Photochemistry at the Active Site of the Carbon Monoxide Inhibited Form of the Iron-Only Hydrogenase (CpI)

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In biological systems, reversible hydrogen oxidation is catalyzed by the enzyme hydrogenase, which is found in a variety of organisms including aerobic, anaerobic, sulfate reducing, and methanogenic bacteria. Hydrogenases play important roles in the physiological processes of these bacteria by producing reducing equivalents via hydrogen oxidation or regenerating oxidized electron acceptors through proton reduction and thus constitute a key component in biological energy recycling.[1,2] Recently, the X-ray crystal structures of two Fe-only hydrogenases have been presented revealing that the site of reversible hydrogen oxidation exists as a unique 2Fe site (Figure 1).[3,4] The remarkable efficiency of the hydrogen producing enzymes has stimulated investigations regarding synthetic analogues.[5−9]

The inhibition of the bidirectional Fe-only hydrogenase from *Clostridium pasteurianum* (CpI) by carbon monoxide has been well characterized by kinetic and spectroscopic methods and the results have had mechanistic implications.[10−13] Initial investigations of carbon monoxide inhibition of CpI revealed that it acts as a competitive inhibitor of hydrogen oxidation and that inhibition can be reversed by illumination with light.[10,11] Our most recent studies indicate that carbon monoxide causes the reversible inhibition of hydrogen oxidation or proton reduction.[14] This single binding event results in a [2Fe] subcluster that is coordinately saturated with strong ligands (carbon monoxide, cyanide, and sulfur), representing a chemically rational mechanism for carbon monoxide inhibition of reversible hydrogen oxidation.[15] Additionally, these results strongly implicate the involvement of a terminal Fe site of the 2Fe active site cluster in the mechanism of reversible hydrogen oxidation, considering the observed competitive manner of carbon monoxide inhibition.

We have structurally characterized the photolytic chemical behavior that results when the carbon monoxide inhibited form of CpI hydrogenase is illuminated. Data were collected on a crystal of the carbon monoxide inhibited state of CpI first in the absence of illumination. After an essentially complete data set was accumulated, data were again collected over the same range of ϕ space on the same crystal under constant illumination with a helium/neon laser.[16] The result was two X-ray diffraction data sets (before and during laser illumination) that were isomorphous to one another such that small differences that occur during carbon monoxide photolysis could be observed in difference Fourier electron density maps.[17]

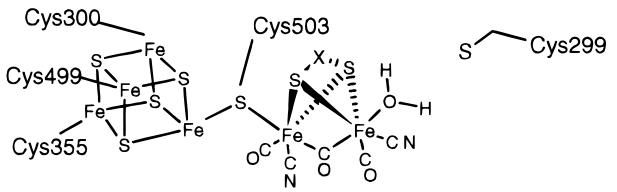

Figure 1. The active site "H cluster" in *C. pasteruianum* and associated Cys residues. The multiple light atom thiolate-bridging moiety of unknown composition is indicated by X.

The calculation of F_o(illuminated) − F_o(nonilluminated) electron density maps reveals significant changes in electron density at the H cluster (Figure 2A) during illumination. Most notable is the presence of a large peak of negative electron density at the position where the exogenously added carbon monoxide molecule had been previously modeled. The peak indicates a change in electron density at that position, corresponding to the loss of the carbon monoxide ligand. Additionally, evaluation of $F_o - F_{calc}$ electron density maps calculated using data sets before and during illumination indicates the presence of a smaller peak of electron density at this site during illumination (Figure 2B). The appearance of a large peak of negative difference electron density in F_o(illuminated) − F_o(nonilluminated) electron density maps together with the positive differences observed at this site in $F_o - F_{calc}$ omit maps suggest that either photochemical cleavage is incomplete and a percentage of carbon monoxide remains at the site or possibly carbon monoxide is displaced by a water molecule.[17]

Another significant feature of the F_o(illuminated) − F_o(nonilluminated) difference map is found near the distal iron atom in the [2Fe] subcluster. Two areas of electron density flank the iron atom, corresponding to overlapping spheres of positive and negative electron density. The two half-spheres correspond to movement of the iron atom upon illumination and photolysis of the terminal carbon monoxide ligand. The movement of the iron atom occurs from the negative electron density feature toward the positive electron density feature and suggests that upon photolysis there is a decrease in the distance between the terminal iron atom and the carbon atom of the bridging carbonyl group.

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(16) Crystals of the CO-treated form of the enzyme prepared as previously described [Lemon, B. J.; Peters, J. W. *Biochemistry* **1999**, *38*, 12969−12973]. X-ray diffraction data were collected at the Stanford Synchrotron Radiation Laboratory beamline 9-1 at $\lambda = 0.78$ Å. The data were processed using MOSFLM [Leslie, A. G. W. In *CCP4 and ESF-EACMB Newsletter on Protein Crystallography* **1992**, 26] and scaled using SCALA of the CCP4 suite of computer programs [Collaborative Computational Project No. 4: *Acta Crystallogr.* **1994**, *D50*, 760]. Data collected in the manner described above and in the text generated two data sets (before and during illumination) that were 90% complete from 20 to 1.8 Å resolution with 4-fold redundancy and an R_{merge} of 5.5%. Model fitting and refinement were accomplished using the programs O [Jones, T. A.; Zou, J. Y.; Cowan, S. W.; Kjeldgaard, M. *Acta Crystallogr. A* **1991**, *47*, 110−9] and XPLOR [Brünger, A. T.; Kuriyan, J.; Karplus, M. *Science* **1987**, *235*, 458−460].

(17) The refined carbon monoxide inhibited model of CpI has an R_{cryst} of 20.6 and an R_{free} value of 24.8 with relative mean standard deviations from ideality for the bond angles and bond distances of 2.42° and 0.015 Å respectively. The photolyzed model has an R_{cryst} value of 20.8 and an R_{free} value of 26.4 with relative mean standard deviation from ideality for the bond angles and bond distances of 2.40° and 0.014 Å, respectively. Both structures have the same solvent structure with 711 water molecules and have an overall rms deviation of 0.02 Å for corresponding Cα atoms. The refined *B*-factor for an O modeled in the photolyzed model is 25.0 and the refined *B*-factors for CO modeled in the photolyzed model are 20.1 for C and 32.1 for O.

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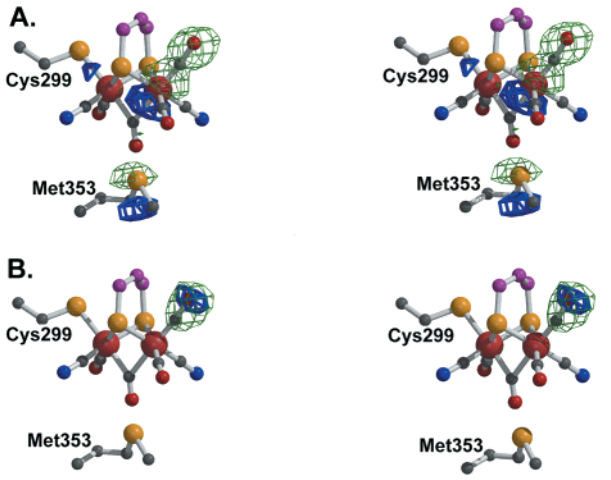

Figure 2. Wall-eyed stereoviews of the 2Fe subcluster of the active site H cluster and methionine 353 in the CO-inhibited state. (A) F_o-(illuminated) − F_o(nonilluminated) electron density map contoured at $+5\sigma$ (bold navy blue) and -5σ (thin green) and (B) $F_o - F_c$ maps for the illuminated (bold navy blue) and nonilluminated (thin green) diffraction data contoured at 3σ. The CO-inhibited structure with atoms at the terminal ligand exchange site omitted was used in the structure factor and phase calculation. Atoms are color coded with Fe in rust (large spheres), S in yellow, O in red (small spheres), N in blue, and C in gray. The unknown moiety bridging the two sulfur atoms is shown in magenta.

This change is consistent with the refinement of the iron-bridging carbonyl bond length from 2.09 Å in the carbon monoxide-bound form versus 1.85 Å in the photolyzed model. The presence of the terminally bound carbon monoxide molecule presents a situation where the two carbonyl groups trans to each other compete for the back-bonding electrons of the terminal iron atom. Loss of the terminally bound carbonyl group increases the back-bonding of electrons from the iron to the remaining carbonyl group, thus increasing the bond strength and decreasing the bond length. This terminal carbonyl ligand at a position trans to the bridging carbonyl ligand is more chemically labile than the other terminal carbonyls native to the active site that are positioned trans to sulfur atoms.

Also evident in the difference maps is a change in the orientation of an adjacent methionine residue with respect to the [2Fe] subcluster. Curiously, the Sδ of Met353 is approximately 3.1 Å away from the oxygen of the bridging carbonyl group in the native structure of CpI and is conserved in all Fe-only hydrogenases for which the deduced amino acid sequence has been reported.[18] The close proximity of the Met353 Sδ and the O of the bridging carbonyl of the 2Fe subcluster is somewhat unexpected due to the absence of hydrogen bonding. Neither moiety would likely be protonated and therefore their interaction would not be favorable. The change in electron density shows the movement of the Sδ away from the carbonyl group in the photolyzed model. This would be consistent with less favorable interaction between the Sδ of the Met353 side chain due to a more electron rich bridging carbonyl group.

These results indicate the presence of a ligand-exchangeable coordination site on the distal iron atom of the [2Fe] subcluster. This site, or the availability of this site for binding during catalysis, is proposed to be a salient feature of the mechanism of hydrogen oxidation and proton reduction. Inorganic molecules, especially metal−carbonyl complexes that can be protonated or bind molecular hydrogen or hydrides, have been useful in exploring potential mechanisms for hydrogen activation. Although the arrangement of a coordinating carbon monoxide ligand trans to a terminally bound water molecule is uncommon, it has been seen in $W(CO)_3(PR_3)_2(H_2O)$.[19] Interestingly, it has been shown by Kubas et al. that H_2 can displace a H_2O ligand on this molecule, and this displacement is actually favored at room temperature.[19] The presence of multiple π acceptor carbon monoxide ligands on the iron atoms of the [2Fe] subcluster affects the electronic nature of the iron atoms such that they are more electrophilic in character. This attribute facilitates reversible binding of molecular H_2 and its deprotonation by proximal bases, i.e., heterolytic cleavage to a hydride and a proton.[7] When H_2 binds to CO-containing metal centers it becomes acidic, with pK_a values approaching zero or less for cationic centers, easily low enough to protonate thiols.[20] It is likely that the diiron site attains a positive charge to facilitate deprotonation of H_2.[7]

A possible mechanism for H_2 oxidation would be the displacement of the terminally bound water molecule by H_2 binding and subsequent hydride formation upon deprotonation of the H_2 by an adjacent cysteine residue.[7] During proton reduction initial hydride formation could again occur at this site and upon addition of an additional proton yield hydrogen gas via a transient H_2 ligand. In a scenario involving only the terminal Fe2 site in reversible hydrogen oxidation the function of Fe1 would be to tune the chemistry of the Fe2 site with carbon monoxides and cyanides bound and the terminal site trans to a bridging carbonyl that is appropriately labile for effective enzyme turnover. However, formation of a hydride by direct protonation at this site would represent the reaction of a metallo Lewis acid and a proton (two Lewis acids), which would be rare although not unprecedented.[21] Other possible scenarios for protonation at the 2Fe subcluster include initial hydride formation at the Fe−Fe bond, which can be readily protonated to form a hydride-bridged species,[22−24] or protonation at the Fe-bridging sulfur atoms.[25] The bridging H could migrate to a terminal position upon reduction and subsequent hydride formation.

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