Enzyme Mechanisms

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Carbon Monoxide Dehydrogenase Reduces Cyanate to Cyanide

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Abstract: *The biocatalytic function of carbon monoxide dehydrogenase (CODH) has a high environmental relevance owing to its ability to reduce CO_2. Despite numerous studies on CODH over the past decades, its catalytic mechanism is not yet fully understood. In the present combined spectroscopic and theoretical study, we report first evidences for a cyanate (NCO^-) to cyanide (CN^-) reduction at the C-cluster. The adduct remains bound to the catalytic center to form the so-called CN^--inhibited state. Notably, this conversion does not occur in crystals of the Carboxydothermus hydrogenoformans CODH enzyme ($CODHII_{Ch}$), as indicated by the lack of the corresponding CN^- stretching mode. The transformation of NCO^-, which also acts as an inhibitor of the two-electron-reduced C_{red2} state of CODH, could thus mimic CO_2 turnover and open new perspectives for elucidation of the detailed catalytic mechanism of CODH.*

Carbon monoxide dehydrogenase (CODH), which catalyses the reversible transformation between CO and CO_2, is a pivotal enzyme of the Wood–Ljungdahl metabolic pathway that allows microorganisms such as *Carboxydothermus hydrogenoformans* (*Ch*) and *Moorella thermoacetica* to grow on H_2 and CO_2 as an electron and carbon source, respectively, or to oxidise CO in order to use it as electron source.[1] Furthermore, the reverse process, that is, the enzymatic reduction of CO_2, is of particular interest since it may serve as a template for the bioinspired development of catalysts for CO_2 degradation.

The enzyme consists of two monomeric units, each bearing three iron–sulphur clusters (D, B, and C, from the protein surface to the inner core of the protein). The cubane-shaped [4Fe-4S] D-cluster is coordinated by two cysteine residues from each subunit, so that the homodimeric enzyme exhibits one D-cluster, two B-clusters, and two [$NiFe_4S_4OH_x$] active sites (C-cluster). The C-cluster is known to adopt at least four different oxidation states. C_{ox} and C_s are the most oxidized inactive states, and differ by the nature of the bridging ligand (S^- or OH^-) between the Ni and the Fe_1 atom.[2] One-electron reduction of C_s/C_{ox} is proposed to lead to the C_{red1} active state, which binds CO at the Ni ion. Interactions with the terminal OH_x ligand of the nearby Fe_1 ion are essential for CO turnover and subsequent oxidation to CO_2 with formation of the two-electron-reduced C_{red2} state.[3] Re-oxidation most likely occurs by single-electron transfer from the C-cluster to the B-cluster via a transiently formed C_{int} state.[4]

Despite a large number of biochemical, spectroscopic, and crystallographic investigations, the detailed mechanism of the catalytic process of CODH remains unclear. The gain of deeper insight is hindered by the difficulty in trapping substrate-bound intermediate or pure redox states. Thus, as an alternative approach to overcome this limitation, inhibitors mimicking the substrates have been employed. While CN^- has been widely studied as an analogue for CO,[5] much less work has been reported on the binding of cyanate (NCO^-), which is considered to be an analogue of CO_2.[2b,5] Most importantly, recent high-resolution structures ($d_{min} <$ 1.1 Å) suggest that the binding of CO_2 and NCO^- to crystals of the *Carboxydothermus hydrogenoformans* CODH enzyme ($CODHII_{Ch}$) leads to the formation of a Ni-bound carboxylate and a carbamoyl (H_2NCO^+), respectively.[5b] In analogy to the reduction of CO_2 to CO, NCO^- reduction to CN^- by CODH was recently postulated. This is supported by the reduction of NCO^- to CN^- detected in nitrogenases and the observation of a slow oxidative turnover of *n*-butylisocyanide into *n*-butylisocyanate in $CODHII_{Ch}$, as the corresponding reverse reaction.[5b,6] To further elucidate the degradation of NCO^- by $CODHII_{Ch}$, we employed IR spectroscopy to probe the substrate bound to the C-cluster of the enzyme in solution and in the crystalline state.

The dithionite (DT)-reduced $CODHII_{Ch}$ enzyme was exposed to a solution of 6 mM of NCO^- (or isotopically labelled $N^{13}CO^-$), corresponding to a concentration that is around two times higher than the estimated K_d.[7] The IR spectrum obtained from these samples displayed two bands in the region between 2270 and 2000 cm^{-1}. The band at 2168 cm^{-1} (2110 cm^{-1} with $N^{13}CO^-$) is readily attributed to the stretching mode of NCO^- in solution (Figure 1).[8] The second band at 2110 cm^{-1} (2065 cm^{-1} with $N^{13}CO^-$) is at the same position as the one observed for the $CODHII_{Ch}$–CN adduct, for which it could be unambiguously assigned to the C≡N stretching of the Ni-bound cyanide.[9] The active role of C_{red2} state of the C-cluster in the reduction of NCO^- to CN^- was emphasized by two control experiments (see Figure S2 in the Supporting Information). A DT-reduced Ni-deficient $CODHII_{Ch}$ (misassembled C-cluster) exposed to NCO^- did not afford a band at 2110 cm^{-1}, which corresponds to the bound CN^-. Moreover, the dithiothreitol (DTT)-reduced $CODHII_{Ch}$ (C_{red1} state) did not display a band at 2110 cm^{-1} after incubation with NCO^-. Finally, the CN^- band disap-

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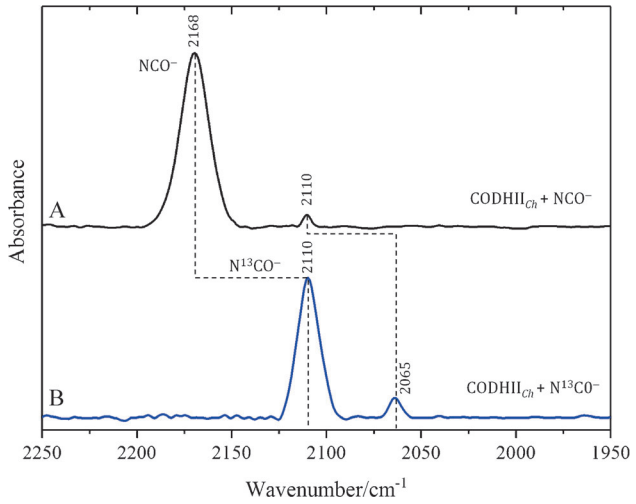

Figure 1. IR spectra of CODHII$_{Ch}$ after incubation with NCO⁻ (A) or N^{13}CO⁻ (B) measured at 10 °C in solution.

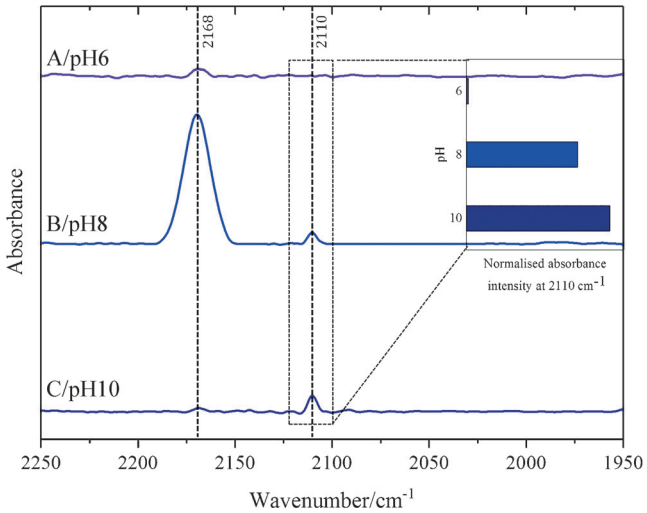

Figure 2. IR spectra of CODHII$_{Ch}$ incubated with NCO⁻ measured at 10 °C in solution at pH 6.0 (A), pH 8.0 (B), and pH 10.0 (C). Inset: Normalized intensity of the C≡N stretching band as a function of pH value. Note that the buffer exchange procedure leads to variations of the residual NCO⁻ concentration from experiment to experiment.

pears after incubation under CO atmosphere at 60 °C, a typical procedure used to reactivate CN⁻-inhibited CODH (see Figure S1).[9]

Additional control experiments were carried out to exclude CN⁻ as a contaminant of the NCO⁻ or as product of NCO⁻ reduction to CN⁻ mediated by possibly released metals ions (Fe or Ni) from the enzyme (see Figure S2). First, the IR spectra of aqueous NCO⁻ in the presence of DT did not reveal any bands at 2080 cm^{-1} and 2093 cm^{-1}, which correspond to CN⁻ and HCN in aqueous solution, respectively. Second, a solution containing $NiCl_2$/$FeCl_2$ with a 1:10 ratio (same metal stoichiometry as in CODH: 1 Ni to 10 Fe ions per monomer) and DT was exposed to NCO⁻. Again, under these conditions, no signals were observed at 2080 cm^{-1} and 2093 cm^{-1}. Thus, free CN⁻ can be ruled out as a contaminant. Consequently, these spectroscopic data demonstrate that under the present experimental conditions, NCO⁻ is converted into CN⁻ at the C-cluster.

The intensity of the C≡N stretching of the bound cyanide varies with the pH value, with the strongest signal observed at pH 10.0 (Figure 1). Lowering the pH to 8.0 causes a moderate intensity decrease whereas at pH 6.0, the band can hardly be detected anymore. Note that at this pH value, no extra band was observed except for the band of the dissolved cyanate anion. This variation in intensity can be interpreted by taking into account the pH-dependence of the reduction potential of DT (see Figure S3), which decreases with increasing pH. Consequently, whereas the reduction potential of the C_{red1}/C_{red2} couple has been determined to be −530 mV,[2a] one can anticipate a higher amount of C_{red2} state at high pH, thus resulting in a higher CN band intensity. This observation underpins the active role of the C_{red2} state of the C-cluster in NCO⁻ reduction. For a quantitative comparison, the integral intensity of the 2110 cm^{-1} band was related to the respective amide II band intensity, taken as a measure for the amount of enzyme, and plotted in the inset of Figure 2.

We subsequently extended the IR spectroscopic studies to the NCO⁻-bound state of a single CODHII$_{Ch}$ crystal. However, in contrast to the measurements in solution, no signal due to the CN⁻ enzyme could be observed at 2110 cm^{-1} (Figure 3). Furthermore, increasing the incubation time in NCO⁻ solution from 30 min to 16 h did not yield a band at this position. The absence of this band in the spectrum of the crystal is not due to intrinsically lower spectral quality. This is demonstrated by the comparison with the spectrum of CODHII$_{Ch}$ crystals exposed to CN⁻ solution, which clearly displays the characteristic 2110 cm^{-1} band of the Ni-bound cyanide (Figure 3). Thus, we conclude that transformation of NCO⁻ into CN⁻ only occurs in the enzyme in solution and is impaired in the crystalline state.

According to the crystal structure obtained from CODHII$_{Ch}$ after NCO⁻ binding, cyanate is likely to be converted

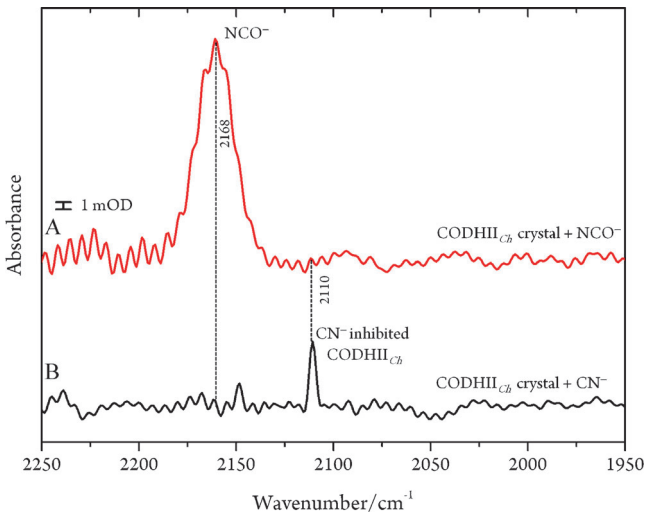

Figure 3. IR spectra of a single CODHII$_{Ch}$ crystal in presence of aqueous NCO⁻ (A) and CN⁻ (B) measured at 10 °C. The sinusoidal modulations in the spectra are related to the formation of standing waves of the incident IR radiation within the plan parallel CaF_2 windows in the sampling cell.

into a carbamoyl, as judged from bond lengths and angles.[5b] QM/MM calculations carried out in the present study predict the characteristic vibrational modes of the carbamoyl moiety to be at 1598, 1432, and 1293 cm^{-1},(1595 (−3), 1395 (−35), and 1263 (−30) cm^{-1} with $N^{13}CO^-$). Unfortunately, these bands positions fall into a spectral region of saturating absorption such that their detection is impossible.

The conversion of the Ni-bound carboxylate into the final product CO requires the uptake of two protons from the solution, followed by the release of a water molecule from the active site. In our previous study, we provided evidence for a redox-state dependent "valve" that controls the directionality of the proton channel.[9]

Whereas in the C_{red1} state, only proton transfer to the solution is possible, the directionality is reversed in the C_{red2} state to provide protons for the degradation of CO_2 to CO. Although we cannot rule out CO turnover inside the crystals, we were not able to detect the analogous CN^- formation. The origin of the discrepancy in the NCO^- reduction behaviour between solution and crystalline phase might originate from crystal lattice effects that prevent CN^- formation or substantially increase its time of formation (> 16 hours).

Interestingly, the process in solution can be compared to the biogenesis of the CN^- ligands in [Ni-Fe] hydrogenases involving the HypE accessory protein, which binds a carbamoyl group and subsequently converts it after formal dehydration to a CN^- group that will be transferred to an iron center.[10]

Experimental Section

Buffers and reagents are anoxically prepared in a glovebox. The inhibition procedures described below are performed under the same anaerobic conditions. 6 μL of 400 μM $CODHII_{Ch}$ are diluted in 500 μL of buffer A (Tris 50 mM pH 8, NaCl 100 mM) containing 4 mM dithionite (DT) and are incubated for 45 minutes. Subsequently, cyanate is added to the sample and left to react with $CODHII_{Ch}$ for 45 minutes under gentle stirring. The $CODHII_{Ch}$ sample is subsequently washed five times with Buffer A and reductant in order to remove cyanate excess and finally concentrated to 10 μL prior to injection into the transmission cell. NCO^-- and CN^-- bound $CODHII_{Ch}$ crystals were prepared as described earlier.[11] IR spectra were recorded on a Tensor 27 spectrometer (Bruker) equipped with a liquid nitrogen-cooled MCT photovoltaic detector using a spectral resolution of 2 cm^{-1}. The sample compartment was purged with dried air, and the sample was held in a temperature-controlled (10 °C) gas-tight IR cell for liquid samples (volume ≈ 10 μL, 50 μm path length) equipped with CaF_2 windows. Single $CODHII_{Ch}$ crystals were measured anaerobically at 10 °C in a N_2 purged cryostat (THMS600, Linkam Scientific) with a HYPERION 3000 FTIR microscope (Bruker) in the transmission mode. Spectra were base-line-corrected by using the OPUS software from Bruker. Spectra were normalized with respect to the intensity of amide II band. CO activity measurements were performed as described before.[12] QM/MM calculations was performed according to the procedure already established for the CN-inhibited state of $CODHII_{Ch}$.[9] The recently published structure (PDB ID: 4UDY)[11a] was used as input data. Titratable amino acids were protonated according to the pH value of 7.0. The structural arrangement and protonation state of His93 required special attention. Its double protonated state and the conformation with higher occupancy (65 %) were chosen, since the NCO ligand was modelled as a carbamoyl moiety. In the QM/MM setup, the system was divided in three layers. The QM part was treated with DFT employing the BP86 functional with the 6-31G* basis set for non-metal sections and def2-TZVP for metals. It consists of a total number of 155 atoms, specifically 1) the $[NiFe_3S_4]$ C-cluster and the iron Fe_1; 2) Cys 295, Cys 333, Cys 446, Cys 476, Cys 526, and His 261, coordinating the Fe atoms of the cluster; 3) the NH_2CO non-proteic ligand; 4) additional residues as His 93, Lys 563, Gln 332, Ile 567, and four water molecules interacting in different way with the system (e.g. hydrogen bonding, steric hindrance). The second layer was made up of protein residues and water molecules included in a sphere of 15 Å around the Ni atom of the C-cluster in monomer A, and the atoms in this section were treated at the MM level, described with the CHARMM force field, and allowed to move during the optimization cycles. The third layer includes the remaining atoms and was held fixed during the QM/MM geometry optimization. Vibrational frequencies of the QM part in the optimized conformation were then computed following the procedure described in Ref. [9].

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Conflict of interest

The authors declare no conflict of interest.

Keywords: biocatalysis · carbon monoxide dehydrogenase · cyanate · enzyme mechanisms · infrared spectroscopy

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Communications

Enzyme Mechanisms

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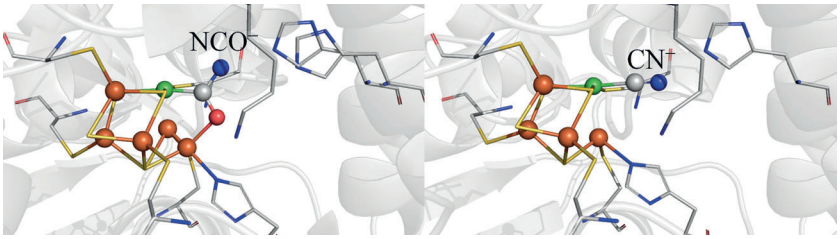
Carbon Monoxide Dehydrogenase Reduces Cyanate to Cyanide



Take-O-way: By combining spectroscopic and theoretical studies, the C-cluster of carbon monoxide dehydrogenase (CODH) was found to catalyze the reduction of cyanate to cyanide. The adduct remains bound to the catalytic center to form the so-called CN^--inhibited state. The transformation of NCO^- could thus mimic CO_2 turnover and open new perspectives for elucidation of the detailed catalytic mechanism of CODH.