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Hydrogenases play an important role in the metabolism of many microorganism, by performing reversible oxidation of hydrogen: $H_2 \leftrightarrow 2\ H^+ + 2\ e^-$.[1] Three classes are known, depending on the metal content of the active site: [NiFe], [FeFe], and [Fe] hydrogenases. The [FeFe] hydrogenases have been extensively studied by experimental and theoretical methods.[1,2,3] Crystal structures are available for [FeFe] hydrogenase from two bacteria.[4,5] The active site consists of a ferredoxin-like [4Fe4S] cluster, connected via a Cys residue to a binuclear Fe_2 cluster, where each Fe ion has one CO and one CN^- ligand. In addition, there are three bridging groups, another CO, and a dithiolate ligand (cf. Figure 1). The iron ion distal to the [4Fe4S] cluster (Fe_d) has a vacant coordination site that is believed to be the reactive centre. In some oxidized structures, a water molecule is found in this site.

The nature of the dithiolate ligand has been much discussed. It was originally tentatively assigned as $CH_2(CH_2S^-)_2$.[4ab] However, several groups have suggested that $NH(CH_2S^-)_2$ is a more likely interpretation,[2,4c,6] because the amine group could act as a proton acceptor during the catalytic cycle.[3] Recently, a new crystal structure was presented together with density functional theory (DFT) calculations, indicating that the dithiolate ligand actually is $O(CH_2S^-)_2$.[5] The authors compared six ligands $X(CH_2S^-)_2$ with $X = CH_2$, NH, NH_2^+, S^-, SH, and O, and showed that a 200-atom model of the active site with $O(CH_2S^-)_2$ had the lowest relaxation energy when optimized in vacuum starting from the crystal structure.

Unfortunately, such an approach strongly depends on small errors in the crystal structure and the theoretical method, as is illustrated by the large relaxation energies involved, over 200 kJ/mol. A more satisfactory approach is to include the DFT calculations already in the crystallographic refinement so that each structure becomes an ideal compromise between DFT and crystallography. This is possible with the quantum-refinement approach developed in our group,[7] in which the molecular mechanics force field, used to supplement the crystallographic raw data, is replaced by more accurate DFT calculations for the active site of the protein. Thereby, the structure can be improved and the nature of atoms can be determined, e.g. the protonation state of metal ligands.[8] In this paper, we use this approach to decide which form of the dithiolate ligand fits the crystallographic raw data best.

The calculations are based on the 1.39 Å structure of [FeFe] hydrogenase from *C. pasteurianum*, 3c8y.[5] We used the QM system $Fe_2(CO)_3(CN)_2(H_2O)(SCH_2XCH_2S)(CH_3S)_4(Fe_4S_4)$ (CH_3SH), with $X = CH_2$, NH, NH_2^+, and O, where the hydrogen atom of NH pointed either up or down relative to the Fe cluster (Figure 1). This model includes both the catalytic Fe_2 cluster, the connected [4Fe4S] cluster, and a model of Cys-299, which may accept or donate a hydrogen bond to the X group. In keeping with the previous combined

crystallographic and DFT work,[5] we studied the cluster in the H_{ox} ($Fe^{II}Fe^I$; $Fe^{II}_2Fe^{III}_2$) state, which gives a net charge of -3 for all X groups except NH_2^+ (-2). We employed the BP86[9] DFT method and the def2-SV(P) basis set,[10] using the broken-symmetry approach.[11] The models had a surplus of one unpaired electron: Two pairs of antiferromagnetically coupled high-spin Fe ions were considered for the [4Fe4S] subcluster along with two low-spin Fe ions in the binuclear subsite, both with significant spin, but most on Fe_d.[3] Test calculations with other models, functionals, or basis sets gave similar results (see the supporting information, SI). The calculations were sped up with the resolution-of-identity approximation.[12]

Several values of the w_A weight factor between the QM and crystallographic energy functions were tested (see the SI), but they gave similar results. Therefore, only results for $w_A = 1$ (which gave the lowest values for the R_{free} factor) are presented in Table 1. All five X groups gave very similar Fe–Fe (2.54 Å) and Fe–ligand distances. Only the rather weakly bound H_2O ligand shows differences larger than 0.02 Å. In the NH_2^+ and NH_{down} structures, the X group forms a rather strong hydrogen bond to the water ligand (1.59 and 1.74 Å, respectively). Therefore, the Fe–O bond is essentially broken (2.46 and 2.40 Å) and the two hydrogen atoms of water points towards the CN and CO ligands of Fe_d (see Figure 1).

On the other hand, in the NH_{up} and O structures, the water ligand forms a hydrogen bond to the X group via one of its hydrogen atoms (1.57 and 1.65 Å). Thereby, the Fe–O bond is strengthened (2.15 and 2.12 Å). The CH_2 structure is intermediate, because the CH_2 group can only form a weak H–O interaction (1.95 Å), giving an intermediate Fe–O bond (2.21 Å). In the original crystal structure, the Fe–O distance was 2.38 Å.

In the calculations with $X = CH_2$, NH_2^+, and NH_{up}, the SH group of Cys-299 accepts a hydrogen bond from the X group, with H–S distances of 2.93, 2.97, and 2.93 Å, respectively (Figure 1a). For the two other systems, the SH group of Cys-299 instead donates a hydrogen bond to X with H–N/O distances of 2.11 Å (Figure 1b).

Owing to these small differences in the geometry, there are only small differences in the crystallographic R factors. However, both the R_{free} and R factors are lowest for the NH_2^+ structure. Likewise, the residue (real-space) R factor is lowest for this structure (Table 1), indicating that the NH_2^+ structure fits the experimental electron density better than the other structures. This is also confirmed by the electron-density maps, which show smaller differences for NH_2^+ than for the other ligands (Figure 2).

However, if we compare the quantum-refined structures with the same structures optimized in a vacuum (which shows the preferred structure of the active site), it can be seen that the strain energy (i.e. the QM energy difference of the quantum

Table 1. Results of the quantum refinement calculations.[1]

X	Fe–O	R_{free}	Residue R	ΔE_{QM1}	Δr
CH_2	2.21	0.141833	0.480	144	0.59
NH_2^+	2.46	**0.141727**	**0.406**	163	**0.40**
NH_{down}	2.40	0.141806	0.418	**142**	0.45
NH_{up}	2.15	0.141809	0.462	154	0.61
O	2.12	0.141844	0.472	**142**	0.66
Crystal	2.38	0.142551	0.419		

[1] Fe–O distance and Δr in Å, ΔE_{QM1} in kJ/mol.

system optimized in vacuum or in the crystal, ΔE_{QM1} in Table 1), is lowest for the NH_{up} and O structures (142 kJ/mol), whereas it is slightly higher for the NH_2^+ structure (166 kJ/mol). If we instead compare the sum of the differences in the Fe–ligand bond distances for structures optimized in vacuum and in the crystal (Δr in Table 1), it can be seen that the NH_2 structure shows the lowest deviation (0.40 Å). The latter two comparisons are complicated by the fact that the water ligand dissociates in vacuum for all complexes. Therefore, we perform the vacuum calculations with the Fe–O distance fixed to the value observed in the quantum refinements. We also delete the Cys-299 model, because it otherwise may move to form hydrogen bonds with other atoms in the structure. The results also depend somewhat on the QM model used, although the structures with N atoms always give the best results (see the SI). Thus, it is clear that the quality estimates indicate that a N atom is more favorable in the dithiolate ligand than C or O atoms.

It is notable that the QM energies of the NH_{down} and NH_{up} states are directly comparable (they contain the same atoms). The energies indicate that NH_{down} is the more stable conformation, by 0–19 kJ/mol.

Consequently, we conclude that the quantum-refinement calculations do not support the suggestion that the dithiolate ligand should be $O(CH_2S^-)_2$. On the contrary, the quality criteria in Table 1 indicate that central atom instead is nitrogen. These results are in accordance with recent EPR measurements, as well as studies of the role of the HydG maturation factor.[13] The reason why the present results differ from the previous DFT study,[5] which was based on the same crystal structure, is that we use structures that are an optimum compromise between DFT and crystallography and that we direct measure how well the structures fit the crystallographic raw data (not the crystal coordinates, which are the result of an involved process of model building and refinement, involving a molecular mechanics potential), in the form of crystallographic R factors.

Supporting Information Available: Detailed description of the method, optimized Fe–ligand distances and quality criteria with three different QM systems, and results of calculations with the B3LYP method or larger basis sets. This material is available free of charge via the Internet at http://pubs.acs.org.

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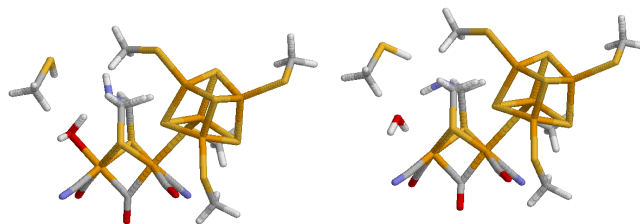
Figure 1. Quantum-refined structures of the states with $X = NH_{up}$ (left) and NH_{down} (right).
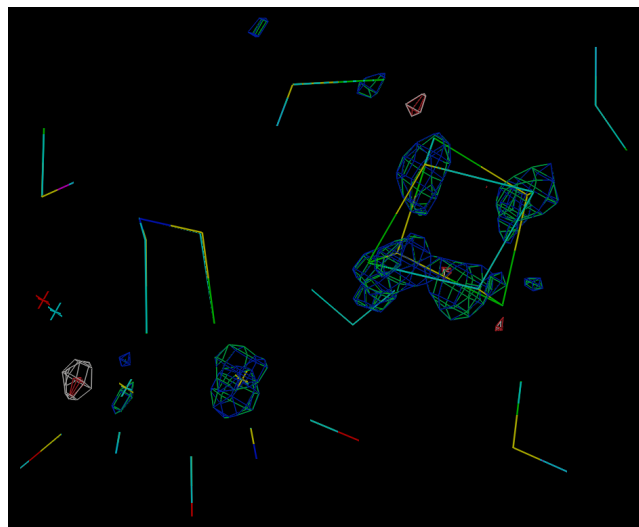
Figure 2. Electron density $f_o - f_c$ difference maps of the states with $X = NH_2^+$ (atomic colors; red and blue densities) and O (cyan atoms; white and green densities). The densities (4.0 σ) are marked with red and white (negative) or blue and green (positive). Note the larger white volume at the lower left.

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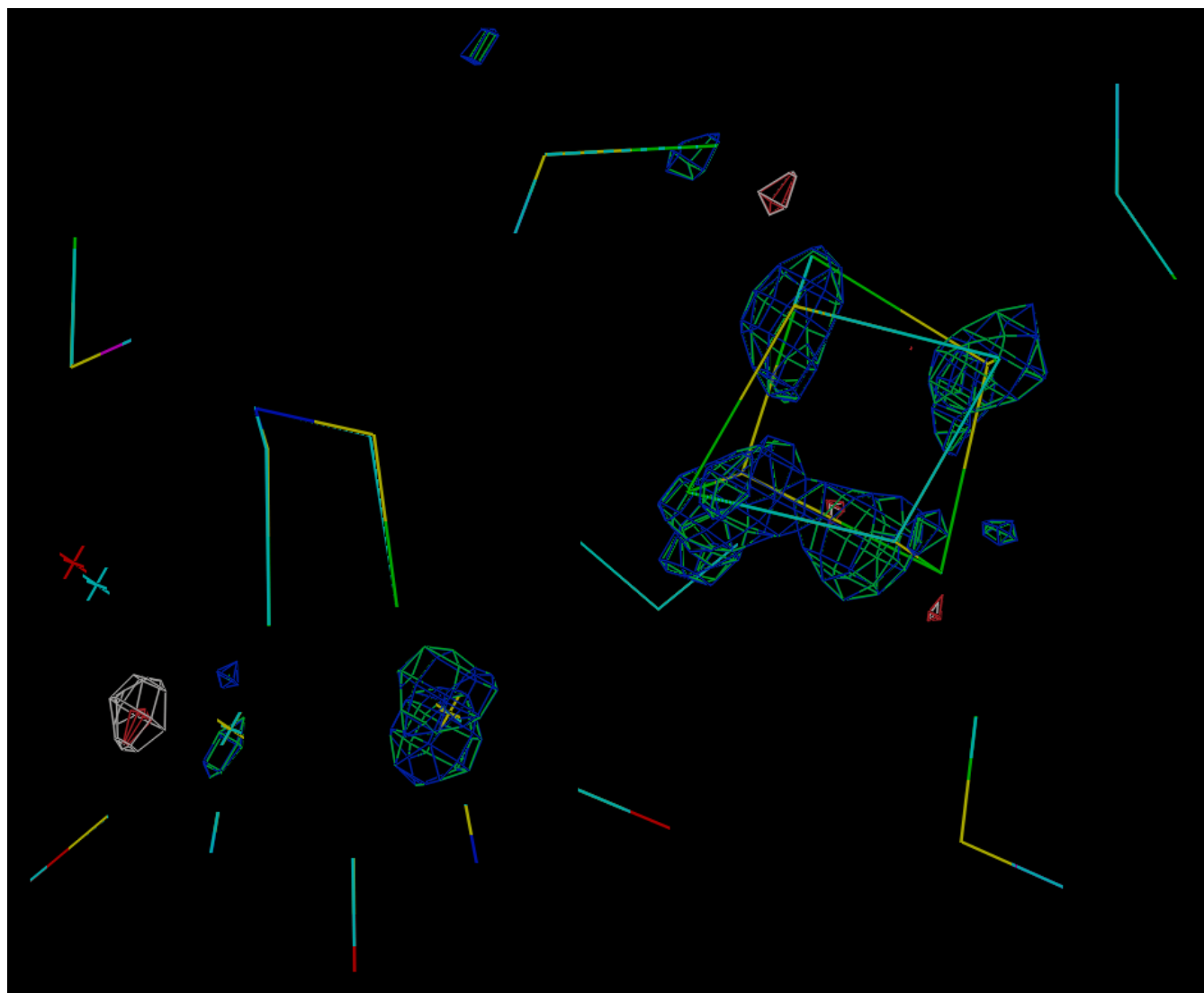
The active site of the [FeFe] hydrogenases contains two Fe ions bound to one Cys ligand, three CO molecules, two CN^- ions, and a dithiolate ligand. The nature of the latter has been much discussed and it has been suggested that it contains C, N, or O as the bridgehead atom. Most experimental studies indicate N, whereas a recent DFT study of a crystal structure indicated an O atom. Here, we perform quantum refinement on the same crystal structure with five different models of the dithiolate ligand, $X(CH_2S^-)_2$, with $X = CH_2$, NH_2^+, NH (in two conformations), and O, and show that structures with a N bridgehead atom actually fits the crystallographic raw data best. Quantum refinement is standard crystallographic refinement where the molecular mechanics force field, normally used to supplement the experimental raw data to give a more chemical structure, is replaced by more accurate DFT calculations for the active site. Thereby, we obtain structures that are an ideal compromise between DFT and crystallography.