoA. The CO derived from this ACS split reaction is subsequently oxidized to CO₂ by CODH/ACS generating the electrons for reduction of the methyl-group to methane (33, 75, 85). Detail metabolic function of the monofunctional CODHs remain unknown like CO-utilizing acetogen, however it was suggested that some of these enzymes play electron supplier to methanogenesis pathway through CO-oxidation (30, 77). Notably, some autotrophic methanogen such as *Methanosarcina barkeri* produce substantial amounts of H₂ during carboxydotriohic growth, suggesting CO oxidation by CODH/ACS and/or the monofunctional CODH coupled H₂ production by hydrogenase (84).

CO-utilizing Sulfate reducing microbes

The ability to utilize CO was found in several sulfate reducing microbes (72, 89, 90). Monofunctional CODH genes were identified on the genomes of many sulfate reducing bacteria. It was suggested that for most sulfate reducing microbes this CO-utilizing ability derived from the CODHs was for detoxification of CO. The reasons are as follows; first most CO-utilizing

sulfate reducing microbes except for *Desulfotomaculum carboxydovorans* and *Archaeoglobus fulgidus* are quite sensitive to high level of CO in gas phase (4-20%) (40, 90). Second it has been shown that *Desulfovibrio vulgaris* produce CO during normal growth. Under this condition, CO concentration could build up to 6000 ppm in the head space (135). *D. carboxydovorans* and *A. fulgidus* grow in the high-level CO of ~80% and ~100%, respectively. Compared to CO-sensitive sulfate reducing microbes, *D. carboxydovorans* and *A. fulgidus* have larger number of CODHs, suggesting these CODHs can be considered to the cause of the ability (61). How sulfate reducing microbes couple CO oxidation to energy conservation remains unclear, however, in *A. fulgidus* VC-16, cultivation experiments showed that oxidation of CO to CO₂ was coupled to sulfate reduction (40). Taking full advantage of sulfate reducing microbes which can grow under high level of CO such as *D. carboxydovorans*, these microbes are good candidate for biosulfurization processes of sulfate containing waste water using synthesis gas as substrates.

CODH and CODH/ACS

CODHs from *M. thermoacetica*, *R. rubrum*, and *C. hydrogenoformans* have been most extensively studied. Since Yagi first identified an enzyme that catalyzes the oxidation of CO to CO₂ (150), mainly because of its extreme oxygen sensitivity, it took numerous years to purify CODH to homogeneity (97). When the *M. thermoacetica* CODH was purified to homogeneity, it appeared to contain 2 mol of nickel per mol of $\alpha\beta$ dimeric enzyme (95). Many researches have also focused on CODH from *R. rubrum*. In addition to enzyme analyses (10, 11, 67, 120, 128), *in vivo* analyses of CODH such as transcriptional analysis of CODH genes were performed using gene disruption and replacement system of this organism (9, 57, 108). In 2001, from *C. hydrogenoformans*, CODH-I and CODH-II were purified. It was shown that these enzymes are

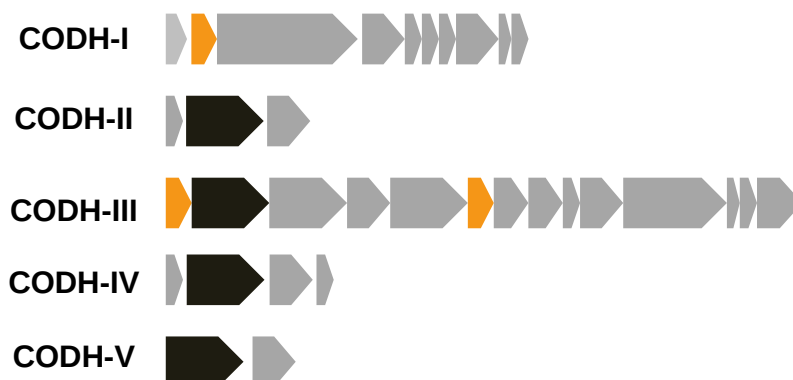
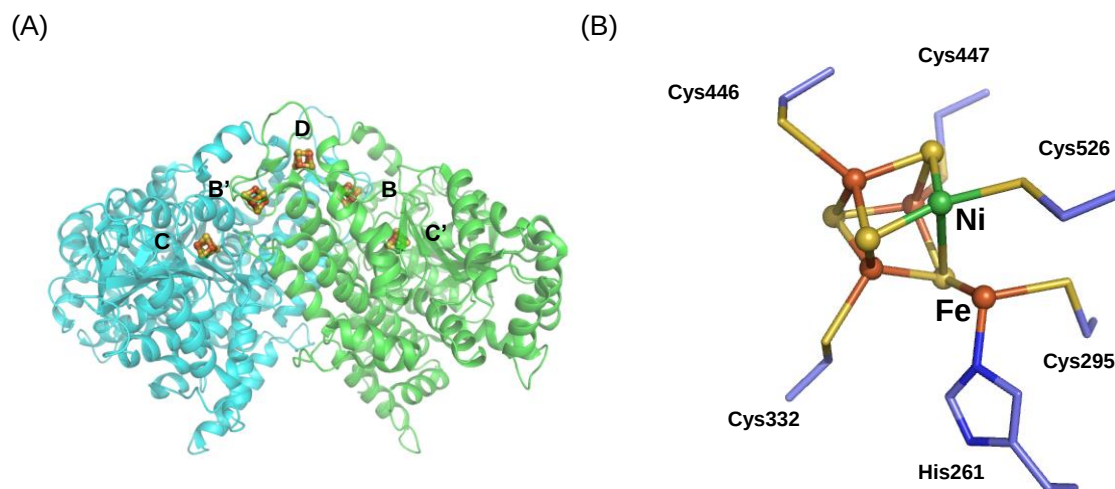
composed homo-dimer and contains 1.3 to 1.4 and 1.7 Ni atom, 20 to 22 and 20 to 24 Fe atoms, respectively (125). Both enzymes oxidize CO very efficiently i.e. k_{cat}/K_m values at optimal temperature of $1.3 \cdot 10^9$ and $1.7 \cdot 10^9 \text{ M}^{-1}\text{CO s}^{-1}$, respectively. In addition, immunogold labeling analysis revealed both CODHs were associated with inner cytoplasmic membrane (125). These early studies of CODHs demonstrated CODH is complex metal enzyme like hydrogenase. Thus, in order to reveal metal binding system various spectroscopy such as ESR and Mossbauer has been applied (67, 97).

Recently crystal structures of several CODHs from *C. hydrogenoformans*, *R. rubrum*, *M. thermoacetica* and *M. barkeri* were determined, and the basic structures of CODHs were similar (17, 22, 23, 35, 53, 124). In their structure, CODH constitute homo-dimetric structure where five metal-clusters called B, C and D were bound (Fig. 1-1). In this chapter, residue numbers are based on the structure of *C. hydrogenoformans* CODH-II, unless otherwise noted. The conventional [4Fe-4S] cluster (D-cluster) bridges two subunits covalently via two cysteines (Cys³⁹ and Cys⁴⁷) from each subunit. Another [4Fe-4S] cluster, called cluster B-cluster is located 10 Å away from D-cluster bounded by Cys⁴⁸, Cys⁵¹, Cys⁵⁶ and Cys⁷⁰. The active site, called C-cluster is bound by five cysteines (Cys²⁹⁵, Cys³³³, Cys⁴⁴⁶, Cys⁴⁷⁶ and Cys⁵²⁶) and one histidine (His²⁶¹), is located 11 Å away from cluster B'. C-cluster is composed of unique [Ni-Fe-S] cluster (52). In *M. barkeri* CODH structure, additional one [4Fe-4S] cluster was contained (35). The residues coordinating these clusters are highly conserved between CODHs (68).

Applications of CODH-I and CODH-II from *Carboxydotherrmus hydrogenoformans*

C. hydrogenoformans is a thermophilic anaerobic CO-utilizing bacterium isolated from a hot swamp of Kunashir Island (123). This bacterium is a model for hydrogenogenic CO-utilizing microbes, as discussed. Its complete genome was sequenced in 2005 (149), as a

result, this bacterium contains five genes encoding CODHs, termed CODH-I to CODH-V (Fig. 1-2). Several functions for the enzymes are proposed based on their gene context and in physiological experiments (125, 149); CODH-I, energy conservation conjugated with a proton-pumping hydrogenase; CODH-II, NADH generation; CODH-III, carbon fixation in the acetyl-CoA pathway; and CODH-IV, oxidative stress response. The physiological function of CODH-V remains unknown. Among CODH-I to -V, CODH-I and CODH-II are well characterized. CODH-I and CODH-II show almost similar characteristics. The primary difference between CODH-I and CODH-II was that CODH-II was strongly inhibited by CO and CN⁻, suggesting CODH-II is more suited to CO oxidation than CODH-I (136).



CODHs are potentially efficient catalysts to be feasible for an industrial CO₂ reduction system because; i), CODHs can catalyze the reduction of CO₂ to CO without over-potential whereas inorganic catalysts used for CO₂ activation and reduction require excessive over-potential (6, 88, 98, 109), ii) reaction product, CO is an industrially important chemical. Many studies have focused on converting the greenhouse gas CO₂ into valuable organic molecules by artificial catalysts and/or enzymes, and a desired goal is to reduce CO₂ to CO using solar energy (147). Since the first report of CO₂ photo-reduction by *Inoue et al.* (48), many studies have focused on photo-catalysts titanium dioxide (TiO₂) and many CO₂ photo-reduction systems have been constructed using the TiO₂ (62, 69, 82). Among CODHs, CODH-I is the model catalysts in CO₂ photo-reduction systems (Fig. 1-3). The reasons are in a previous report, CODH-I absorbed onto an electrode catalyzed the rapid and reversible conversion of CO₂ and CO (88, 136). In addition, CODH-I is the only enzyme which can interact with TiO₂ (147, 148). However, CODH-I over-expression system, which is essential for application CODH-I to CO₂ photo-reduction system, remain to be constructed.

CODHs are also considered to be model for construction of artificial CO₂ reducing catalysts (7). C-cluster, activity center of CODH, is composed of unique [Ni-Fe-S] cluster and the structure, especially the metal binding system in C-cluster, has provided significant information for construction of artificial catalysts. Among CODHs, CODH-II is the model catalysts of artificial catalysts because detailed structures and reaction mechanisms of CODH-II are revealed (31, 53, 106). Based on the CODH-II C-cluster's structure, several mimetic model artificial catalysts have been constructed. For example, a cubanoid structure, $[(\text{tdt})\text{NiFe}_3\text{S}_4(\text{LS}_3)]^{3-}$ (LS3: a semirigid trithiolate ligand, tdt: toluene-3,4-dithiolate), mimicked one of the key C-cluster features, i.e. the square planar and four-coordinate Ni²⁺ at one corner of the cubanoid was constructed (121). In addition, recently several complexes mimicking the

Ni^{2+} -Exo- Fe^{2+} component structure of the CODH-II C-cluster were constructed (46). However Ni-binding mechanism in C-cluster, which would be the most important for reactivity of C-cluster remains to be revealed.

The Aim of Study

Therefore, in Chapter 2, firstly I improved the over-expression system of CODH-II for further analysis and application of CODH-II. Secondly I established the over-expression system of CODH-I for CO_2 photo-reduction systems. In Chapter 3, in order to provide useful insight to construct artificial catalysts, I investigated Ni-binding mechanism in C-cluster using CODH-II.

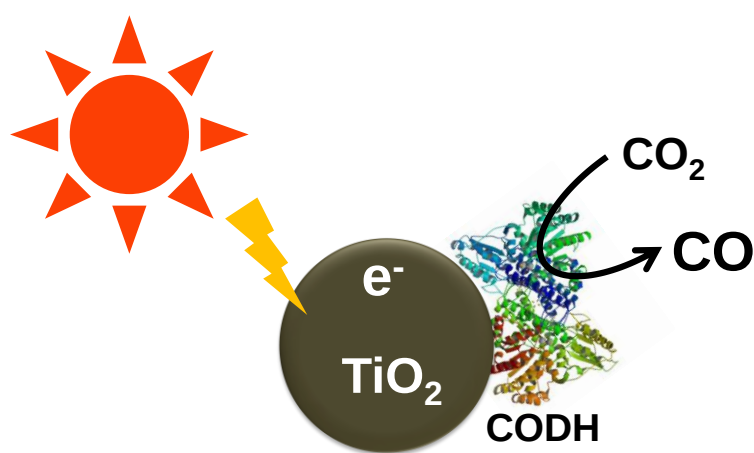


Figure 1-3 A CO_2 photo-reduction system using a CODH attached to TiO_2 nanoparticle
(149)

Chapter 2

Over-expression systems for carbon monoxide dehydrogenases (CODHs)

There are wide interests in application of CODH to industrial CO₂ reduction, i.e. photo-reduction of CO₂ and model of artificial CO₂ reducing catalysts. As discussed in Chapter 1, the model enzyme of the former is CODH-I and of the latter is CODH-II from *C. hydrogenoformans*. These CODH applications requires efficient over-expression systems as purification of native-CODH requires a lot of effort, especially cultivation of CO-utilizing microbes such as *C. hydrogenoformans* with a CO-purge. However, CODH-I over-expression system remains to be constructed. Additionally, CODH-II over-expression system contains some issues to be solved, described below.

In general, heterologous production of active metalloprotein in genetically-tractable organisms such as *Escherichia coli* has met with limited success, due to complex maturation processes to assemble the complex activity center and/or its extreme oxygen sensitivity. For example, heterologous production of active hydrogenase has required numerous efforts (127). Hydrogenase catalyzes the reversible reaction of $\text{H}_2 \rightleftharpoons 2\text{H}^+ + 2\text{e}^-$ and is the most efficient catalyst for biological H₂ production (32, 60). The activity center of hydrogenase is composed of complex metal clusters (Ni-Fe cluster and Fe-Fe cluster), and correct assemble of the activity center require many maturation process by hydrogenase maturation proteins (8, 81, 107, 139). Recently, several heterologous expression systems of [Ni-Fe] hydrogenase have been established (25, 122, 141). These are accomplished through the co-expression of multiple plasmids carrying the entire or large part of hydrogenase maturation genes. Notably, active

2. Over-expression systems for carbon monoxide dehydrogenase

[Ni-Fe] hydrogenase from *Pyrococcus furiosus* was successfully expressed using unique plasmids where catalytic subunit genes and maturation genes were induced under anaerobic condition using *P. furiosus* hydrogenase promoter (*Phya*) (122).

In early stage of CODH over-expression studies, several studies aimed to express *acsA* and *acsB* encoding β and α subunit of CODH/ACS from *M. thermoacetica*, respectively (71). Establishment of an efficient over-expression system for the CODH in genetically-tractable organisms was difficult which also hold true for all metalloproteins in general. Roberts et al. cloned and expressed these genes and expressed in *E. coli* (99). However, the enzyme was inactive and it was demonstrated that the recombinant enzyme lacked native tetrameric structure. Morton et al. also attempted to express, however, recombinant enzyme appeared to lack C-cluster (78). In 2001, the expression systems for *acsA* and *acsB* genes in *E. coli* have been successfully constructed (71). In the over-expression system, the recombinant CODH/ACS show essentially equivalent character to native enzyme. Briefly, the recombinant CODH/ACS was expressed under anaerobic condition and high-concentration of Ni^{2+} , and after purification of the recombinant enzyme was incubated in high concentration of Ni^{2+} overnight under Argon or CO (Ni-activation). Recently, *cooS-II* gene encoding CODH-II from *C. hydrogenoformans* was reported (53, 106). Notably, Jeoung et al. reported the recombinant CODH-II (Rec-CODH-II) showed CO oxidation activity that was nearly equal to native CODH-II (52, 53). In this expression system, *cooS* gene was induced under anaerobic condition and high-concentration of Ni^{2+} using medium bottles. Novel future was for correct assemble of [Fe-S] clusters, in this system pRKISC plasmid carrying [Fe-S] cluster biosynthesis genes and Na_2S which is a sulfide source for [Fe-S] clusters and reductant for reduction of the media. However this over-expression has some issues to be solved. Mainly the system inevitably produces the hydrogen sulfide derived from Na_2S and is not suited for large-scale systems.

2. Over-expression systems for carbon monoxide dehydrogenase

Therefore, in Chapter 2-1 I improved the CODH-II over-expression system toward large-scale and simple system, and in Chapter 2-2 I constructed CODH-I over-expression system. Presently, several maturation genes of CODH-I such as *cooC* has been identified, thus using a Ni-insertion maturation gene co-expression, I attempted to construct CODH-I over-expression system.

Chapter 2-1

A simple and large scale over-expression method for carbon monoxide dehydrogenase-II from thermophilic bacterium *Carboxydotherrmus hydrogenoformans*.

Introduction

CODHs are strictly anaerobic metalloenzymes; CODHs have 3 to 5 [4Fe-4S] clusters and the activity center is composed of novel [Ni-Fe-S] clusters. Over-expression systems for CODHs in *E. coli*, as with metalloprotein in general, have met with limited success due to the complexity of their maturation process that is required to assemble the activity center. In 2001, the expression systems for *acsA* and *acsB* genes encoding β and α subunit of CODH/ACS of *M. thermoacetica*, respectively, using *E. coli* have been successfully constructed (71). The recombinant CODH/ACS was expressed under anaerobic condition with the medium containing high-concentration of Ni^{2+} . In addition, after purification, the recombinant enzyme was incubated in high concentration of Ni^{2+} overnight under Argon or CO atmosphere (Ni-activation). As-isolated recombinant enzyme (before Ni-activation) contained a full complement of [Fe-S] clusters while less content of Ni (1.6) than expected (2.0). The Ni-activated enzyme showed exhibited CO oxidation activity of 280 U/mg and significant CO exchange activity. In addition, redox and spectroscopic properties of as-isolated and Ni-activated enzyme are nearly indistinguishable from those of native enzyme. In the report, Loke *et al.* concluded the recombinant CODH/ACS was essentially equivalent to native enzyme (71).

Recently, the expression system for *cooS-II* gene encoding CODH-II catalytic subunit from *C. hydrogenoformans* in *E. coli* has been reported (53, 106). Notably, Jeoung *et al.* reported the

recombinant CODH-II (Rec-CODH-II) shows CO oxidation activity that is nearly equal to native CODH-II (52, 53). In this expression system, host cell harboring *cooS* gene was cultivated with medium containing high-concentration of Ni^{2+} using medium bottles. The cells were cultivated aerobically with air purged, and subsequently, the gas was switched to pure N_2 with IPTG induction. After the induction, cells were cultivated under anaerobic condition. Additionally, for correct assemble of [Fe-S]clusters, pRKISC plasmid carrying [Fe-S] cluster biosynthesis genes and Na_2S which is a sulfide source for [Fe-S] clusters and reductant for reduction of the media. However, this over-expression has some issues to be solved. Firstly, the expression system requires a strict anaerobic condition with a bottle closed by screw capped butyl rubber septum. Moreover, the system inevitably produces the hydrogen sulfide derived from Na_2S which is a sulfide source for [Fe-S] clusters and reductant of the media. Therefore, this culture method is not suitable for large scale culture. Given the tremendous utility of Rec-CODH-II, I attempted to establish the large scale over-expression system for CODH-II using a fermentor and characterize the purified Rec-CODH II.

Materials and methods

Construction of expression vector

C. hydrogeniformans Z2901 (DSM 6008) was purchased from DSM (Germany) and cultivated as described previously (19, 123). Genome DNA was isolated from the cells using DNeasy Blood & Tissue Kits (Qiagen Chatsworth, CA). All primer sequences used for cloning and mutagenesis of *cooS-II* gene are shown in Table 2-1-1. The *cooS-II* gene was amplified from the genomic DNA of *C. hydrogeniformans* using PCR with the primers CODH-II_Fw1_NdeI and CODH-II_Rv1_BamHI. The PCR product was cloned into PTA2 plasmid using Target Clone TM -Plus- kit (TOYOBO, Osaka, Japan). The Quick Change

site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to introduce mutations into the *cooS*-II gene. Two primers, delta_cooS II_Fw and delta_cooS II_Rv, were used. PCR products were treated with the *DpnI* restriction enzyme and transformed. A plasmid was isolated and nucleotide sequencing confirmed resulting in pTCODH-II_NA. The mutated *cooS*-II gene was amplified from the pTCODH-II_NA DNA using PCR with the primers CODH-II_Fw1_NdeI and CODH-II_R_Rv1_BamHI. The product was cloned into the *NdeI* /*BamHI* sites of the pET28a(+) vector (Novagen, Madison, WI).

Heterologous expression and purification of recombinant CODH-II

The N-terminal (His)₆-tagged CODH-II was expressed in *E. coli* Rosetta2 (DE3) (Novagen, Madison, WI) harboring the pRKISC plasmid (a generous gift from Y. Takahashi, Saitama University, Saitama, Japan) (79). All culture media used in this study are shown in Table 2-1-2. The transformant was cultured aerobically at 30°C in a 10 L fermentor (Sanki Seiki, Osaka, Japan) with 5 L of modified TB medium containing 0.02 mM NiCl₂, 0.1 mM FeSO₄, 2 mM L-cysteine, 50 µg/ml kanamycin, 50 µg/ml chloramphenicol and 12.5 µg/ml tetracycline and purged with air. When the culture reached an OD₆₀₀ of 0.6-0.7, the gas was switched to pure N₂. After 30 min, 0.5 mM NiCl₂, 1 mM FeSO₄, 50 mM KNO₃, 2 mM L-cysteine and 0.2 mM IPTG were added to induce expression. After 20-22 h, the cells were harvested aerobically. The cell pellet was frozen for storage at -80 °C until needed. The following purification steps were performed in an anoxic glove box (COY Laboratory Products Inc., Grass Lake, MI) under an atmosphere of 95% N₂/ 5% H₂ at 25 °C, (except for a cell disruption step) and all buffers used in this study were made anaerobic by autoclaving (121°C, 20 min) and bubbling by pure N₂ for 30 min. The cells were disrupted using a French press (5501-M, Ohtake Factory, Tokyo, Japan) and the cell free extract was centrifuged at 7,100 g for 30 min at 25 °C. The supernatant was applied to Cosmogel His-Accept column (Nacalai Tesque, Kyoto, Japan). After extensive

washing with buffer A (50 mM Tris-HCl, pH 8.0, 300 mM NaCl) containing 20 mM imidazole

Table 2-1-1 Primers used in this study.

Name	Sequence (5' → 3')
CODH-II_Fw1_NdeI	CATATGGCTAAGCAAAATTTAAAGTCTAC
CODH-II_Rv1_BamHI	GGATCCGAGCTCACCATGGTAATCCCAGGCC
delta_cooS-II_Fw	CACACAGGAACCCATGTGGAGGACCGGTGGC
delta_cooS-II_R1	CACCGGTCCTCCACATGGGTTCCTGTGTG
CODH II_Seq1	TGGGTTGTGATGCCGATGC
M13_Fw	GTAAAACGACGGCCAG
M13_Rv	CAGGAAACAGCTATGAC
T7_pro	TAATACGACTCACTATAGGG
T7_ter	GCTAGTTATTGCTCAGCGG

Table 2-1-2 Culture media used in this study.

Name	Composition
LB medium	10 g/l trypton, 5 g/l yeast extract, 5 g/l NaCl, pH7.0
TB medium	12 g/l trypton, 24 g/l yeast extract, 8.7 ml/l 100 % glycerol
Modified TB medium	12 g/l trypton, 24 g/l yeast extract, 8.7 ml/l 100 % glycerol, 0.02 mM NiCl ₂ , 0.1 mM FeSO ₄ /7H ₂ O, 2 mM cysteine

and 1 mM dithionite, CODH-II was eluted with buffer A containing 300 mM imidazole and 1 mM dithionite. The eluent was subsequently subjected to Amicon Ultra (30 kDa MWCO, Millipore, Boston, MA) filtration and equilibrated in 50 mM Tris-HCl pH 8.0 and 2 mM dithionite to remove imidazole. Purity of the Rec-CODH-II was analyzed using 10 % (W/V) SDS-PAGE.

As a control, CODH-II was also expressed according to the previous method (52, 53). The transformant was cultured aerobically at 30°C in a 1.0 L Pyrex medium bottle with 500 mL of modified TB medium containing 0.02 mM NiCl₂, 0.1 mM FeSO₄, 0.1 mM Na₂S, 50 µg/ml kanamycin, 50 µg/ml chloramphenicol and 12.5 µg/ml tetracycline and purged with air (Table 2-1-2). When the culture reached an OD₆₀₀ of 0.6-0.7, the medium was aerated by pure N₂. After 30 min, 0.5 mM NiCl₂, 1 mM FeSO₄, 50 mM KNO₃, 2 mM L-cysteine and 0.2 mM IPTG were added to induce expression. After 20-22h, the cells were harvested aerobically. The cell pellet was frozen for storage at -80 °C until needed. The following purification steps were performed by same method as described above.

Enzyme assay

CO oxidation activity was assayed based on the CO dependent reduction of oxidized methyl viologens (MV ox) as described previously at the optimal growth temperature for *C. hydrogenoformans* (70°C) (125). One ml assay solution containing 20 mM MV ox and 2 mM dithiothreitol in 50 mM HEPES-NaOH pH 8.0 was filled in screw-capped cuvette sealed with a silicon septum with CO bubbling. Reactions were initiated by injecting Rec-CODH-II with a gas-tight syringe (HAMILTON Bonaduz AG, Switzerland). One unit of activity was defined as the reduction of 2 µmol of methyl viologen per minute.

CO₂ reduction activity was assayed at 25°C according to a previous report with minor modification with the increase in absorbance at 419 nm (27). Standard curve was prepared by

titrating samples of a saturated CO solution at 25 °C in the anaerobic chamber and the titration was performed with measuring spectral changes between 400 nm and 460 nm in assay cuvette (27). Assays were performed in 2 ml quartz cuvettes sealed by silicon septum with screw cap without free headspace (27). The assay solution contained 50 mM MOPS pH7.5, 0.2 mg/ml hemoglobin, 50 mM NaHCO₃ and various reductants (methyl viologen reduced with 0.3 mM dithionite, 2 mM dithionite and 5 mM Ti(III) citrate, respectively). This reaction solution was equilibrated for 2 min at 25 °C in anaerobic chamber until no spectral changes was occurred. Reaction was initiated by addition of Rec-CODH-II. The production of CO from CO₂ by Rec-CODH-II was determined by monitoring the increase in absorbance at 419 nm which is caused by the formation of carboxyhemoglobin. One unit of activity was defined as 1 µmol CO production per minute.

The reductants were added to the mixture from stock solution prepared in the anaerobic chamber. Stock solutions of dithionite and dithiothreitol were prepared freshly in water as 1 M concentration. Eighty three mM Ti(III) citrate was prepared according to a previous report (105), : 1 ml TiCl₃ (Merck) was dissolved in 4.8 ml 0.5 M Na₃-citrate and buffered by adding 6.2 ml 1 M Tris-HCl pH 8.0.

Spectrum of the Rec-CODH-II was recorded as described previously at room temperature (125). All UV-visible spectra were obtained on an Ultraspec 2100 *pro* spectrophotometer (Amersham biosciences, Little Chalfont Bucks, UK) with screw-caped quartz cuvette (1 cm path length, Hellma GmbH & Co) sealed with silicon septum.

Determination of protein concentration

Protein concentration of Rec-CODH-II was determined by BIO RAD PROTEIN ASSAY Kit (BIO-RAD Laboratories, Hercules, CA) with BSA as a standard.

SDS-PAGE analysis

The denatured samples were loaded on the gel with Protein Ladder One -Triple-color- (250 kDa, 130 kDa, 95 kDa, 72 kDa, 55 kDa, 43 kDa, 36 kDa, 28 kDa, 17 kDa, and 10 kDa) (Nacalai Tesque, Kyoto, Japan)

Results and discussion

After the induction with IPTG, transformed strain was cultured with additional L-cysteine instead of Na₂S as reductant and sulfide source, under anaerobic condition with N₂ purging. The catalytic subunit gene of CODH-II (*cooS-II*) from *C. hydrogenoformans* was expressed in *E. coli* and purified using a Ni affinity column. A one-step affinity purification produced apparent homogenous recombinant Rec-CODH-II as judged by single band at approximately 60 kD in SDS/PAGE analysis (Fig. 2-1-1). The purified Rec-CODH-II showed 8,500-9,600 U/mg of CO oxidation activity which was slightly higher than that of the Rec-CODH-II prepared in according to the previous method (Table 2-1-3) (53). Expression level was also slightly higher than that by the previous method (Table 2-1-3). Cell yield was 3.60 g/L nearly equal to that obtained (Table 2-1-3). UV-visible absorption spectra of Rec-CODH-II were similar to that of native-CODH-II under the various conditions I examined (Fig. 2-1-2). The ratio of absorbance at 419 nm to 280 nm was 0.43, which is close to that of the native-CODH-II (0.45) (52), suggesting that the Rec-CODH-II had a full complement of [4Fe-4S] clusters. Therefore, Rec-CODH-II was successfully expressed in this large expression system without addition of Na₂S.

CO₂ reduction activity was measured by monitoring the formation of carboxyhemoglobin at 25°C. Because hemoglobin lacks stability at higher temperatures, It was not able to determine the optimum temperature for the CO₂ reduction activity of this thermophilic enzyme. Various reductants including dithionite, Ti(III) citrate and dithionite

reduced methyl viologen were suitable for electron donor like *R. rubrum* CODH (Table 2-1-4) (27). Rec-CODH-II. Rec-CODH-II showed a highest CO₂ reduction activity of 16.9 U/mg at 25 °C using 0.3 mM dithionite reduced methyl viologen as a reductant, which is 1/10 of *R. rubrum* CODH (27).

In conclusion, I established the simple and large scale over-producing system for Rec-CODH-II. Moreover this system may provide safety since the system is free from hydrogen sulfide caused by Na₂S.

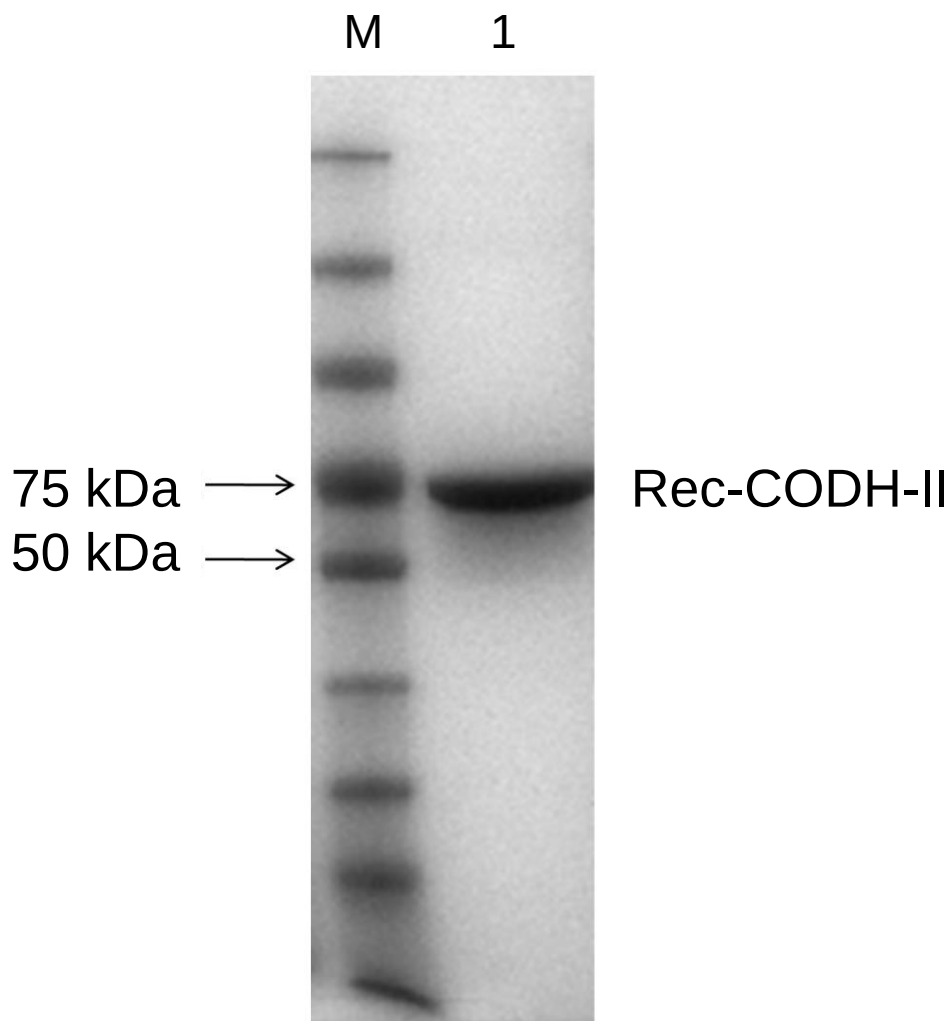


Figure 2-1-1 Ten percent (w/v) SDS/PAGE analysis. M, MW markers; Lane 1, Rec-CODH-II.

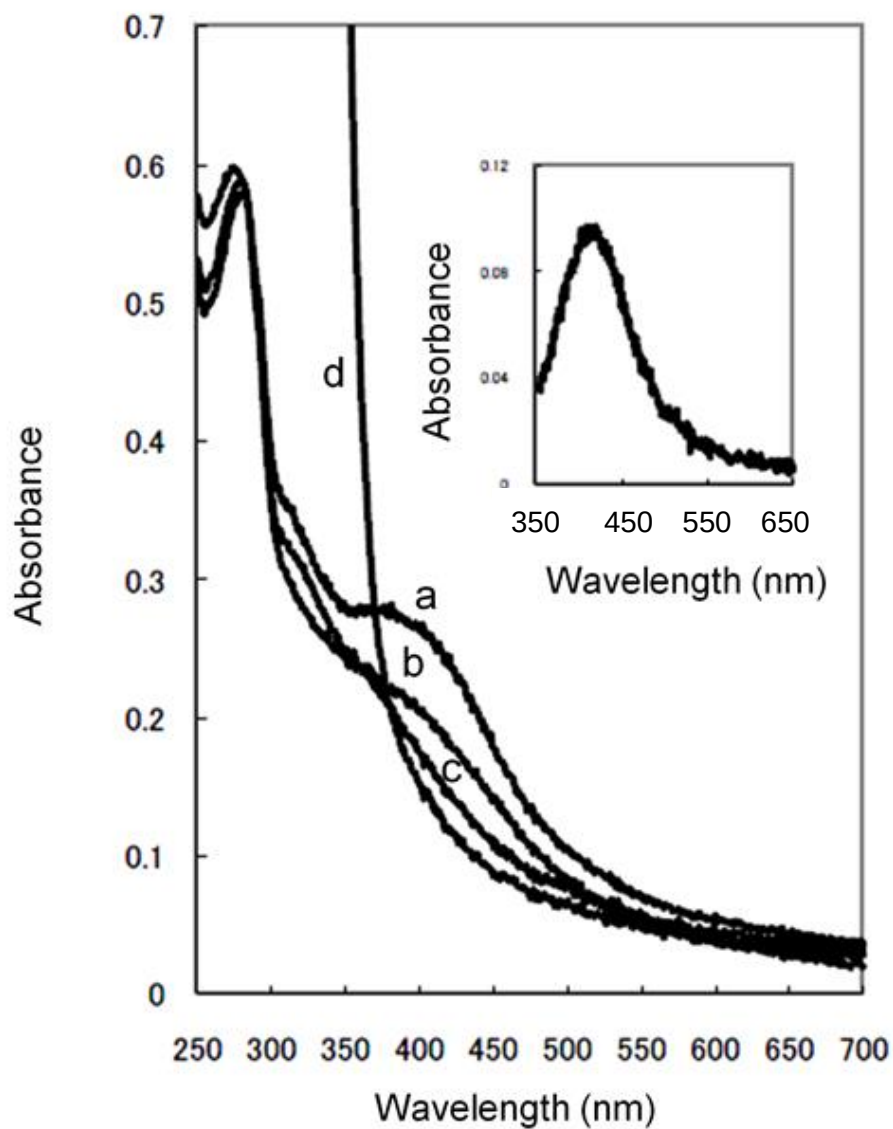


Figure 2-1-2 UV-visible absorption spectra of Rec-CODH-II. Condition for each curve: a, as-isolated; b, oxidized with air; c, reduced with pure CO; d, reduced with 2 mM dithionite under N_2 . Inset; a difference spectrum of condition a minus condition c.

Table 2-1-3 Comparison of the expression methods

	This study	Jeoung and Dobbek 2007
CO oxidation activity (U/mg)	8,500-9,600	8,070
Cell yield (g/L)	3.60	3.00
Expression level (mg/L)	2.61	1.76

Table 2-1-4 CO₂ reduction activity of Rec-CODH-II using various reductants

Reductants	Specific activity (U/mg)	Relative activity (%)
2 mM dithionite	0.33	1.0
2 mM dithionite plus		
0.1 mM methyl viologen	13.0	40
0.2 mM methyl viologen	15.4	47
0.3 mM methyl viologen	16.9	52
2 mM Titanium(III) citrate	2.45	7.5
4 mM Titanium(III) citrate	3.97	12
6 mM Titanium(III) citrate	4.50	14

Chapter 2-2

Over-expression of carbon monoxide dehydrogenase-I with an accessory protein co-expression: a key enzyme for carbon dioxide reduction

Introduction

CODHs have three to five [4Fe-4S] clusters, one of which is known to be the active center denoted C-cluster, a unique [Ni-Fe-S] cluster. CODHs are expected to be used as catalysts for an industrial CO₂ reduction system where the reaction product CO is an industrially important chemical. CODHs can catalyze the reduction of CO₂ without over-potential; whereas inorganic catalysts used for CO₂ reduction require excessive over-potential. Several over-expression systems in *E. coli* which is an essential step for industrial applications of CODHs have been reported (53, 71, 106). The complexity of the maturation processes that are required to assemble the C-cluster makes the establishment of an efficient over-expression system for CODHs difficult.

Presently, several accessory genes have been identified in correct assemble of the CODHs C-cluster. In *R. rubrum*, the *cooFSCTJ* operon contains the gene for a ferredoxin-like protein (*cooF*), the structural gene for CODH (*cooS*), and the gene for an accessory protein (*cooCTJ*). Several studies have shown that CooCTJ products are involved in Ni insertion into the C-cluster during maturation. Briefly, CooJ has been shown to bind Ni atom. The precise role of CooT, however, remains unknown (58). CooC is an essential accessory protein for the Ni insertion using ATP hydrolysis energy (49, 55, 56). On the genome of the *C. hydrogenoformans*, three genes encoding CooC-type proteins (CooC1 to CooC3) were identified. Several biochemical and structural studies on CooC1 from *C. hydrogenoformans* suggested that two monomers bind

one Ni^{2+} with micro-molar level of affinity, inducing dimerization of CooC1 (56). CooC1 from *C. hydrogenoformans* shows sequence homology to the Mrp/MinD protein family of SIMIBI-class ATPases (56, 65, 73). These proteins are characterized by the presence of a deviant Walker A motif (P-loop) which are constitute of a highly conserved lysine residues (underlined in XKGGXXK[T/S]). A mutation of the lysine resulted in the disturbance of the formation of a stable dimer with ATP and abolishment of the ATPase activity, but has no effect on the affinity of CooC1 for nickel (55). In addition to the conserved Walker A motif, alignment analyses suggested CooCs proteins have a highly conserved CXC motif which containing two conserved cysteine residues (Fig. 2-2-2). Disruption or substitution of the two conserved cysteines, i.e. C112A and C114S in *C. hydrogenoformans cooC1*, completely abolished the ability to bind nickel. This demonstrates that the CXC motif is the nickel-binding site of CooC1 (55).

C. hydrogenoformans is contains five genes encoding CODHs, termed CODH-I to CODH-V. Several functions for the enzymes are proposed based on their gene context and in physiological experiments; CODH-I, energy conservation conjugated with a proton-pumping hydrogenase; CODH-II, NADH generation; CODH-III, carbon fixation in the acetyl-CoA pathway; and CODH-IV, oxidative stress response. The physiological function of CODH-V remains unknown. Among CODH-I to -V, CODH-I and CODH-II are well characterized. CODH-I and CODH-II show almost similar characteristics. The primary difference between CODH-I and CODH-II was that CODH-II was strongly inhibited by CO and CN^- , suggesting CODH-II is more suited to CO oxidation than CODH-I (136). CODH-I is the model catalysts in CO_2 photo-reduction systems. The reasons are in a previous report, CODH-I absorbed onto an electrode catalyzed the rapid and reversible conversion of CO_2 and CO (88). Secondly, CODH-I is the only enzyme which can interact with TiO_2 (147). However, the over-expression system for

CODH-I remains to be established, as it is a critical issue for the development of a practical and reproducible CO₂ reduction system. CODH-I constitutes a CODH/hydrogenase gene cluster that includes a gene encoding the CooC homologue (*cooC3*) (Fig 2-2-2). Therefore, through the co-expression of its accessory protein CooC3, we investigated an over-expression system for *C. hydrogenoformans* CODH-I, a key enzyme for CO₂ reduction.

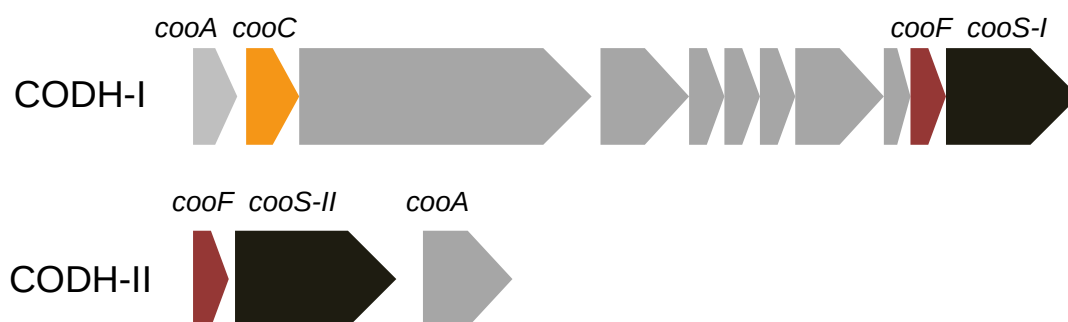


Figure 2-2-1 Gene structure comparison between *C. hydrogenoformans* CODH-I and CODH-II. ORFs coding CODH structural gene (*cooS*) was indicated in black. ORFs coding Ni insertion maturation gene (*cooC*) was indicated in yellow. ORF coding ferredoxin gene (*cooF*) was indicated in red. ORFs coding except for *cooS* and *cooC*. was indicated in gray (149).

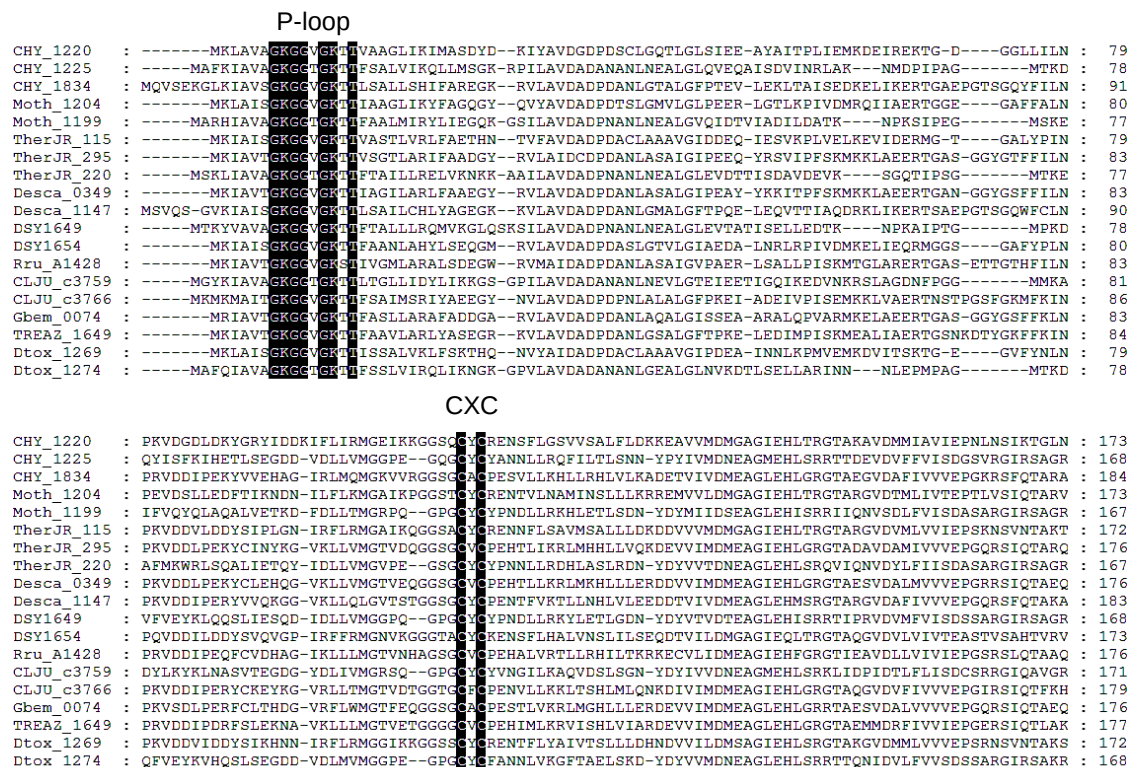


Figure 2-2-2 Amino acid sequence alignment of CooC homologs. The N-terminal P-loop and CXC motifs are shaded black. The proteins included in the alignment are as follows. CHY_1220, 1225, 1834, CooC1-3 from *Carboxydotherrhus hydrogenofomans*; Moth_1199, 1204, CooC1 (AcsF), CooC2 from *Moorella thermoacetica*; TherJR_115, 220, 290, CooC1-3 from *Thermincola potens* JR; Desca_0349, 1147, cooC1, 2 from *Desulfotomaculum carboxydivorans*; Dsy1649, 1654, cooC1,2 from *Desulfitobacterium hafniense*; Rru_A1428; CooC from *Rhodospirillum rubrum*, CLJU_c3759, 3766, CooC1,2 from *Clostridium lijundahlii*; Gbem_0074; CooC from *Geobacter bemidliensis*; TREA_1649, CooC from *Treponema azotonutricium*; and Dtox_1269, 1274, CooC from *Desulfotomaculum acetoxidans*.

Materials and methods

Construction of expression and co-expression vectors

C. hydrogeniformans Z2901 (DSM 6008) was purchased from DSM (Germany) and cultivated as described previously (19, 123). Genome DNA was isolated from the cells using DNeasy Blood & Tissue Kits (Qiagen Chatsworth, CA). All primer sequences used for cloning and mutagenesis of *cooS-II* gene are shown in Table 2-2-1. To construct an expression vector for CODH-I, the CODH-I structural gene *cooS-1* was amplified using PCR from the genomic DNA of *C. hydrogeniformans* using KOD ver2 with the primers CODH-I_Fw1_NdeI and CODH-I_Rv1_XhoI. The product was cloned into the *NdeI* /*BamHI* sites of the pET28a(+) vector (Novagen, Madison, WI) and named pKSI.

To construct the co-expression vector of *CooC3*, *cooC3* was amplified using PCR from the genomic DNA of *C. hydrogeniformans* using KOD ver2 with the primer pair *CooC_Fw_NdeI* and *CooC_Rv_XhoI*. The product was cloned into the *NdeI* and *XhoI* sites of the pETDUET(+) vector (Novagen, Madison, WI) and named pKC3 which doesn't contain His-tag

Table 2-2-1 Primers used in this study.

Name	Sequence (5' →3')
CODH-I_Fw1_NdeI	GGGCATATGAGTAATTGGAAAAATTCCT
CODH-I_Rv1_XhoI	GGGCTCGAGTTAAATCCCCAACCCCCTGC
CooC_Fw_NdeI	GGGCATATGCAGGTGTCTGAAAAGGGAC
CooC_Rv_XhoI	GGGCTCGAGCTAACAATTCTGCCTTTTCACTTC
T7_pro	TAATACGACTCACTATAGGG
T7_ter	GCTAGTTATTGCTCAGCGG

Heterologous expression and purification of recombinant CODH-I

The pKSI plasmid encoding recombinant CODH-I (Rec-CODH-I) with six histidine residues at the N-terminus was transformed into *E. coli* Rosetta2 (DE3) harboring the pRKISC plasmid (79). The transformant was cultivated aerobically at 28°C in a 10 L fermentor (Sanki Seiki, Osaka, Japan) with 5 L of TB medium containing appropriate antibiotics (50 µg/ml kanamycin, 50 µg/ml chloramphenicol and 12.5 µg/ml tetracycline), 43.5 ml 100% glycerol, 0.02 mM NiCl₂, 0.1 mM FeSO₄ and 2 mM L-cysteine and purged with air (Table 2-1-2). When the culture reached an OD₆₀₀ of 0.6-0.7, the gas was switched to pure N₂. After 30 min, 0.5 mM NiCl₂, 1 mM FeSO₄, 50 mM KNO₃, 2 mM L-cysteine and 0.5 mM IPTG were added to induce expression. After 20 h, the cells were harvested aerobically. The cell pellet was frozen for storage at -80 °C until needed. The following purification steps were performed in an anoxic glove box (COY Laboratory Products Inc., Grass Lake, MI) under an atmosphere of 95% N₂/ 5% H₂ at 25°C, (except for the cell disruption step) and all buffers used in this study were anaerobically made by autoclaving (121°C, 20 min) and bubbling by pure N₂ for 30 min. The cells were disrupted using a French press (5501-M, Ohtake Factory, Tokyo, Japan) and the cell free extract was centrifuged at 7,100 g for 30 min at 25°C. The supernatant was applied to a Cosmogel His-Accept column (Nacalai Tesque, Kyoto, Japan). After washing with buffer A (50 mM Tris-HCl, pH 8.0, 300 mM NaCl) containing 20 mM imidazole and 2 mM dithionite, CODH-I was eluted with buffer A containing 300 mM imidazole and 2 mM dithionite. The eluent was subsequently subjected to Amicon Ultra (30 kDa MWCO, Millipore, Boston, MA) filtration and equilibrated with 50 mM Tris-HCl pH 8.0 and 2 mM dithionite to remove imidazole. Purity of the Rec-CODH-I was analyzed using 10 % (W/V) SDS-PAGE.

Heterologous co-expression of *cooC3* and purification of Rec-CODH-I

The plasmid pKC3 encoding Rec-CooC3 was transformed into *E. coli* Rosetta2 (DE3)

harboring the pRKISC and pKSI plasmids. The transformant was cultivated aerobically at 28°C in a 10 L fermentor (Sanki Seiki, Osaka, Japan) with 5 L of TB medium containing appropriate antibiotics (50 µg/ml kanamycin, 50 µg/ml chloramphenicol, 12.5 µg/ml tetracycline and 50 µg/mL streptomycin), 43.5 ml 100% glycerol, 0.02 mM NiCl₂, 0.1 mM FeSO₄ and 2 mM L-cysteine and purged with air (Table 2-1-2). When the culture reached an OD₆₀₀ of 0.6-0.8, the gas was switched to pure N₂. After 30 min, 0.5 mM NiCl₂, 1 mM FeSO₄, 50 mM KNO₃, 2 mM L-cysteine and 0.5 mM IPTG were added to induce expression. After 20 h, the cells were harvested aerobically. The Cell pellet was frozen for storage at -80 °C until needed. The following purification steps were performed in an anoxic glove box (COY Laboratory Products Inc., Grass Lake, MI) under an atmosphere of 95% N₂/ 5% H₂ at 25°C, (except for the cell disruption step) and all buffers used in this study were made anaerobic by autoclaving (121°C, 20 min) and bubbling by pure N₂ for 30 min. The cells were disrupted using a French press (5501-M, Ohtake Factory, Tokyo, Japan) and the cell free extract was centrifuged at 7,100 g for 30 min at 25°C. The supernatant was applied to a Cosmogel His-Accept column (Nacalai Tesque, Kyoto, Japan). After washing with buffer A (50 mM Tris-HCl, pH 8.0, 300 mM NaCl) containing 20 mM imidazole and 2 mM dithionite, CODH-I was eluted with buffer A containing 300 mM imidazole and 2 mM dithionite. The eluent was subsequently subjected to Amicon Ultra (30 kDa MWCO, Millipore, Boston, MA) filtration and equilibrated in 50 mM Tris-HCl pH 8.0 and 2 mM dithionite to remove imidazole. Purity of the Rec-CODH-I was analyzed using 10 % (W/V) SDS-PAGE. Additionally, expression of CooC3 was analyzed using SDS-PAGE. SDS-PAGE was performed in a 10% (W/V) polyacrylamide gel.

***In vitro* Ni-reconstitution**

In vitro Ni-reconstitution of Ni-deficient Rec-CODH-I was performed at 25°C in an anoxic glove box (5, 50). Activation was begun by mixing 0.5 mg of the enzyme with the

activation mixture containing 50 mM HEPES buffer and, 0.5, 1.0 or 2.0 mM NiCl₂. After 1.5h, an appropriate amount of enzymes obtained from the activation mixture was assayed for CO oxidation activity.

Enzyme assays

CO oxidation activity was assayed based on the CO dependent reduction of oxidized methyl viologens (MV ox) as described previously at the optimal growth temperature for *C. hydrogenoformans* (70°C) (125). One ml assay solution containing 20 mM MVox and 2 mM DTT in 50 mM HEPES-NaOH pH 8.0 was filled in screw-capped cuvette sealed with either a silicon septum under CO bubbling into the atmosphere. Reactions were initiated by injecting Rec-CODH-II with a gas-tight syringe (HAMILTON Bonaduz AG, Switzerland). One unit of activity was defined as the reduction of 2 µmol of methyl viologen per minute.

Spectrum of the Rec-CODH-II was recorded as described previously at room temperature (125). All UV-visible spectra were obtained on an Ultraspec 2100 *pro* spectrophotometer (Amersham biosciences, Little Chalfont Bucks, UK) with screw-caped quartz cuvette (1 cm path length, Hellma GmbH & Co) sealed with butyl or silicon septum.

Metal content analysis

Determination of the metal content of the enzyme solutions was performed at the Nitto Bunseki center (Osaka, Japan) by inductively coupled plasma mass spectrometry (ICP-MS). Enzyme samples were equilibrated with 50 mM Tris-HCl buffer at pH 8.0 containing 2 mM sodium dithionite before shipment for metal analysis.

Determination of protein concentration

Protein concentration of Rec-CODH-II was determined by BIO RAD PROTEIN ASSAY Kit (BIO-RAD Laboratories, Hercules, CA) with BSA as a standard.

SDS-PAGE analysis

The denatured samples were loaded on the 10% (W/V) gel with Protein Ladder One -Triple-color- (250 kDa, 130 kDa, 95 kDa, 72 kDa, 55 kDa, 43 kDa, 36 kDa, 28 kDa, 17kDa, and 10 kDa) (Nacalai Tesque, Kyoto, Japan)

Computational analysis

Multiple sequence alignments were constructed using CLUSTAL W (131). The alignments were examined and columns with gaps were trimmed prior to phylogenetic reconstructions. Maximum-likelihood phylogenetic analysis was performed using PhyML ver. 3 (37).

Results

Heterologous expression of *cooS-I* and and purification of its recombinant protein

The catalytic subunit gene of CODH-I (*cooS-I*) from *C. hydrogenoformans* was expressed in *E. coli* and purified using a Ni affinity column (Fig. 2-2-3). A one-step affinity purification produced apparent homogenous recombinant Rec-CODH-I protein (Rec-CODH-I) as judged by single band at approximately 60 kD in SDS/PAGE analysis (Fig. 2-2-5). The purified Rec-CODH-I exhibited CO oxidation activity of 2,270 U/mg, which was 14% of the native-CODH-I (Table 2-2-2).

UV-visible Spectroscopy of the Rec-CODH-I

To determine the reason for the low CO oxidation activity of the Rec-CODH-I produced with this method, the UV visible absorption spectra were determined using various conditions; and were compared to those of native-CODH-I previously reported (125). The spectra of the isolated Rec-CODH-I were similar to that of native-CODH-I (Fig. 2-2-4). The ratio of absorbance at 420 nm to 280 nm (0.38) was close to the native-CODH-I (0.40). This suggested the Rec-CODH-I had a full complement of [4Fe-4S] clusters. Both CO and dithionite rapidly reduce the native CODH-I. In contrast, the [4Fe-4S] clusters of the Rec-CODH-I were rapidly

reduced by dithionite but only slightly reduced by CO in accordance with the low CO oxidation activity of the enzyme (Fig. 2-2-4 insets).

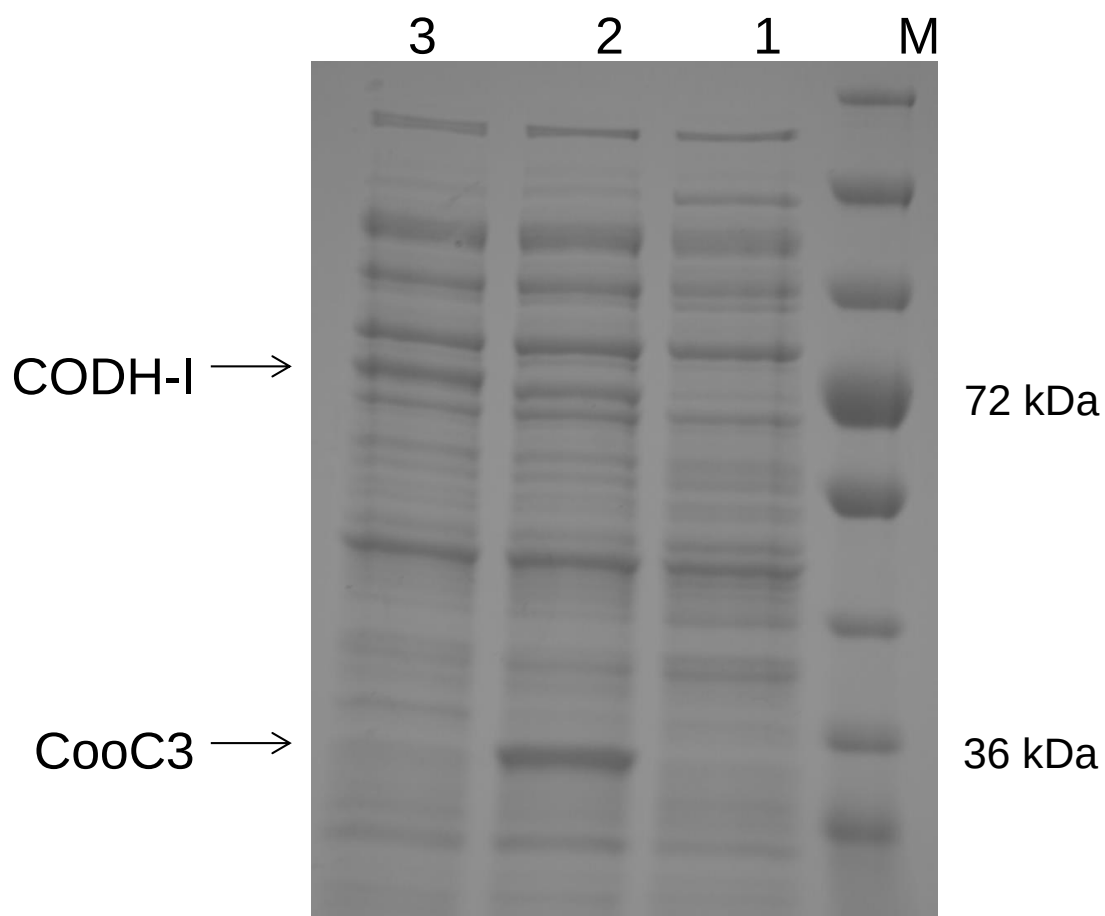


Figure 2-2-3 Ten percent SDS/PAGE analysis of crude extract produced using the co-expression method. M, MW markers; lane 1, Crude extract without over-expression of CODH-I and CooC3 genes; lane 2, Crude extract with CooC3 gene co-expression; lane 3, Crude extract without CooC3 gene co-expression

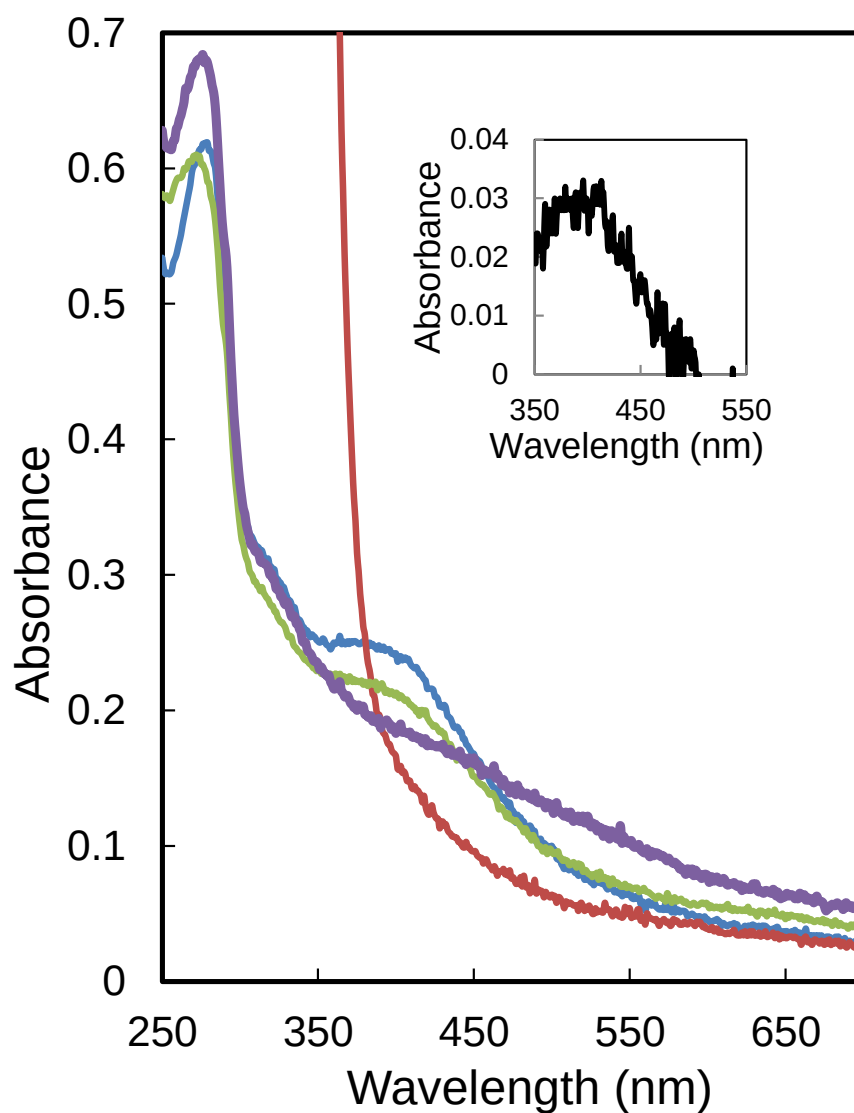


Figure 2-2-4 UV-visible absorption spectra of Rec-CODH-I. The UV-visible absorption spectra of purified Rec-CODH-I are shown. For each curve: blue line, as-isolated; red line, reduced with 2 mM dithionite under N_2 ; green line, reduced with pure CO; purple line, oxidized with air. Inset: difference spectrum of as-isolated minus CO-reduced.

Effect of *cooC3* co-expression

A genome analysis of *C. hydrogenoformans* show the presence of three CooC homologs, termed CooC1, CooC2 and CooC3. In the genome of *C. hydrogenoformans*, CooT and CooJ homologs are not present (149). The CooC3 gene is positioned close to a structural gene of CODH-I (*cooS-I*) and constitutes a hydrogenase/CODH gene cluster. The primary sequence alignment of CooC3 and other CooCs homologs showed conserved walker A (P-loop) and highly conserved region containing two cysteines (CXC motifs), resembling the structure of well-characterized CooC, i.e. *R. rubrum* CooC and *C. hydrogenoformans* CooC1 (Fig. 2-2-2). Thereby, CooC3 is inferred to insert Ni^{2+} into the CODH-I C-cluster coupled with ATP hydrolysis. I investigated the effect of *cooC3* co-expression on the structural constitution of CODH-I. The expression of CooC3 was identified as evidenced by a band at approximately 30 kDa in the crude extract fraction using SDS/PAGE analysis (Fig. 2-2-5). One-step affinity purification produced a nearly homogenous Rec-CODH-I as evidenced by a band at approximately 60 kDa (Co-Rec-CODH-I). A band of 30 kDa, which is the size agrees with that of CooC3 was identified in the same fraction as Co-Rec-CODH-I, while the corresponding band was not detected in the purified Rec-CODH-I fraction without co-expression of CooC3 (Fig. 2-2-5). A weak band at 30 kDa, which is nearly the same size of CooC3, was identified in the enzyme fraction while the band was not identified in the enzyme fraction produced using not-co-expressed method (Fig. 2-2-5). The presence of the 30kDa band suggested that the protein–protein interaction between CODH-I and CooC3 co-purified CooC3 indirectly by His-tag affinity chromatography because CooC3 does not have His-tag. In addition, there was high correlation with IPTG concentration and Co-Rec CODH-I activity. When the Rec-CODH-I reached the highest specific activity (Fig. 2-2-6), *E. coli* cells were immediately harvested under anaerobic condition. The purified Co-Rec-CODH-I exhibited CO oxidation activity at 8,060

U/mg, which is approximately 355% of the Rec-CODH-I and 48% of native-CODH-I (Table 2-2-2).

In vitro Ni-reconstitution was performed with various concentrations of Ni^{2+} . However, Ni-reconstitution decreased in the CO oxidation activity of Rec-CODH-I (Table 2-2-2). The CO oxidation activities of the Rec-CODH-I were 76% (0.5 mM), 74% (1.0 mM) and 68 % (1.5 mM) of the not-treated Rec-CODH-I, respectively.

Table 2-2-2 Activities of Rec-CODH-I prepared with various expression methods

	CO oxidation activity (U/mg)	Relative activity (%)
Native-CODH-I	16,800	100
Without Ni activation	2,270	14
With Ni activation		
0.5 mM	1,730	10
1.0 mM	1,690	10
2.0 mM	1,540	9.2
<i>cooC3</i> co-expression	8,060	48

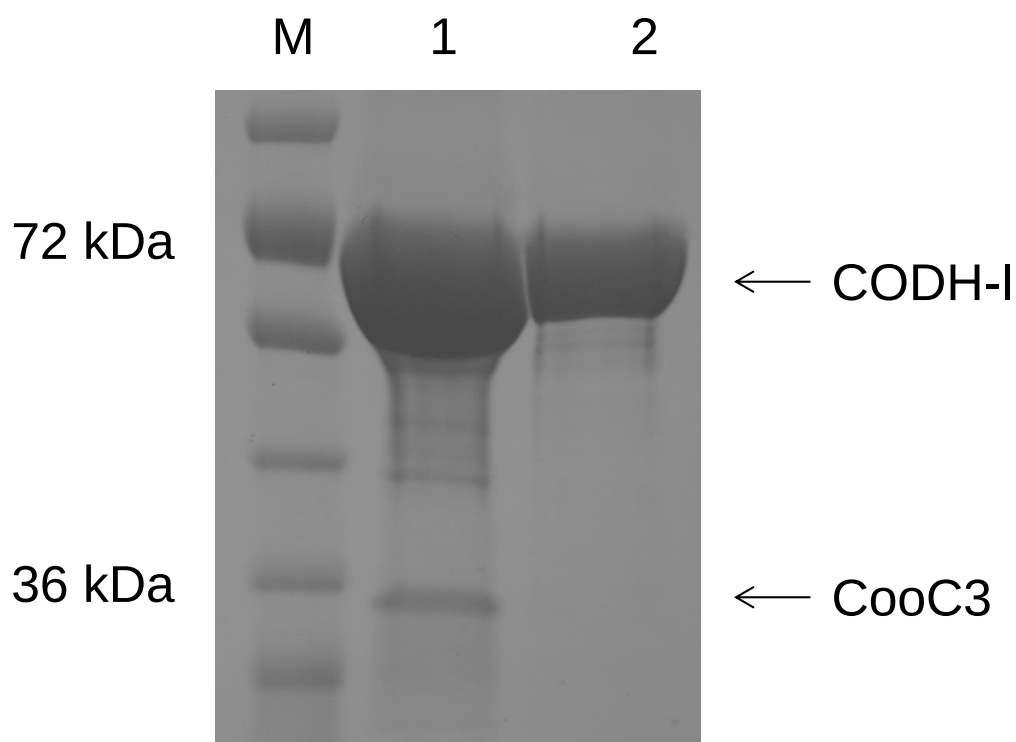


Figure 2-2-5 SDS/PAGE analysis of enzyme fraction produced using the co-expression method. M, MW markers; lane 1, Enzyme fraction produced using the co-expression method; lane 2, enzyme fraction without CooC3 gene co-expression

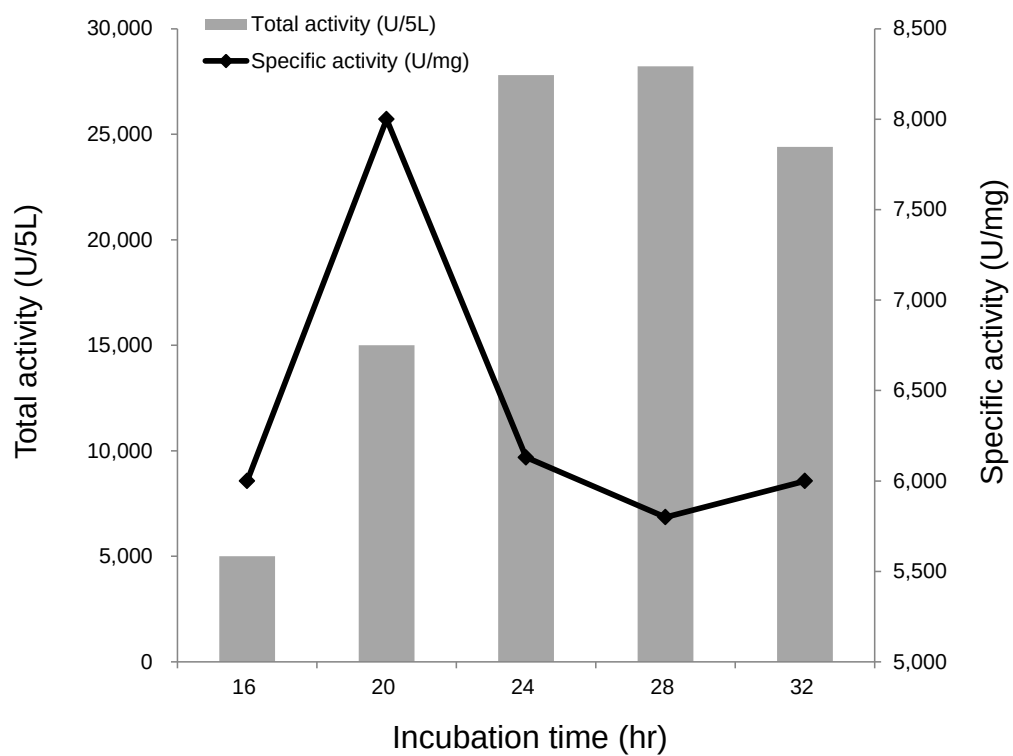


Figure 2-2-6 Activity monitoring of Rec-CODH-I expressed in *E. coli*

Metal content analysis

The metal content of CoRec-CODH-I was compared with those of Rec-CODH-I and Native-CODH-I (Table 2-2-3) (125). The amounts of Fe atoms per monomer of CoRec-CODH-I and Rec-CODH-I in this study (9.94 and 9.52 respectively) were the same level as that from native-CODH-I (9.92). This also suggests that the Rec-CODH-I had a full complement of [4Fe-4S] clusters. The amounts of Ni atom of these Rec-CODH-I (0.10), while Ni content in native-CODH-I was reported to be (0.71), which was not contradicted with their CODH activity levels.

Table 2-2-3 Metal contents of Rec-CODH-I prepared with various expression

	Ni content (mol/mol CODH)	Fe content (mol/mol CODH)
Native-CODH-I	0.71	9.92
Without Ni activation	0.10	9.52
With Ni activation		
0.5 mM	ND	ND
1.0 mM	ND	ND
2.0 mM	ND	ND
cooC3 co-expression	0.33	9.94

ND, Not determined

Discussion

In the present study, an efficient active CODH-I over-expression system was established utilizing co-expression of accessory protein CooC3. This was achieved through co-expression of accessory protein CooC3. A large part of Rec-CODH-I lacked Ni^{2+} , and co-expression was effective in inserting Ni^{2+} into the Rec-CODH-I. Firstly UV spectra of as isolated Rec-CODH-I was similar to that of wild type. Secondly Fe^{2+} content of Rec-CODH-I was same as that of Co-Rec-CODH-I and Native-CODH-I. Finally co-expression resulted in activity increase and the Ni^{2+} content increase. However, Co-Rec-CODH-I exhibited only 48% of the CO oxidation activity of native-CODH-I. The reasons are as follows; i) As CODH activity levels coincided with the concentration of Ni^{2+} in the recombinant protein, lower activity of Co-Rec-CODH-I is attributed to the lower concentration of Ni^{2+} compared to native CODH-I. Further, over-expression of CooC3 may increase the Ni^{2+} content and CODH activity. ii) The existence of several accessory proteins strongly suggest that more factors would be required for full activity of Co-Rec-CODH-I, e.g. *cooF* encoding ferredoxin (113). iii) Due to CooC which was identified in enzyme fraction, specific activity of enzymes was underestimated. Incubation of apo-enzymes with high concentrations of constituent metals (*in vitro* reconstitution) is frequently effective in inserting the metals (134). However, *in vitro* Ni-reconstitution was not efficient for Rec-CODH-I (Table 2-2-3). In a previous report, insertion of Ni^{2+} into the Ni-deficient CODH from *R. rubrum* was dependent upon the presence of a low-potential one-electron reductant (28), suggesting insertion of Ni^{2+} into Rec-CODH-I also required activation of the C-cluster using the lower-potential.

In contrast to Rec-CODH-I, a recombinant CODH-II, a paralogous enzyme of CODH-I in *C. hydrogenoformans*, which was expressed in *E. coli* in the presence of the same concentrations of Ni^{2+} as CODH-I and showed a comparable activity to that of the native

CODH-II. This strongly suggested that CODH-II does not require accessory proteins to integrate Ni^{2+} within the structure. In fact, there is no *CooC* homolog located close to the CODH-II gene (Fig 2-2-1), indicative that the Ni-incorporation mechanism differs depending on the type of CODHs.

Heterologous co-expression of *CooC* homolog termed *acsF* in *M. thermoacetica* showed no increase in CO oxidation activity of *M. thermoacetica* CODH (71), which constitute ACS/CODH operon with acetyl-CoA synthase (ACS) and complexes with ACS in vivo. However, I identified one more *CooC* homolog in the ACS/CODH gene cluster in addition to *acsF*. As well as the active site of CODHs, the active site of ACSs called A-cluster contains Ni^{2+} (17, 23). Combined, *CooC* may have protein specificity in Ni^{2+} insertion i.e. *CooC3* specific to CODH-I; *AcsF* specific to ACSs. To support this hypothesis, I investigated the phylogeny of *CooC* and the distribution of *CooC*. *CooCs* were phylogenetically divided into two major groups (Group 1 and Group 2) (Fig. 2-2-7). In addition, I found two kinds of ACS/CODH gene clusters, i.e. the clusters containing only one *CooC*, and clusters containing two *CooC* homologs. In the latter clusters, one *CooC* belongs to Group 1 while the other belongs to Group 2. All of the *CooCs* positioned in hydrogenase/CODHs belonged to Group 1, and the *acsF* belongs to Group 2. This suggested *CooC* homologs were divided into two major groups where one has the function of inserting Ni^{2+} into CODHs and the other the function of insertion into ACSs (Fig 2-2-8). ACS/CODH gene clusters of *Thermincola potens* JR contain only one *CooC* homologs. This suggests these *CooC* homolog insert Ni^{2+} into both CODHs and ACSs.

To my knowledge, this is the first report to construct an over-expression system of CODH-I in *E. coli* by applying the co-expression of *cooC*. Considering that purification of native-CODH-I requires a lot of effort, especially cultivation of *C. hydrogenoformans* with a CO-purge, my finding may be helpful for the application of CODH-I to CO_2 reduction systems.

These results also show the possibility that this method may be applied to other CODHs as well as to complex metalloenzyme.

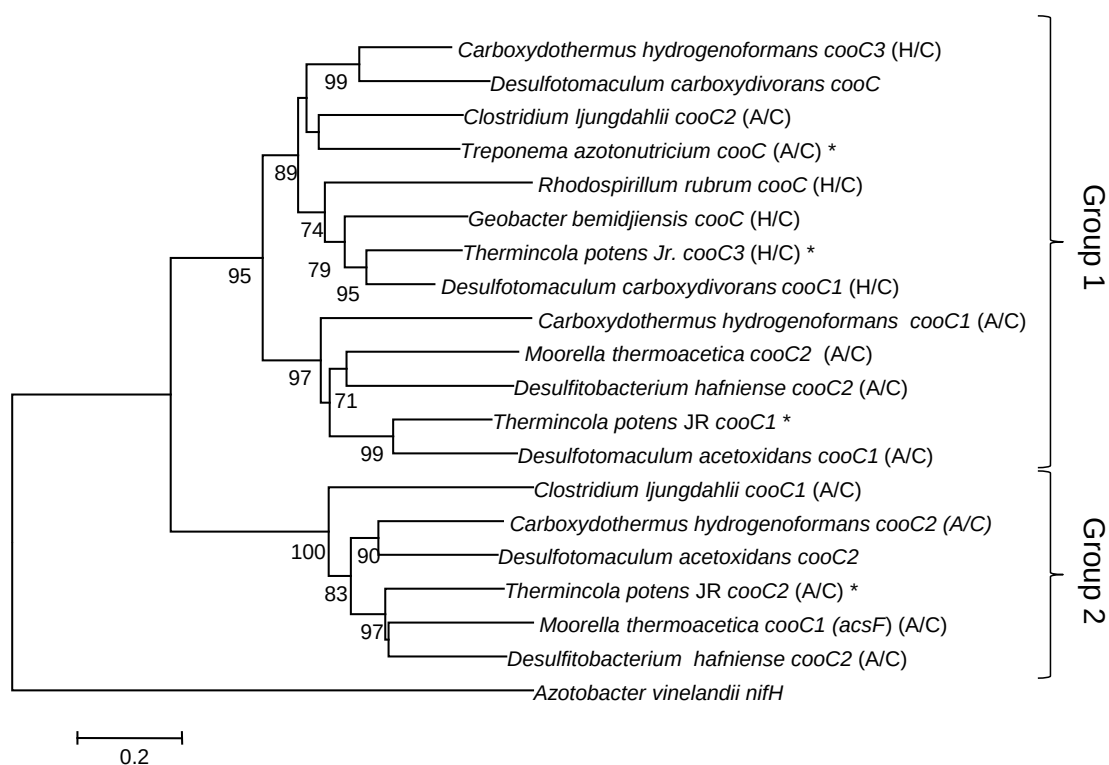


Figure 2-2-7 Phylogenetic tree of CooC homologs. A phylogenetic tree of CooC homologs is constructed with the neighbor-joining method. CooC homologs positioned in ACS/CODH gene clusters and hydrogenase/CODH gene clusters are indicated in (A/C) and (H/C), respectively. CooCs from *Treponema azotonutricium* and *Thermincola potens* JR, that have ACS/CODH gene clusters containing only one CooC homolog are highlighted by an asterisk. Only bootstrap supports equal to or more than 70% are indicated.

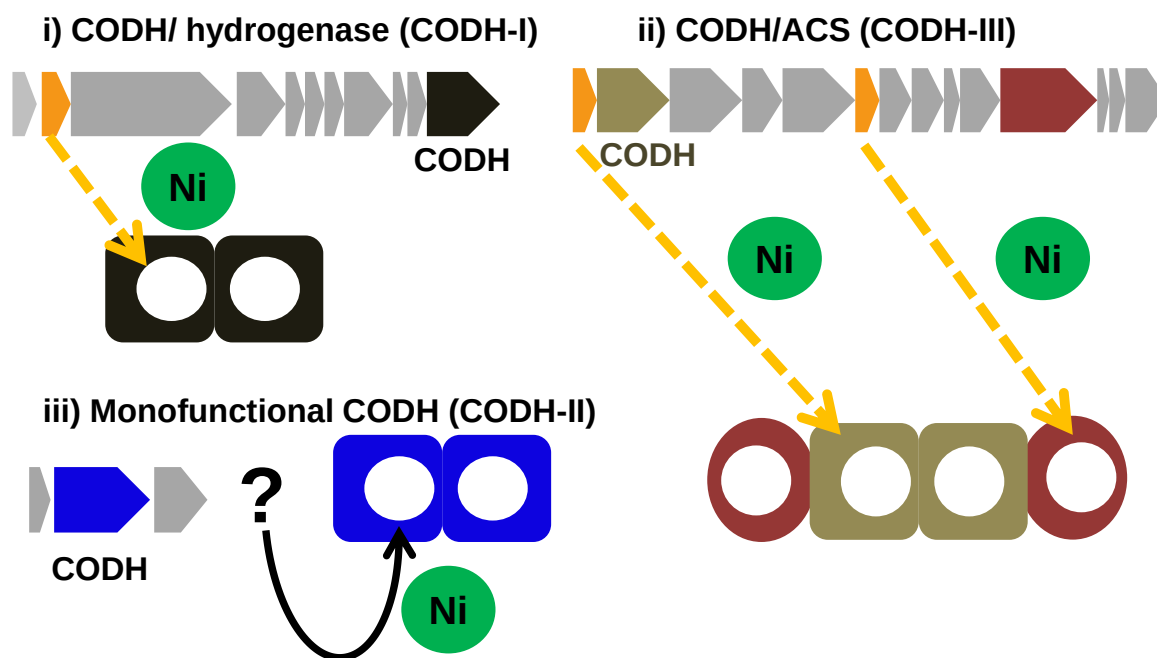


Figure 2-2-8 Ni insertion specificity of CODHs. CooC homologs were divided into two major groups where one has the function of insertion Ni^{2+} into CODHs and the other has the function of insertion into ACSs.

Chapter 3

Cysteine 295 indirectly affects Ni coordination of carbon monoxide dehydrogenase-II C-cluster

Introduction

CODHs are phylogenetically related and are considered as a key enzyme for microbial CO metabolisms; however, they vary in terms of subunit composition, catalytic activities and possibly their metabolic roles (85, 97). While a little phylogeny and evolution of CODHs is known, recent phylogenetic analyses of CODH genes demonstrated CODH showed extensive diversity, being divided into six distinct clades (130). In addition, several studies indicated many organisms acquire CODH genes by horizontal gene transfer, mainly because of many examples of CODH clades that are composed of organisms which are not related to one another phylogenitically and codon usage analysis (130). Recent genome studies also have revealed among CODH encoding organisms, more than 40% organisms encode multiple CODHs and, except for a few model enzymes, the biochemical character and metabolic role of these CODHs remains unknown (130). Finally, in addition to physiological activity i.e. CO oxidation and CO₂ reduction activity, CODHs show further activities including hydorrrogenase activity, trinitrotoluene reduction activity, n-butyl isocyanate reduction and hydroxylamine reduction activity (41, 47, 54, 125). These results also suggested CODHs have extensive diversity.

In contrast to the diversity, basic structures of CODHs are similar (17, 22, 23, 35, 53, 124). CODH constitute homo-dimetric structure where five metal-clusters called B, C and D were bound. The conventional [4Fe-4S] cluster (D-cluster) bridges two subunits covalently via two cysteine residues from each subunit. Another [4Fe-4S] cluster, called B-cluster is located 10 Å

away from D-cluster bounded by four cysteine residues. The active site, called C-cluster is located 11 Å away from cluster B'. C-cluster is composed of unique [Ni-Fe-S] cluster that is linked by conserved ligands: five cysteines and one histidine residues. Additional [4Fe-4S] clusters called E and F clusters were found in a CODH from methanogenesis archaea *M. barkeri* (35). These clusters are linked in proteins by highly conserved amino acid residues such as a cysteine and histidine (68).

Like other clusters, the C-cluster structure, which is the activity center, is conserved in CODH structures from some bacteria, *C. hydrogenoformans*, *R. rubrum*, *M. thermoacetica*, and an archaeon *M. barkeri*. The C-cluster ligands are important for their activity as well as metal coordination, e.g., the His²⁶¹ ligand is the central residue required for proper assembly of the Ni into the C-cluster (118) and substitution of Cys ligands cause Fe atom deficient. Structural studies show His²⁶¹ directly coordinates one of the Fe atoms in the cluster with the Cys²⁹⁵ in CODH-II (corresponding His²⁶⁵ in CODH of *R. rubrum* (CODH_{Rr}) (Table 3-1). In this chapter, residue numbers are based on the structure of *C. hydrogenoformans* CODH-II, unless otherwise noted (Table 3-1). It is very unlikely that this His residue is a ligand for the Ni²⁺ in all structures because it is over 4 Å away. In view of the importance of the His²⁶¹ for Ni coordination, the role of the ligand at position 295 is of interest to further understand of C-cluster.

C. hydrogenoformans contains five genes encoding CODHs designated CODH-I to CODH-V on the genome (149). Several functions for the enzymes are proposed based on their gene context and on physiological experiments; CODH-I constitute CODH/ hydrogenase gene cluster and the metabolic role is energy conservation conjugated with a proton-pumping hydrogenase; CODH-II is monofunctional CODH, which doesn't constitute gene cluster with hydrogenase and acetyl-CoA synthase (ACS). A biochemical study suggested the metabolic role of CODH-II is NADH generation; CODH-III constitute CODH/ACS gene cluster, and the role

3. Cysteine 295 indirectly affects Ni coordination

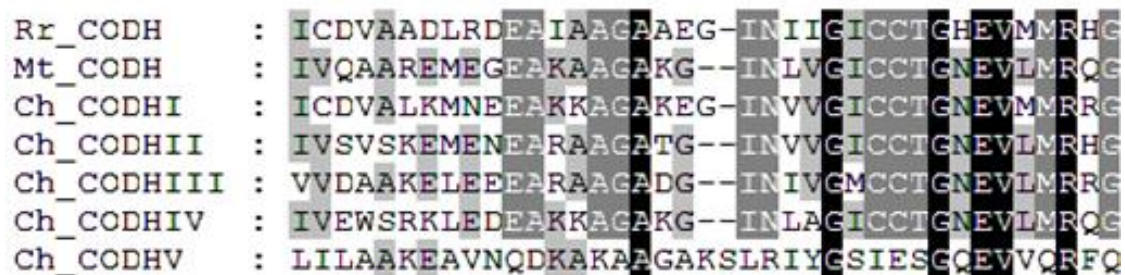
is carbon fixation in the acetyl-CoA pathway; and CODH-IV constitute a gene cluster with oxidative stress response gene, suggesting CODH-IV play a role in oxidative stress response metabolism. The physiological function of CODH-V remains unknown.

In CODH-V, several alignment analyses suggest the conserved ligand Cys²⁹⁵ is replaced by Glu (66, 68) (Fig. 3-1). Considering the important role of His²⁶¹ for Ni-binding and substitution of the ligand positioned 295 in CODH-V, the role of ligand positioned 295 is interesting. Thus to evaluate the role of Cys²⁹⁵ in CODH-II, I constructed CODH-II variants of the Fe1 ligands (C295A, C295E mimicked for CODH-V and H261A) and compared their properties.

Table 3-1 Selected residues in CODHs that coordinate C-cluster.

Residue	<i>M. thermoacetica</i> CODH	<i>C. hydrogenoformans</i> CODH-II	<i>C. hydrogenoformans</i> CODH-V	<i>R. rubrum</i> CODH
H1	H283	H261	H259	H265
C1'	C316	(C294)	(I294)	(C299)
C1	C317	C295	E295	C300
C2	C355	C333	C338	C338
C3	C470	C446	C449	C451
C4	C500	C476	C480	C481
C5	C550	C526	C521	C531

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Rr_CODH      : ICDVAADLRDEAIAAGAAEG--INIIGICCTGHEVMMRHG
Mt_CODH      : IVQAAREMEGEAKAAGAKG--INLVGICCTGNEVIMRQG
Ch_CODHI     : ICDVALKMNEEAKKAGAKEG--INVVGICCTGNEVMMRRG
Ch_CODHII    : IVSVSKEMENEARAAGATG--INVVGICCTGNEVIMRHG
Ch_CODHIII   : VVDAAKELEEEARAAGADG--INIVGMCCTGNEVIMRRG
Ch_CODHIV    : IVEWSRKLEDEAKKAGAKG--INLAGICCTGNEVIMRQG
Ch_CODHV     : LILAAKEAVNQDKAKAAGAKSLRIYGSIESGQEVVQRFQ
  
```

Fig. 3-1 Alignment of amino acid sequences for the CODHs. Residues conserved in the sequences are indicated by black backgrounds, whereas residues conserved in >80 and 60 >% of the proteins examined are indicated by white type on a dark gray background and by black type on a light gray background, respectively. The Cys²⁹⁵ residues are indicated by black closed circle.

Materials and methods

Site-directed mutagenesis, heterologous expression and purification of recombinant CODH-II variants

All primer sequences used for cloning and mutagenesis of *cooS-II* gene are shown in Table 3-2. The pET28-a based CODH-II expression plasmid used for mutagenesis experiments was previously described in 2-1. The Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to introduce C295A, C295E, and H261A mutations into the *cooS-II* gene. For construction of CODH-II C295A, delta_cooSII_C295A_Fw and delta_cooSII_C295A_Rv were used, for C295E, delta_cooSII_C295E_Fw and delta_cooSII_C295E_Rv were used and for CODH-II H261A, delta_cooSII_H261A_Fw and delta_cooSII_H261A_Rv were used.

The N-terminal (His)₆-tagged CODH-II was expressed in *E. coli* Rosetta2 (DE3) (Novagen, Madison, WI) harboring the pRKISC plasmid (a generous gift from Y. Takahashi, Saitama University, Saitama, Japan) (79). The transformant was cultured aerobically at 30°C in a 10 L fermentor (Sanki Seiki, Osaka, Japan) with 5 L of modified TB medium containing 0.02 mM NiCl₂, 0.1 mM FeSO₄, 2 mM L-cysteine, 50 µg/ml kanamycin, 50 µg/ml chloramphenicol and 12.5 µg/ml tetracycline and purged with air (Table 2-1-2). When the culture reached an OD₆₀₀ of 0.6-0.7, the gas was switched to pure N₂. After 30 min, 0.5 mM NiCl₂, 1 mM FeSO₄, 50 mM KNO₃, 2 mM L-cysteine and 0.2 mM IPTG was added to induce expression. After 20-22h, the cells were harvested aerobically. The Cell pellet was frozen for storage at -80 °C until needed. The following purification steps were performed in an anoxic glove box (COY Laboratory Products Inc., Grass Lake, MI) under an atmosphere of 95% N₂/ 5% H₂ at 25 °C, (except for a cell disruption step) and all buffers used in this study were anaerobically made by autoclaving (121°C, 20 min) and bubbling by pure N₂ for 30 min. The cells were disrupted using

Table 3-2 Primers used in this study.

Name	Sequence (5' → 3')
delta_cooS-II_C295A_F1	TTAACGTGGTAGGTATTTGTGCCACGGGCAACGAGGTTTTGATGC
delta_cooS-II_C295A_R1	GCATCAAAACCTCGTTGCCCCGTGGCACAAATACCTACCACGTTAA
delta_cooS-II_C295E_F1	ATTAACGTGGTAGGTATTTGTGAAACGGGCAACGAGGTTTTGATGC
delta_cooS-II_C295E_R1	CATCAAAACCTCGTTGCCCCGTTTCACAAATACCTACCACGTTAAT
delta_cooS-II_H261A_F1	GTGGCCGTTTCATGGGGCTAACCCGGTCCTGTC
delta_cooS-II_H261A_R1	GACAGGACCGGGTTAGCCCCATGAACGGCCAC
CODH II_Seq1	TGGGTTGTGATGCCGATGC
M13_Fw	GTAAAACGACGGCCAG
M13_Rv	CAGGAAACAGCTATGAC
T7_pro	TAATACGACTCACTATAGGG
T7_ter	GCTAGTTATTGCTCAGCGG

3. Cysteine 295 indirectly affects Ni coordination

a French press (5501-M, Ohtake Factory, Tokyo, Japan) and the cell free extract was centrifuged at 7,100 g for 30 min at 25 °C. The supernatant was applied to Cosmogel His-Accept column (Nacalai Tesque, Kyoto, Japan). After extensive washing with buffer A (50 mM Tris-HCl, pH 8.0, 300 mM NaCl) containing 20 mM imidazole and 2 mM dithionite, CODH-II was eluted with buffer A containing 300 mM imidazole and 2 mM dithionite. The eluent was subsequently subjected to Amicon Ultra (30 kDa MWCO, Millipore, Boston, MA) filtration and equilibrated in 50 mM Tris-HCl pH 8.0 and 2 mM dithionite to remove imidazole. Purity of the Rec-CODH-II was analyzed using 10 % (W/V) SDS-PAGE.

Metal analysis

Determination of the metal content of the enzyme solutions was performed at the Nitto Bunseki center (Osaka, Japan) by inductively coupled plasma mass spectrometry (ICP-MS). Enzyme samples were equilibrated in 50 mM Tris-HCl buffer at pH 8.0 containing 2 mM sodium dithionite before shipment for metal analysis.

Enzyme assays

CO and H₂ oxidation activity was assayed based on the CO dependent reduction of oxidized methyl viologens (MV ox) as described previously at the optimal growth temperature for *C. hydrogenoformans* (70°C) (125). One ml assay solution containing 20 mM MV ox and 2 mM DTT in 50 mM HEPES-NaOH pH 8.0 was filled in screw-capped cuvette sealed with either a silicon septum under CO bubbling into the atmosphere. Reactions were initiated by injecting Rec-CODH-II with a gas-tight syringe (HAMILTON Bonaduz AG, Switzerland). One unit of activity was defined as the reduction of 2 μmol of methyl viologen per minute.

CO₂ reduction activity was assayed at 25°C according to a previous report with minor modification with the increase in absorbance at 419 nm (27). Standard curve was prepared by titrating samples of a saturated CO solution at 25 °C in the anaerobic chamber and the titration

was performed with measuring spectral changes between 400 nm and 460 nm in assay cuvette (27). Assays were performed in 2 ml quartz cuvettes sealed by silicon septum with screw cap without free headspace (27). The assay solution contained 50 mM MOPS pH7.5, 0.2 mg/ml hemoglobin, 50 mM NaHCO₃ and various reductants (methyl viologen reduced with 0.3 mM dithionite, 2 mM dithionite and 5 mM Ti(III) citrate, respectively). This reaction solution was equilibrated for 2 min at 25 °C in anaerobic chamber until no spectral changes was occurred. Reaction was initiated by addition of Rec-CODH-II. The production of CO from CO₂ by Rec-CODH-II was determined by monitoring the increase in absorbance at 419 nm which is caused by the formation of carboxyhemoglobin. One unit of activity was defined as 1 µmol CO production per minute.

NH₂OH reduction activity was assayed based on NH₂OH dependent oxidation of reduced methyl viologen at 40°C, according to a previous report with minor modification (41, 87). Assays were performed anaerobically in a 1.0 ml assay mixture containing 100 mM MOPS-NaOH (pH 7.5), 10 mM methyl viologen, and 100 mM NH₂OH. The assay solution was reduced to give an absorbance at 578 nm of near 1.0 with 100 mM sodium dithionite solution. The reaction was started adding the enzyme. A no-enzyme control assay was also performed with no significant decrease in A₅₇₈. One unit of activity was defined as the reduction of 1 µmol of NH₂OH per minute. CO₂ reduction activity was assayed at 25°C as described previously (27).

Stock solution of dithionite was prepared freshly in water as 1 M concentration. 83 mM Ti(III) citrate was prepared as reference as following (105): 1 ml TiCl₃ was dissolved in 4.8 ml 0.5 M Na₃-citrate and buffered by adding 6.2 ml 1 M Tris-HCl pH 8.0.

Computational analysis

Multiple sequence alignments were constructed using CLUSTAL W (131). The alignments were examined and columns with gaps were trimmed prior to phylogenetic reconstructions.

Maximum-likelihood phylogenetic analysis was performed using PhyML ver. 3 (37).

Results

Heterologous expression of *codS-II* and purification of its recombinant protein

CODH-II variants from *C. hydrogenoformans* was expressed in *E. coli* and purified. A one-step affinity purification produced apparent homogenous Rec-CODH-IIs as judged by single band at approximately 60 kD in SDS/PAGE analysis (Fig. 3-2).

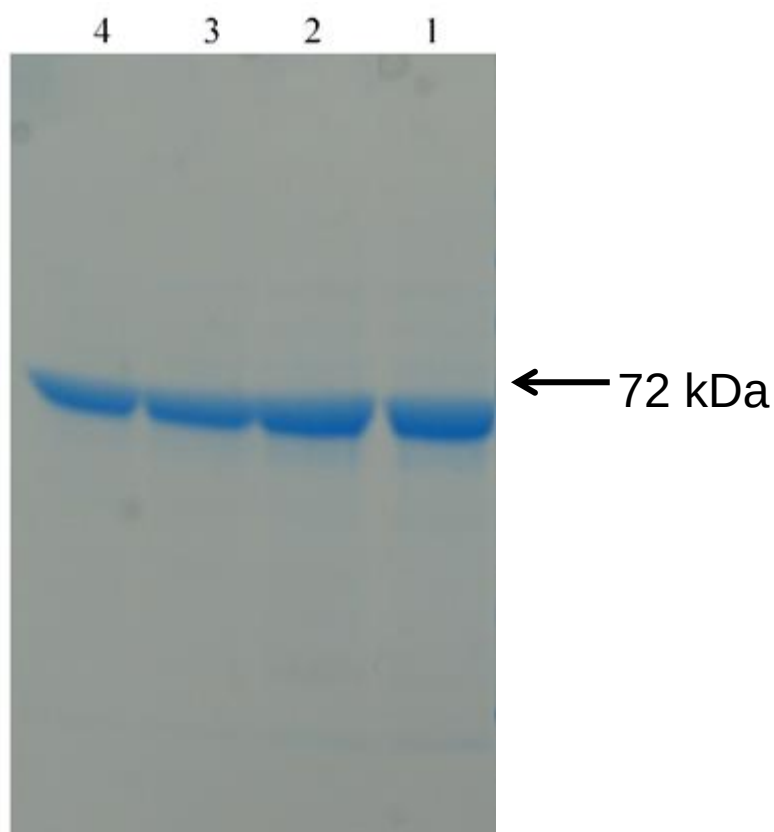


Fig. 3-2 SDS/PAGE analysis of CODH-II variants. lane 1, Wild type; lane 2, C295A; lane 3, C295E and lane 4, H261A.

Metal analysis

The metal content of CODH variants were compared with that of wild type (Table 3-3). The amounts of Fe and Ni atoms per monomer of wild type in this study (7.5Fe and 0.90Ni, respectively) were lower than that expected from CODH-II structure (10Fe and 1Ni, respectively) (53). C295A, C295E and H261A contained nearly the same amount of iron atoms respectively (Table 3-3). Wild type CO oxidation activity (8,900 U/mg) obtained in this study was lower compared to that of the previous report (10,000-14,000U/mg), suggesting that on a part of recombinant enzymes, the [Fe-S] and/or [Ni-Fe-S] cluster degraded during purification process. The variants showed a very low level of nickel compared with wild type enzyme (Table 3-3).

UV-visible absorption spectra of CODH-II variants

UV-visible absorption spectra of CODH-II variants were recorded using several redox conditions, and were compared to the wild-type CODH-II. The spectra of as-isolated variants were identical to the wild-type (Fig. 3-3). The spectra of A420/A280, which is unique absorbance for CODH-II C-cluster, were similar to wild-type CODH-II (125). For the wild-type CODH-II, bleaching of the [Fe-S] cluster shoulder occurs after treatment with CO or dithionite (125). In contrast, the bleaching was observed when variants were treated with dithionite but not CO, e.g., CODH_{Rf} H265V (Fig. 3-2 insets), suggesting the [Fe-S] clusters of variants were not reduced by CO.

(A)

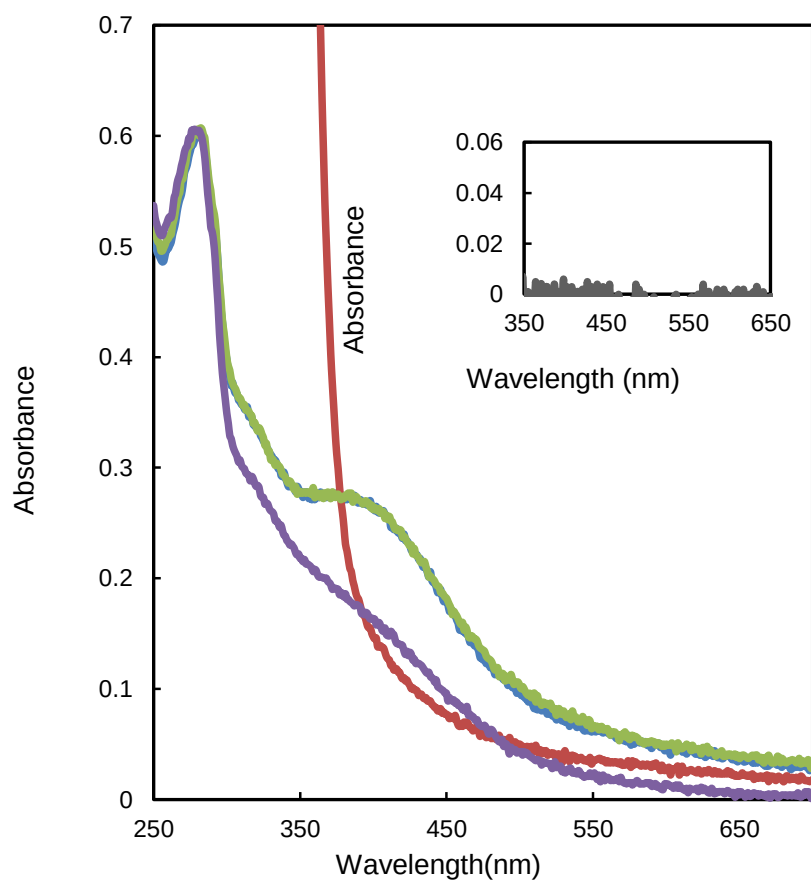


Figure 3-3 UV-visible absorption spectra of CODH-II variants. The UV-visible absorption spectra of purified CODH-II variants in 50 mM Tris-HCl pH 8.0 are recorded. (A) CODH-II C295A, (B) CODH-II C295E and (C) CODH-II H261A. Conditions for each curve: blue line, as-isolated; red line, reduced with 2 mM dithionite under N_2 for 2 minutes; green line, reduced with pure CO for 5 minutes; and purple line, oxidized with air for 5 minutes. Insets; a difference spectrum of condition as-isolated minus CO-reduced.

(B)

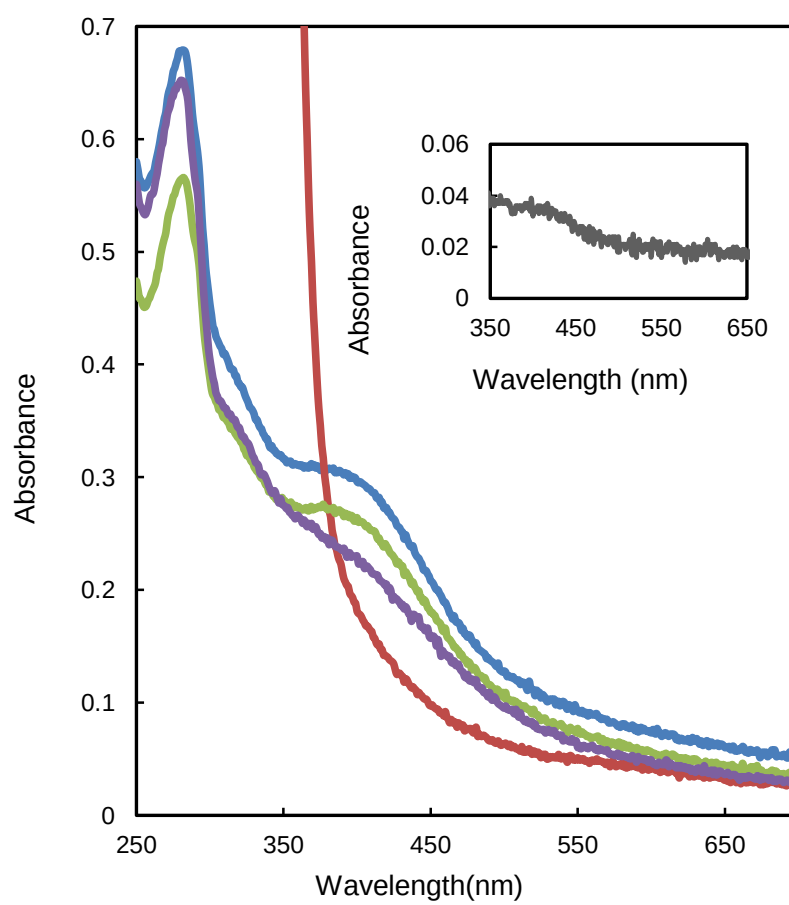


Figure 3-3 Continued.

(C)

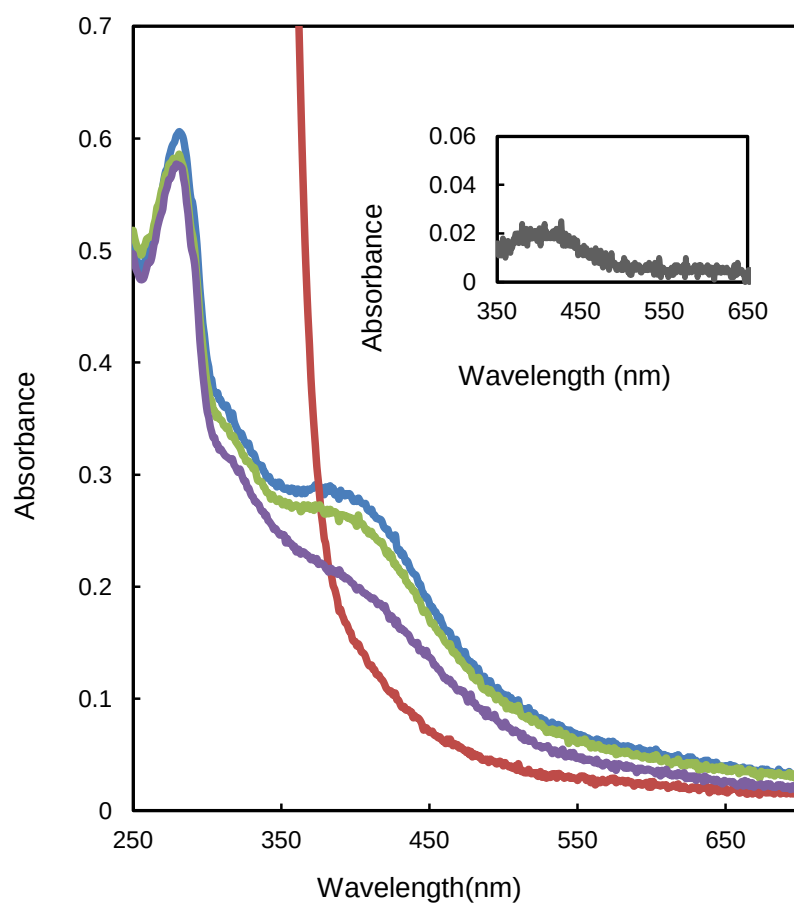


Figure 3-3 Continued.

Table 3-3 Metal contents of CODH variants.

Recombinant protein	Metal content	
	Ni (mol/mol CODH)	Fe (mol/mol CODH)
W.T.	0.90	7.5
C295A	0.078	8.9
C295E	0.076	9.2
H261A	0.082	8.8

Activity of CODH-II variants

The activities of CODH-II variants were determined. All substitutions resulted in decreased CO oxidation activity, and an increase in NH₂OH reduction activity (Table 3-4). C295A and H261A showed no CO oxidation activity and C295E showed 0.02% of the wild-type, respectively. The NH₂OH reduction activities of C295A, C295E and H261A were 171, 343, and 614% of the wild-type, respectively. Although the optimal growth temperature of *C. hydrogenoformans* is 70°C, we determined NH₂OH reduction activities of variants at 40°C, because reduced methyl viologen lacks stability at higher temperatures. And under the condition, all variants showed no CO₂ reduction and H₂ oxidation

Distribution of the replacement at position 295

A phylogenetic tree was constructed using the sequence of Cdh from *Archaeoglobus fulgidus* as an out group (Fig. 3-4). Protein phylogeny of the deduced amino acid sequence from the *cooS* genes, which is the catalytic subunit of CODHs, was divided into two major groups (Group 1 and Group 2) supported with robust (100%) bootstrap values (Fig. 3-4). Group 1 contained well-characterized CODHs including CODH-II and CODH_R. In contrast, Group 2 included CODHs like sequences whose properties and physiological functions are not known. Group 2 was further divided into two major clades (Clade A and Clade B) supported with robust (100%) bootstrap values (Fig. 3-3). The substitution of the ligand positioned 295 was identified in some sequence belonging in Group 2.

Table 3-4 Specific activities of CODH variants.

	CODH-II _{Ch}				CODH _{Rr}	
	W.T.	C295A	C295E	H261A	W.T. ^a	H265V ^a
CO oxidation (units/mg)	8,900	N.D.	1.8	N.D.	7,000	0.67
H ₂ oxidation (units/mg)	N.D.	N.D.	N.D.	N.D.	0.04	0.05
CO ₂ reduction (units/mg)	4.0	N.D.	N.D.	N.D.	4.3	0.003
NH ₂ OH reduction (units/mg)	0.7	1.2	2.4	4.3	1.0	28.8

CODH-II_{Ch}; CODH-II from *C. hydrogenoformans*, CODH_{Rr}; CODH from *R. rubrum*

N.D.; not detected, ^a; the values are taken from Ref. (41)

3. Cysteine 295 indirectly affects Ni coordination

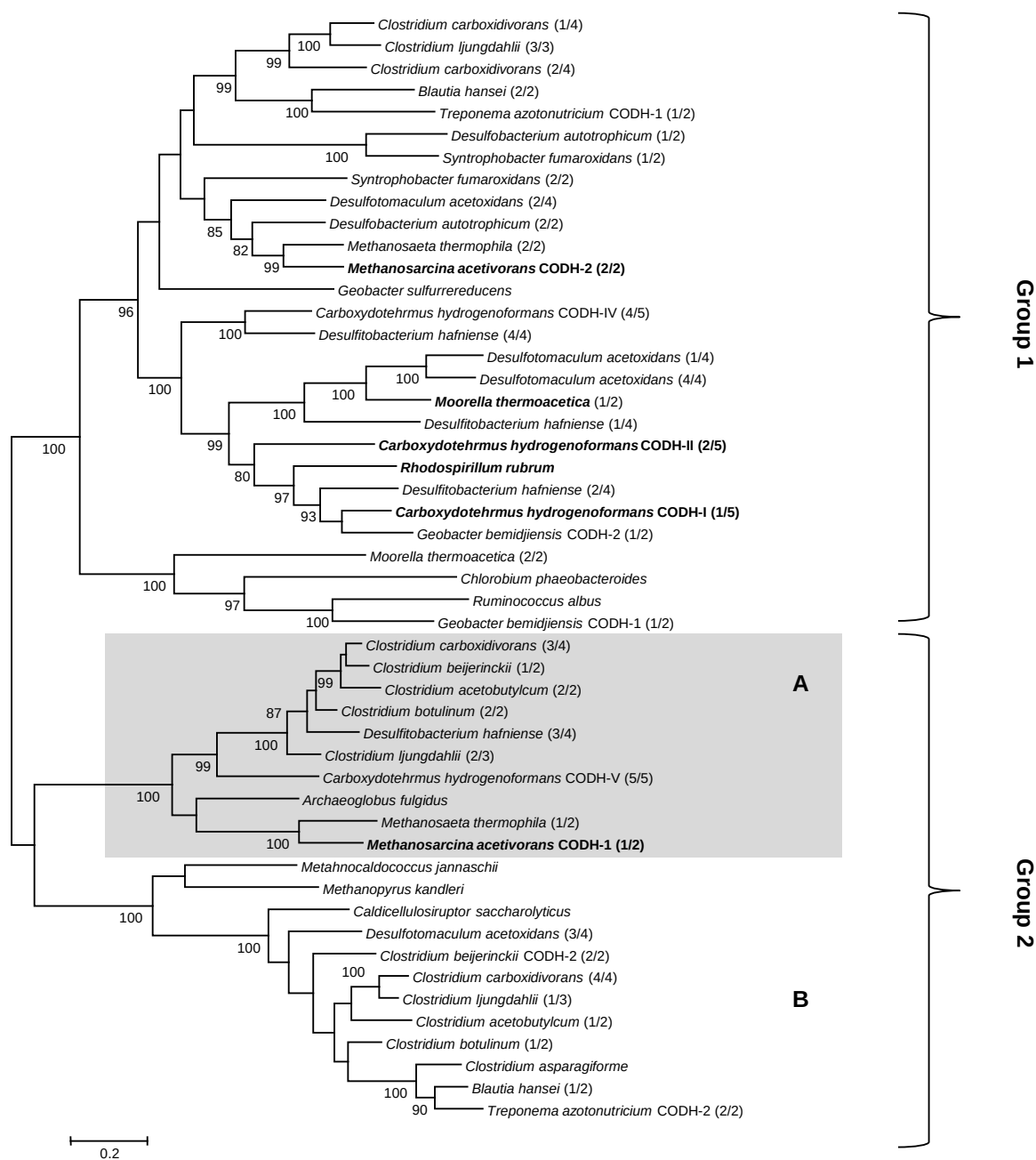


Figure 3-4 Phylogenetic tree of CooS homologs. A phylogenetic tree of CooS homologs is constructed using the maximum-likelihood method. Major clades (labeled A) of CooS sequences are highlighted in gray boxes. CODHs biochemically or structurally characterized are indicated in bold letters. For species having more than one CODH, the number of the CODH out of the total is given parenthetically. Only bootstrap supports equal to or more than 80% are indicated.

Discussion

In previous reports, several CODH_{Rr} variants of the C-cluster ligands were constructed and examined for the activities and metal contents (Table 3-5) (41, 51, 118, 119). Among the variants, only Val substitution for the His²⁶¹ (corresponding His²⁶⁵ in CODH_{Rr}) resulted in a decrease in Ni content (118). Therefore, His²⁶¹ was thought to be the only critical residue required for proper assembly of the Ni into the C-cluster. The activity resulting from this substitution decreases CO oxidation activity, like other substitutions. In addition, this substitution results in an increase in NH₂OH reduction activity.

This study for the first time demonstrates that, Cys²⁹⁵ also plays a critical role in Ni coordination in the C-cluster where these ligands directly coordinate Fe1. ICP-MS analysis demonstrated the substitution revealed the decrease of Ni content and no major change of Fe content (Table 3-3). In addition, the UV-visible absorption spectrum of the as-isolated C295A in this study and C-cluster-miss CODH-II spectrum (52), where only C-cluster was missed and B and C clusters were assembled, suggests the substitution had no major effect on the ability of CODH-II to assemble a full complement of [Fe-S] clusters (Fig. 3-2). Thus, Cys²⁹⁵ and His²⁶¹ were strongly suggested to coordinate the Fe1 directly, playing central roles for Ni coordination together in the C-cluster. Interestingly, an Ala substitution for Cys⁵²⁶, which seems to coordinate the Ni directly, did not have a major effect on Ni content (51). The exact role of His²⁶¹ and Cys²⁹⁵ in Ni coordination remains unknown. The lack of these Fe1 ligands probably alters the electronic structure as well as the geometry around the active site so that putting Ni into the cube is disrupted. The crystal structure presently reported provides for the common feature of C-clusters that allows the configurations of Fe₃-S₄-Ni moiety in the C-clusters to be asymmetric and apparently distorted by virtue of the link to Fe1 (53). Our data suggest that the correct configuration of Fe1 coordinated by His²⁶¹ and Cys²⁹¹ is essential for Ni-insertion into the

C-cluster.

CODH-II C295A exhibited no CO oxidation activity and, alternatively, exhibited an increased level of NH_2OH reduction activity (Table 3-4). This was similar to CODH-II H261A and previously from CODH_{Rr} H265V (41). Hybrid cluster proteins (HCPs) also exhibit NH_2OH reduction activity (144). HCP, first reported in 1989, many research have focused on the activity center called hybrid cluster. Recent structural studies revealed hybrid cluster is composed of a novel $[\text{4Fe-2S-2O}]$ cluster (3, 4, 74). Despite the wealth of spectroscopic and structural information on HCP, the precise metabolic role of HCPs remains unknown (1, 13). These suggested the reactivity of Cys²⁹⁵ or His²⁶¹ substituted C-cluster was similar to that of hybrid cluster of HCPs. The reasons are as follows; i) in previous reports, a structurally-based sequence alignment between the HCP and CODH-II highlights the close structural similarity between these enzymes. In fact, all the cysteine and histidine cluster ligands and many of the residues contributing to the strong hydrophobicity are conserved (4). Secondly, HCPs showed hydroxylamine reduction activity, while the metabolic role of HCPs remains unknown.

CODH-II C295E showed similar properties compared to the variants. Specifically, UV-visible absorption spectrum, metal content and activity change of CODH-II C295E are quite similar to C295A and H261A. This suggested that the CODH-V C-cluster lacks Ni and consequently differs in its properties from well-characterized CODHs. Additionally, a few studies concerning Group 2 suggest the physiological function was different from the well-characterized CODHs (76). This study would provide important insight into Group 2 CODHs like sequences whose property and physiological functions are not well known.

3. Cysteine 295 indirectly affects Ni coordination

Table 3-5 Metal contents and activities in *R. rubrum* CODH mutants.

Recombinant Protein	Metal content		Activity			
	Ni (mol/mol CODH)	Fe (mol/mol CODH)	CO oxidation (U/mg) ¹	CO ₂ reduction (U/mg) ²	NH ₂ OH reduction (U/mg) ³	H ₂ Oxidation (U/mg) ⁴
WT	0.85(1)	8.97(1)	6,230(1)	4.3(2)	1.0(4)	0.04(4)
	0.97(2)	8.3(2)	4,000(2)			
	0.97(3)	8.9(3)	77,00(3)			
H265V	0.24(2)	7.9(2)	0.67(2)	0.003(2)	28.8(4)	0.05(4)
C531A	0.85(1)	8.9(1)	6.9(1)	N.D.	0.85(4)	8.28(4)
	0.85(3)	8.9(3)				
C338A	0.87(1)	7.29(1)	<0.1(1)	N.D.	N.D.	N.D.
C451A	0.92(1)	6.58(1)	<0.1(1)	N.D.	N.D.	N.D.
C451S	0.84(1)	7.59(1)	86.3(1)	N.D.	N.D.	N.D.

U, 1 $\mu\text{mol CO}$ ¹, NH_2OH ³ and H_2 ⁴ consumption per minute, ² 1 $\mu\text{mol CO}$ production per minute

N.D. , Not determined

(1), Jeon *et al.* ; (2), Spangler *et al.* 1998; (3), Staples *et al.* 1999; (4), Heo *et al.* 2002

Chapter 4

Integration and outlook

In this study, firstly I investigated over-expression system of carbon monoxide dehydrogenases (CODHs) in a thermophilic carboxydophilic bacterium *Carboxydotherrmus hydrogenoformans*, which are potentially efficient catalysts to be feasible for an efficient CO₂ reduction system. In Chapter 2-1, I improved CODH-II over-expression system. The system is suitable for large scale culture and may provide safety since the system is free from hydrogen sulfide caused by Na₂S. Further detailed structural analysis towards the construction of artificial catalysts will be easy using this CODH-II over-expression system. In Chapter 2-2, I constructed an over-expression system of CODH-I, which is a key enzyme for CO₂ reduction, in *E. coli* through co-expression of Ni insertion gene *cooC*. This finding will be helpful for the application of CODH-I to CO₂ photo-reduction systems. Additionally, these studies suggested that CooC have protein specificity in Ni²⁺ insertion among CODHs and ACSs.

Secondly, I investigated Ni binding system in CODH-II activity center using the CODH-II over-expression system. I demonstrated one of C-cluster ligands Cys²⁹⁵ indirectly and His²⁶¹ together affect Ni-coordination in the C-cluster. The exact role of His²⁶¹ and Cys²⁹⁵ in Ni coordination remains unknown. It is very unlikely that this Cys residue is a ligand for the Ni - in all structures it is over 4 Å away. The lack of these Fe1 ligands probably alters the electronic structure as well as the geometry around the active site so that putting Ni into the cube is disrupted. EPR and/or EXAFS study may reveal further detailed Ni and Fe1 state in the catalytic center. Based on these information, efficient and artificial CO₂ reducing catalysts mimicking CODH-II C-cluster will be constructed in near future, This study also would provide

important insight into CODH-V and several CODHs whose property and physiological functions are not well known. In several CODHs including CODH-V, several alignment analyses suggest the conserved ligand Cys²⁹⁵ is replaced by Glu. This study suggested that the C-cluster of the CODHs lacks Ni and consequently differs in its properties from well-characterized CODHs.

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