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Resolving the CO/CN Ligand Arrangement in CO-Inactivated [FeFe] Hydrogenase by First Principles Density Functional Theory Calculations

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The currently presumed assignment of CO/CN ligands in the structure of the active cluster in CO-inactivated [FeFe] hydrogenase is shown to be inconsistent with the available IR data in the enzyme from *Clostridium pasteurianum* I. A different arrangement has the correct qualitative and quantitative features, reproducing the observed line spacing and intensities and the observed line shift consequent to inactivation with labeled ^{13}CO instead of ^{12}CO. The new assignment is also consistent with the observed change from rhombic to axial symmetry of the electron paramagnetic resonance **g** tensor upon inactivation.

Hydrogenase enzymes have been the focus of intense studies in the past decade.[1,2] Many anaerobic microorganisms, e.g., methanogenic archaea and acetogenic, nitrogen-fixing, photosynthetic, sulfate-reducing bacteria, produce or consume H_2 as part of their metabolic cycles. Understanding the structures and mechanisms of the hydrogenases is important for its biological implications and also in the quest for biological or bioinspired synthetic catalysts for H production and for its oxidation[3] in fuel cells.

The relationship between the structure of the active site and its mechanism is essential for understanding metalloenzyme action. Significant landmarks in the study of [FeFe] hydrogenase (Fe_2H_2ase; Figure 1a) were X-ray studies of the active form[4−6] from *Clostridium pasteurianum* I (CpI) and *Desulfovibrio* desulfuricans (DdHase) and later of the CO-inhibited form[7] (Fe_2H_2ase-CO) for CpI. We focus here on Fe_2H_2ase-CO (from CpI) obtained by exposing the oxidized state to CO.[8,9] Lemon and Peters[7] determined its structure to 2.4 and 1.8 Å resolutions, finding that the exogenous CO binds to the distal Fe (with respect to the Fe_4S_4 cubane) as in Figure 1b. That X-ray resolution is at best only marginally sufficient to determine such fine structural features as the assignment of the unusual CO/CN ligands in the active cluster.[6] The currently accepted assignment[6] of the CO/CN ligands, which is now widely accepted as definitive,[10] has served in theoretical studies as a fundamental input to

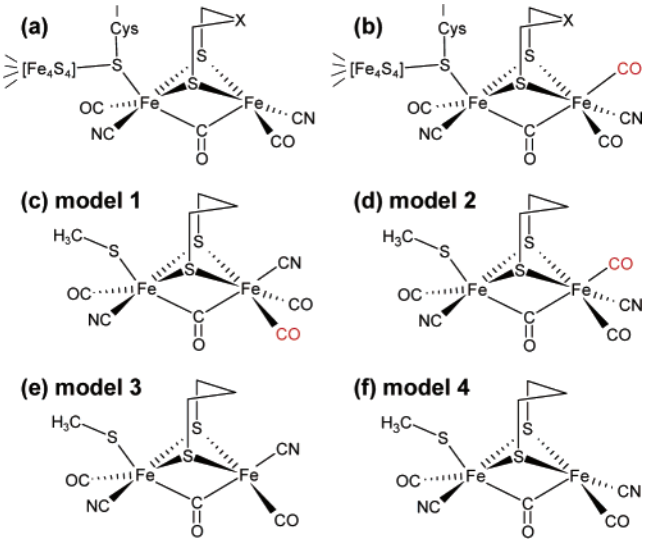

Figure 1. (a) Structure of the active site in Fe_2H_2ase[4,5] and (b) assumed CO-inhibited form[7] of CpI. Parts c−f are our computational models. The unknown bridging ligand X was proposed to be either PDT (X = CH_2) or DTN[16] (X = NH). The exogenous CO is highlighted in red.

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validate the computations.[11,12] So far, however, there is no direct evidence to support that assignment. Theoretical studies[12] of the IR frequencies of Fe_2H_2ase-CO did not reproduce the observed line spacing and did not address any of the other qualitative aspects of the IR spectra such as intensities and, in particular, the *isotopic shift* consequent to inhibition with ^{13}CO in place of ^{12}CO.[13−15]

We present a detailed and critical *theoretical* treatment of the IR data of Fe_2H_2ase-CO, showing that the original assignment[7] (Figure 1b) is inconsistent with the available IR data,[13,14] failing to reproduce the correct spacing of the modes and the overall line shape. More importantly, the isotopic shift is qualitatively incorrect in the accepted structure. Our calculations suggest that the discrepancies originate from the assumed assignment of the CO/CN ligands, and a different assignment is found that is uniquely consistent with both the IR data and the X-ray studies.

The models we consider (Figure 1c,d) are in the Fe^{II}/Fe^{I} mixed-valence oxidation state and differ only in the arrangement of the CO/CN ligands. We focus on the Fe_2 part of the Fe_2H_2ase; the Cys-bridged cubane is replaced by a thiomethyl ligand, a simplification that is known to have little effect on the relevant spectral region.[12] Model 2 has the structure of Fe_2H_2ase-CO that is accepted by most research groups. It has the exogenous CO in the cis position with respect to the bridging thiolates. In contrast, model 1 has the arrangement that we find to be in agreement with experiment, with the CN in the position cis to the bridging S atoms and the exogenous CO trans to a bridging thiolate. This result is independent of the nature of X (see Figure 1). All other possible arrangements of the CO/CN ligands were considered but do not fit well and are not reported here. The computational methods are discussed in the Supporting Information.

In Figure 2, we report the results for models with a propanedithiolate (PDT) bridge (see Figure 1). Figure 2a shows the experimental IR spectra of Fe_2H_2ase-CO,[14] focusing on the CO/CN bands. The ^{12}CO spectrum has a μ-CO mode at 1810 cm^{-1}, two terminal CN modes at 2077 and 2095 cm^{-1}, and a central band of terminal CO's. These three terminal CO modes generate a single, high-frequency, high-intensity line at 2017 cm^{-1} and two closely spaced lower-intensity lines at 1974 and 1971 cm^{-1}. The line at 1971 cm^{-1} is not affected by the ^{13}CO labeling, while two other lines are shifted as indicated by the black arrows. These observations are consistent with a picture of inhibition taking place on the distal Fe, with the two terminal CO's on this Fe being coupled, while the fixed mode is associated with the single terminal CO on the proximal Fe. (It is presumably remote enough to be insensitive to changes on the distal Fe.)

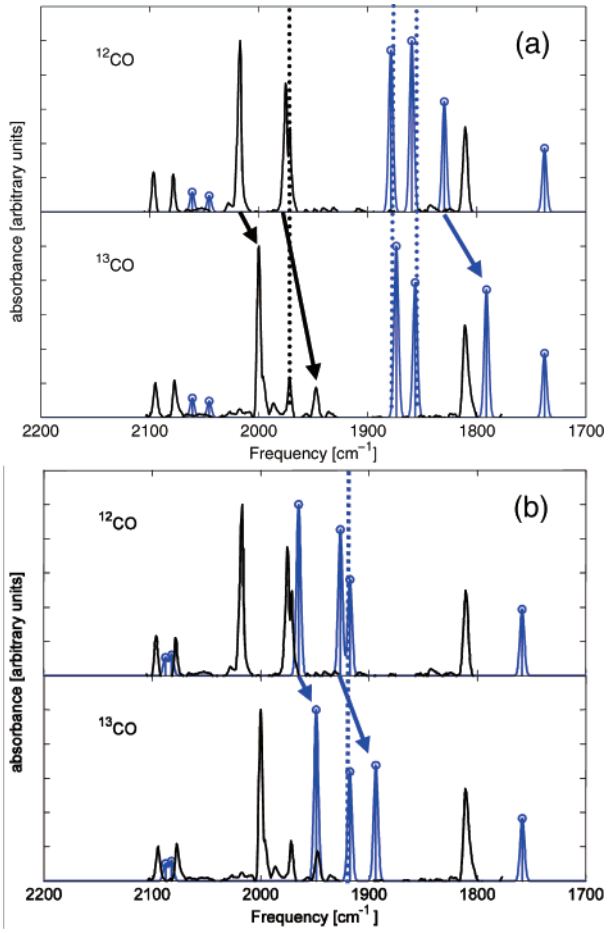

Figure 2. Experimental spectra (black line; reproduced with permission from ref 14) and simulated spectra of Fe_2H_2ase-CO models having a PDT bridge (blue line): (a) model 2 and (b) model 1. A Gaussian broadening of 2 cm^{-1} is used. Dashed lines mark the terminal CO modes unchanged by labeling, and the arrows show the modes that shift consequent to labeling of the exogenous CO with ^{13}C.

Figure 2a also shows the simulated spectra of model 2. The agreement with experiment is poor for the spacing between the modes and their intensities, especially for the terminal CO modes. Moreover, the isotopic shift is incorrect; only one of the terminal CO modes shifts appreciably instead of two as in experiment. Last, the computed spectrum is red shifted by more than ~100 cm^{-1} with respect to experiment, a large deviation but not uncommon in first principles computations of IR spectra.

The corresponding spectra of model 1 (Figure 2b) show that seemingly mild structural changes have a profound impact on the vibrational spectra. The overall deviation from experiment is reduced from ~100 to 50 cm^{-1} (compare with Figure 2a); the line shapes are in very good qualitative and quantitative agreement; and the spacing between the CO modes is practically identical with the experimental spacing. The spectrum of the ^{13}CO-labeled structure is in particularly good agreement with experiment, reproducing quantitatively the two-line shift.

The isotopic shifts in the IR spectra of the CO-inhibited form of [FeFe] hydrogenase are thus consistent only with the structure of model 1. Moreover, replacing the bridging PDT by dithiomethylamine (DTN) does not change any of

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the qualitative features of the IR spectrum (see the Supporting Information). Including nearby groups, such as cubane Fe_4S_4, does not change this conclusion.

The internal energy difference between models 1 and 2 is small, 3.3 kcal/mol in favor of model 1, and the vibrational modes differ only by $\sim$50 cm^{-1}, Therefore, the difference in the isotopic shift must originate from *subtle* differences in the electronic structure. A minimal requirement for vibrational coupling between the two CO groups is to have molecular orbitals that couple the two groups via the distal Fe, i.e., having significant orbital density simultaneously on the two CO's and on the Fe. Such a computation indicating that the coupling is stronger in model 1 than in model 2 is reported in the Supporting Information.

Further validation of our assignment comes from the electron paramagnetic resonance (EPR) measurements in CpI[9,17] and DdHase,[18] which show a change from a rhombic to an axial **g** tensor upon inactivation with CO. There is a clear difference in symmetry between models 1 and 2. Model 1 has a local symmetry plane on the distal Fe, defined by the two Fe atoms and the C atom of the distal CN. This local symmetry can explain the axial signal. The *z* principal axis of the **g** tensor must lie in the symmetry plane with symmetrically equivalent *x* and *y* axes at 45° to the symmetry plane. In contrast, model 2 has no symmetry and cannot have an axial **g** tensor. In the active-ready state (model 4), there is also no such symmetry and the signal should be rhombic, as observed experimentally. In the Supporting Information, we report computed spin density distributions that clearly support these arguments. In a recent paper, Fiedler and Brunold[11] have calculated the EPR response of model systems. For the active-ready state, they get good agreement with experiment, but for the CO inactivated model, they were not able to reproduce the axial symmetry of CpI and DdHase. For CpII, however, they do find good agreement with experiment, which indicates an asymmetric arrangement of the diatomic ligands in CpII, as in model 2.

In the active-ready state, the lowest-energy configuration has the distal CN trans to μ-CO (model 3), having a total energy of 1.15 kcal/mol less than that of model 4. However, in the native enzyme, the arrangement of model 4 is presumably further stabilized by a H bond to the side-chain N of Lys358[4] in CpI. A reasonable estimate of the bond strength is on the order of $\sim$2 kcal/mol because N atoms form a weak bond (compared with 4.6 kcal/mol in water) and there may also be geometrical constraints in the enzyme. Therefore, the configuration of model 4 should be more stable only by $\sim$1$-$1.5 kcal/mol, rendering it thermally accessible at room temperature. The contribution of the configuration of model 3 to the EPR spectra of Bennett et al.[9] was negligible because the measurements were taken at 15$-$17 K. We have calculated the reaction path for the rearrangement from model 4 to model 3 and found that, in the absence of a stabilizing H bond, there is no barrier for the rearrangement. These results are consistent with earlier studies showing lability of the ligands in synthetic models.[2,19] Therefore, we argue that it is plausible for the binding of the exogenous CO to be as in model 1, but the mechanism leading to that configuration from the active-ready configuration is presently unknown.

We now show that our results are consistent with the known reversibility of the inhibition. Reactivation of Fe_2H_2-ase-CO was achieved either by repeated sparging[9] with Ar and H_2 (chemical activation) or by exposure to a He$-$Ne laser light[14] (photolytic activation). In chemical activation, the typical time scale for the process was $\sim$10 min. Assuming a reaction rate of $\sim^1/_{10}$ min^{-1} and an Arrhenius prefactor of 10^{13} s^{-1} results in an activation barrier of $\sim$22 kcal/mol for CO dissociation. In our systems, the binding energy of the exogenous CO is 24.6 kcal/mol for model 1 and 22.4 kcal/mol for model 2. The known reversibility also indicates that CN^- does not dissociate, presumably because of the hydrophobic nature of the enzyme in the near vicinity of the active site. Our results suggest that reactivation involves dissociation of the exogenous CO in model 1, coupled with rearrangement of the CN ligand moving from model 3 to the more favorable cis position of model 4. The system thus returns reversibly to the active-ready state.

We conclude that the original CO/CN ligand assignment (model 2) of the CO-inhibited CpI and DdHase is inconsistent with the available IR and EPR data and should be replaced by that of model 1. This, in turn, implies that the mechanism of inhibition must be more complex than direct binding of the exogenous CO to the vacancy on the distal Fe because the added CO is not in the original vacancy position. The inactivation mechanism is presently unknown and is the subject of ongoing research in our group. Our results may also be important for the understanding of inactivation by O_2[20] because both CO and O_2 are apolar and may use the same diffusion channel.[21]

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Supporting Information Available: Discussion of the computational methods and additional computations. This material is available free of charge via the Internet at http://pubs.acs.org.

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