O$_2$-tolerant CO dehydrogenase via tunnel redesign for the removal of CO from industrial flue gas

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Ni–Fe carbon monoxide dehydrogenases (CODHs) are nearly diffusion-limited biocatalysts that oxidize CO. Their O$_2$ sensitivity, however, is a major drawback for industrial applications. Here we compare the structures of a fast CODH with a high O$_2$ sensitivity (ChCODH-II) and a slower CODH with a lower O$_2$ sensitivity (ChCODH-IV) (Ch, Carboxydothermus hydrogenoformans). Some variants obtained by simple point mutations of the bottleneck residue (A559) in the gas tunnel showed 61–148-fold decreases in O$_2$ sensitivity while maintaining high turnover rates. The variant structure A559W showed obstruction of one gas tunnel, and molecular dynamics supported the locked position of the mutated side chain in the tunnel. The variant was exposed to different gas mixtures, from simple synthetic gas to sophisticated real flue from a steel mill. Its catalytic properties remained unchanged, even at high O$_2$ levels, and the efficiency was maintained for multiple cycles of CO detoxification/regeneration.

Large amounts of carbon monoxide (CO) are emitted from both natural sources and human activity$^1$ (for example, annually over 21.9 billionNm$^3$ of CO-containing flue gases from the South Korean steelmaking company POSCO)$^{2,3}$. CO, the most abundant air pollutant found in the atmosphere other than CO$_2$ according to the Organisation for Economic Co-operation and Development database (https://data.oecd.org/air/air-and-ghg-emissions.htm#indicator-chart), provides sufficient carbon and energy for converting waste gases into fuels and chemicals through a clean and sustainable method. One of the most ideal ways to convert inexpensive, abundant and recyclable CO into fuels and chemicals is the employment of carbon monoxide dehydrogenases (CODHs) capable of oxidizing CO to give CO$_2$ in the manner of bacteria and archaea$^4$. Practical applications of these CODHs, which show remarkable substrate selectivity and viability in gas mixtures even with metal-catalyst inhibitors (sulfur compounds, aromatics, halogens, tar and potentially reactive nitrogen species)$^5$–$^8$, are promising; however, they remain very limited due to the diminished activity of CODHs in the presence of atmospheric oxygen (O$_2$).

The two CODH families are classified by the metal species of the active site$^9$–$^{10}$, namely, Mo–Cu CODHs with a dinuclear [(CuSMo(O)O) and (CuSMo(O)OH)$_2$] (ref. $^{11}$) cluster (EC 1.2.5.3) and Ni–Fe CODHs with a distinctive [NiFe$_3$S$_4$OH]$^-$ cluster (EC 1.2.5.4); these catalyse a moderate rate of aerobic CO conversion ($93.3$ s$^{-1}$) (ref. $^{12}$) or a very high rate of anaerobic CO conversion ($31,000$ s$^{-1}$) (ref. $^{13}$), respectively. Unlike the highly O$_2$-sensitive CODHs, two Ni–Fe CODHs from Carboxydothermus hydrogenoformans (ChCODH-IV)$^9$ and Desulfovibrio vulgaris (DvCODH)$^{14}$ that are less O$_2$ sensitive show unique features and provide intriguing insights into how the active-site environment is changed by O$_2$ exposure. For the effective and widespread use of CODHs in industrial fields, the inevitable disadvantage of a poor activity under aerobic conditions must be overcome. This barrier motivated us to address the issue of the O$_2$ sensitivity of ChCODH-II, which has the highest known CO oxidation rate ($31,000$ s$^{-1}$) (ref. $^{14}$), for practical applications in treating pollutant CO flue gases.

Here we demonstrate a substrate tunnel modification of ChCODH-II using a rational approach based on protein sequences and structures from Ni–Fe CODHs, which leads to greatly decreased O$_2$ sensitivity of the enzyme during oxidation of CO to give CO$_2$. By analysing substrate tunnels close to the active site (C cluster) of ChCODH-II, we discovered a common bottleneck of substrate tunnels in Ni–Fe CODHs, which included acetyl-CoA synthase–CODH complexes involved in the Wood–Ljungdahl pathway. Our mutational and kinetic experiments revealed that under near-atmospheric conditions, the redesigned ChCODH-II variants achieved approximately 100-fold decreases in O$_2$ sensitivity relative to that of ChCODH-II wild type (WT) while maintaining excellent catalytic performances. In addition, the CO of real industrial flue gas was completely removed by the engineered enzymes even under atmospheric O$_2$ conditions, which strongly indicates the potential for application as a CO-removal biocatalyst in industrial fields$^{2,3}$.

Results

A bottleneck for the O$_2$ response in ChCODHs. To explore key regions for O$_2$ sensitivity in Ni–Fe CODHs, we compared the protein sequences and analysed the phylogenetic trees of O$_2$-sensitive ChCODH-II and the less O$_2$-sensitive ChCODH-IV with five forms (ChCODH I–V) as the genomic context$^{15}$ (Fig. 1a,b and Supplementary Fig. 1). Five residues (D94, E101, R187, Y558 and Q559) of ChCODH-IV are quite different from those of ChCODH-II. These residues are conserved in DvCODH. Moreover, analysis of the structures and gas tunnels in Ni–Fe CODHs suggests that of the two residues Y558 and Q559 located along the tunnel, only Y558 is predicted to participate in gas substrate transport in the less O$_2$-sensitive ChCODH-IV (Fig. 1c and Supplementary Fig. 2). In O$_2$-sensitive ChCODH-II, the corresponding residues A559 and M560 are positioned similarly (Supplementary Fig. 2). Similar to Y558 in ChCODH-IV, the side chain of the tunnel-forming

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A559 was also oriented towards the substrate tunnel, whereas the side chain of M560 faced away from the tunnel.

In addition, we realized that residue A559 forms a triad with its neighbouring residues (V565 and I580) along the junction of the substrate tunnels (Fig. 1d). Interestingly, the substrate tunnels with triad residues appear to be non-selective paths where water molecules and xenon-coordinating residues coexist (Supplementary Fig. 3). This suggests that various molecules (which include water, CO and O₂) can access the active site through putatively non-selective substrate tunnels. Much to our surprise, the triad near the active site is universal in structurally similar Ni–Fe CODHs, even in the different bifunctional acetyl-CoA synthase–MtCODH (Mt, Moorella thermoacetica) subunit (Supplementary Table 1). Moreover, this narrowest position at the conserved triad is likely to act as a bottleneck in gas tunnels that critically affect the transport rate of substrates depending on the tunnel characteristics, such as diameter and dynamic fluctuation14 (Supplementary Fig. 4a,b). We therefore hypothesized that the selective obstruction of the common bottleneck point in the gas tunnel would decrease the O₂ sensitivity of CODH by blocking O₂ transfer into the active site of the C- cluster. Accordingly, by...
substituting amino acids and experimentally demonstrating decreased O₂ sensitivity, we designed a number of variants displaying different characteristics at the bottleneck point. The results illuminate the relationships between tunnel characteristics and the O₂ sensitivity of CODH- or CODH-harbouring cells.

O₂ sensitivity and catalytic properties of ChCODH variants. First, to determine the respective effects of bulk, charge, polarity and hydrophobicity at the putative key positions 559 and/or 560, 565 and 580 of ChCODH-II, we constructed and purified a number of single and double variants in Escherichia coli (Supplementary Table 2 and Supplementary Fig. 5). The variants were screened based on their specific activities of CO oxidation and their residual activities after O₂ exposure (Supplementary Table 3). The effects of the mutations showed a simple and clear correlation between the aromatic side chains and decreased O₂ sensitivity, except for A559S (Fig. 2a).

After exposure to dissolved O₂ (10 μM), the residual activities of the variants with aromatic groups, A559H, A559W, A559Y (>90%), A559F and A559P (40–50%), sulfur-containing A559M (58%) and hydroxyl-containing A559S (90%) were detected, whereas ChCODH-II WT exhibited zero residual activity, which shows a complete loss of activity. Additionally, compared with that of WT, the specific activities of A559F, A559H, A559S, A559W and A559Y for the substrate CO were increased by approximately 1.38–2.5-fold (Supplementary Table 3). The amino acid side chains at the 559 position seemed to have a strong impact on the O₂ sensitivity and CO oxidation in ChCODH-II.

Figure 2b demonstrates the remarkable effects of mutations to bulkier amino acids on improving the resistance to O₂. The engineered A559W and A559H ChCODH-II showed an over 50% residual activity due to the notably lower O₂ sensitivity, even in the presence of 38.2–92.9 μM O₂, whereas the WT showed only a 50% residual activity at 0.63 μM O₂, which suggested an approximately 60.6–147.5-fold improvement in the half-maximal inhibitory O₂ concentration (Supplementary Table 4). The O₂ sensitivity of the engineered variant of ChCODH-II was much lower than those of the naturally less O₂-sensitive ChCODH-IV and DvCODH, which would enable its application to industrial flue gas that contains 1.5–6.5 μM O₂ (0.1–0.5%). Table 1 indicates that most ChCODH-II variants have high catalytic rates (k_{cat} ≈ 2,000 s⁻¹ at 30°C) similar to the value (k_{cat} ≈ 1,500 s⁻¹ at 20°C) reported by Ragsdale and co-workers, which suggests that the engineering of enzymes did.
not disturb the catalytic activity. Moreover, the ChCODH-II variants were as robust as the WT enzyme up to 70 °C (Supplementary Table 5), which presents the additional advantage of higher activities and stabilities even at relatively high temperatures.

Regarding the catalytic properties with CO as a substrate, the apparent Michaelis constant ($K_{\text{app}}$) for CO in ChCODH-II WT was estimated to be 20 μM, which is slightly higher than the value of 8 μM reported by Dobbek and co-workers, but is similar to the value of 18 μM reported by Meyer and co-workers. For the variants, the $K_{\text{app}}$ value of 24 μM CO for A559Y showed a similar CO affinity to that of the WT (20 μM), whereas other mutants exhibited a slightly decreased CO affinity. Often, mutations that show a decreased O$_2$ binding exhibit a corresponding decrease in substrate binding (for example, RuBisCo (ref. 18)).

To uncover the reasons for the low O$_2$ sensitivities of the variants, positive mutations (tryptophan and histidine) were introduced at Y558, and the residual activities of the ChCODH-IV variants were observed (Fig. 2c). These results showed that replacing tyrosine with tryptophan or histidine at 558 also decreased the O$_2$ sensitivity. Therefore, it is predicted that low O$_2$-sensitive ChCODH-II and Y558A to reconfirm the results for the ChCODH-II variants. As expected, Y558A was very sensitive to O$_2$, in fact, as sensitive as ChCODH-II, which thus indicates that the role of Y558 (ChCODH-IV) is similar to that in the engineered A559 mutants (ChCODH-II) as a key site for decreased O$_2$ sensitivity. This supported the hypothesis that the substitution of tyrosine for alanine decreases O$_2$ sensitivity but does not change the catalytic properties of Y558A relative to those of ChCODH-IV WT (Table 1). With reference to the data on the reduced O$_2$ sensitivities of the ChCODH-II variants, positive mutations (tryptophan and histidine) were introduced at Y558, and the residual activities of the ChCODH-IV variants were observed (Fig. 2c). These results showed that replacing tyrosine with tryptophan or histidine at 558 also decreased the O$_2$ sensitivity in ChCODH-IV, as is the case for the ChCODH-II A559 variants. Consequently, we concluded that the 559 or 558 position in either ChCODH-II or IV was the commonly shared key site that affected O$_2$ sensitivity.

For the stable and efficient conversion of CO in the presence of O$_2$, we further estimated the stability of engineered A559W as an oxidizing biocatalyst (Fig. 2d). At 10 μM O$_2$, the relative activities of ChCODH-IV and A559W of ChCODH-II were stably maintained over time, but the activity of ChCODH-II WT rapidly diminished. These results revealed that the variant A559W exhibited a stable activity at 10 μM O$_2$ despite the rapid O$_2$ inactivation of ChCODH-II WT and the low CO conversion of ChCODH-IV. It is well-known that most Ni–Fe CODHs are inactivated by O$_2$ within only a few minutes, and inactivation is more severe in the absence of O$_2$ scavengers, such as dithiothreitol (DTT) and dithioerythritol (DTE). Furthermore, variants exposed to O$_2$ for several hours can efficiently undergo CO conversion at higher conversion rates than can ChCODH-IV, which has a low specific activity (~90 U mg$^{-1}$). Therefore, it is predicted that low O$_2$-sensitive ChCODH-II variants will provide a good performance in removing CO from industrial waste gas mixtures that contain O$_2$.

### Table 1 | Catalytic properties of ChCODH variants

| CODH, variant | Substrate | Specific activity (U mg$^{-1}$) | $K_{\text{app}}$ (mM)$^a$ | $k_{\text{cat,app}}$ (s$^{-1}$)$^b$ | $k_{\text{cat,app}}/K_{\text{app}}$ (s$^{-1}$ mM$^{-1}$) | $K_{O_2,c}^b$ | $K_{O_2,c}^b/K_{\text{app}}$ |
|---------------|-----------|-------------------------------|---------------------|-----------------|-------------------------------|----------|------------------|
| ChCODH-II     | CO        | 1,300 ± 108.5                 | 0.020 ± 0.002       | 1,500 ± 670     | 75,800 ± 3,840               | 0.012 ± 0.001 | 1,600 ± 45.2     |
|               | EV        | 900 ± 28.2                    | 1.8 ± 0.1           | 1,000 ± 15.7    | 560 ± 15.1                   |           |                  |
| A559H         | CO        | 2,100 ± 118.0                 | 0.061 ± 0.002       | 2,300 ± 378     | 37,000 ± 991                 | 5.4 ± 0.8  | 11.5 ± 2.2       |
|               | EV        | 1,900 ± 63.5                  | 2.1 ± 0.1           | 2,300 ± 719     | 1,100 ± 22.9                 |           |                  |
| A559S         | CO        | 1,900 ± 57.6                  | 0.041 ± 0.003       | 2,000 ± 61.6    | 50,000 ± 2,790               | 2.9 ± 0.8  | 14.6 ± 2.9       |
|               | EV        | 2,200 ± 53.5                  | 2.1 ± 0.1           | 2,600 ± 98.2    | 1,200 ± 370                  |           |                  |
| A559Y         | CO        | 2,000 ± 118.8                 | 0.024 ± 0.004       | 1,500 ± 78.1    | 62,000 ± 8,360               | 0.6 ± 0.1  | 38.8 ± 0.1       |
|               | EV        | 1,700 ± 62.8                  | 1.9 ± 0.1           | 1,800 ± 75.6    | 1,100 ± 19.3                 |           |                  |
| A559W         | CO        | 1,800 ± 110.0                 | 0.034 ± 0.002       | 1,800 ± 49.5    | 53,000 ± 2,300               | 1.2 ± 0.1  | 27.1 ± 0.7       |
|               | EV        | 2,000 ± 13.2                  | 2.0 ± 0.1           | 2,100 ± 38.8    | 1,000 ± 40.4                 |           |                  |
| ChCODH-IV     | EV        | 85 ± 1.2                      | 1.3 ± 0.1           | 87 ± 0.3        | 67 ± 2.1                     | ND       | ND               |
| Y558A         | EV        | 68 ± 0.4                      | 1.1 ± 0.1           | 63 ± 0.1        | 57 ± 0.7                     | ND       | ND               |
| Y558H         | EV        | 85 ± 3.8                      | 0.9 ± 0.1           | 86 ± 0.1        | 99 ± 2.8                     | ND       | ND               |
| Y558S         | EV        | 36 ± 2.2                      | 1.4 ± 0.1           | 34 ± 1.4        | 25 ± 2.7                     | ND       | ND               |
| Y558W         | EV        | 99 ± 0.5                      | 11 ± 0.1            | 110 ± 1.4       | 100 ± 2.4                    | ND       | ND               |

$^a$Specific activities were determined at 20 mM ethyl viologen (EV) in the presence of HEpES buffer saturated with CO (30 °C, pH 8). Values are the means ± standard variation, n = 3. $^b$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) and five different CO concentrations (0.01–0.16 mM) to the Hanes–Woelfli equation using SigmaPlot 10.0. All the enzymatic activities were determined in triplicate (see details in Methods). $^c$The values of $K_{\text{app}}$ were calculated from $V_{\text{cat,app}}$ for EV and $V_{\text{cat,app}}$ for CO.
variants, we solved the crystal structures of the variants A599W (Protein Data Bank (PDB) 7XDM), A599H (PDB 7XDN) and A599S (PDB 7XDP) (Fig. 3a and Supplementary Table 6). Given that the activity loss of most CODHs under aerobic conditions is closely related to the alteration or destruction of the active site C cluster, which is the most O₂-sensitive metal site⁹,¹⁴,²⁰, to maintain the structural integrity of the C cluster is vital to decrease O₂ sensitivity. Thus, we determined whether the structural integrity of the Fe–S clusters in the three variants was maintained and compared the anaerobic structure of A599W (PDB 7XDM) with the O₂-exposed structure of A599W (PDB 7XXR) prepared under aerobic conditions (Fig. 3a and Supplementary Fig. 6). When we examined the positions and the interacting residues around the B, C and D clusters of the variants, they were highly similar root mean squared deviation (r.m.s.d.) values: PDB 7XDM 0.296 Å, PDB 7XXR 0.333 Å, PDB 7XXN 0.325 Å and PDB 7XXP 0.291 Å for the 633 Cr atoms; PDB 7XXM 0.400 Å, PDB 7XXR 0.598 Å, PDB 7XXN 0.384 Å and PDB 7XXP 0.357 Å for all atoms to those of ChCODH–II WT (PDB 1SU7). Similarly, the r.m.s.d. between aerobic and anaerobic A599W structures (PDB 7XXR and 7XDM) was 0.17Å (633 Cr atoms) and 0.432 Å (all atoms), as shown in Fig. 3a. Additionally, the omit maps (Fo–Fc (Fo, experimentally measured amplitude; Fc, model-based amplitude)) and Fe anomalous difference maps were similar to each other, which suggests that the Fe–S clusters of the variants have similar architectures to those of ChCODH–II WT and no loss of Fe and Ni (Supplementary Table 7 and Supplementary Figs. 6 and 7). However, the only local environments around the A559 mutation sites that affect the characteristics of the gas tunnel were significantly changed compared with those of ChCODH–II WT. In the A599W and A599H variants, the side chain of I580 was pushed away through the incorporation of the bulkier residue W559 or H559 instead of alanine, and the position of V582 was also slightly changed by making additional hydrogen bonds with W559 or H559. In the A599S variant, neither hydrogen bonding with V582 residues nor flipping of the I580 side chain was observed. Only the side chain of V582 moved towards S559 to form the additional van der Waals interaction.

The incorporation of W559, H559 or S559 made the gas tunnel in the variants narrower than that of ChCODH–II WT (Fig. 3b and SupplementaryFig. 4c). When we measured the radius of the gas tunnel at the bottleneck point of A599W using the CAVER program,¹¹ it was reduced by approximately 1.02 Å compared with that of ChCODH–II WT (1SU7). Consequently, we deduced that the obstructed tunnels of the ChCODHs would not allow the transfer of CO₂. Thus, the less O₂-sensitive biocatalyst A559W was shown to be the most efficient biocatalyst for the removal of gaseous CO and CO₂, CH₄ and H₂. In particular, the lack of severe inactivation by O₂ and unknown trace impurities in the industrial flue gases BFG, COG and LDG suggests that a less O₂-sensitive ChCODH variant can be used as a suitable and widely applicable biocatalyst for CO-containing flue gases from a variety of industrial environments.

Finally, to evaluate the performance of the low-O₂-sensitive ChCODH–II variant for the removal of gaseous CO, we tested the variants as an oxidizing biocatalyst in 50% (v/v) CO-saturated 0.2 M N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) (HEPES, pH 8) with or without dissolved 170 μM O₂ (−13.4%, v/v). Figure 4b indicates that ChCODH–II WT and A559W readily consumed CO within 2.5 hours under anaerobic conditions, whereas under O₂, only A559W consumed CO within 3 hours. This experiment confirmed that A559W, unlike ChCODH–II WT, can sufficiently and selectively consume CO without severe O₂ inactivation. Under the aerobic conditions, A559W seemed to traverse a lag phase in half-an-hour, probably until the enzyme reactivation by CO could overcome O₂-induced arrest. Ni–Fe CODHs can be recovered when CO or dithionite is added.¹² A plausible explanation is that CO can serve as a weak reductant for A559W recovery in the manner of dithionite-treated ChCODH–II.¹²,¹³ Furthermore, we observed the reversibility of the inactivation of A559W after a short-term exposure to air (Supplementary Fig. 12). ChCODH–II WT is almost irreversibly inactivated by O₂. In contrast, the activity of the variant A559W towards CO reversibly and rapidly recovers from the inactive state caused by air exposure. This reversibility of the O₂ inhibition could be very interesting for industrial applications. However, the exact mechanism of this remarkable reversibility is currently unclear and needs to be further explored. 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%, v/v). The variant A559W displayed an impressive capability to remove the total amount of CO (53.4%) from LDG (Fig. 4c,d), but no CO consumption was observed for ChCODH–II WT. Even under atmospheric conditions of approximately 250 μM O₂ (−20%, v/v), CO uptake of 33% was observed for A559W (Supplementary Fig. 13). Moreover, we compared the CO consumptions of variants A559H, A559S and A559W using 50% (v/v) CO-saturated buffer and LDG-saturated buffer (Supplementary Fig. 14). A559W had the fastest CO removal rate in the presence of dissolved O₂ (−13.4%, v/v). This may be because the catalytic efficiency of A559H and A559S is lower than that of A559W, as shown in Table 1. We considered A559W to be the most efficient biocatalyst for the removal of CO. Thus, the less O₂-sensitive biocatalyst A559W was shown to enable the enhanced CO bioconversion under near-atmospheric conditions and demonstrated outstanding performance in complete CO removal from steel-mill flue gas (LDG).

In addition to an efficient CO consumption, A559W must have the sustainable capacity to be reused and recycled for the treatment of industrial flue LDG. Immobilized biocatalysts can be employed as a simple reuse and recovery method for cost savings in industrial
Fig. 3 | Structures of less O₂-sensitive ChCODH-II variants. a, Bottleneck residues of ChCODH-II variants and metal clusters of A559W. Structures of ChCODH-II WT and the A559 variants are superimposed (top row). Dotted lines and arrows denote hydrogen bonds and van der Waals interactions, respectively. The clusters of aerobic and anaerobic A559W are superimposed (bottom row). Fe, orange; S, yellow; Ni, green. b, Superimposition of ChCODH-II WT and A559W (highlighted in red). c, Distances between tunnel-forming residues A559/W559 and F608. d, Distance fluctuations between F608 and A/W559 in the MD simulation. Each local fluctuation (y axis) in the WT (orange) and A559W (blue) was calculated. Values of the r.m.s.d of Cα atoms of WT (grey) and A559W (black) calculated during 8 ns are shown on the right. The vertical arrows indicate the maximum and minimum distances in WT (orange) and A559W (blue) after the equilibrium state at 3 ns (violet vertical line; see Supplementary Fig. 9).
CODH-II WT (squares) and A559W (reverse triangles) under near-atmospheric conditions (170 μM O2 (~13.4%, v/v)), only A559W utilized CO as a substrate. The data represent the mean ± s.d., as determined from n = 3 independent experiments. 

Conclusions

Resistance to atmospheric O2 exposure is a rare feature among enzymes that express CO dehydrogenase activity. To date, the only known aerobic CODH is the Mo–Cu CODH enzyme. In contrast, Ni–Fe CODHs are highly sensitive to O2, and even after repeated reuse, and might be adaptable for future industrial applications. Figure 5 shows that the conversion rate of CO by immobilized A559W is maintained through repeated cycles of reuse and in the presence of O2. The immobilized A559W was incubated at room temperature (r.t.) using flue LDG for the first ten cycles (Fig. 5a,b), and LDG with dissolved 170 μM O2 (~13.4%, v/v) was then used for the second ten cycles (Fig. 5a,c). In the absence of O2, there was no loss of conversion rate during ten cycles, which indicates that the enzyme is still active after repeated uses and is not inhibited by the product CO2 or other gas impurities from LDG. In the presence of O2, ten cycles of the reaction also exhibited complete oxidation of CO to give CO2, which indicates that the immobilized A559W is unaffected by O2 interference. The results suggested that this engineered variant can efficiently remove CO from steel-mill flue gases that contain varying levels of CO and O2, and might be adaptable for future industrial applications.

In summary, our study presents the key discoveries that one position is enough to decrease the sensitivity to O2 and that the second, unaffected tunnel seems to be highly specific for CO/CO2 rather than O2; it provides some reasons as to why all anaerobes have such highly O2-sensitive CODHs even though one point mutation would be enough to drastically improve their CODHs by conferring a faster turnover and decreased O2 sensitivity but probably less efficient in vivo under a lower CO environment and it suggests that the slow CODHs, ChCODH-IV and DvCODH, which are less sensitive to O2. Thus, the use of low-O2-sensitive ChCODH biocatalysts with a marked increase in rate efficiency would provide the advantage of an efficient CO conversion for various waste gases and syngases that contain O2, for example, biomass and plastics in industry or applied green chemistry. Based on our analyses and a rational approach, we selected different strategic points in the enzyme to generate variants able to maintain the high activity rates of CODH-II combined with the low O2 sensitivity of CODH-IV. This study thus points towards an effective avoidance of the rapid O2 inactivation of Ni–Fe CODHs through an increased selectivity of the gas tunnel. Catalytic activity and decreased O2 sensitivity are often considered to be trade-offs, but the ChCODH-II variants show how to overcome such limits.

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next step in applying these designed biocatalysts will be coupling on an electrode or direct transfer of the reducing power to another system (for example, CO₂ fixation into formate). Alternatively, these variants could be implanted in anaerobic bacteria to increase their performance as bioconverters. This work provides concrete proof that CODHs can be tailored for industrial or gas-cleaning processes.

Methods

Gene synthesis and cloning. The genes encoding CODH-II (CHY_0085), CODH-IV (CHY_0736) and CODH-II (CHY_0085), and its variants was induced by the addition of 0.2 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) supplemented with 0.5 mM NiCl₂, 1 mM FeSO₄ and 100 μg ml⁻¹ DTE and resazurin were omitted from the washing and elution steps during the purification protocol. Cell lysates were centrifuged and purified by Ni-NTA affinity chromatography (Qiagen) with a standard buffer that contained 2 mM DTE and 2 μM resazurin, but DTE and resazurin were omitted from the washing and elution steps during the purification protocol. Cell lysates were centrifuged and purified by Ni-NTA affinity chromatography (Qiagen) with a standard buffer that contained 2 mM DTE and 2 μM resazurin, but DTE and resazurin were omitted from the washing and elution steps during the purification protocol. The purified proteins were subjected to SDS-PAGE on 12% gels, all the variants were detected as a single soluble band whose size (69 kDa) corresponded to the calculated size of the proteins (67 kDa) with the His₆-tag (2 kDa). Western blot analyses using anti-His-tag antibodies were also carried out to confirm the presence of the His tag and putative degradation of the His-tagged protein. For the crystallization of CODH-II variants (A559W, A559H and A559S) under anaerobic conditions, further purification was anaerobically performed in an anoxic glove box (model B, COY Laboratory Products Inc.) by size-exclusion chromatography (HiLoad 16/60 Superdex 200 prep grade, GE Healthcare Bio-Sciences), which was previously equilibrated with a buffer that contained 20 mM Tris-HCl pH 7.5 and 3 mM DTT. For crystallization of the aerobic A559W variant, the protein purification step using Ni-NTA resin was conducted in a similar manner to that above but under aerobic conditions (outside the anaerobic chamber). Further purification by size-exclusion chromatography was also performed aerobically with a buffer that contained 20 mM Tris-HCl pH 7.5 and 3 mM DTT.

Size-exclusion chromatography with multilangle light scattering. Size-exclusion chromatography with multilangle light scattering experiments on the CODH-II A559W variant were performed aerobically using a fast protein liquid chromatography (FPLC) system (GE Healthcare) connected to a Wyatt MiniDAWN TRESO XL-8 instrument and a Wyatt Optilab REX differential refractometer. A Superdex 200 10/300 GL (GE Healthcare) gel-filtration column was pre-equilibrated with a buffer that contained 20 mM Tris-HCl pH 7.5 and 3 mM DTT and normalized using ovalbumin protein from hen egg white (Sigma-Aldrich). Proteins were injected (1 μg), and elution was performed at a flow rate of 0.4 ml min⁻¹. Data were analysed using the Zimm model for static light-scattering analysis.
Activity measurement. CO oxidation activity was measured at 30 °C after saturation with CO using screw-cap cuvettes with a CO head space. The activity was assayed by observing the CO-dependent reduction of oxidized EV (E_vox) (ref. 33) and 1.84 Å 1° oscillation at the 11C and 5C beamlines of the Pohang Light Source with a wavelength of 1.0000 Å. The structure determination was initiated by injecting each reaction buffer with different CO concentrations (0.01–0.16 mM) into the enzyme (0.1–0.2 μg). The absorbance change at 578 nm was spectrophotometrically monitored at 30 °C in an anoxic glove box. The kinetic parameters (k_cat,app and K_M,app) for CO and EV_vox were calculated by the Hanes–Woolf equation (Supplementary Fig. 16). All of the enzymatic activities were determined in triplicate. K_determinations, IC50 values (Supplementary Fig. 17) were calculated using nonlinear regression (four-parameter logistic curve). [S] is the concentration of the substrate CO. Subsequent K_determinations were calculated using equation (1) (ref. 32):

\[ K_d = \frac{IC50}{1 + \frac{[S]}{K_d}} \]  

Crystallization and structural determination. To solve the crystal structures of the CODHs, the reaction was initiated by injecting recombinant CODHs (0.1–0.2 μg) into a 10-mg ml−1 CHO-saturated buffer that corresponded to 0.91 mM CO (99.998% according to Henry’s law) at 1 °C. The head-space rubber-sealed cuvette that contained 0.5–8 mM EV in 50 mM HEPES/NaOH buffer (pH 8) was equilibrated with CO for 1 h. To monitor the initial velocity of the CODHs, the reaction was initiated by an injection of recombinant CODHs (0.1–0.2 μg). For measurement of the kinetic parameters towards CO, two CO-saturated buffers that corresponded to 0.91 mM CO (99.998% according to Henry’s law) and 45.5 μM CO (5% CO (v/v)/95% N2 (v/v)) according to Henry’s law) were used at 30 °C. Three CO concentrations (10 μM, 20 μM and 40 μM) and two CO concentrations (80 μM and 160 μM) that each contained a final concentration of 20 mM EV in 50 mM HEPES pH 8 buffer were immediately prepared before the reaction by diluting two CO-purged buffers of 0.0455 mM and 0.91 mM, respectively. The enzyme reaction was initiated by injecting each reaction buffer with different CO concentrations (0.01–0.16 mM) into the enzyme (0.1–0.2 μg). The absorbance change at 578 nm was spectrophotometrically monitored at 30 °C in an anoxic glove box. The kinetic parameters (k_cat,app and K_M,app) for CO and EV_vox were calculated by the Hanes–Woolf equation (Supplementary Fig. 16). All of the enzymatic activities were determined in triplicate. K_determinations, IC50 values (Supplementary Fig. 17) were calculated using nonlinear regression (four-parameter logistic curve). [S] is the concentration of the substrate CO. Subsequent K_determinations were calculated using equation (1) (ref. 32):

\[ K_d = \frac{IC50}{1 + \frac{[S]}{K_d}} \]  

Crystallographic and refinement statistics. The crystals of the CODHs (2–4 Å and 1.84 Å 1° oscillation at the 11C and 5C beamlines of the Pohang Light Source with 3.07% (v/v) polyethylene glycol 3350 and 53 sodium and 54 chloride ions for the WT, 20,924 water molecules and 66 sodium and 55 chloride ions for the A559W mutant, 19,875 water molecules and 53 sodium and 54 chloride ions for the A559H mutant and 20,045 water molecules and 53 sodium and 54 chloride ions for the A559S mutant were grown under aerobic conditions using the sitting drop method by mixing 0.2 μl of reservoir solution that consisted of 0.1 M HEPES/NaOH pH 7.0, 200 mM MgCl2 and 25% (w/v) polyethylene glycol 3350. The crystals were transferred to a solution that contained the reservoir solution 50 ml of stock solution flushed with CO gas for 1 h (ref. 29). When the activity was measured, trace O2, which was trapped in the mobile head space of the experiment, was removed by flushing with CO gas for 30 min (ref. 29). When the activity was measured, trace O2, which was trapped in the mobile head space of the experiment, was removed by flushing with CO gas for 30 min (ref. 29).

The structural factors were deposited in the Protein Data Bank (PDB 7XDM, 7XDN and 7XDP) as a probe32. Subsequent manual model building was performed using the Coot program33, and restrained refinement was carried out using PHENIX (version 1.19.2, 4158) and CCP4 refmac5 in the absence of hydrogen atoms. The atomic coordinates and structure factors were deposited in the Protein Data Bank (PDB 7EMR).
proteins. A small aliquot of these immobilized proteins was eluted to check the specific activity of the protein in the same manner described in the protein purification section above. In addition, we measured the apparent units of protein per immobilization bead volume owing to the difficulty of determining the exact amounts of immobilized proteins.

The reaction buffer for the enzyme reaction was anaerobically prepared in a rubber-capped serum bottle (115 ml) with 95 ml of buffer solution (40 mM EV, 200 mM HEPES, pH 8), transferred from the anaerobic chamber, and then purged with LDG for 1 h. The rubber-capped serum bottle (115 ml) with 82.3 ml of reaction buffer (40 mM EV, for a 95 ml final volume, 200 mM HEPES, pH 8) was transferred from the anaerobic chamber and then purged with LDG for 1h. Then, 12.7 ml of the O$_2$-purged buffer was injected into the LDG-purged reaction buffer (final O$_2$ concentration of 170 ppm) in the reaction buffer was analysed by GC. As in the previous experiments using TM score software (https://zhanggroup.org/TM-align). Phylogenetic trees for the assessment of the topological similarity of protein structures were analysed using the CD-Search tool at the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/). Structural alignments for the assessment of the topological similarity of protein structures were analysed using TM score software (https://zhanggroup.org/TM-align). Phylogenetic trees were constructed using MEGA 10 software (https://www.megasoftware.net/) with the neighbour-joining method (1,000 replicates). Bootstrap values (>90%) are displayed at the branch points. Non-redundant reference sequences (1,710 proteins) of Ni–Fe CODHs belonging to CoDHi (the number in clusters of orthologous groups) from eggNOG 5.0 (https://www.ncbi.nlm.nih.gov/). 90% are displayed at the branch points. The challenging in silico description of carbon monoxide oxidation as catalyzed by molybdenum–copper CO dehydrogenase. Front. Chem. 6, 630 (2019).

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Author contributions

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

Competing interests

The authors declare no competing interests.

Additional information

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Data collection: Data were collected using the Agilent Technologies Cary WinUV Kinetics Application V.5.0.0.999 and the Agilent Chemstation software Version 8.04.03. Crystal Data were collected using the Macromolecular Crystallography Data Collector software (MxDC).

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