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Binding of Exogenously Added Carbon Monoxide at the Active Site of the Iron-Only Hydrogenase (CpI) from *Clostridium pasteurianum*[†,‡]

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ABSTRACT: A site for the binding of exogenously added carbon monoxide has been identified at the active site of the Fe-only hydrogenase (CpI) from *Clostridium pasteurianum*. The binding and inhibition of carbon monoxide have been exploited in biochemical and spectroscopic studies to gain mechanistic insights. In the present study, we have taken advantage of the ability to generate an irreversibly carbon monoxide bound state of CpI. The crystallization and structural characterization of CpI inhibited in the presence of carbon monoxide indicates the addition of a single molecule of carbon monoxide. The ability to generate crystals of the carbon monoxide bound state of the hydrogenase that are isomorphous to those of the native enzyme has allowed for a direct comparison of the crystallographic data and an unambiguous identification of the site of carbon monoxide binding at the active site of CpI. Carbon monoxide binds to an Fe atom of the 2Fe subcluster at the site of a terminally bound water molecule in the as crystallized native state of CpI that has been previously suggested to be a potential site of reversible hydrogen oxidation. Binding of carbon monoxide at this site results in an active site that is coordinately saturated with strong ligands (S, CO, and CN), providing a rational potential mechanism for inhibition of reversible hydrogen oxidation at the active site of CpI.

Hydrogenases catalyze reversible hydrogen oxidation according to the reaction:

$$H_2 \rightleftharpoons 2e^- + 2H^+$$

These enzymes are expressed mainly in microorganisms where their function is either to dispose of electrons accumulated during bacterial fermentation (hydrogen evolution) or to couple hydrogen oxidation to the energy yielding metabolic processes (hydrogen uptake). Hydrogen does not accumulate to any appreciable extent in the anaerobic niches where the microorganisms occur. The hydrogen evolved by the fermentative bacteria is utilized directly by the hydrogen-oxidizing bacteria to generate reduced electron carriers. Hydrogenases are often classified on the basis of their metal content and exist in NiFe, NiFeSe, Fe-only, and metal-deficient forms (*1*−*3*). Of the metal-containing hydrogenases, the Ni containing are most often associated with hydrogen oxidation and those that contain only Fe are most often associated with hydrogen evolution.

Deduced primary amino acid sequence alignments of known Fe-only hydrogenases show considerable identity and similarity, indicating that they all share a common architecture (*4*−*9*). As in the case of the NiFe hydrogenase, there is

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[‡] Atomic coordinates have been deposited in the Brookhaven Protein Data Bank, codes 1c4a (native) and 1c4c (CO bound).

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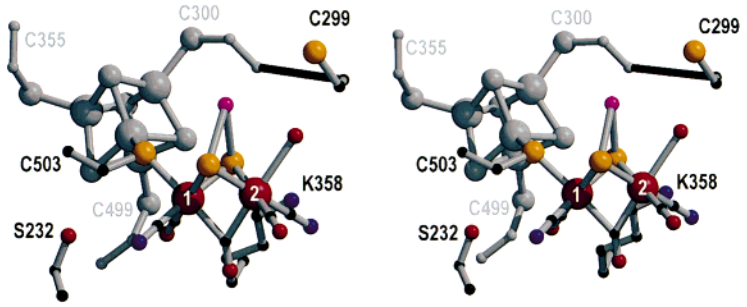

FIGURE 1: Stereoview of a ball-and-stick representation of the native, as crystallized, 2Fe subcluster of the active site cluster and selected amino acid residues in its immediate polypeptide environment. Atoms are color coded with Fe in rust, S in yellow, O in red, N in blue, and C in black. The as of yet undetermined S-bridging moiety is shown in magenta.

considerable interest in the structure of the metal-containing prosthetic group located at the active site of the enzyme. Structures of the Fe-only hydrogenases from both *Clostridium pasteurianum* and *Desulfovibrio desulfuricans* have been determined by X-ray diffraction methods (*10*, *11*). The structure of the *C. pasteurianum* enzyme reveals that the active site cluster exists as a regular [4Fe-4S] cubane bridged to a 2Fe cluster through a thioether linkage supplied by a cysteine sulfur. The 2Fe cluster is coordinated by, in addition to the cysteine sulfur, two additional sulfur atoms, five diatomic ligands (believed to be both carbon monoxide and cyanide), and a terminally bound water molecule (Figure 1). The assignment of these diatomic ligands was based on, in addition to the X-ray diffraction data, the presence of these ligands in the NiFe hydrogenase (*12*) and Fourier transform infrared spectrscopic studies that indicate the presence of these diatomic ligands at the active site of the Fe-only hydrogenases (*13*).

Although the salient features are similar and the structures of the active site domains are essentially superimposable, there are some key differences in comparing the 2Fe-containing subclusters of active sites of the Fe-only hydrogenases *C. pasteurianum* (CpI) and that of *D. desulfuricans* (DdH). In both of the structures, CpI and DdH, sulfur atoms that serve to bridge the Fe atoms appear to be bridged themselves by a covalent linkage of multiple light atoms. In the structure of DdH this moiety has been assigned as a propane linkage. In the as crystallized state of CpI the active site has been modeled with five diatomic ligands believed to be either carbon monoxide or cyanide. These include four terminal ligands to the Fe atoms and a single diatomic ligand that acts to bridge the two Fe atoms. In contrast, the structure of DdH has been modeled with carbon monoxide and cyanide ligands in the analogous positions of the terminal ligands of CpI; however, an asymmetrically coordinated water molecule has been assigned at the site of the bridging diatomic ligand of CpI. The results of recent FTIR studies indicate that both terminal carbon monoxide and cyanide ligands bound to Fe and suggest the presence of a bridging carbon monoxide atom in an oxidized state in aerobically purified Fe-only hydrogenase from *Desulfovibrio vulgaris* prior to reductive activation (*13*). Another notable difference in the active sites is the absence of the terminally bound water molecule in the DdH active site which has an apparent vacant coordination site at this position. The experimental scenarios under which the respective Fe-only hydrogenases are crystallized suggest that the structure of the *C. pasteurianum* enzyme represents an oxidized or resting state and the structure of the *D. desulfuricans* represents a reduced or turnover state. This would be consistent with a proposed mechanism of reversible hydrogen oxidation involving displacement of the terminally bound water molecule.

Table 1: Data and Refinement Statistics

	native data	CO-bound data
Data Statistics		
a (Å)	133.7	133.5
b (Å)	84.0	83.8
c (Å)	55.7	55.6
resolution range (Å)	20.0−2.4	20.0−2.4
observations	176088	234583
unique reflections	24218	24879
R_{merge} (%)	0.068 (0.181)[a]	0.052 (0.130)[a]
completeness	90.4 (74.5)[a]	97.7 (79.1)[a]
Refinement Statistics		
resolution range (Å)	20.0−2.4	20.0−2.4
total reflections ($F > 1\sigma F$)	23019	24021
R_{cryst}	19.9	19.3
R_{free}	25.4	24.0
rms of bond distances (Å)	2.54	2.48
rms of bond angles (deg)	0.010	0.010

[a] Statistics for the highest resolution shell (2.51−2.40 Å) are indicated in parentheses. $R_{merge} = \sum_{hkl}[\sum_i(|I_{hkl,i} - \langle I_{hkl}\rangle|)]/\sum_{hkl,i}\langle I_{hkl}\rangle$, where I_{hkl} is the intensity of an individual measurement of the reflection with indices *hkl* and $\langle I_{hkl}\rangle$ is the mean intensity of that reflection.

Although multiple carbon monoxide molecules serve as ligands to the 2Fe cluster, additional carbon monoxide has been shown to bind to CpI both in a manner that results in the inhibition of reversible hydrogen oxidation and in a manner that does not (*1*, *14*−*18*). The most recent studies report that the addition of carbon monoxide to CpI when poised in either an oxidized or a reduced state results in the irreversible binding of carbon monoxide indicated by the appearance of a specific axial EPR signal. When this enzyme is assayed in the absence of carbon monoxide, the axial EPR signal remains but the enzyme is fully active. In contrast, when carbon monoxide is added to the enzyme at high concentrations under turnover conditions, it results in complete and irreversible inhibition in a state of the enzyme that has not been characterized by spectroscopic methods (*1*, *14*−*16*). In the present study, we have been able to crystallize and structurally characterize a state of CpI with exogenously added carbon monoxide bound.

EXPERIMENTAL PROCEDURES

The Fe-only hydrogenase (CpI) from *C. pasteurianum* was purified as described previously (*19*). The carbon monoxide inhibited form of the enzyme was generated by addition of

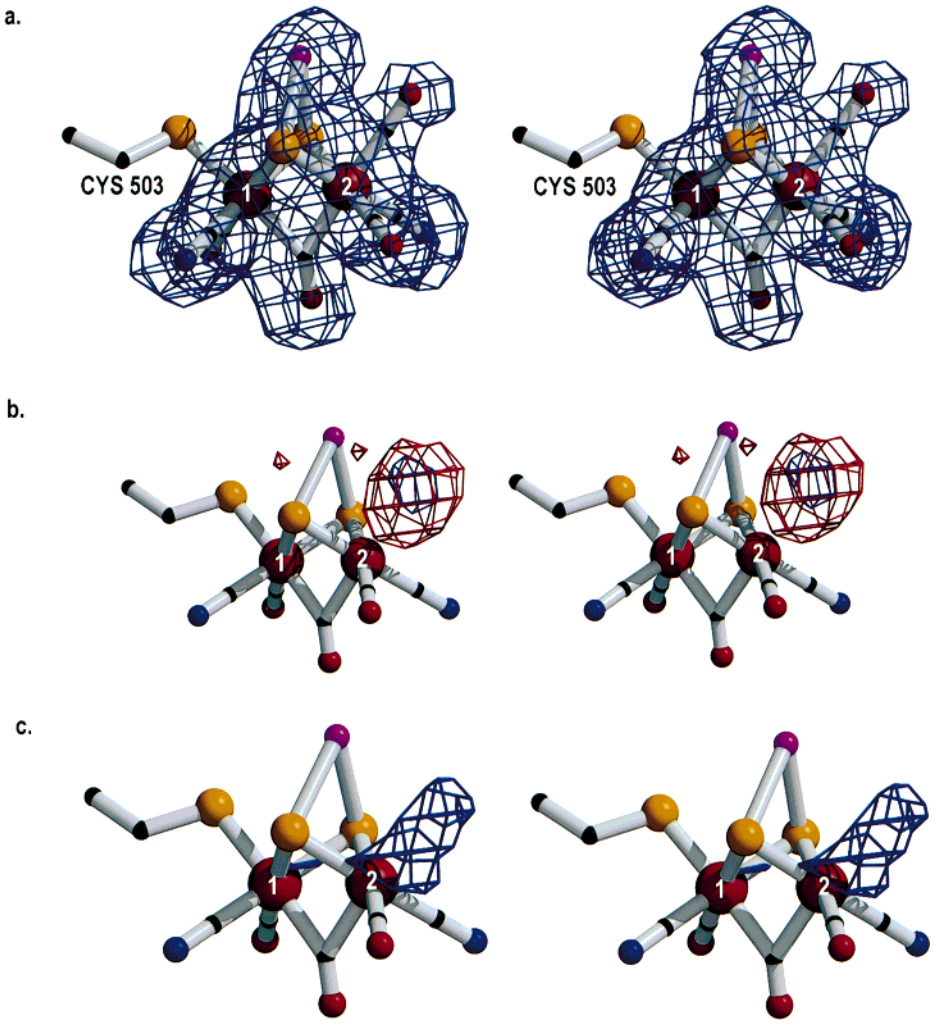

FIGURE 2: (a) Stereoview of 2.4 Å $F_o - F_c$ electron density maps contoured at 3σ in which the active site 2Fe subcluster has been omitted in the calculation. (b) Stereoview of 2.4 Å resolution $F_o - F_c$ electron density maps contoured at 3σ in which the terminal water ligand was omitted from the native structure (blue) and the carbon monoxide was omitted from the inhibited structure (red). (c) Stereoview of 2.4 Å resolution F_o(CO inhibited) $- F_o$(native) electron density maps contoured at 5σ. The atom color scheme is the same as in Figure 1.

carbon monoxide (UHP grade 99.99%, Matheson) to a final concentration of 37.5% to a sealed serum vial containing CpI at a concentration of 52 mg/mL in the presence of 2 mM sodium dithionite, followed by incubation at room temperature for 5 min. To maintain turnover conditions (the catalytic state), the enzyme was treated with carbon monoxide in the presence of sodium dithionite at a concentration of 2 mM. Inhibition was monitored in these samples by the loss of hydrogen evolution activity, and the addition of carbon monoxide resulted in greater than 90% inhibition in concentrated protein samples (*1*, *14*). Crystallization of the native and carbon monoxide inhibited forms of the enzyme was accomplished using the microcapillary batch diffusion method (*20*) with 20 μL aliquots of protein incubated with 30 μL of a precipitating solution of 25% poly(ethylene glycol) 4000, 0.1 M sodium acetate (pH 4.6), and 0.2 M ammonium acetate.

Although the crystallization conditions were identical to those previously described (*10*), the tetragonal crystals originally observed could not be generated in the native or inhibited states of the enzyme and instead a new crystal form was reproducibly observed. This new crystal form appears in ~2−3 weeks and exists in a crystal morphology that can be described as flattened rhombs in contrast to the laths observed in the previously characterized tetragonal form (*10*). The symmetry of these crystals has no apparent relationship to the previously observed form and belongs to the orthorhombic space group $P2_12_12$ with one molecule in the asymmetric unit with a Matthews coefficient of 2.6 (*21*) translating to a solvent content of 52.7%. Although the crystals were of slightly inferior diffraction quality to that of the tetragonal form, they were of sufficient quality to permit the collection of good quality data at 2.4 Å resolution.

The cell parameters were very similar in the case of the native and carbon monoxide bound forms such that the crystals in each state were isomorphous to each other, allowing direct comparison of the data and the interpretation of F_o(CO bound) $- F_o$(native) electron density maps. Data were collected using a RU H3R X-ray source equipped with a RAXIS IIc detector. The data were processed in DENZO and scaled using SCALEPACK (*22*). The native structure was solved in the orthorhombic crystal form by molecular replacement using AMORE (*23*) with the previously described native structure as a search model. The original AMORE solution for the native form resulted in a correlation factor of 49.9% and an *R* factor of 41.6%. The model fit to the data in both the native and carbon monoxide structures was accomplished by simulated annealing, positional, and

B factor refinement of X-PLOR (*24*). Randomization by simulated annealing refinement using an initial temperature of 4000 K was employed to remove bias such that a cross-validation could be introduced into the refinement (*24*). Slight alterations to improve the model fit were introduced in the O program (*25*).

RESULTS AND DISCUSSION

Previous studies have established that the activation of H_2 by hydrogenase occurs by a heterolytic cleavage mechanism involving the formation of a hydride and a proton (*16*). We have recently proposed that hydride formation in CpI during either hydrogen oxidation or proton reduction occurs at an Fe site found to be weakly coordinated by a water molecule in what is believed to be an oxidized state of the enzyme (*10*). This proposal was based on the ligand composition of the active site cluster and the availability of proton donors/acceptors.

In the present study, we have addressed carbon monoxide binding to CpI with the structural characterization of CpI with exogenously added carbon monoxide bound at the active site. The structure of CpI in its native and CO-bound state has been determined in a new orthorhombic crystal form by molecular replacement methods (*23*). The structures have been refined to 2.4 Å resolution (Table 1). Density in H cluster omit maps of the carbon monoxide inhibited state indicates peaks of electron density of similar size to those of the previously assigned diatomic ligands (Figure 2a). This peak in the electron density maps was distinct from the peak present as a bound water molecule in the native structure. This can be distinguished quite clearly when electron density maps of both the native and bound forms of CpI in which the ligand at this position is omitted are directly compared at the same contour level (Figure 2b). In this form, crystals of the carbon monoxide bound CpI were isomorphous with that of the native CpI crystals, allowing direct comparisons and calculation of F_o(CO bound) $-$ F_o(native) electron density maps of excellent quality (Figure 2c). These maps indicate a feature of positive difference electron density consistent with the replacement of water observed in the previously characterized presumed oxidized state by carbon monoxide in the carbon monoxide bound state (Figure 2c). This peak of electron density is by far the largest feature in these difference maps, indicating that the binding by carbon monoxide is not accompanied by additional conformational changes in the protein structure. Refinement of carbon monoxide at this position resulted in geometry and temperature factors that are on the order of those observed for the other diatomic ligands.

Under the conditions of crystallization of the native CpI enzyme, although reductant (2 mM sodium dithionite) was present at the beginning of the crystallization, we assume that over the crystallization (2−3 weeks) this reductant would be consumed, leaving the enzyme in an oxidized or resting state. In the work of Fontecilla-Camps and co-workers, crystals of DdH were obtained under a constant atmosphere of H_2 and in the absence of external electron acceptors and are believed to represent the structure of the active site poised in a reduced state (*11*). The main differences in the active sites of CpI and DdH include (1) the presence of a terminally bound water molecule in CpI which is replaced by an open coordination site in DdH and (2) the presence of a bridging diatomic ligand in the structure of CpI where an asymmetrically bound water molecule has been assigned in DdH. It seems reasonable that the difference observed at the terminal site could be attributable to differences in oxidation state, and this would support a mechanism of reversible hydrogen oxidation involving displacement of the bound water molecule. However, in the absence of a direct biochemical and biophysical characterization of the hydrogenase crystals, it is impossible to make a definitive assignment of the as crystallized states of CpI and DdH to previously characterized states of the enzymes. The difference at the bridging site, assigned as a diatomic ligand in CpI and a water molecule in DdH, could also be mechanistically significant. Some possible explanations for this difference include differences in bacterial species (a detailed spectroscopic characterization of DdH has not been reported) and differences in oxidation state, and it cannot be excluded that the as crystallized state of CpI represents a state in which a single exogenous diatomic molecule (either CO or O_2) has been bound.

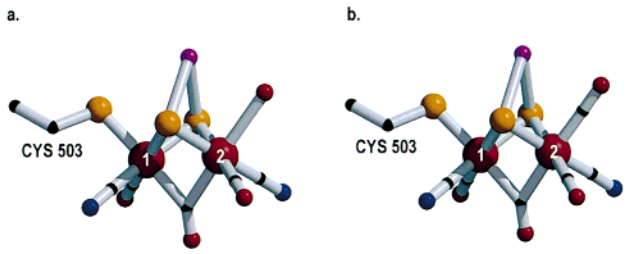

FIGURE 3: Comparison of the 2Fe subcluster of the H cluster in the (a) native and (b) CO-inhibited forms. For clarity, the clusters are depicted in the same orientation and color scheme as in Figure 2.

The observation that carbon monoxide can bind to the active site of CpI in both an inhibitory and a noninhibitory manner has been interpreted to indicate that two molecules of carbon monoxide are bound at separate sites on the enzyme. In this scenario one molecule is bound irreversibly at the active site under conditions in which the enzyme is poised in the oxidized or reduced state but is not in its catalytic state involving enzyme turnover. This state of the enzyme has been characterized as an irreversibly carbon monoxide bound but not inhibited state since the active enzyme retains the specific $g = 2.07$ axial EPR signal. In the presence of small concentrations of oxygen a similar noninhibitory binding effect with analogous spectroscopic properties is observed (*26*). The addition of carbon monoxide under conditions in which the enzyme is turning over and evolving hydrogen results in complete and irreversible inhibition of enzyme activity. These results have been interpreted to indicate that CpI binds two molecules of carbon monoxide, one at the site of reversible hydrogen oxidation (inhibitory) and one at a separate site that does not result in enzyme inhibition (*1*, *14*).

Although the previous work of Adams and co-workers (*1*, *14*) suggests that two molecules bind to CpI in the fully inhibited state, in the present study the binding by only a single carbon monoxide molecule is observed. As previously mentioned, a possibility that cannot be excluded from consideration is that the as crystallized native state of CpI represents a form that has bound a single exogenous diatomic ligand (either CO or O_2), and this state may represent the

carbon monoxide or oxygen bound but not inhibited state of CpI. A scenario along these lines may be supported by the differences observed in the CpI and DdH active site structures. However, the crystallizations were conducted under anaerobic conditions, and it is unclear at this point where either additional carbon monoxide or oxygen could have been introduced into crystallization for binding.

In the results presented here a single molecule of carbon monoxide has been bound, resulting in an active site that is coordinately saturated with strong ligands (S, CO, and CN) and likely represents an inhibited form of the enzyme (Figure 3). This is consistent with the loss of hydrogen evolution activity observed in samples examined prior to crystallization. The addition of carbon monoxide by displacement of the terminally bound water molecule would be a reasonable manner of inhibition of reversible hydrogen oxidation if such a mechanism involves the displacement of the bound water molecule at this terminal site during hydride formation. However, a definitive assignment of the states of the enzyme observed in the crystal structures with the states previously characterized by biochemical and spectroscopic methods requires direct examination of the crystals by these methods, and these difficult experiments are a major effort in our ongoing research.

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