n-Butyl Isocyanide: A Structural and Functional Analog of Carbon Monoxide for Carbon Monoxide Dehydrogenase from *Clostridium thermoaceticum*†

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Received August 9, 1995

Carbon monoxide dehydrogenase (CODH) from *Clostridium thermoaceticum* is a Ni−FeS metalloenzyme that catalyzes two separate reactions of CO metabolism. These reactions involve three metal clusters on CODH called centers A, B, and C. CO is oxidized to CO_2 at a Ni−FeS cluster called center C. Acetyl-coenzyme A (acetyl-CoA) synthesis from a methyl group, CO, and CoA occurs at another Ni−Fe-S cluster called center A. This communication demonstrates that *n*-butyl isocyanide (*n*-BIC) mimics both CO metabolizing activities. It was found to compete with CO in the CO oxidation reaction and to act as a slow substrate in a *n*-BIC/acceptor oxidoreductase activity. *n*-BIC caused EPR spectral changes at centers A, B, and C similar to those elicited by CO, albeit at greatly reduced rates. An early intermediate in the *n*-BIC reaction with center C was observed by EPR spectroscopy that is likely to be important in CO oxidation.

CODH is the key enzyme in a CO and CO_2 fixation pathway called the reductive acetyl-CoA pathway that involves the condensation of two one-carbon units to form the acetyl group of acetyl-CoA.[1,2] This pathway is unusual because the final steps involve enzyme-bound organometallic intermediates. The enzyme contains two Ni, 11−14 Fe, and ~14 inorganic sulfides per 150 kDa dimer which are parceled into centers A, B, and C, all of which are reduced by CO.[3,4] CODH interconverts CO and CO_2 (the CODH activity) at center C and activates and condenses a methyl group, CO, and CoA to form the C−C and C−S bonds of acetyl-CoA at center A.[5] The mechanism of acetyl-CoA synthesis at center A involves a bimetallic mechanism in which both Fe and Ni play catalytic roles.[6,7] The methyl group derives from a methylnickel intermediate.[7] The carbonyl group is donated by an end-on Fe−CO complex[6,8,9] with a minimal structure, [Ni−X−$Fe_{3-4}S_4$]−C≡O, where X is an unknown bridging ligand between Ni and Fe.[10] The Fe−CO adduct is characterized by two EPR signals collectively called the "Ni−Fe−C signal" with *g*-values at 2.074, 2.028 and 2.062, 2.047, 2.028.[11] The center A−CO adduct has been shown to be catalytically competent in acetyl-CoA synthesis.[9,12,13] Center B is a typical Fe_4S_4 cluster with *g*-values at 2.04, 1.94, and 1.89 and a redox potential of −420 mV.[3,14] It mediates electron transfer between CO and external electron acceptors.[12] Center C is another Ni−Fe-containing cluster with an unknown structure.[9,15] The existence of significant similarity between the sequences[16,17] and the EPR spectra[3,18] of the CO oxidation centers of the *C. thermoaceticum* and *Rhodospirillum rubrum* CODHs hint that center C has a conserved structure and function in these two enzymes.

Since isocyanides (RNC)[19,20] are isoelectronic with CO and have been studied as CO analogs for heme proteins,[21−25] several isocyanides were tested as CO analogs for CODH.[26] Isocyanides are members of a limited class of stable organic compounds that formally possess a divalent carbon. They can be nucleophiles, electrophiles, carbenes, radical acceptors, and pseudohalogens.[20] The presence of a nonbonding pair of electrons in the sp-hybridized orbitals on the terminal carbon enables isocyanides to behave as strong carbon ligands for transition metals. Besides the increased bulk of isocyanides, the main difference between CO and RNC ligands is that isocyanides have a 34-fold larger dipole moment than CO. Since the negative charge is on the terminal carbon, isocyanides have a tendency to form cationic species and can stabilize high oxidation states of metal ions. CO characteristically stabilizes the lower oxidation states of metal ions.

Treatment of CODH with *n*-BIC markedly altered the EPR spectra of as-isolated CODH.[27] Before reaction with *n*-BIC, the only EPR spectrum observed was from center C, with *g*-values of 2.01, 1.81, and 1.65 (Figure 1A). Treatment of this enzyme with 0.3 mM *n*-BIC rapidly (k_{obs} > 1 s^{-1} at 4 °C) converted center C into a paramagnetic form, called C*, with *g*-values of 2.28, 2.16, and 2.081 (g_{av} = 2.187) (Figure 1B). This spectrum was similar to that obtained by treating CODH with anions like azide and thiocyanate.[28,29] As CODH was incubated longer with *n*-BIC, the C* state converted (k_{obs} ~ 0.04 min^{-1} at 20 °C) to a state called C′,[12] with *g*-values at 1.97, 1.86, and 1.73 (Figure 1C). The C′ state has been observed on reaction of CODH with CO and other reductants.[3] In earlier freeze-quench EPR studies of the reaction of CODH with CO,

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† Supported by the National Institutes of Health (GM39451).

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(26) We did not observe reaction of CODH with phenylisocyanide.
(27) CODH was purified as previously described (Ragsdale, S. W.; Clark, J. E.; Ljungdahl, L. G.; Lundie, L. L.; Drake, H. L. *J. Biol. Chem.* **1983**, *258*, 2364−2369) to a specific activity measured at 55 °C of 393 units mg^{-1} in the CO oxidation reaction and 0.035 (25 °C) and 0.297 (55 °C) units mg^{-1} in the exchange reaction between CO and [1-^{14}C]acetyl-CoA. One unit is defined as 1 μmol of CO oxidized or exchanged per minute. Dithionite was removed using a Penefsky column (Penefsky, H. S. *J. Biol. Chem.* **1977**, *252*, 2891−2899).
(28) A similar EPR signal from reaction of azide and thiocyanate with CODH was shown to derive from an anion−center C adduct.[29]
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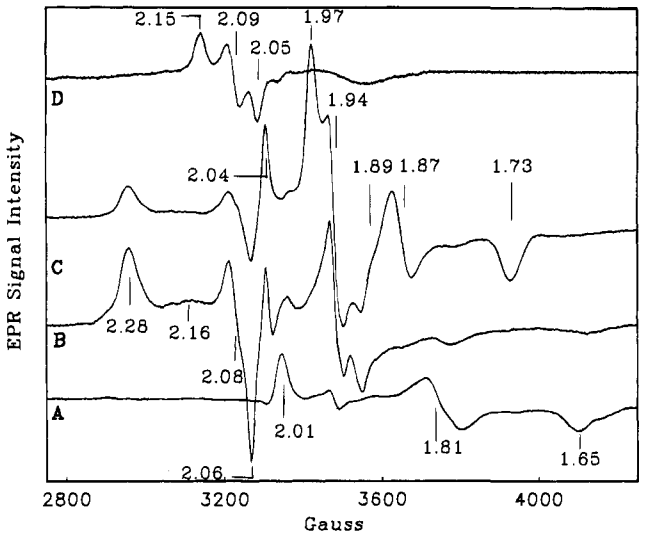

Figure 1. EPR spectra of the *n*-BIC adduct with CODH. (A) CODH as isolated (72 μM, final) in 50 mM Tris-HCl, pH 7.6. EPR parameters: temperature, 10 K; microwave power, 40 mW; microwave frequency, 9.4476 GHz; gain, 2×10^4; modulation amplitude, 10 G; modulation frequency, 100 kHz. This spectrum quantitates to 0.36 spins/mol. (B) CODH (72 μM final) treated with 300 μM (final) of *n*-BIC at 25 °C for 30 s before being frozen and stored in liquid nitrogen. (C) Same as (B) except incubated for 30 min with *n*-BIC before freezing. The spectrum assigned to the *n*-BIC−CODH adduct quantitates to 1.0−1.2 spins/mol, and the total spectrum quantitates to 3.2 spins/mol. (D) CODH (72 μM final) treated with 600 μM (final) of *n*-BIC at 25 °C for 15 min before being frozen and stored in liquid nitrogen. EPR parameters: temperature, 80 K; microwave power, 100 mW. This spectrum quantitates to 0.15 spins/mol.

the decay of center C appeared to mirror the formation of the C′ state ($k_{obs} \sim 400$ s^{-1} at 4 °C).[12] Thus, *n*-BIC reacts with center C ~10^5-fold slower than CO. A C* intermediate was not detected earlier in the freeze-quench EPR studies of the reaction of CODH with CO.

As shown in Figure 1, *n*-BIC also was capable of reducing center B ($k_{obs} \sim 3$ min^{-1} at 20 °C), strongly suggesting that it could be a functional CO analog. This was confirmed by reacting *n*-BIC with CODH in the presence of high-potential electron acceptors.[30] With saturating concentrations of *n*-BIC, the specific activity was found to be 10 nmol min^{-1} mg^{-1} with methylene blue and 60 nmol min^{-1} mg^{-1} with thionin as electron acceptors.[31]

n-BIC inhibited the oxidation of CO[32] by methyl viologen (Figure 2), with a K_i value of 1.66 mM. The K_m for CO and V_{max} values at 25 °C were 0.22 mM and 91 units mg^{-1}, respectively. The V_{max} value was independent of the concentration of *n*-BIC, and the steady-state velocity did not decrease as a function of time of incubation with *n*-BIC. Thus, *n*-BIC was classed as a rapid binding competitive inhibitor versus CO. Other inhibitors of CO oxidation that have been previously studied include cyanide and thiocyanate. Cyanide is a potent, slow-binding competitive inhibitor of CO oxidation, with a K_i below 10 μM.[33] Thiocyanate is a partial mixed inhibitor with respect to CO, with pH-dependent inhibition constants of 160 and 320 mM at pH 7.3.[29] Neither cyanide nor thiocyanate appears to be a substrate of CODH.

(30) The oxidized product of the *n*-BIC reaction is currently being identified. Two-electron oxidation would yield *n*-butyl isocyanate.

(31) No reduction of methyl viologen and benzyl viologen by *n*-BIC was detected. This was unexpected, since *n*-BIC can reduce the low-potential centers of CODH. Given the slow reaction with the high-potential acceptors and the high driving force (~500 mV), reaction with the viologens is likely to occur but to be too low to be detectable.

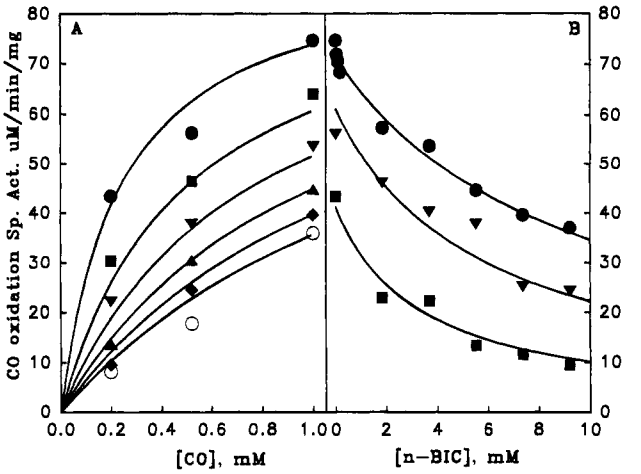

Figure 2. Inhibition of CO oxidation by *n*-BIC. The assay mixture contained 50 mM Tris-HCl, pH 7.6, 2 mM dithiothreitol, 10 mM methyl viologen, and varying amounts of CO and *n*-BIC at 25 °C. The reaction was initiated with 10 μL of a 0.49 mg/mL solution of CODH. (A) Concentrations of *n*-BIC were (●) 0, (■) 1.84, (▼) 3.68, (▲) 5.52, (◆) 7.36, and (○) 9.2 mM. (B) Concentrations of CO were (●) 1, (▼) 0.52, and (■) 0.2 mM. The solid lines were generated from the equation for a competitive inhibitor: $v = V_{max}[CO]/\{[CO] + K_m[(1 + [n\text{-BIC}])/K_i]\}$. The following values were determined by a nonlinear least-squares fitting procedure: $K_i = 1.66 \pm 0.23$ mM, $K_m^{CO} = 0.22 \pm 0.035$ mM, and $V_{max} = 91 \pm 5$ units/mg.

n-BIC not only reacted with CODH at the CO oxidation site; it also reacted with center A. At higher temperatures, where the EPR signals from centers B and C are not observed due to relaxation broadening, a rhombic EPR signal with *g*-values at 2.15, 2.09, and 2.05 was observed (Figure 1D). This signal is similar to that of the adduct between CO and center A, which has been called *the Ni−Fe−C EPR signal.* It also has a low-spin quantitation, as has been observed with the *Ni−Fe−C signal.* It would have been informative to determine the effect of *n*-BIC on the exchange reaction between CO and acetyl-CoA, a diagnostic reaction for acetyl-CoA synthesis at center A. However, when the exchange reaction was performed in the presence of *n*-BIC, the extent, not the rate, of exchange decreased with the *n*-BIC concentration, indicating that *n*-BIC reacts with acetyl-CoA. Therefore, we were unable to determine the effect of *n*-BIC on center A's reactivity.

In summary, *n*-BIC can act as a structural and functional analog of CO that can reduce centers A, B, and C of CODH and elicit EPR signals analogous to those observed when CODH is reacted with CO. These properties and its slow but analogous kinetics beg further studies aimed at better understanding substrate binding and intra- and intermolecular electron transfer reactions of CODH.

JA9527129

(32) CO oxidation was followed at 25 °C essentially as described before (Seravalli, J.; Kumar, M.; Lu, W.-P.; Ragsdale, S. W. *Biochemistry* **1995**, *34*, 7879−7888). The reduction of methyl viologen by CO was followed in an assay mixture containing a saturated solution of CO (0.90 mM) (*The Merck Index*, 11th ed.; Budavari, S., Ed.; Merck & Co.: Rahway, NJ, 1989; pp 274−275), 10 mM methyl viologen, 50 mM Tris-HCl buffer, pH 7.6, and 2 mM dithiothreitol. The complete time course was followed at 604 nm (the molar extinction coefficient (ϵ) was 13.9 mM^{-1} cm^{-1}). One milliliter of assay mixture was sparged with CO for 10 min at 25 °C, 1−10 μL of a 9.2 mM stock solution of *n*-BIC was added (Olson, J. S.; Gibson, Q. H. *J. Biol. Chem.* **1971**, *246*, 5241−5253), and the reaction was initiated with CODH. For studies of the inhibition of CO oxidation, *n*-BIC was added prior to addition of enzyme.

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