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Structural Insights into the Active-Ready Form of [FeFe]-Hydrogenase and Mechanistic Details of Its Inhibition by Carbon Monoxide

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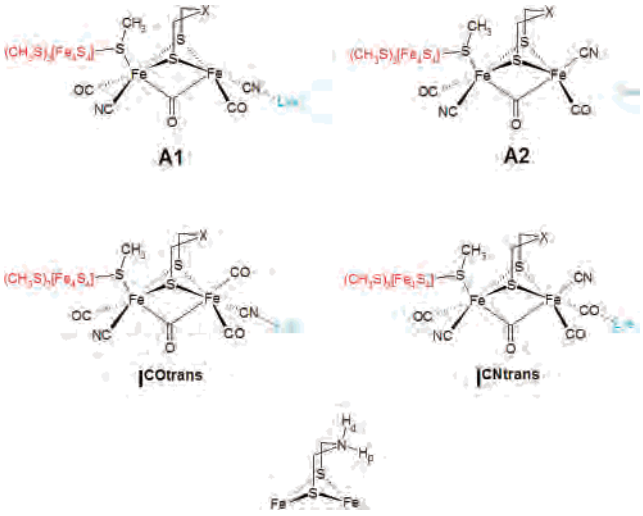
[FeFe]-Hydrogenases harbor a {2Fe3S} assembly bearing two CO and two CN^- groups, a μ-CO ligand, and a vacant coordination site trans to the μ-CO group. Recent theoretical results obtained studying the *isolated* {2Fe3S} subsite indicated that one of the CN^- ligands can easily move from the crystallographic position to the coordination site trans to the μ-CO group; such an isomerization would have a major impact on substrates and inhibitors binding regiochemistry and, consequently, on the catalytic mechanism. To shed light on this crucial issue, we have carried out hybrid QM/MM and free energy perturbation calculations on the whole enzyme, which demonstrate that the protein environment plays a crucial role and maintains the CN^- group fixed in the position observed in the crystal structure; these results strongly support the hypothesis that the vacant coordination site trans to the μ-CO group has a crucial functional relevance both in the context of CO-mediated inhibition of the enzyme and in dihydrogen oxidation/evolution catalysis.

[FeFe]-Hydrogenases catalyze the reversible oxidation of molecular hydrogen: $H_2 \leftrightarrows 2H^+ + 2e^-$. X-ray crystallographic studies on [FeFe]-hydrogenases from *Clostridium pasteurianum*[1a] (CpH) and *Desulfuvibrio desulfuricans* (DdH)[1b,c] have shown that the active site of these enzymes contains a {2Fe3S} subsite encompassing three CO and two CN^- ligands. The Fe atoms of the subsite are bridged by two S atoms of a 1,3-propanedithiolate (PDT) or a related di(thiomethyl)amine (DTMA) unit. One of the Fe atoms shares a cysteinyl sulfur ligand with a classical Fe_4S_4 cluster; the resulting iron−sulfur complex is usually referred to as the H cluster, and the two Fe atoms of the {2Fe3S} subsite are termed proximal (Fe_p) or distal (Fe_d), depending on their positions with respect to the Fe_4S_4 cluster. Notably, the cyanide ligand bound to Fe_d is in close proximity to a lysine side chain (Lys-237 in DdH; see Chart 1), and it also forms a hydrogen bond with an amide group of the protein backbone (NH group of Ile203 in DdH).

Chart 1. Schematic View of the QM System in the QM/MM Models Discussed in the Present Paper[a]



[a] The distal (H_d) and proximal (H_p) positions for the amine H atoms in the DTMA-containing binuclear subsite are also shown at the bottom of the chart. The atoms represented in black and red are part of the QM system in our QM/MM models; Cys-178, which is also part of the QM system, has been omitted for clarity. X can be either a CH_2, NH_p, or NH_2^+ group, depending on the nature of the bidentate ligand in the model. Lys corresponds to the Lys-237 residue in DdH. The models investigated in ref 5 include only the atoms drawn in black.

A direct metal−hydrogen interaction at the subsite is required for the enzymatic activity of [FeFe]-hydrogenases. On the other hand, binding of a fourth (exogenous) CO molecule to the Fe_d ion leads to the formation of a CO-inhibited form of the enzyme (**H_{ox}-CO**). Experimental and computational studies have converged toward the assignment of a Fe^IFe^{II} state to the CO-inhibited binuclear subcluster,[2] while there is still much debate about the disposition of CO and CN^- ligands around Fe_d in this form. In particular, X-ray

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investigations suggest that the binuclear subsite in its active-ready state has a vacant coordination site trans to the CO group bridging Fe_p and Fe_d (Chart 1, species **A1**);[1] thus, one would expect that both substrates (H^+ and H_2) and inhibitors (CO) would bind trans to the μ-CO group.

The availability of CpH and DdH crystal structures and the supposed lack of mobility for the endogenous ligands in the H cluster represented fundamental bases for recent advances in the experimental[3] and theoretical[4] investigation of [FeFe]-hydrogenase synthetic models. At the same time, in a recent density functional theory (DFT) study on models of the *isolated* {2Fe3S} subcluster, Zilberman et al. proposed that the active-ready state could easily interconvert to a form with a CN^- group trans to μ-CO and a vacant coordination site trans to a S atom of the bidentate ligand (species **A2** in Chart 1).[5] The interaction between this hypothetical form and exogenous carbon monoxide would give rise to a different CO-inhibited H cluster isomer (adduct $\mathbf{I^{CNtrans}}$ as opposed to $\mathbf{I^{COtrans}}$; see Chart 1), which is characterized by a CN^- group trans to the μ-CO ligand. In fact, computation of theoretical IR bands of $\mathbf{I^{COtrans}}$ and $\mathbf{I^{CNtrans}}$ indicated that only the latter isomer is consistent with the experimental IR data available on $\mathbf{H_{ox}}$**-CO**.[5] Moreover, $\mathbf{I^{COtrans}}$ was computed to be significantly less stable than $\mathbf{I^{CNtrans}}$, while **A1** was essentially as stable as **A2**. These results, as well as qualitative considerations of the electron paramagnetic resonance (EPR) properties of the various models,[5] supported the hypothesis that an isomer corresponding to $\mathbf{I^{CNtrans}}$ can be formed upon CO inhibition. More importantly, the possible coexistence of two different forms (**A1** and **A2**) of the active-ready enzyme could influence the regiochemistry of substrate binding to the H cluster, a fact that would have important bearings on the catalytic mechanism of dihydrogen oxidation and evolution, as well as on the design of bioinspired models of the H cluster.

In light of these considerations, we have carried out a study of the active-ready and CO-inhibited forms of [FeFe]-hydrogenase using combined quantum and molecular mechanics (QM/MM) calculations, which take the whole enzyme (DdH) into consideration. In addition, we have taken dynamic and entropic effects into account by quantum mechanical thermodynamic cycle perturbation (QTCP).[6] The whole H cluster and Cys-178 were included in the QM region, while the rest of the protein was modeled at the MM level (see the Supporting Information for details). The labels adopted for the various species are those reported in Chart 1, with the only difference that the subscripts "PDT", "DTMA", and "$DTMAH^+$" have been added to specify which bidentate ligand was included in the models (i.e., propanedithiolate, di(thiomethyl)amine, and di(thiomethyl)-ammonium, respectively; see Chart 1). $DTMAH^+$ was considered only for the active-ready form of the enzyme because recent computational studies indicate that the DTMA moiety is unprotonated in the CO-inhibited enzyme.[7]

For the PDT-containing version of the CO-inhibited enzyme, the calculated energies of the isolated QM systems (i.e., energies from single-point calculations in a vacuum on the QM portion of QM/MM-optimized enzyme models) indicate that $\mathbf{I_{PDT}^{CNtrans}}$ is 30 kJ mol^{-1} more stable than $\mathbf{I_{PDT}^{COtrans}}$, in qualitative agreement with the previous DFT data.[5] However, QTCP calculations on the whole enzyme show that the protein environment stabilizes the conformation characterized by a CO group trans to the μ-CO group and the Fe_d-bound CN^- ligand in close proximity to Lys-237. In fact, $\mathbf{I_{PDT}^{COtrans}}$ turned out to be 14 ± 1.5 kJ mol^{-1} more stable than $\mathbf{I_{PDT}^{CNtrans}}$ at the QTCP level.[8] The QTCP energy difference is slightly reduced when DTMA-containing models of the enzyme are considered: $\mathbf{I_{DTMA}^{COtrans}}$ was 6 ± 0.5 kJ mol^{-1} more stable than $\mathbf{I_{DTMA}^{CNtrans}}$. In any case, these results clearly highlight the role of the protein environment in the preferential stabilization of the $\mathbf{I^{COtrans}}$ form of the cofactor.

More information about the feasibility of the formation of $\mathbf{I^{CNtrans}}$ upon CO-mediated inhibition can be gained from a QTCP analysis of the active-ready state of the enzyme (which accepts CO to form the CO-inhibited form). The energy difference between $\mathbf{A1_{PDT}}$ and $\mathbf{A2_{PDT}}$ (see Chart 1) is very small when single-point QM calculations carried out in a vacuum are considered. In fact, $\mathbf{A2_{PDT}}$ was 2 kJ mol^{-1} more stable than $\mathbf{A1_{PDT}}$, in good agreement with the previous DFT data.[5] However, the QTCP calculations on the whole enzyme give considerably different stabilities: In fact, $\mathbf{A1_{PDT}}$ turned out to be as much as 117 ± 12 kJ mol^{-1} more stable than $\mathbf{A2_{PDT}}$. Such a large difference between the vacuum and QTCP results is mainly due to the fact that the single-point vacuum calculations ignore all interactions with the surrounding enzyme, i.e., electrostatic interactions between the enzyme active site and the protein environment. In fact, the active-ready and CO-inhibited forms of the H cluster are negatively charged (-2 or -3, depending on the protonation state of the bidentate ligand in the binuclear subsite); thus, long- and short-range electrostatic interactions between the H cluster and its protein environment are expected to give way to relevant energy contributions and can significantly affect the energetics of CO/CN^- ligand redisposition in the enzyme active site. In particular, the QM/MM and QTCP computations on $\mathbf{A1_{PDT}}$ take into account the hydrogen bond between the NH group of Ile-203 and the Fe_d-bound CN^-

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(8) Reported error estimates represent half of the hysteresis in the molecular mechanics free-energy perturbations.

group and also the interaction between the same cyanide group and the Lys-237 side chain. This lysine residue is strictly conserved among [FeFe]-hydrogenases (sequence alignment in the Supporting Information), and its amine group should be protonated at physiological pH; in turn, the CN^- ligand has a strong anionic character, also due to the overall -3 (or -2, see above) charge of the H cluster. Therefore, the resulting Lys−NC−Fe interaction has an ionic nature, which is strongly attenuated when the CN^- group is repositioned in the $\mathbf{A2_{PDT}}$ state. Notably, replacement of PDT with DTMA does not lead to any qualitative changes in the QTCP energies: $\mathbf{A1_{DTMA}}$ remains 73 ± 6.5 kJ mol^{-1} more stable than $\mathbf{A2_{DTMA}}$.

Finally, the possibility of a transient protonation of the DTMA amine group prior to CO inhibition[7] was also considered. Also in this case, $\mathbf{A1_{DTMAH+}}$, i.e., the conformer of the active-ready enzyme showing a vacant coordination site trans to μ-CO, is 76 ± 13 kJ mol^{-1} more stable than the alternative isomer $\mathbf{A2_{DTMAH+}}$ at the QTCP level. Notably, in the latter enzyme conformation, the Fe_d-bound CN^- group can establish a stabilizing ion-pair interaction with the NH_2^+ group of $DTMAH^+$. However, this does not lead to any substantial reduction in the stability gap between the two conformers. This is due to long-range electrostatic interactions and short-range nonbonding interactions between the H cluster and the rest of the protein, which stabilize the **A1** conformation; as for the short-range nonbonding interactions, previous QM/MM studies have shown that there are unfavorable interactions between H_d in $DTMAH^+$ (see Chart 1) and the protein residues surrounding it, thus leading to amine inversion in models with an unprotonated DTMA residue.[9] Such interactions, which should exist also when DTMA is protonated, are expected to be enhanced by the movement of the Fe_d-bound CN^- group toward the NH_2^+ group in $DTMAH^+$ because the cyanide ligand would give rise to additional steric clashes in that case.

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In conclusion, the **A1** conformation of the active-ready enzyme is strongly stabilized by the protein environment, irrespective of the nature of the bidentate ligand included in the binuclear subsite. Because the active-ready enzyme is expected to be the CO acceptor during CO inhibition of the enzyme, our results strongly support the hypothesis that the CO-inhibited form of [FeFe]-hydrogenase corresponds to a conformation with a terminal CO group on Fe_d trans to the μ-CO ligand. In fact, the $\mathbf{I^{COtrans}}$ species is more stable than $\mathbf{I^{CNtrans}}$ when the H-cluster environment is explicitly taken into account.

Direct calculations of EPR parameters indicate that it might not be possible to distinguish between the alternative conformations of the CO-inhibited enzyme solely on the basis of their magnetic properties (see the Supporting Information). Moreover, computation of IR absorption peaks at the DFT level shows that assignment of a specific geometry to the CO-inhibited enzyme can be a difficult task when experimental and DFT data are compared because calculated IR frequencies are highly sensitive to the exchange-correlation functionals (see the Supporting Information).

The reliability of the QTCP study presented here is due to the fact that all energy computations take into account both enthalpic and entropic effects in fully solvated all-atom models of the enzyme; as a result, not only do our data give insights into the issue of the CO/CN^- ligand arrangement in the CO-inhibited form of [FeFe]-hydrogenase but they also support the hypothesis that the protein environment is able to maintain a vacant coordination site trans to μ-CO for substrate binding on Fe_d, when the enzyme is in its active-ready state.

Supporting Information Available: Details of the QM/MM and QTCP approaches adopted here, relative stabilities and optimized geometries of the various QM/MM H cluster models, alignment of [FeFe]-hydrogenases aminoacidic sequences, analysis of computed IR absorption peaks, and computed EPR parameters. This material is available free of charge via the Internet at http://pubs.acs.org.

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