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



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ABSTRACT

A unique [Ni–Fe–S] cluster (C-cluster) constitutes the active center of Ni-containing carbon monoxide dehydrogenases (CODHs). His²⁶¹, which coordinates one of the Fe atoms with Cys²⁹⁵, is suggested to be the only residue required for Ni coordination in the C-cluster. To evaluate the role of Cys²⁹⁵, we constructed CODH-II variants. Ala substitution for the Cys²⁹⁵ substitution resulted in the decrease of Ni content and didn't result in major change of Fe content. In addition, the substitution had no effect on the ability to assemble a full complement of [Fe–S] clusters. This strongly suggests Cys²⁹⁵ indirectly and His²⁶¹ together affect Ni-coordination in the C-cluster.

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1. Introduction

Ni-containing carbon monoxide dehydrogenases (CODHs) are phylogenetically related; however, they vary in terms of their metabolic role, subunit composition, and catalytic activities [1,2]. CODHs physiologically catalyze oxidation of CO or reduction of CO₂ in an active center called the C-cluster [1–3]. The C-cluster is conserved in CODH structures from some bacteria, *Carboxydotherrmus hydrogenoformans*, *Rhodospirillum rubrum*, *Moorella thermoacetica*, and an archaeon *Methanosarcina barkeri*. The C-cluster is composed of a unique [Ni–Fe–S] cluster that is linked by conserved ligands: five cysteines and one histidine residue (Fig. 1) [4–8].

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 [9]. Structural studies show His²⁶¹ directly coordinates one of the Fe atoms in the cluster (Fe1) with the Cys²⁹⁵ in CODH-II (corresponding His²⁶⁵ in CODH of *Rhodospirillum rubrum* (CODH_{Rr}) [6]. Hereafter, residue numbers are based on the structure of *C. hydrogenoformans* CODH-II, unless otherwise noted. It is very unlikely that this His residue is a ligand for the Ni in all structures because it is over 4 angstroms 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 is a thermophilic anaerobic CO-utilizing bacterium. *C. hydrogenoformans* contains five genes encoding CODHs designated CODH-I–CODH-V on the genome [10]. Several functions for the enzymes are proposed based on their gene context and on physiological experiments [10–12]; 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. In CODH-V, several alignment analyses suggest the conserved ligand Cys²⁹⁵ is replaced by Glu [3,13]. To evaluate the role of Cys²⁹⁵ in CODH-II, we constructed CODH-II variants of the Fe1 ligands (C295A, C295E mimicked for CODH-V and H261A) and compared their properties.

2. Materials and methods

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

The pET28-a based CODH-II expression plasmid used for mutagenesis experiments was previously described [14]. The Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to introduce C295A, C295E, H261A and H261V mutations into the *cooS*-II gene. The oligonucleotide primer pairs were as follows: for C295A, 5'-TTAACGTGGTAGGTATTTGTGCCACGGG-CAACGAGGTTTTGATGC-3' (forward), and 5'-GCATCAAAACCTCGT TGCCCGTGGCACAAATACCTACCACGTTAA -3' (reverse), C295E; 5'-

Abbreviations: NH₂OH, hydroxylamine; CoA, coenzyme; PCR, polymerase chain reaction; IPTG, isopropyl β-D-1-thiogalactopyranoside.

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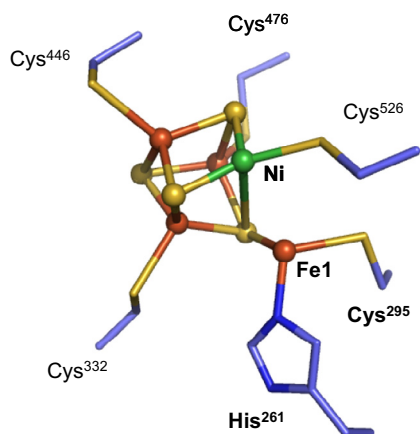


Fig. 1. C-cluster structure of *C. hydrogeniformans* CODH-II. The C-cluster is linked with residues His²⁶¹, Cys²⁹⁵, Cys³³², Cys⁴⁴⁶, Cys⁴⁷⁶ and Cys⁵²⁶. Fe atoms are colored in red, sulfur atoms are in yellow, and a Ni atom is in green.

ATAACGTGGTAGGTATTTGTGAAACGGGCAACGAGGTTTTGATG-3' (forward) and 5'-CATCAAAACCTCGTTGCCGTTTCACAAATACCTAC CACGTTAAT-3' (reverse); H261A, 5'-GTGGCCGTTTCATGGGGCTAA CCCGGTCCTGTC-3' (forward), 5'-GACAGGACCGGGTTAGCCCATGA ACGGCCAC-3' (reverse); and for H261V, 5'-AATGTGGCCGTTTCATG GGGTGAACCCGGTCCTGTCCGA-3' (forward) and 5'-TCGGACAGGA CCGGGTTACCCCATGAACGGCCACATT-3' (reverse). Heterologous expression and purification of the CODH-II variants were performed as described previously [14]. Briefly, N-terminal (His)₆-tagged CODH-II variants were expressed in *Eschericia coli* Rosetta2 (DE3) (Novagen, Madison, WI) harboring the pRKISC plasmid [15]. *E. coli* cells were cultivated in TB medium at 30 °C aerobically to an OD₆₀₀ of 0.6–0.7. Then, 0.2 mM IPTG was added and the cells were cultivated anaerobically for 20–22 h. The cells, 6–8 g (wt/vol), containing recombinant CODH-II variants were disrupted using a French press. The His-tagged enzymes were purified using a Cosmogel His-Accept column (Nacalai Tesque, Kyoto, Japan) in an anoxic glove box (COY Laboratory Products Inc., Grass Lake, MI) in an atmosphere of 95% N₂/5% H₂ at 25 °C. Buffer A (50 mM Tris-HCl, pH 8.0, 300 mM NaCl) containing 20 mM imidazole and 2 mM dithionite was used for washing, buffer A containing 300 mM imidazole and 2 mM dithionite was used for elution.

2.2. 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.

2.3. Enzyme assays

CO and H₂ oxidation activity was assayed based on the CO and H₂ dependent reduction of oxidized methyl viologens at 70 °C, respectively, as described previously [11]. One unit of activity was defined as the reduction of 1 μmol of CO 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 [16,17]. 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 [14]. Briefly, production of CO from CO₂ with the enzyme was monitored with the formation of carboxyhemoglobin. One unit of activity was defined as 1 μmol CO production per minute. UV visible absorption spectra of the CODH-II variants were recorded at room temperature as described previously [11]. Enzyme samples were equilibrated in 50 mM Tris-HCl buffer at pH 8.0 before activity and UV-visible absorption analysis.

2.4. Computational analysis

Multiple sequence alignments were constructed using CLUSTAL W [18]. The alignments were examined and columns with gaps were trimmed prior to phylogenetic reconstructions. Maximum-likelihood phylogenetic analysis was performed using PhyML ver. 3 [19].

3. Results

3.1. Metal analysis

The metal content of CODH variants were compared with that of wild type (Table 1). 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) [6]. C295A, C295E and H261A contained nearly the same amount of iron atoms, respectively (Table 1). Wild type CO oxidation activity (8900 U/mg) obtained in this study was lower compared to that of the previous report (10,000–14,000 U/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 1).

3.2. 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. 2). The spectra of A420/A280, which is unique absorbance for CODH-II C-cluster, were similar to wild-type CODH-II [14]. For the wild-type CODH-II, bleaching of the [Fe-S] cluster shoulder occurs after treatment with CO or dithionite [14]. In contrast, the bleaching was observed when variants were treated with dithionite but not CO, e.g., CODH_R H265 V (Fig. 2 insets), suggesting the [Fe-S] clusters of variants were not reduced by CO.

3.3. Activity of CODH-II variants

The activities of CODH-II variants were determined. All substitutions resulted in decreased CO oxidation activity and an increase

Table 1
Metal content 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

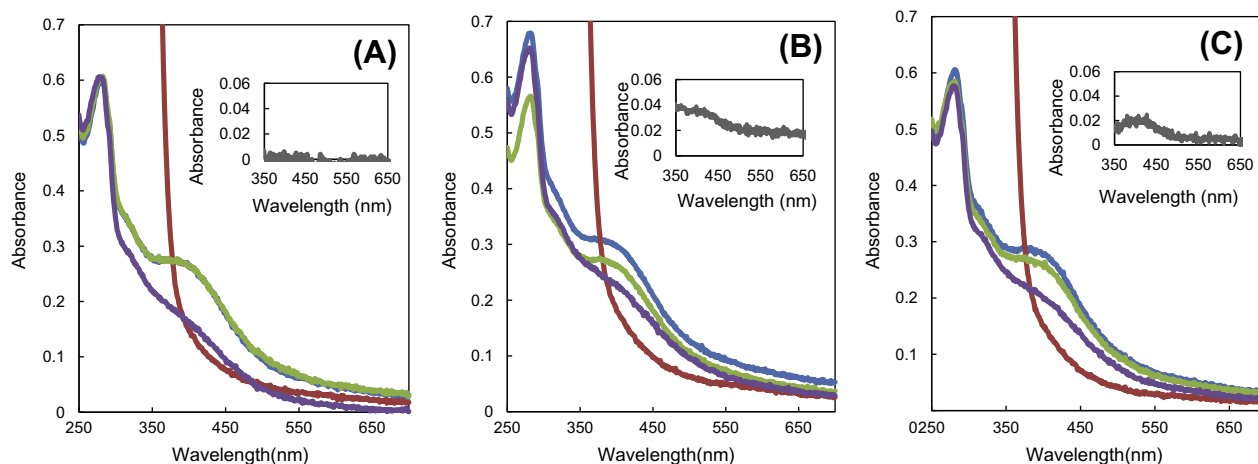


Fig. 2. UV–visible absorption spectra of CODH-II variants. The UV–visible absorption spectra of purified CODH-II variants in 50 mM Tris–HCl, pH 8.0 are recorded. (A) CODH-II C295A, (B) CODH-II C295E and (C) CODH-II H261A. Conditions for each curve: blue line, as-isolated; red line, reduced with 2 mM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

Table 2
Specific activity of CODH variants.

	CODH-II _{Ch}				CODH _{Rr}	
	W.T.	C295A	C295E	H261A	W.T. ^a	H265V ^a
CO oxidation (units/mg)	8900	N.D.	1.8	N.D.	7000	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. [9].

in NH₂OH reduction activity (Table 2). C295A, 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. 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.

3.4. 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). 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). Group 1 contained well-characterized CODHs including CODH-II and CODH_{Rr}. 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).

4. Discussion

In previous reports, several CODH_{Rr} variants of the C-cluster ligands were constructed and examined for the activities and metal contents [9,16,20,21]. Among the variants, only Val substitution for the His²⁶¹ (corresponding His²⁶⁵ in CODH_{Rr}) resulted in a decrease in Ni content [9]. 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 1). In addition, the UV–visible absorption spectrum of the as-isolated C295A in this study and C-cluster-miss CODH-II spectrum [22], 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. 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 [9]. 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 [4–8]. 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 NH₂OH reduction activity (Table 2). This was similar to CODH-II H261A and previously from

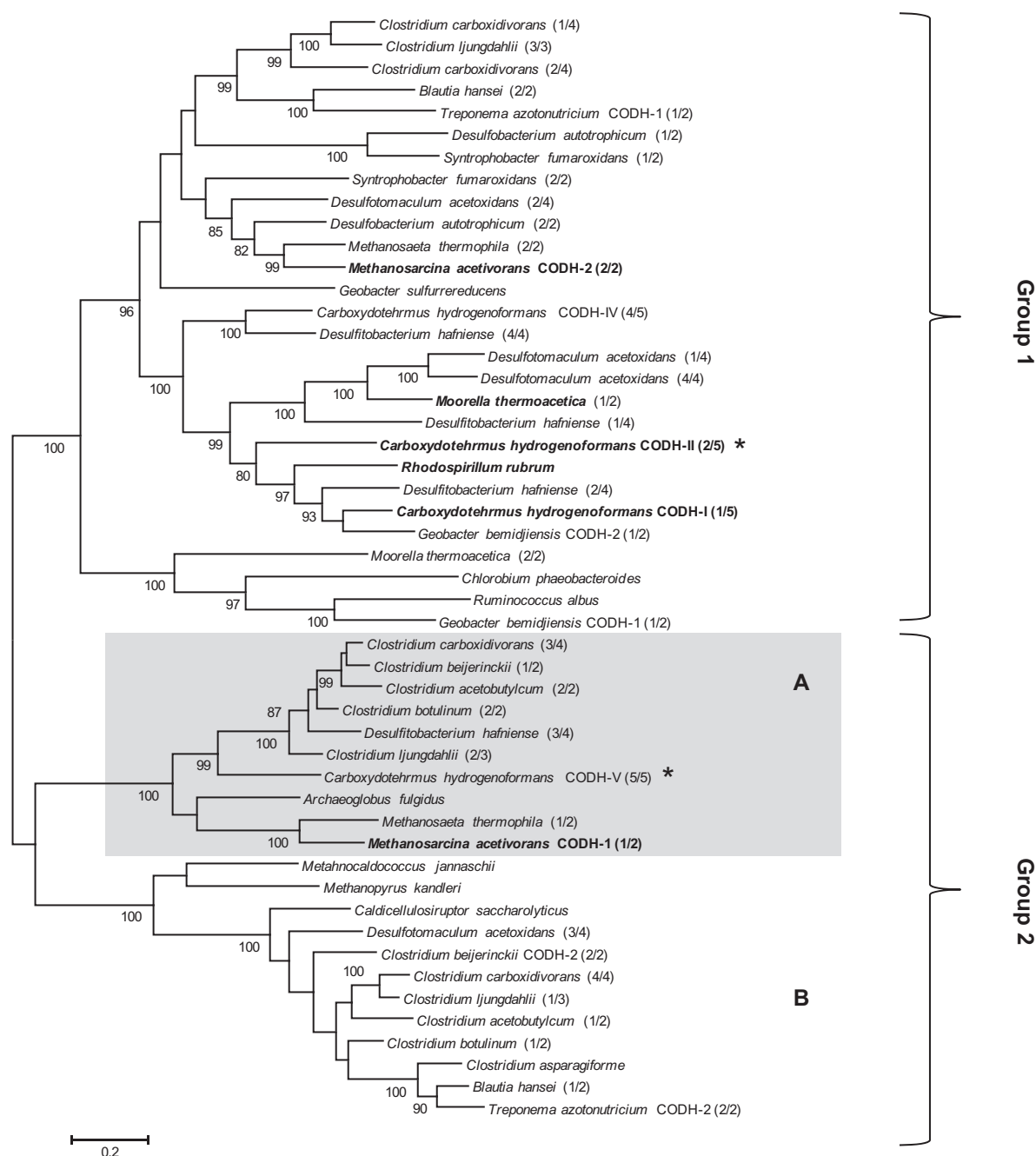


Fig. 3. Phylogenetic tree of CooS homologs. A Phylogenetic tree of CooS homologs is constructed using the maximum-likelihood method. Major clades (labeled A) of CooS sequences are highlighted in gray boxes. CODHs biochemically or structurally characterized are indicated in bold letters. CODH-II and CODH-V from *C. hydrogenoformans* is highlighted by an asterisk. 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.

CODH_R H265V [16]. C-cluster structure is similar to hybrid cluster structure of hybrid cluster proteins (HCPs), and HCPs show NH₂OH reduction activity [23,24]. These suggested the reactivity of Cys²⁹⁵ or His²⁶¹ substituted C-cluster was similar to that of hybrid cluster.

CODH-II C295E showed similar properties compared to the variants, suggesting 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 [25,26]. This study would provide important insight into Group 2 CODHs like sequences whose property and physiological functions are not well known.

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