Tunnel-redesigned O₂-tolerant CO dehydrogenase for removal of CO in real flue gas

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Abstract

Carbon monoxide dehydrogenase (CODH)-catalyzed oxidation of CO to CO₂ provides a promising means of removal of toxic and waste CO from industrial flue gas despite of the lack of active and stable enzymes in the atmosphere. Herein we present rationally and selectively redesigned *Ch*CODH-II (*Carboxydotherrnus hydrogenoformans*) variants by engineering gas tunnels in order for O₂-tolerant CODHs to catalyze efficient CO oxidation under oxygen (O₂). Using the redesigned *Ch*CODH-II A559W and A559H variants showing 42- and 128-fold elevation of O₂ tolerance, respectively, complete CO removal was achieved under a near-atmospheric condition. Moreover, these variants efficiently removed CO from industrial flue gas (Linz–Donawitz converter Gas: LDG) discharged from a steel mill despite the high O₂ level (13.4%) during successful and repeated reuse after immobilized on Ni-NTA agarose beads. Our study will provide insights into redesigning the transformation of O₂-sensitive CODHs into tolerant enzymes for use as workhorses for conversion of toxic or waste gases into safe or value-added chemicals.

Main

Large amounts of CO pollutants are emitted from natural sources as well as man-made processes¹ (e.g. annually over 219 billion Nm³ of CO-containing flue gases from POSCO)^{2,3}. Carbon monoxide (CO), the most abundant air pollutant found in the atmosphere other than CO₂ according to the OECD database (<https://stats.oecd.org>, air emissions source in 2017), can provide sufficient carbon and energy sources for converting waste gas to fuels and chemicals through a clean and sustainable method. To convert cheap, abundant and recyclable CO resources² into fuels and chemicals, one of the most ideal CO utilizations is the employment of carbon monoxide dehydrogenases (CODHs) capable of oxidizing CO to CO₂ in the manner of bacteria and archaea⁴. Practical applications of these CODHs showing remarkable substrate selectivity and viability in gas mixtures even with metal-catalyst inhibitors (sulfur, chlorine, and tar)⁵ are promising; however, they remain very limited due to the diminished activity for CODHs² under an atmospheric oxygen (O₂) presence.

Two CODH families classified as metal species of active site^{2,6}, namely Mo–Cu CODHs with a dinuclear [CuSMo(O)OH] cluster (E.C.1.2.5.3) and Ni–Fe CODHs with a distinctive [NiFe₃S₄OH_x] cluster (E.C.1.2.7.4), catalyze a moderate rate of aerobic CO conversion (93.3 s⁻¹)⁷ or a very high rate of anaerobic CO conversion (31,000 s⁻¹)⁸, respectively. Unlike highly O₂-sensitive CODHs, only two O₂-tolerant Ni–Fe CODHs from *Carboxydotherrnus hydrogenoformans* (*Ch*CODH-IV)⁶ and *Desulfovibrio vulgaris* (*Dv*CODH)⁹ show unique features and intriguing insights in terms of both structures and the amino acids' electrochemical properties against O₂ exposure. For effective and widespread use of CODHs in industrial fields, these enzymes must overcome the inevitable disadvantage of poor activity under the aerobic condition. This barrier motivated us to tackle the issue of the O₂ sensitivity of *Ch*CODH-II with the known highest CO oxidation rate (31,000 s⁻¹)⁸ for practical applications treating pollutant CO flue gases.

Herein we demonstrate the substrate tunnel modification of *Ch*CODH-II (*Carboxydotherrmus hydrogenoformans*) using a rational approach based on protein sequences and structures from Ni–Fe CODHs, which leads to highly improved O₂ tolerances in the oxidation of CO to CO₂. By analyzing substrate tunnels close to the active site (C-cluster) of *Ch*CODH-II, we discovered the common bottleneck of substrate tunnels in Ni–Fe CODHs, even those including acetyl-CoA synthase/CODHs. Our mutation and kinetic experiments revealed that approximately 100-fold-increased O₂ tolerance relative to that of the *Ch*CODH-II wild type (WT) was achieved for the redesigned *Ch*CODH-II variant under a near-atmospheric condition while maintaining excellent catalytic performances. In addition, the CO of real industrial flue gas was completely removed by the engineered enzyme even under the atmospheric O₂ condition, which strongly indicates a potential application as CO-removal biocatalysts in industrial fields^{2,3}.

Results

A key spot for O₂ response in *Ch*CODHs

To explore key regions for O₂ sensitivity in Ni–Fe CODHs, we analyzed the phylogenetic trees and protein sequences between O₂-sensitive *Ch*CODH-II and O₂-tolerant *Ch*CODH-IV: five forms (*Ch*CODH I–V) as genomic contexts¹⁰ (Fig. 1a–b and Supplementary Fig. S1). Five residues (D94, E101, R187, Y558, Q559) of *Ch*CODH-IV are quite different from those of *Ch*CODH-II, but are conserved in *Dv*CODH. Moreover, analysis of the structures and gas tunnels of Ni–Fe CODHs suggests that only two residues, Y558 and Q559 located along tunnels, can participate in substrate transfer for O₂-tolerant *Ch*CODH-IV (Supplementary Table S1–2 and Fig. S2). In the substrate tunnels of O₂-labile *Ch*CODH-II, the respective two residues of *Ch*CODH-IV (Y558 and Q559) correspond to the positions of A559 and M560 in a similar fashion of localization (Fig. 1c and Supplementary Fig. S2). The orientation of the side chain at A559 in *Ch*CODH-II (Y558 in *Ch*CODH-IV) was predicted to be more crucially involved in substrate molecule transport in tunnels than M560, due to the residue's opposite orientation.

In addition, we realized that residue A559 forms a triad with its neighboring residues (V565 and I580) along the junction of substrate tunnels (Fig. 1d). Much to our surprise, the triad close to the active site is universally present in structurally similar Ni–Fe CODHs, even in the different bifunctional acetyl-CoA synthase (ACS)/*Mt*CODH subunit. Moreover, this narrowest position in analyzed substrate tunnels (Supplementary Fig. S2) is more likely to act a key spot in gas tunnels that critically affects the transport rate of substrates depending on tunnel characteristics such as diameter¹¹. We therefore hypothesized that O₂ transfer inside *Ch*CODH-II and IV is affected by different characteristics of the substrate bottleneck point as a common route, while gas substrates pass into the active site of the C-cluster. Accordingly, by substituting amino acids and experimental demonstrations of O₂ tolerance, we designed various variants displaying different characteristics at bottleneck points. The results facilitate understanding of the relationships between tunnels' characteristics and the O₂ stress response of CODH or CODH-harboring cells.

O₂ tolerance and catalytic properties of ChCODH variants

First, to determine the respective effects of bulk, charge, polarity, and hydrophobicity at putative hot-spot positions 559 and/or 560, 565, and 580 of *Ch*CODH-II, we constructed and purified a number of single and double variants in *E. coli* (Supplementary Table S3–4 and Fig. S3). The variants were spectrophotometrically screened by measuring specific activities by CO oxidation and residual activities after O₂-exposure and inactivation (Supplementary Table S4). The effects of the mutations showed a simple and clear correlation between the side-chain aromatic and O₂ tolerance (Fig. 2a). After exposure to dissolved O₂ (10 µM), the residual activities of aromatic groups A559H, A559Y, A559W (> 90%), A559F, A559P (40–50%), A559M (58%), and A559S (90%) were detected respectively, while *Ch*CODH-II WT exhibited zero residual activity due to the complete loss of activity. Also, compared to WT, the specific activity for substrate CO was increased by about 1.5–2 fold for A559S, A559H, A559F, A559Y, and A559W. The amino-acid side chains at the 559 position seemed to have a strong impact on O₂ sensitivity response as well as CO oxidation in *Ch*CODH-II.

Figure 2b demonstrates the remarkable effects of bulky aromatic mutations in improving the resistance to O₂. The engineered A559W and A559H *Ch*CODH-II showed over 50% residual activity due to significant O₂ tolerances, even in the presence of 25–76.5 µM O₂, while the WT showed only 50% residual activity at 0.6 µM O₂, which suggested that a roughly 41.7–127.5-fold improvement in O₂ tolerance was achieved. The O₂ tolerance of the engineered variant of *Ch*CODH-II was much higher than those of naturally O₂ resistant *Ch*CODH-IV and *Dv*CODH, which would enable application to industrial flue gas containing 1.5–6.5 µM of O₂ (0.1–0.5%). Table 1 indicates that all of the *Ch*CODH-II variants have high catalytic rates ($k_{\text{cat}} \approx 2,000 \text{ s}^{-1}$ at 30°C), and thus suggests that the engineering of enzymes did not disturb the catalytic activity. By contrast, *Ch*CODH-IV and its variants presented 100-fold-lower k_{cat} than that of *Ch*CODH-II. These results suggest that the tunnel characteristics of *Ch*CODHs would partially affect enzymatic activity.

As for V565 and I580 comprising the junction triad, mutations at these sites were ineffective for O₂ tolerance, thus indicating that the A559 position is a key site for O₂ access in *Ch*CODH-II (Supplementary Table S4). Meanwhile, among the mutations of M560, which is another unique position in *Ch*CODH-II and IV, the M560Q variant showed only slightly improved O₂ tolerance (Supplementary Fig. S4), suggesting that this site is not crucial for O₂ tolerance.

As shown in Fig. 2c, we examined the reverse changes of O₂ tolerances in *Ch*CODH-IV and Y558A in order to reconfirm the results for the *Ch*CODH-II variants. As expected, Y558A was very sensitive to O₂, as much as *Ch*CODH-II in fact, thus indicating that the role of Y558 (*Ch*CODH-IV) is similar to that of the engineered A559 variants (*Ch*CODH-II) as a key site for O₂ tolerance. This supported the hypothesis that the substitution of tyrosine for alanine affects O₂ tolerance but does not change the catalytic properties of Y558A relative to *Ch*CODH-IV WT (Table 1). According to the data for the improved O₂-tolerant

*Ch*CODH-II variants, when positive mutations (tryptophan, histidine) were introduced to Y558, residual activities of the *Ch*CODH-IV variants were observed (Fig. 2c). These results showed that tyrosine substitutions for tryptophan and histidine at 558 are also relevant to O₂ tolerance in *Ch*CODH-IV, as is the case for the *Ch*CODH-II A559 variants. Consequently, we concluded that the 559 or 558 position was the commonly shared key spot affecting O₂ tolerance.

For stable and efficient conversion of CO under the influence of O₂, we further estimated the stability of engineered A559W as an oxidizing biocatalyst (Fig. 2d). Under 10 μM O₂, the remaining activities of *Ch*CODH-IV and A559W were quite stably maintained as time courses, but *Ch*CODH-II's rapidly diminished. These results revealed that the *Ch*CODH-II variants exhibited stable activities under the 10 μM O₂ concentration in spite of the rapid O₂ inactivation of *Ch*CODH-II WT and the low CO conversion of *Ch*CODH-IV. It is well known that most Ni–Fe CODHs are quickly inactivated by O₂ within only a few minutes^{8,9}, which inactivation is more severe in the absence of O₂ scavengers such as dithiothreitol (DTT) and dithioerythritol (DTE)¹². Further, variants exposed to O₂ for several hours can efficiently undergo CO conversion at higher conversion rates than can *Ch*CODH-IV harboring low specific activity (≈90 U/mg). Therefore, it is predicted that O₂-tolerant *Ch*CODH-II variants will give good performances in the utilization of industrial waste gas mixtures containing O₂.

Structural analysis of O₂-tolerant *Ch*CODH-II A559W

To uncover how *Ch*CODH-II variants are O₂-tolerant, we resolved the crystal structure of the A559W variant (PDB ID: 7ERR, Fig. 3a and Supplementary Table S5). By considering that the activity loss of most CODHs under the aerobic condition is highly related to alteration or destruction of the active site C-cluster, which is the most O₂-sensitive metal site^{6,9,13}, maintaining the structural integrity of the C-cluster under the aerobic condition is vital for tolerance. Thus, having prepared protein samples under the aerobic condition, we determined whether the structural integrity of the C-cluster in the A559W variant was maintained. When we examined the interacting residues around the C and D clusters of the A559W variant, they were highly similar to those of *Ch*CODH-II WT (PDB ID: 3B53), which was anaerobically prepared with DTT addition. Also, the positions of the C and D clusters were similar to each other (Fig. 3a), suggesting that the C cluster of the A559W variant has a similar architecture to that of *Ch*CODH-II WT. In the case of properties for the gas tunnel around Trp559, they were changed significantly due to the bulky and aromatic side chain of the Trp559 residue (Supplementary Fig. S5). The side chain of Ile580 was pushed away through the incorporation of bulky Trp559 instead of alanine, and the position of Val582 also was slightly changed (Supplementary Fig. S5). As a consequence of Trp559 incorporation, the gas tunnel in the A559W variant became narrower compared to that of *Ch*CODH-II WT (Fig. 3b). When we measured the radius of the gas tunnel at the bottleneck point using the CAVER program¹⁴, it was reduced by about 0.4 Å compared to *Ch*CODH-II WT.

To estimate the structural rigidity on each tunnel of *Ch*CODH-II WT and A559W, we calculated and compared distances between two residues (A/W559 and F608) across the tunnel by MD simulation (Fig.

3c). The distance and standard deviation (S.D.) values of A559W was slightly decreased to 7.545 and 0.175 Å than WT's, respectively. The present analysis shows decreases of local fluctuation in A559W's tunnel, reflecting that A559W by tunnel constriction is more rigid and likely to limit O₂ accessibility than WT. In case of O₂-tolerant NiFe hydrogenases^{15–18}, it is supported that constriction of the tunnels protects their active sites from oxygen attacks by serving as a barrier for molecular oxygen intrusion. Therefore, these results suggest possibilities that the tryptophan at 559 position is critical for rigidity of tunnel and affect for oxygen access to the active site of *Ch*CODH-II.

Efficient removal of industrial flue gas (Linz–Donawitz converter Gas: LDG) from steel mill under near-atmospheric condition

Next, we assessed the activity of engineered A559W for different CO mixtures from synthetic and industrial flue gases (Fig. 4a). Compared to the low-content CO (synthetic gas 1, 5% balanced N₂), the relative activities of A559W were similarly observed as a deviation up to 3.6% towards other CO mixtures: synthetic gas 1 (100%), gas 2 (95%), gas 3 (101%), Blast–Furnace Gas (BFG, 91%), Coke–Oven Gas (COG, 99%), and LDG (96%) respectively. This indicates that *Ch*CODH-II A559W has consistent and sufficient performances selectively for gaseous CO mixtures irrespective of the low CO content and multi-component mixtures such as CO₂, CH₄, and H₂. Especially, no severe inactivation by O₂ and unknown trace impurities in industrial flue gases of BFG, COG, LDG suggests that O₂-tolerant *Ch*CODH variant can be used as a suitable and usual biocatalyst for CO-containing flue gases from a variety of industrial environments.

Finally, to evaluate the O₂-tolerant *Ch*CODH-II variant's performance for removal of gaseous CO, we tested A559W as an oxidizing biocatalyst in 50% CO-saturated 0.2 M HEPES (pH 8) with or without dissolved 170 µM O₂ (≈13.4%). Figure 4b indicates that *Ch*CODH-II and A559W readily consumed CO within 2.5 hrs under the anaerobic condition, whereas under O₂, only A559W consumed CO within 3 hrs. This experiment confirmed that A559W, unlike *Ch*CODH-II WT, is able to sufficiently and selectively consume CO without severe O₂ inactivation. A559W under the aerobic condition seemed to traverse a lag phase in a half-hour, probably until enzyme reactivation by CO could overcome O₂-induced arrest. Ni–Fe CODHs can be recovered when CO or dithionite is amended¹⁹. A plausible explanation is that CO can serve as a weak reductant for A559W recovery, in the manner of dithionite-treated *Ch*CODH-II^{6,9,19}. Further, we also often observed reactivation of CODHs by CO in short-O₂-exposure enzymes appearing in a lag phase (data not shown). Finally, for practical application purposes, we monitored the CO utilization of A559W using real LDG flue gas from a steel mill in the presence of dissolved O₂ (≈13.4%). The variant A559W impressively displayed the capability to remove the total amount of CO (53.4%) from the LDG (Fig. 4c–d), but no CO consumption was observed in *Ch*CODH-II WT. Even under the atmospheric condition around 250 µM O₂ (≈20%), CO uptakes of 33% were observed in A559W. Thus, this oxidizing biocatalyst of A559W was shown to enable the first CO bioconversion under a near-atmospheric condition, and demonstrated outstanding performance in its complete CO consumption using a steel mill flue gas (LDG).

In addition to the efficient CO consumption, A559W must have the sustainable capacity to reuse and recycle for industrial flue LDG. Immobilized biocatalyst can be employed as a simple reuse and recovery manner for cost savings in industrial applications. Figure 5 shows that the conversion rate of CO by immobilized A559W is maintained regardless of repeated cycles for reuse and the condition of O₂ presence. The immobilized A559W was incubated at room temperature using flue LDG for the first 10 cycles (Fig. 5a), and consecutively LDG with dissolved 170 μ M O₂ ($\approx$ 13.4%) was used for the second 10 cycles. In case of O₂ absence, no loss of conversion rate during 10 cycles represents that the enzyme is still active after repeated uses and not affected by inhibitions of product CO₂ and other gas impurities from LDG. Under the presence of O₂, 10 cycles of the reaction also exhibited complete oxidation of CO to CO₂, indicating that the immobilized A559W is unaffected by O₂ interference. The results suggested O₂-tolerant CODH through immobilization could be operational stable and recyclable without any severe inactivations by flue LDG and harsh O₂ conditions.

Conclusions

This study thus points the way toward efficient avoidance of rapid O₂ inactivation of Ni–Fe CODHs. It is often considered that catalytic activity and O₂ tolerance are tradeoffs, but the *Ch*CODH-II variants show how to overcome such limits. Thus, the use of O₂-tolerant *Ch*CODH-II would provide the advantage of efficient CO conversion for various waste gases and syngases containing O₂. (e.g. biomass, plastics). Moreover, O₂-tolerant Ni–Fe CODHs could be applied to treatment of harmful CO in daily life (e.g. removal of toxic CO from cigarette smoke)².

In summary, for the first time, we have demonstrated that O₂-tolerant CO-oxidizing enzymes can efficiently and effectively convert a flue CO gas mixture without any inactivation. It is noteworthy as well, that the bottleneck of substrate tunnels in Ni–Fe CODHs was routinely discovered through bioinformatic and structural analysis. To characterize the recombinant *Ch*CODH-II and IV variants from site-directed mutagenesis, the O₂ tolerances, tunnel characteristics, and kinetic parameters were determined. This knowledge may prove useful in future efforts towards the development of more efficient carbon monoxide catalysts that are less inhibited by O₂. Further, the feasibility of the *Ch*CODH variants was evaluated for construction of a CO-utilization system utilizing a CO-containing flue gas from a steel-making process as a cheap, abundant, and sustainable feedstock.

Methods

Gene synthesis and cloning

The genes coding for *Ch*CODH-II (CHY_0085), *Ch*CODH-IV (CHY_0736), and *Dv*CODH (DvU_2098) with *Nde*I and *Bam*HI restriction sites were optimized, synthesized and sequence-verified by Macrogen (Korea) based on their GenBank sequences (accession no. NC_007503 and NC_002937). The resulting fragments were cloned into pET28a (Novagen) containing a His-tag and an additional thrombin-cutting site. Site-

directed variants were constructed using the Quick Change site-directed mutagenesis method from Stratagene through *Pfu* DNA polymerase. PCR products were incubated for 60 min at 37°C with *DpnI* (2 U/μl) to remove the parental plasmid. The *DpnI*-treated plasmid was purified and transformed into the *E. coli* DH5α strain. Point mutations were verified by DNA sequencing. The resulting pET28a-*Ch*CODH-II, pET28a-*Ch*CODH-IV, pET28a-*Dv*CODH and all of the variant plasmids were transformed into *E. coli*-BL21-harboring pRKISC²⁰.

Protein expression and purification

Cultures were grown in a modified TB medium with addition of 0.02 mM NiCl₂, 0.1 mM FeSO₄, 2 mM L-cysteine, kanamycin (50 μg/mL) and tetracycline (10 μg/mL) at 37°C. When the culture reached an OD₆₀₀ of 0.4–0.6, expression of recombinant CODHs and variants was induced by addition of 0.2 mM IPTG supplemented with 0.5 mM NiCl₂, 1 mM FeSO₄, and 50 mM KNO₃ at 30°C after N₂-fluxing. The resulting cell pellets after harvesting were stored at –70°C. All of the purification steps were conducted in an anoxic glove box (model B, COY Laboratory Products Inc., Michigan, USA) under an atmosphere of 95% N₂/5% H₂ at room temperature. Sonication was anaerobically carried out on ice.

Cell lysates were centrifuged and purified by Ni-NTA affinity chromatography (Qiagen) with standard buffer containing 2 mM dithioerythritol (DTE) and 2 μM resazurin, but DTE and resazurin were omitted from the washing and elution steps when O₂-inactivation assaying. Protein concentrations were determined using the Bradford method. When the purified proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis on 12% gels, all of the samples were detected as a single band, the sizes of which approximately corresponded to the calculated sizes of proteins with the His₆-tag. If required, Western blot analyses using anti-His-tag antibodies were also carried out to confirm the purity of the proteins. For crystallization of the A559W variant, further purification was performed by size-exclusion chromatography (HiLoad 16/600 Superdex 200 prep grade, GE Healthcare Bio-Sciences, PA, USA), which was previously equilibrated with a buffer containing 20 mM Tris-HCl pH 7.5 and 3 mM DTT.

Size-exclusion chromatography with multi-angle light scattering (SEC-MALS)

SEC-MALS experiments on the *Ch*CODH-II A559W variant were performed using an fast protein liquid chromatography (FPLC) system (GE Healthcare) connected to a Wyatt MiniDAWN TREOS MALS instrument and a Wyatt Optilab rEX differential refractometer. A Superdex 200 10/300 GL (GE Healthcare) gel-filtration column was pre-equilibrated with buffer containing 20 mM Tris-HCl pH 7.5 and 3 mM DTT and normalized using ovalbumin protein. Proteins were injected (1 mg) at a flow rate of 0.4 ml/min. Data were analyzed using the Zimm model for static light-scattering data fitting and graphed using EASI graph with a UV peak in the ASTRA V software (Wyatt), as shown in Supplementary Fig. S6.

Activity measurement

CO oxidation activity was measured at 30°C, after having saturated the concentrations of CO using screw-cap cuvettes with CO head space. The activity was assayed by observing the CO-dependent reduction of oxidized ethyl viologen (EV_{ox} , $\epsilon_{578} = 10,000 \text{ M}^{-1} \times \text{cm}^{-1}$). A screw-cap cuvette contained 2 mL of reaction buffer: 20 mM EV_{ox} , 0–250 μM O_2 , 50 mM HEPES/NaOH buffer pH 8 with saturated CO (from 50 mL stock solution flushed with CO gas for 1 hr)²¹, and reactions were started by enzyme injection.

To test the effect of O_2 inactivation, we measured the residual activity of enzyme in the presence of appropriate O_2 concentrations. Before assaying the residual activity, the CODHs were incubated for 2 min by O_2 addition, and then the reaction mixture was diluted by 200–400 times, resulting in the final remaining O_2 being below 0.6 μM . One unit of CODH activity was defined as the amount of enzyme required to reduce 1 μmol of EV_{ox} per min at 30°C and pH 8.

Kinetic analysis

For calculation of the kinetic parameters, CO-saturated buffer corresponding to 0.91 mM CO (99.998% according to Henry's law) was used at 30°C. CO was purged into the headspace of a rubber septum-stopped cuvette containing 20 mM EV in HEPES/NaOH buffer (pH 8) for 1 hr. To monitor the initial velocity of the CODHs, the reaction was initiated by an injection of recombinant CODHs. The absorbance change at 578 nm was spectrophotometrically monitored at 30°C in an anoxic glove box. The kinetic parameters (k_{cat} and K_m) were calculated by the Hanes–Woelf equation (Supplementary Fig. S7). All of the enzymatic activities were determined in triplicate.

Crystallization and structural determination

Crystals of the ChCODH-II A559W variant were grown using the sitting drop method under the aerobic condition by mixing 0.2 μL of protein (5 mg/ml) with 0.2 μL of reservoir solution consisting of 0.1 M HEPES/NaOH pH 7.0, 50 mM $MgCl_2$, and 25% (w/v) polyethylene glycol 3350. The crystals were transferred to a solution containing the reservoir solution and 5% glycerol for cryoprotection. Data were collected at 100 K in 1° oscillations at the 5C beamline of the Pohang Light Source (PLS). Protein crystal was diffracted to a resolution of 2.2 Å, and the diffraction data were processed and scaled using the *HKL2000* software package²². The crystal for the A559W variant belonged to space group *C2*, with unit-cell parameters of $a = 112.1 \text{ Å}$, $b = 75.1 \text{ Å}$, and $c = 70.8 \text{ Å}$. The structure was resolved according to the molecular replacement method that uses the ChCODH-II WT model (PDB ID: 3B53) as a probe²³. Subsequent manual model building was performed using the *Coot* program,²⁴ and restrained refinement was carried out using the *CCP4* program²⁵. Several rounds of model building, simulated annealing, positional refinement, and individual *B*-factor refinement were performed using the *Coot* and *CCP4* programs. Table S6 lists the refinement statistics. The atomic coordinates and structural factors were deposited in the Protein Data Bank (PDB code 7ERR).

Molecular dynamics simulation of ChCODH-II WT and A559W

The structure of A559W (PDB ID: 7ERR) was used for simulation, and the wild type structure was predicted using Discovery Studio software (BIOVIA Corp., USA). The standard dynamic cascade using the NPT ensemble was performed for molecular dynamics simulation with the CHARMM force field. The molecular dynamics simulation was carried out for energy minimization, heating, equilibration and production. For minimization, first steepest descent algorithm with the max step of 1,000 and the RMS gradient of 1.0 was used. For more detailed minimization, the conjugate gradient algorithm with the max step of 2,000, and the RMS gradient of 0.3 were applied. The temperature was raised from 50 to 300 K for 10 ps at time step of 2 fs. Equilibration was conducted for 1 ns at time step of 2 fs and 300 K, and production was done for 3 ns at time step of 2 fs and 300 K.

GC analyses

The amounts of CO and CO₂ in the head phase were measured using an Agilent 7890B gas chromatograph equipped with a thermal conductivity detector (GC-TCD) and a carbon molecular sieve column (Carboxen 1000 with 60/80 mesh, Supelco, Park, Bellefonte, PA, USA). During the GC-TCD analysis, the initial oven temperature was 35°C for 5 min, which was gradually increased to 225°C at a rate of 20°C·min⁻¹. The injector and detector temperatures were 250 and 225°C, respectively, and the flow rate of the carrier gas (Ar) was maintained at a constant rate of 30 mL·min⁻¹. A rubber-sealed serum bottle (115 mL) containing 20 mL head space (CO of 446 µmol) and 95 mL of reaction buffer (20 mM EV_{ox}, 0–200 mM O₂, 200 mM HEPES/NaOH buffer pH 8 with purged CO [50% balanced N₂] or LDG gas for 1 hr) was adopted for the reactions, which were started by enzyme injection (50U) at RT and 150 rpm of shaking. For the enzyme reusability of A559W, each cycle of reaction was initiated at room temperature and pH 8, by the addition of the immobilized A559 enzyme (500 U/mg immobilized protein) into LDG-purged buffer with 40 mM EV_{ox}. The flue gases of BFG (CO, 25.45%; CO₂, 23.56%; H₂, 3.75%; O₂, 0.01%; N₂, 46.71%), COG (CO, 6.15%; CO₂, 2.42%; CH₄, 24.16%; C_mH_n, 3.07%; H₂, 55.4%; O₂, 0.1%; N₂, 8.63%), LDG (CO, 53.17%; CO₂, 18.51%; H₂, 1.43%; O₂, 0.11%; N₂, 26.77%, and unknown 0.1%) were acquired from Hyundai Steel Co (Korea).

Bioinformatics analysis

Searches for amino acid sequence homologs and multiple sequence alignments were performed using BLAST and ClustalW, respectively. Conserved domains (CDs) and clusters of orthologous groups of proteins were analyzed using the CD-Search tool at the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov>). Structural alignments for assessment of the topological similarity of protein structures were analyzed using TM-score software (<https://zhanglab.ccmb.med.umich.edu/TM-score>). Phylogenetic trees were constructed using MEGA 10 software with the neighbor-joining method (1,000 replicates). The substrate tunnel analyses were carried out using a tool of CAVER3.0 software (<http://www.caver.cz>).

Data and materials availability

All data are available in the main text or the supplementary materials. The atomic coordinates and structural factors for the *ChCODH-II* A559W variant have been deposited in the Protein Data Bank under the accession code 7ERR. Other data are available from the corresponding author upon reasonable request.

Declarations

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Authors' Contributions

Y.H.K. and S.M.K. conceived and planned all of the experiments. S.M.K. performed the bioinformatic analysis and gene cloning. S.M.K. and J.H.L. achieved the biochemical characterization, the kinetic analysis, and the feasibility evaluation, all under the supervision of Y.H.K. S.M.K. and S.H.K. engineered the gas tunnels and performed their structural analysis. Y.H.K. and S.M.K. wrote the manuscript. H.H.L. and Y.H. determined the crystal structure. Y.H.K. and H.H.L. reviewed the manuscript.

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Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Catalytic properties of *Ch*CODH variants

CODH variant	Specific Activity ^a (U·mg ⁻¹)	K_m^{EV} (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m^{EV} (s ⁻¹ ·mM ⁻¹)
<i>Ch</i> CODH-II	$9.0 \times 10^2 \pm 28.2$	1.8 ± 0.1	$1.0 \times 10^3 \pm 15.7$	$5.6 \times 10^2 \pm 15.1$
A559H	$1.9 \times 10^3 \pm 63.5$	2.1 ± 0.1	$2.3 \times 10^3 \pm 71.9$	$1.1 \times 10^3 \pm 22.9$
A559S	$2.2 \times 10^3 \pm 53.5$	2.1 ± 0.1	$2.6 \times 10^3 \pm 98.2$	$1.2 \times 10^3 \pm 37.0$
A559Y	$1.7 \times 10^3 \pm 62.8$	1.9 ± 0.1	$1.8 \times 10^3 \pm 75.6$	$1.1 \times 10^3 \pm 19.3$
A559W	$2.0 \times 10^3 \pm 13.2$	2.0 ± 0.1	$2.1 \times 10^3 \pm 38.8$	$1.0 \times 10^3 \pm 40.4$
<i>Ch</i> CODH-IV	$8.5 \times 10 \pm 1.2$	1.3 ± 0.1	$8.7 \times 10 \pm 0.3$	$6.7 \times 10 \pm 2.1$
Y558A	$6.8 \times 10 \pm 0.4$	1.1 ± 0.1	$6.3 \times 10 \pm 0.1$	$5.7 \times 10 \pm 0.7$
Y558H	$8.5 \times 10 \pm 3.8$	0.9 ± 0.1	$8.6 \times 10 \pm 0.1$	$9.9 \times 10 \pm 2.8$
Y558W	$9.9 \times 10 \pm 0.5$	1.1 ± 0.1	$1.1 \times 10^2 \pm 1.6$	$1.0 \times 10^2 \pm 2.4$

^a Specific activities were determined at 20 mM ethyl viologen (EV) concentration in the presence of HEPES buffer saturated with CO (30°C, pH 8). Values are the means $\pm$ standard variation, n = 3.

* Kinetic data were assayed at 30°C, pH 8. The kinetic parameters were calculated by fitting the initial rates obtained at seven different EV concentrations (0.5–8 mM) to the Hanes–Woolf equation using a

lineage and are assumed to be insensitive and to possess unique mechanisms against molecular O₂. The phylogenetic trees showed orthologous relationships based on the amino sequences of the CODH proteins. The sequence alignments were performed with the ClustalW 1.4 program, and the trees were constructed using MEGA 10 software with the neighbor-joining method (1,000 replicates). Bootstrap values (> 90%) are displayed at the branch points. (b) Partial sequence alignments of CODH proteins. Five residues (D94, E101, R187, Y558, Q559) are conserved in ChCODH-IV and DvCODH but are quite different from those of ChCODH-II, whereas the cysteine residues for the five metal clusters are highly conserved in all of the Ni–Fe CODHs with exceptions, such as DvCODH with a different [Fe₂S₂] cluster²⁶. Abbreviations: ChCODH-I, *Carboxydotherrmus hydrogenoformans* (W011344718); ChCODH-II, *C. hydrogenoformans* (WP011343033); ChCODH-IV, *C. hydrogenoformans* (W011343666); DvCODH, *Desulfovibrio vulgaris* (WP010939375); RruCODH, *Rhodospirillum rubrum* (W011389181). (c) Identified gas tunnels in ChCODH-II (PDB: 1SU6) and IV (PDB: 6ELQ) structures. ChCODH-II shows the highest activity toward CO but is more O₂ sensitive than ChCODH-IV. The two putative substrate gas tunnels ranging through the homodimers of ChCODH-II and IV, respectively, are quite similar. The homodimeric ChCODH-II and IV contain five metal clusters: [NiFe₃S₄] cluster at catalytic sites (C-cluster), [Fe₄S₄] cubane-type cluster (B-cluster), and canonical [Fe₄S₄] cluster in dimer interface (D-cluster), unlike the variable [Fe₂S₂] cluster of DvCODH, implying that the catalytic mechanisms of ChCODHs proceed similarly due to the identical metal cluster contents. (d) Common bottleneck point of gas tunnels near active site of Ni–Fe CODHs. A branch of the gas tunnels depicted in the inset ends at cluster C directly above the apical coordination site of the Ni ion.

Figure 2

Figure 2

(a) Residual activities of A559X variants after exposure to 10 μ M dissolved O₂ addition for 2 mins. In the absence (blue) or presence (orange) of O₂, CO oxidation was catalyzed by ChCODH-II variants. Abbreviations: WT, wild type; C, A559C; D, A559D; E, A559E; F, A559F; H, A559H; M, A559M; N, A559N; P, A559P; Q, A559Q; S, A559S; T, A559T; W, A559W; Y, A559Y. (b) O₂ tolerances of Ni-Fe CODHs and variants. The effect of the O₂ concentration on Ni-Fe CODHs (open) or engineered ChCODHs (closed) was observed: ChCODH-II (squares), ChCODH-IV (circles), DvCODH (triangles) or ChCODH-II A559H (diamonds), A559W (reverse triangles). See the Methods section. (c) O₂-tolerance changes of ChCODH-IV and its variants. The effect of the O₂ concentration on ChCODH-IV (open) or variants (closed) was reversely measured: ChCODH-II (squares), ChCODH-IV (circles) or ChCODH-IV Y558A (triangles), Y558H

(diamonds), Y558W (reverse triangles). No activities of ChCODH-IV Y558S or DvCODH W552A were observed. (d) Time course of O₂ influence on ChCODHs. Under the O₂ presence at 10 μ M, the residual activities of ChCODH-II (squares), A559W (reverse triangles), and ChCODH-IV (circles) were monitored for 3hrs.

Figure 3

(a) Crystal structure of ChCODH-II A559W variant. The overall structures and metal clusters of the A559W variant (PDB ID: 7ERR, light blue) and ChCODH-II WT (PDB ID: 1SUF, light orange) are highly similar. In the right panel, the overall structures of the A559W variant and ChCODH-II WT (PDB ID: 1SUF) are aligned, and the interacting residues for the C and D clusters are represented as sticks. (b) Superimposition of ChCODH-II and A559W. The steric collision between the tunnels of ChCODH-II and tryptophan of A559W indicates that A559 reduces tunnel accessibility through the bulky aromatic residue tryptophan. Encountering a steric crash is highlighted in red color. The radius of the gas tunnel was predicted by the CAVER program¹⁴. (c) Local fluctuation of ChCODH-II and A559W tunnels. The distance changes between the residues across the tunnel indicates relative constriction of A559 tunnel than WT's. Each local fluctuation of WT (orange) and A559W (blue) was calculated by MD simulation. Two residues across the tunnel in ChCODH- WT and A559W were displayed. A559 and W559 (highlighted in red) of ChCODH-II WT and A559W were corresponded to opposite F608. Each distance was calculated to 9.584 and 6.269 Å in WT and A559W, respectively.

Figure 4

Figure 4

(a) Gas mixture influences on ChCODH-II A559W. Using synthetic mixture and industrial flue gases, the relative activities of A559W were determined. The tested gas mixtures have the following molar compositions: synthetic gas 1 (CO, 5%; N₂, 95%), gas 2 (CO, 5%; CO₂, 12%; N₂, 83%), gas 3 (CO, 6%; CO₂, 12%; CH₄, 6%; H₂, 6%; N₂, 70%), real flue BFG (CO, 25.45%; CO₂, 23.56%; H₂, 3.75%; O₂, 0.01%; N₂, 46.71%), real flue COG (CO, 6.15%; CO₂, 2.42%; CH₄, 24.16%; C_mH_n, 3.07%; H₂, 55.4%; O₂, 0.1%; N₂, 8.63%), real flue LDG (CO, 53.17%; CO₂, 18.51%; H₂, 1.43%; O₂, 0.11%; N₂, 26.77%, and unknown 0.1%). (b) CO re-moval by ChCODH-II A559W under 170 μM O₂ (~13.4%). In the absence (open) of O₂, the gaseous CO (50%) with balance N₂ was consumed by either ChCODH-II (squares) or the A559W variant (reverse triangles). Conversely, in the presence (closed) of O₂, only A559W utilized CO as a substrate. (c) CO conversion of ChCODH-II A559W using flue gas (Linz–Donawiz converter gas: LDG) containing 170

$\mu\text{M O}_2$ (~13.4%). The CO consumption of waste LDG from Hyundai's steel mill was carried out in a similar manner by ChCODH-II (squares) and A559W (reverse triangles) under a near-atmospheric condition. (d) GC analysis of reaction products from LDG gas and A559W. The GC chromatograms were obtained after reaction of A559W using flue gas LDG. The products were identified by comparison with the authentic standards (peak 1, H_2 ; peak 2, N_2 ; peak 3, CO; peak 4, CO_2).

a

b

Figure 5

Figure 5

Reusability of immobilized ChCODH-II A559W using flue gas of LDG (a) without O₂ and (b) with 170 μ M O₂ (~13.4%). The CO consumption (circles) of flue LDG by ChCODH-II A559W without O₂ (open) or with O₂ (closed) was equivalent to CO₂ production (triangles). Enzyme with a His₆-tag was immobilized to a Ni–NTA resin for 20 cycles of reuse with no severe losses in conversion rate. The reaction was performed at room temperature using a rubber-sealed serum bottle (115 mL) with 20 mL head space (CO of 446 μ mol) and 95 mL of reaction buffer (40 mM EVox, 170 μ M O₂, 200 mM HEPES/NaOH buffer pH 8 with purged LDG gas for 1 hr). The first 10 cycles of reaction were initiated in the absence of O₂, by the addition of the immobilized A559 enzyme (500 U/mg immobilized protein). After that, the second 10 cycles were consecutively proceeded in the presence of 170 μ M O₂ (~13.4%). The immobilized enzyme was subsequently recovered through a disposable open column, washed twice with 200 mM HEPES/NaOH buffer pH 8, and reused after each reaction.

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