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Theoretical Studies of [FeFe]-Hydrogenase: Infrared Fingerprints of the Dithiol-Bridging Ligand in the Active Site

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An unresolved structural issue for [FeFe]-hydrogenases is the nature of the dithiol-bridging ligand in the diiron subcluster of the active site. The two most probable candidates are 1,3-dithiopropane (propane dithiol, PDT) and di-(thiomethyl)-amine (DTN). In the latter case, the dithiol-bridging ligand is assumed to play a major role in the reaction cycle. We report density-functional theory studies of the differing roles of these dithiol-bridging ligands in the infrared spectra of synthetic models and of computational representations of the diiron cluster of the active site. Our analysis shows distinct spectral features associated with the dithiol-bridging NH mode for compounds having a DTN bridge, which, however, would have been obscured by the H_2O vibrations in existing measurements. However, if indeed nitrogen is present in the dithiol-bridging ligand, a combination of selective deuteration and chemical inactivation with CO would create a unique signature in an accessible region of the infrared spectrum, whose position and intensity are predicted.

I. Introduction

Hydrogenases (H_2ase's) are enzymes that have been the focus of intense studies in the past decade.[1] Many anaerobic micro-organisms, such as methanogenic archaea and acetogenic, nitrogen-fixing, photosynthetic, sulfate-reducing bacteria, produce or consume dihydrogen as part of their metabolic cycles. Understanding the structures and mechanisms of the H_2ase's is important not only for its biological implications but also in the quest for biological or bio-inspired synthetic catalysts for hydrogen production.[2,3]

The structure of the [FeFe]-H_2ase (formerly known as Fe-only H_2ase) was resolved by Peters et al.[4] for *Clostridium pasteurianum* H_2ase I (CpI) and by Nicolet et al.[5] for the

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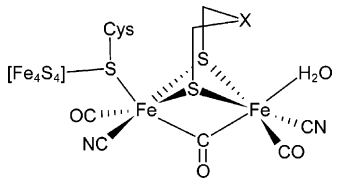

Figure 1. Structure of the active site in [FeFe]-H_2ase. The dithiol-bridging ligand containing X has been suggested to be 1,3-dithiopropane[5] (PDT, X = CH_2) or di-(thiomethyl)-amine[12] (DTN, X = NH).

Desulfovibrio desulfuricans uptake H_2ase (DdHase). It has a turnover rate *in solution* of 10^4-10^6 s^{-1}, and consequently, various groups have tried to design catalytic systems for hydrogen production that exploit either the native form of the enzyme or synthetic analogues.[3,6]

The structure of the Fe−Fe active site is illustrated in Figure 1. Most (but not all) structural features of the active site are now well established, confirmed by several experimental techniques[4,5,7,8] as well as by theoretical calculations.[9−11] Crystallographic studies by Peters et al.[4] (on the CpI enzyme) and by Nicolet et al.[5] (on the DdHase) indicate the existence of a bridge connecting the two μ-thiolates (see Figure 1), but the resolution does not allow a conclusive determination of its nature. Peters et al., based on their 1.8 Å resolution structure, modeled it as a water molecule. Nicolet et al., with a more refined 1.6 Å structure, identified a three-membered chain and initially modeled it as a propane dithiol (PDT) bridge.[5] Later the dithiol bridge was proposed to be an unusual dithiomethyl amine[12] (DTN), based on the proximity of the central atom in the chain to a nearby cysteine sulfur, which suggests the formation of a hydrogen bond. This in turn elicited a mechanistic proposal: the nitrogen atom of this dithiol-bridging ligand could serve as a base to bind a proton during turnover. So far, however, there is no direct evidence for the presence of PDT or DTN in the bridging ligand.

Our computational methods were discussed and validated extensively in ref 13 where we have analyzed synthetic models of [FeFe]-H_2ase. There we show that well-converged calculations of energy, structure and vibrational spectra can accurately reproduce the infrared (IR) spectra of these model systems. We were able to distinguish among various structural isomers, an issue of direct relevance for this paper.

In another study,[14] we have analyzed the structure of the CO-inhibited form of [FeFe]-H_2ase in CpI. We have discovered via first-principles density-functional theory (DFT) calculations a previously unrecognized inconsistency between the presumed arrangement of the CO/CN ligands in the diiron cluster and the experimental IR spectrum. This inconsistency is not present for a different arrangement of these ligands which is still consistent with the resolution of the available X-ray studies. Our new proposed assignment, in contrast to the presumed arrangement, is also consistent with the change in the symmetry of the EPR signal from rhombic to axial upon inactivation with CO. However, there are indications that the currently accepted assignment might be the correct one for the CpII H_2ase enzyme.[15]

Here we address the problem of identifying the dithiol-bridging ligand via first-principles electronic-structure theory. We report DFT studies of the IR spectra of several models of the [FeFe]-H_2ase active site (shown in Figure 2). In the first part of this paper, we analyze the IR spectra of the CO/CN bands in the presence of a PDT, a DTN, and a protonated DTN bridge for various possible configurations of the DTN bridges. We show that a PDT bridge is in best agreement with experiment. However, a DTN bridge is also in good agreement with experiment, but only for singly protonated nitrogen having its single NH bond in a proximal configuration (see Figure 2). In the second part of the paper, we show that compounds having a DTN bridge have a unique, albeit not easily accessible, vibrational signature associated with the NH stretching mode. A combination of specific deuteration and chemical modifications of the active site, such as inactivation with CO, should result in an accessible IR response. In the process, we also take advantage of the insight gained in our previous work about the new proposed arrangement of the CO-inhibited form. Our quantitative analysis of the DTN bridge uncovers clear distinctions between different geometrical arrangements of the dithiol bridge and also reveals a very important and easily accessible low-frequency vibrational band of the NH in the protonated, CO-inhibited form of the enzyme. We also make predictions of the intensities of these modes.

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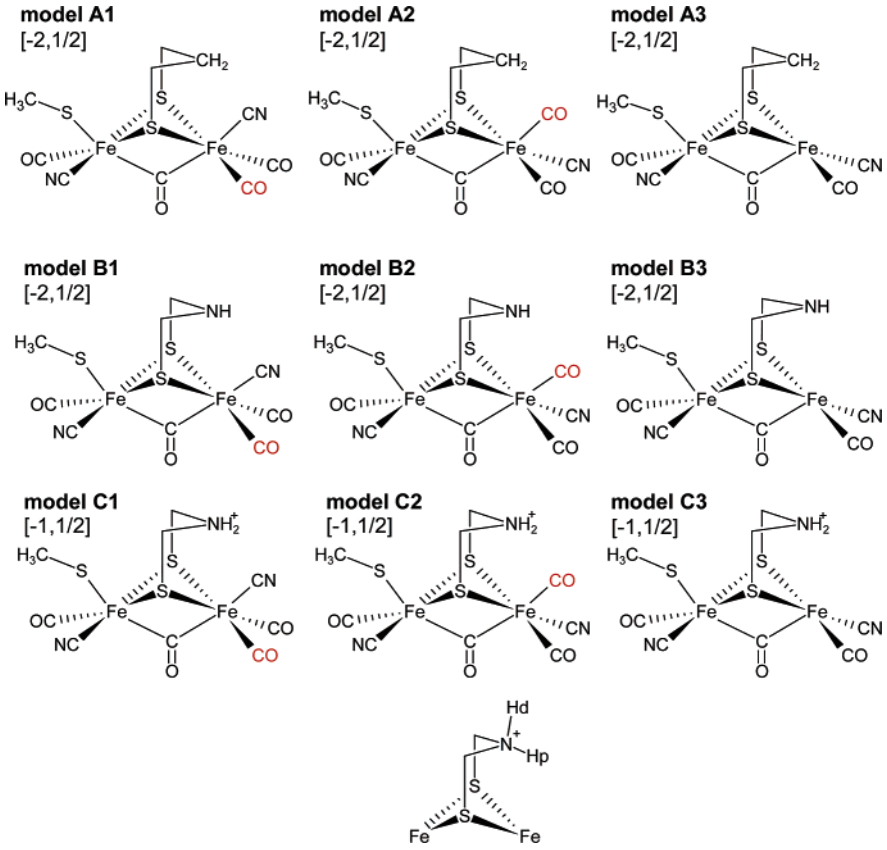

Figure 2. Computational models used in our calculations. The numbers in the square parenthesis are the total charge and the total spin projection on the *z* axis, respectively (see text for details). Also the distal (H_d) and proximal (H_p) positions on the bridging nitrogen are illustrated for models C in the bottom structure. In models B, the NH bond could either be in the distal or proximal positions (with respect to the Fe−Fe site). The exogenous CO in models 1 and 2 is colored in red.

The computational details are presented in Section II, and Section III contains a description of the computational models. In Section IV we analyze the possible manifestations of the dithiol-bridging ligand in the CO/CN region of the infrared spectra. In Section V we analyze the IR spectra of the NH band associated with this putative DTN ligand and propose an experiment aimed at identifying the band. We also analyze an unexpected low-frequency NH band and how the CO/CN band is modified by the presence of a deuterated DTN bridge for all possible configurations of the dithiol bridge and coordination of the distal Fe. We offer conclusions in Section VI.

II. Computational Details

We used DFT[16] with the PBE[17] exchange and correlation functional in a plane-wave code. The active site was shown to be EPR[18] active and to possess spin $^1/_2$. We used spin-unrestricted calculations with ultra-soft pseudopotentials for all the elements, a 30 Ryd kinetic energy cutoff for the pseudowavefunctions, corresponding to a 120 Ryd cutoff for the smooth part of the charge density, and a cutoff of 180 Ryd for the augmented charge density. The molecules were placed in a cubic periodic cell of linear dimension 25 atomic units, large enough for the computed molecular properties to be insensitive to it. The Hessian of the energy with respect to nuclear coordinates was constructed by finite numerical displacement of each atom in sequence in all three Cartesian directions. The IR cross-section was calculated by evaluating the Born effective charges and projecting them onto the normal modes to obtain the transition dipoles.[19] Rigid linear and rotational modes of motion were projected out by standard methods,[20] and the acoustic sum-rule[21] was imposed on the matrix elements of the Born-charge tensors.

Our computational methods were discussed and validated extensively in ref 13, where we have analyzed synthetic models of [FeFe]-H_2ase. There we showed that well-converged calculations of energy, structure, and vibrational frequencies can accurately reproduce the IR spectra of these model systems.

III. Computational Model Systems

Twelve different systems were examined in our study; see Figure 2. Models A1-A2, B1-B2, and C1-C2 are representations of the CO-inhibited form of the enzyme. As mentioned above, we argued in ref 14 that the correct arrangement of the CO/CN ligands in the CO-inhibited form is different from the previously presumed structure, originally suggested on the basis of a qualitative analysis of the X-ray results. In this paper models A1, B1, and C1 have the

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new proposed arrangement whereas models A2, B2, and C2 have the previously assumed arrangement. We analyzed both cases in the current context for the sake of completeness. The structures of models A−C resemble the diiron part of the [FeFe]-H_2ase, having the bridging CO and the CN^- ligands, with the Fe_4S_4 cubane subcluster replaced by a thiol. Similar representations of the active cluster were used in other theoretical explorations of H_2ase.[10,11] Within each group (A, B, or C) the oxidation state was Fe(II)Fe(I) (oxidized state) and group elements differ only by the presence or absence of an exogenous CO ligand attached to the distal Fe (distal with respect to the Cys-thiol) or by the arrangement of the CO/CN ligands.

Models A contain a PDT bridging ligand; A1 and A2 are two different representations of the CO-inhibited form, as discussed above; and A3 is the oxidized active form of the active site, having a vacancy on the distal Fe. Models B differ from models A by the presence of a DTN bridging ligand. The direction of the NH bond can either be distal to the FeFe site (B_d) or proximal (B_p) (see Figure 2), and we shall use the notation $B1_d$, $B2_p$ etc. Models C are protonated versions of model B, having an additional proton bonded to the nitrogen on the putative DTN bridge. Given the proposed mechanistic role of the putative DTN bridge, one cannot exclude it from being in a protonated state in the IR experiments, and the effect of protonation on the IR spectrum is presently unknown. We shall use the notation H_d and H_p when referring to the proton in the distal and proximal positions, respectively (with respect to the Fe−Fe site).

In the Supporting Information we also present calculations for an extension of model C2, having two H_2S and one NH_3 molecules placed such that they mimic the presence of nearby polar residues. There we show that our predictions hold even in the presence of these polar groups.

IV. Signatures of the Putative PDT and DTN Bridging Ligands in the IR Spectrum of the CO/CN Bands

IR studies of H_2ase's have so far[12,22,23] focused on bands of frequencies between ~1800 and 2100 cm^{-1} because it is a null region of the IR spectrum of proteins,[24,25] meaning that any finite response must originate from the unusual CO and CN ligands of the active site. We aim in this section to look for clues in the CO/CN bands that would support either of the two putative dithiol-bridging ligands. To this end we analyze two well-studied configurations of the enzyme, the oxidized active form (models A3, B3, and C3) and the CO-inhibited form (models A1, B1, C1 in the new assignment and A2, B2, C2 in the old assignment).

Overview of the Experimental IR Spectra of the Active and CO-Inhibited Oxidized Form. The IR spectra of the oxidized CO-inhibited form of [FeFe]-H_2ase were studied by several groups[8,22,23,26] focusing on the CO/CN band, with very similar results. Figure 3 is reprinted with permission from ref 23 (Copyright 2002 American Chemical Society), showing the IR spectrum in the oxidized and active form (Figure 3A), as well as the spectrum of the CO-inhibited form (Figure 3B, C). The oxidized active form has a bridging μ-CO band at 1802 cm^{-1}, two terminal CO bands at 1948 and 1971 cm^{-1}, and two terminal CN bands at 2072 and 2086 cm^{-1}. Similarly in the CO-inhibited form there is a bridging μ-CO band at 1810 cm^{-1}, and two terminal CN bands are at 2077 and 2095 cm^{-1}. In Figure 3B we see that the three terminal CO bands are spaced such that there is a single, high-frequency, high-intensity band at 2017 cm^{-1}, followed by two closely spaced lower-intensity bands at 1974 and 1971 cm^{-1}. Comparing this spectrum with that of the ^{13}CO-inhibited form in Figure 3C, it is evident that one band at 1971 cm^{-1} is not affected by the CO-inhibition process or by the ^{13}C labeling. Two other bands are shifted by the ^{13}C labeling, as indicated by the red arrows. These observations are consistent with a picture of CO-inhibition taking place on the distal Fe; the two terminal CO's on this Fe are coupled, as manifested by the two bands in the labeled spectrum that were shifted to lower frequencies with respect to the nonlabeled spectrum. The fixed band must be associated with the single terminal CO on the proximal Fe; it is reasonable to assume that it is remote enough to be insensitive to changes on the distal Fe.

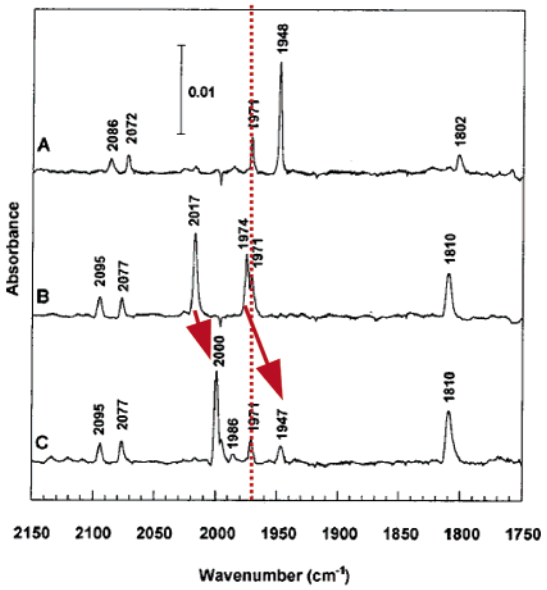

Figure 3. Experimental IR spectra of the CpI H_2ase at 20 K, reprinted with permission from ref 23 (Copyright 2002 American Chemical Society). A is the spectrum of the isolated oxidized enzyme, which presumably either has a vacant site on the distal Fe or binds a water molecule. B is the spectrum of the ^{12}CO-inhibited oxidized Fe(II)Fe(I) form of the enzyme. C is the spectrum of the ^{13}CO-inhibited oxidized Fe(II)Fe(I) form of the enzyme. A dashed red line marks the unchanged terminal CO band, and the two red arrows show the bands that shift consequent to labeling of the exogenous CO with ^{13}C.

Manifestations of the Dithiol-Bridging Ligand in the CO-Inhibited Form. Our previous study[14] has shown that only the arrangement of the CO/CN ligands of A1 produces good agreement with the available experimental IR data for CpI and DdHase and that the originally presumed arrangement, as in A2 (or B2/C2), is in poor agreement with

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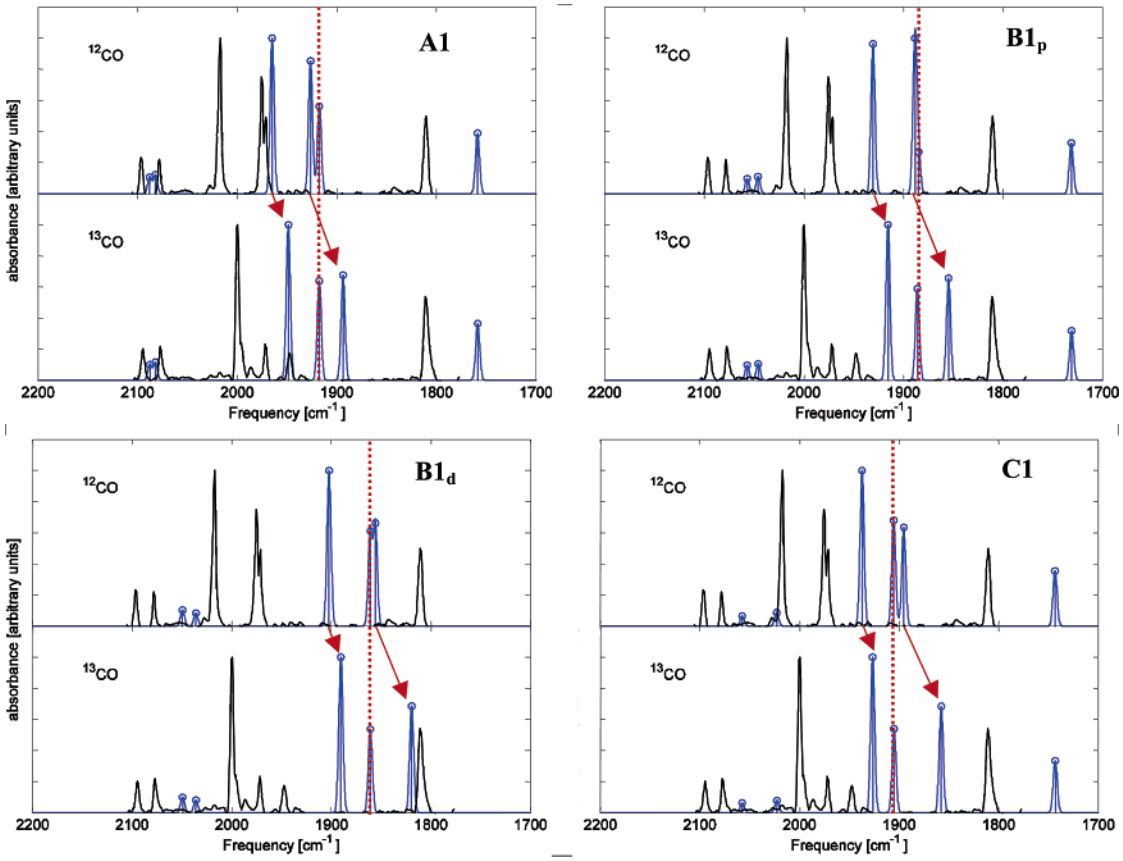

Figure 4. Simulated spectra (blue lines) of models of the CO-inhibited form of [FeFe]-H_2ase, A1, $B1_d$, $B1_p$, and C1, in the presence of an exogenous ^{12}CO and ^{13}CO. A Gaussian width of 2 cm^{-1} is used to broaden the computational mode eigenfrequencies into bands for comparison with the observed spectra. Dashed red lines mark terminal CO bands that are unchanged by the labeling with an exogenous ^{13}CO, and red arrows indicate bands that shift. The experimental spectra (black lines) are reprinted with permission from ref 23 (Copyright 2002 American Chemical Society).

Table 1. Experimental Vibrational Frequencies of the CO/CN Bands and Computed Frequencies of Models A1, $B1_p$, $B1_d$, and C1 in the Presence of an Exogenous ^{12}CO or ^{13}CO[a]

	Experimental		Model A1		Model $B1_p$		Model $B1_d$		Model C1	
	^{12}CO	^{13}CO	^{12}CO	^{13}CO	^{12}CO	^{13}CO	^{12}CO	^{13}CO	^{12}CO	^{13}CO
Bridging CO	1810	1810	1759	1759	1731	1731	1691	1690	1743	1743
Terminal CO's		1947		1894		1855				
	1971	1971	1918	1918	1885	1886	1856	1819	1895	1858
	1974		1926		1888		1861	1861	1905	1905
	2017	2000	1965	1949	1931	1915	1902	1890	1937	1927
CN's	2077	2077	2082	2082	2046	2046	2036	2036	2023	2023
	2095	2095	2087	2088	2057	2057	2050	2050	2058	2058

[a] The result of ^{13}C labeling for each band is shown in the same line as the original ^{12}C frequency, unless the labeling changed the *arrangement* of particular bands. In these cases arrows are added to point to the new positions of the shifted bands.

experiment. In particular, the original arrangement produces an incorrect isotope shift upon inactivation with ^{13}CO. We also showed that changing the dithiol-bridging ligand from PDT (A2 of the current paper) to DTN ($B2_p$ of the current paper) did not cure the discrepancy between the simulated spectrum and experiment. Therefore, we focus now on models A1, B1, and C1 (having the correct ligand arrangement) and examine possible manifestations of the various dithiol-bridging ligands in the CO/CN band of the IR spectrum of the CO-inhibited form.

Table 2. Shift in the Vibrational Frequencies of the Terminal CO's Consequent to Inactivation with ^{13}CO

	experiment	model A1	model $B1_p$	model $B1_d$	model C1
proximal CO	0	0	+1	0	0
lower-frequency distal	−27	−32	−33	−45	−37
higher-frequency distal	−17	−16	−16	−12	−10

The spectra of A1, $B1_{d,\ p}$, and C1 are presented in Figure 4. The IR frequencies and spacing between the modes are listed in Tables 1 and 2. Focusing on the terminal CO band, we observe that all these models have similar qualitative features; the ^{12}CO spectra contain one high-intensity band separated from two closely spaced lower-frequency bands. The isotope shift consequent to inactivation with ^{13}CO has one fixed band (corresponding mostly to the CO on the proximal Fe) and two bands that shift. However, there are also subtle differences that have to be considered. Model C1 is in the poorest qualitative agreement with experiment: the lowest and the highest frequency terminal-CO bands are shifted when using labeled ^{13}CO, whereas in the experiment the two highest frequency bands are shifted (see Figure 3). Also, the shift of the low-frequency band is too large; see Tables 1 and 2. Similar qualitative discrepancies occur for model $B1_d$. Models A1 and $B1_p$ in contrast have qualitatively correct isotope frequency shifts. On the one hand, the spacing between the bands is generally very similar in A1 and $B1_p$ except for the spacing between the CN bands and the terminal CO bands, which is better represented in $B1_p$. On the other hand, all three models having a DTN bridge ($B1_d$, $B1_p$, and C1) deviate from experiment by ∼80−130 cm^{-1} (see Table 1) whereas model A1 has a smaller overall deviation of ∼50 cm^{-1}. Thus, we conclude that both A1 and $B1_p$ agree with experiment and cannot be distinguished on the basis of their respective IR spectra alone. Incidentally, the spacing between the CN bands in A1 is very similar to the experimental spacing in DdHase.[26]

Manifestation of the Dithiol-Bridging Ligand in the Oxidized Active Form. The ground-state configuration of the active state was established by Peters et al.[4] and by Nicolet et al.,[5] showing that the vacancy on the distal Fe is trans to the μ-CO.[27] Having an agreement between two independent studies of the structure of the oxidized active form enables us to focus on the subtle effects of the putative dithiol-bridging ligands.

Figure 5 shows the IR spectra of models A3, $B3_p$, $B3_d$, and C3. All species have the same qualitative features, in agreement with the experimental spectrum. However, the deviation of the simulated spectrum of C3 from experiment is notably smaller than all three other cases. The values of the experimental and computed frequencies are given in Table 3. The line spacing between the different peaks is, on average, much better represented in model C3 than in models A and B. Thus, according to the evidence provided by the IR spectra, model C3 is the best candidate for the oxidized active form of the enzyme.

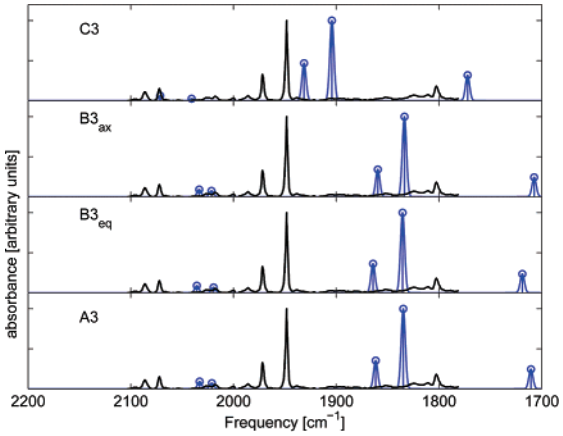

Figure 5. Simulated spectra (blue lines) of models of the oxidized active form of [FeFe]-H_2ase, A3, $B3_d$, $B3_p$, and C3. A Gaussian broadening of 2 cm^{-1} is used. The experimental spectrum (black line) is reprinted with permission from ref 23 (Copyright 2002 American Chemical Society).

The possible presence of a doubly protonated DTN bridge is in agreement with the reaction mechanisms proposed by Liu and Hu.[11] There the nitrogen has an essential role in facilitating proton transfer to the distal Fe. Another proposed mechanism[28] also takes advantage of the nitrogen to bind incoming protons.

Discussion. Our analyses of the IR spectra thus implies that the oxidized active form agrees better with the protonated DTN bridge (model C3), whereas the CO-inhibited form is consistent with either a DTN bridge having a single hydrogen bonded to the nitrogen in the proximal position (model $B1_p$) or a PDT bridge (model A1).

Consequently, our results so far do not provide clear support for either of the putative dithiol-bridging ligands. Thus, there is need for additional experimental information which distinguishes the candidates. We propose such an experiment in the next part of this paper, motivated by this lack of discriminatory power in the IR spectra in the 1800−2100 cm^{-1} range.

V. IR Spectra of the NH Stretch in a Putative DTN Bridge

As mentioned above, IR studies of H_2ase's so far[12,22,23] have focused on bands of frequencies between ∼1800 and 2100 cm^{-1}; it is a null region of the IR spectra of proteins,[24,25] meaning that any finite response must originate from the unusual CO and CN ligands of the active site. However, this null region extends[25] to ∼2800 cm^{-1} and could be exploited for a definite identification of the dithiol-bridging ligand.

The most important difference between the DTN and PDT cases is an additional band at 3400−3500 cm^{-1} corresponding to the stretching mode of the NH bond. This is a typical frequency for hydrogen stretching in amines and is a robust localized mode—the different chemical environment in the native enzyme is expected to shift its position only slightly. Experiments could be focused toward this particular feature

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Table 3. Experimental Vibrational Frequencies of the CO/CN Modes and Computed Frequencies of Models A3, $B3_p$, $B3_d$, and C3[a]

	experimental	model A3	model $B3_p$	model $B3_d$	model C3
bridging CO	1802	1710	1719	1707	1772
terminal CO's	1948 (146)	1835 (125)	1835 (116)	1834 (127)	1904 (132)
	1971 (23)	1862 (27)	1864 (29)	1860 (26)	1931 (27)
CN's	2072 (101)	2021 (59)	2019 (55)	2021 (61)	2041 (110)
	2086 (14)	2033 (12)	2036 (17)	2033 (12)	2071 (30)
average line-spacing deviation	0.0	−15.3	−16.8	−14.5	+3.7

[a] The numbers in parentheses are the spacing between each particular band and the band of lower frequency, for each system. The last line contains the average deviation of the band spacing from the experimental band spacing.

to detect the dithiol-bridging ligand of the [FeFe]-H_2ase. There are, however, two main difficulties to overcome. First, its predicted intensity is weak, roughly 1% with respect to the highest peak of the CO band. Second, the NH band is located well within the OH stretching band of water[24,25] (2800−3600 cm^{-1}) and is likely to be inaccessible to standard measurements in an aqueous environment. However, *selective* deuteration of this NH group would shift the band to ∼2500 cm^{-1}. This could be achieved by allowing the enzyme to turn over in D_2 while suspended in H_2O (as opposed to suspending the enzyme in D_2O). That frequency is conveniently located in the center of the null region of the spectrum and could in principle be observed. The main potential sources of interference would be weak vibrational bands of SH moieties.[24] In the polypeptide binding the active site,[4,5] 12 of the 18 cysteine residues are directly ligated to Fe atoms. Thus, most thiols are probably not protonated. Moreover, as we discuss below, even if protonated SH groups are present, their contribution to an appropriate *difference spectrum* should cancel out. A similar concern might arise from the amide A band, the backbone NH stretch, which is centered around 3300 cm^{-1}. However, these hydrogen atoms are quite unlikely to exchange with deuterons due to the very low concentration of deuterons, D^+, in the solution in a single turn-over experiment. Moreover, even in the unlikely event of a small number of exchanges, their contribution will cancel out in the difference spectrum that we suggest below.

Proposed Experiment and Theoretical Predictions. If a DTN bridge is present, it is likely that the nitrogen's lone pair participates in the enzymatic reaction,[12] serving to bind protons. Since the [FeFe]-H_2ase is reversible, one could have the enzyme turn over in the hydrogen uptake mode with a supply of D_2, resulting in a deuterated dithiol-bridge. Ideally one should have a single or small number of turnovers, using for instance rapid stopped-flow techniques[29] combined with proper control over the temperature[30] and pH[31] and a low deuteron concentration. The enzyme could then be quenched to low temperatures and the IR spectrum measured. However, in the unlikely event of deuteration of other amine groups in the protein, 'false-positive' signals may be detected that do not originate from the active site.

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Table 4. Predicted NH and ND Frequencies for the Various DTN Models

model system	NH frequency [cm^{-1}]	ND frequency [cm^{-1}]
$B1_d$	3477	2537
$B1_p$	3356	2447
$B2_d$	3409	2486
$B2_p$	3437	2504
$B3_d$	3370	2456
$B3_p$	3340	2431

One possible solution for the above difficulties is to take advantage of the fact that the NH/ND vibrations in the active site are expected to be very sensitive to the local chemical environment. In particular, we direct our attention to the CO-inhibited form of the enzyme. Chen et al.[23] used helium−neon laser light to remove the exogenous CO, leaving a vacant site on the distal iron. This is a substantial chemical change in the active site, which is expected to affect the frequencies of the NH/ND vibrations.

First, we focus on models B1−B3 and analyze the change in the NH frequency when going from B1 or B2 to B3. Again, as stated above, we believe that the CO/CN arrangement of B2 is inconsistent with CpI and DdHase IR data, but it might be relevant for CpII and thus we include it in our analysis for the sake of completeness.

Table 4 presents the predicted NH/ND frequencies of models B. We clearly see a strong shift to lower frequencies when the exogenous CO is removed. The shift is sensitive to the orienation of the ND bond: for model B1 it shifts by ∼80 cm^{-1} for the distal position and by 16 cm^{-1} in the proximal. Interestingly, for model B2 the shift trend is inverted, it is about 30 cm^{-1} when the D atom is in the distal position and by ∼70 cm^{-1} in the proximal position.

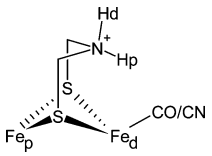
It should be possible to obtain a *difference spectrum*, between the species with and without the exogenous CO, as done by Chen et al.,[23] but in the frequency range of the ND band. This difference is expected to be sensitive only to local modes in the active site, and possible contributions from distant SH modes or other deuterated amines should cancel out. Should the ND vibrational band be observed, it it would be strong evidence for the presence of a DTN bridging ligand (or something quite similar) and also would show that it has a role in the mechanism of hydrogen metabolism.

Next, we turn to the analysis of the protonated analogs (NH_2^+, NHD^+, ND_2^+) of models C1−C3 (cf. Figure 2). As explained in Section I, it is not inconceivable that the bridging nitrogen would be in a protonated state. For these models, both protons on the nitrogen are potentially labile, and we may expect to observe also *double* deuteration. However, due to the limits of the available knowledge of

Table 5. Predicted NH/ND Vibrational Frequencies (in cm^{-1}) of Models C for All Possible Deuteration Substitutions on the Bridging Nitrogen

	model C1		model C2		model C3	
	distal band	proximal band	distal band	proximal band	distal band	proximal band
no deuteration	3324	2833	3345	2501	3310	3079
distal D	2418	2836	2428	2496	2408	3083
proximal D	3323	2078	3344	1892	3318	2267
both positions	2424	2075	2438	1892	2414	2207

Table 6. Selected Distances (Å) for Models B1, C1, B2, and C2[a]



	N−H$_p$	N−H$_d$	N−Fe$_d$	H$_p$−Fe$_d$	H$_d$−Fe$_d$	H$_p$−C
B1$_p$	1.03	−	3.47	3.16	−	2.24
B1$_d$	−	1.02	3.41	−	4.31	−
C1	1.06	1.03	3.35	2.85	4.34	1.96
B2$_p$	1.02	−	3.44	3.12	−	2.38
B2$_d$	−	1.02	3.27	−	4.22	−
C2	1.08	1.03	3.12	2.39	4.05	1.99

[a] In the last column H$_p$−C is the distance between a hydrogen atom in the proximal position and the carbon atom of the CN/CO group of closest proximity in B1, C1 or B2, C2, respectively.

the mechanism, we cannot exclude the possibility that only one of the hydrogen atoms is labile while the other is fixed, resulting only in a *single* deuteration. We have studied all possible cases; the results are summarized in Table 5.

First, we see that the vibrations for the distal position are much higher in frequency than for the proximal position due to the enhanced interaction of the proximal H(D) with the distal CN in model C1, the exogenous CO in model C2, or the vacancy in model C3. Again, we see strong frequency shifts when changing the chemical environment. While only of the order of 20−30 cm^{-1} for the distal band, the proximal band is particularly sensitive, resulting, e.g., in red-shifts of 180 cm^{-1} in model C1 and 375 cm^{-1} in model C2, with respect to model C3 for proximal deuteration. Another convenient feature of these protonated modes is an enhancement in the expected IR intensity, on the order of ∼5−10% of the maximal CO band intensity, comparable to that of the already observed CN modes.[12,22,23]

Understanding the Low-Frequency Proximal NH Band. Table 5 shows very unusual low-lying NH modes for the hydrogen in the proximal position: 2833 cm^{-1} for C1 and 2501 cm^{-1} for C2, compared to a typical NH frequency of ∼3500 cm^{-1}. This band is the result of the unique geometry of the diiron cluster in which there is a significant coupling between the proximal hydrogen and the distal CN in C1 or the exogenous CO in C2. This effect is further illustrated in Table 6 where we present selected distances in and around the NH group and the distal Fe. When comparing C1 to the B1$_p$, or C2 with B2$_p$, clearly a protonated DTN species bends slightly to bring the proximal NH be into closer proximity

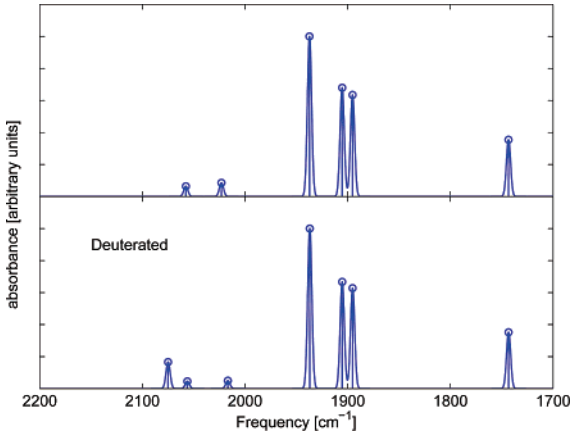


Figure 6. Simulated spectra of model C1 (upper panel) and the doubly deuterated C1 (lower panel). A Gaussian broadening of 2 cm^{-1} is used. A low-frequency (2075 cm^{-1}) proximal ND stretch appears upon deuteration, and the distal CN stretch is shifted to lower frequency (see text for details).

to the C atom of the distal CN group in C1 or the exogenous CO group in C2. The CN−Fe$_d$ distance is reduced by ∼0.1 Å for B1$_p$ vs C1 and by ∼0.3 Å for B2$_p$ vs C2, while the H$_p$−Fe distance is reduced by ∼0.3 Å for B1$_p$ vs C1 and by ∼0.7 Å for B2$_p$ vs C2. This effect is more pronounced for model C2 but is also very important for C1.

The protonation process can be described in terms of a partial charge transfer from electron-'rich' parts to the electron-deficient protonated amine. In the Supporting Information we show that the low-frequency is the result of a combined electrostatic attraction and quantum-mechanical overlap between the H$_p$ and the carbon atom of the CN ligand.

Deuteration-Induced Shifts in the CO/CN Bands. An interesting feature of Table 5 is that the calculated frequency of the proximal ND band in C1 (∼2075 cm^{-1}) is quite close to the frequencies of the CN modes of the nondeuterated C1 (distal CN at 2023 cm^{-1} and proximal at 2058 cm^{-1}, see Table 1). Upon deuteration we calculate a shift of the distal CN band from 2023 to 2016 cm^{-1} (see Figure 6), due to the strong coupling between the distal CN and the proximal ND and to the matching frequencies. Also, an inspection of this band clearly shows that upon deuteration it is no longer a band arising from a localized CN mode but rather a composite band that has a contribution from the proximal ND. A similar effect is present in C2, but there the exogenous CO is the one coupled to the ND mode and thus we observe a shift in the IR band associated with one of the terminal CO's (not shown here). This effect is an additional IR feature that characterizes a DTN bridging ligand and could be easy to detect after any exposure of the enzyme to D_2 in the uptake mode, provided that the nitrogen is protonated.

VI. Conclusions

The current uncertainty in the structure of the active site of the [FeFe]-H_2ase makes it difficult to prove or disprove the proposed participation of the dithiol-bridging ligand in the reaction mechanism for hydrogen formation. In the first part of this paper we presented DFT calculations and

experimental spectra of the CO/CN bands, showing that one cannot favor one bridging ligand over the other. However, in the DTN case we showed that the oxidized active form is better represented by a protonated model (C3) whereas the CO-inhibited form is better represented by a nonprotonated model (B1 or B2) in which the hydrogen of the DTN is in the proximal position, suggesting that the bridging DTN is protonated in the oxidized active form and loses a distal proton upon inactivation with CO.

In the second part, we show that a DTN bridge should have a unique signature at $\sim$3300−3500 cm^{-1} (depending on the active site composition) associated with a localized NH band. Deuteration of this group would reduce its frequency to $\sim$2400−2500 cm^{-1}, well within an accessible window. A protonated NH_2^+ or ND_2^+ is expected to absorb at lower frequencies and with a higher IR intensity. In particular, we predict that the proximal NH band of the CO-inhibited, *protonated* species has a vibrational band at around 2800 cm^{-1}, even without deuteration! In these cases deuteration of the proximal hydrogen would shift its frequency into the CO/CN band, resulting also in a frequency shift of the CN band of C1 or the CO band of C2. The presence of nearby polar protein residues is shown (in the Supporting Information) not to change this observation in a qualitative way. We believe that these spectral signatures are accessible via single-turnover experiments,[29] especially when taking the *difference spectrum* between the species with and without an exogenous CO. Such an experiment could lead to a definitive identification of the dithiol-bridging ligand as DTN and clarification of its role in the mechanism of hydrogen metabolism, should the predicted line shift on deuteration be observed.

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Supporting Information Available: Detailed discussion of the low-frequency proximal NH mode and the impact of nearby protein groups. This material is available free of charge via the Internet at http://pubs.acs.org.

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