The broader potential implications of using a nonphysiological reductant in spectroscopic and mechanistic studies of enzymes are highlighted.

The Nonphysiological Reductant Sodium Dithionite and [FeFe] Hydrogenase: Influence on the Enzyme Mechanism

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ABSTRACT: [FeFe] hydrogenases are highly active enzymes for interconverting protons and electrons with hydrogen (H2). Their active site H-cluster is formed of a canonical [4Fe-4S] cluster ([4Fe-4S]H) covalently attached to a unique [2Fe] subcluster ([2Fe]H), where both sites are redox active. Heterolytic splitting and formation of H2 takes place at [2Fe]H, while [4Fe-4S]H stores electrons. The detailed catalytic mechanism of these enzymes is under intense investigation, with two dominant models existing in the literature. In one model, an alternative form of the active oxidized state Hox, named HoxH, which forms at low pH in the presence of the nonphysiological reductant sodium dithionite (NaDT), is believed to play a crucial role. HoxH was previously suggested to have a protonated [4Fe-4S]H. Here, we show that HoxH forms by simple addition of sodium sulfito (Na2SO3, the dominant oxidation product of NaDT) at low pH. The low pH requirement indicates that sulfur dioxide (SO2) is the species involved. Spectroscopy supports binding at or near [4Fe-4S]H, causing its redox potential to increase by ∼60 mV. This potential shift detunes the redox potentials of the subclusters of the H-cluster, lowering activity, as shown in protein film electrochemistry (PFE). Together, these results indicate that HoxH and its one-electron reduced counterpart HredH+ are artifacts of using a nonphysiological reductant, and not crucial catalytic intermediates. We propose renaming these states as the “dithionite (DT) inhibited” states Hox-DT and Hred-DT. The broader potential implications of using a nonphysiological reductant in spectroscopic and mechanistic studies of enzymes are highlighted.

INTRODUCTION

[FeFe] hydrogenases are highly active metalloenzymes that catalyze the reversible reduction of protons to molecular hydrogen.1,2 Their active site, the H-cluster, comprises a unique diiron subcluster ([2Fe]H) and a canonical [4Fe-4S] subcluster ([4Fe-4S]H), covalently linked by a cysteine thiolate3,4 (Figure 1A and B). The Fe of [2Fe]H that is closest to [4Fe-4S]H is known as the distal Fe (FeD), while the Fe furthest from the cluster is known as the proximal Fe (FeP). In [2Fe]H, the Fe ions are coordinated by two terminal CN− and two terminal CO ligands (one on each Fe), a bridging CO, and a bridging 2-azapropane-1,3-dithiolate (ADT) ligand.5,6 During H2 conversion, the H-cluster goes through a series of redox transitions, where the Fe ions change oxidation states, as well as protonation/deprotonation steps.7−9 While several catalytic intermediate states have been well characterized with a variety of spectroscopic techniques, structural models based on X-ray diffraction data on crystals in spectroscopically defined states are not generally available. Thus, in the absence of structural models supported by experimental data, computational chemistry has played an important role in proposing likely structures of the active site in the catalytic intermediates based on spectroscopic data. However, divergent results from various groups have led to several possible models of the catalytic cycle of [FeFe] hydrogenases.8,10−12 These can be summarized in two main models (here referred to as Model 1 and 2, Figure 1C and D respectively).

The most oxidized state of the active enzyme, Hox, is generally accepted to be the starting point of the catalytic cycle and has a mixed valence of FeP(II)FeD(I) in [2Fe]H and an oxidized [4Fe-4S]H. In Model 1 (Figure 1C), one-electron reduction of Hox is proposed to yield two possible states Hred and HredH+ (in our nomenclature), whose relative population depends on the pH. In Hred, the electron is thought to be localized preferentially on [4Fe-4S]H. In HredH+, the electron is thought to be transferred to the [2Fe]H subcluster (with an FeP(II)FeD(I) configuration) and a proton (from the proton transfer pathway) to bind to the nitrogen in the ADT bridge giving an NH2+.14,15 This process of proton-coupled electronic rearrangement (PCER) of the H-cluster is a crucial component of Model 1. A further one-electron reduction of HredH+ yields...
the H$_{red}^+$ state with a reduced [4Fe-4S]$_{H}$ subcluster. The protonated ADT ligand in both H$_{red}^+$ and H$_{sred}^+$ appears to be able to transfer the proton to Fe$_d$ generating an Fe-bound hydride in the H$_{hyd}$ state. A further one-electron reduction of [4Fe-4S]$_{H}$ gives by proton-coupled electronic rearrangement to give the H$_{sred}^+$ state. The subsequent steps leading to H$_2$ formation are not shown. (D) Catalytic cycle Model 2 in which proton-coupled electron transfer at [4Fe-4S]$_{H}$ converts H$_{ox}$ to H$_{red}^+$, which can engage in further proton-coupled electron transfer to give the terminal hydride-containing H$_{hyd}$ state. H$_{hyd}$ then reacts with an additional proton to generate H$_2$ leaving a protonated H$_{ox}H$ state. Alternatively, H$_{red}^+$ can rearrange to give a less active H$_{red}$ state containing a binding hydride, which proceeds through a low activity pathway. H$_{ox}H$ appears to undergo one-electron reduction to H$_{red}^+$, but this is not included in the catalytic cycle.

The H$_{ox}$ and H$_{red}^+$ states in Model 2 have been reported to accumulate at low pH only in the presence of sodium dithionite (NaDT) (see Supporting Information for further details). NaDT (Na$_2$S$_2$O$_4$, also sodium hydrosulphite) is widely used in biochemistry as an oxygen scavenger and low potential reducing agent ($E^0 = −0.66$ V vs SHE at pH 7 and 25 °C). For example, it is commonly employed to protect metalloproteins from oxidative damage caused by trace amounts of oxygen during purification and handling, or to poised metallocofactors in reduced states for their characterization. However, one of the pitfalls of its use is the failure to consider that NaDT and its oxidation products can engage in side-reactions with the system under study. Several studies on sulfit-reducing enzymes have highlighted how oxidation of NaDT can be a significant source of SO$_3^{2−}$, the substrate for these enzymes, which can bind to the active site and...
complicate the interpretation of spectroscopic studies and activity measurements. In a recent report, during the semisynthetic assembly of the FeMo cofactor of nitrogenase, the donor of the ninth sulfur ligand was found to be the SO$_2^{-}$, generated by the oxidation or degradation of NaDT present in the assay. Numerous studies have reported the interaction of oxidation products of NaDT with various enzymes including nitrite reductase, DMSO reductase, monomethylamine methyltransferase, acetyl CoA synthase, and formation of adducts to flavins and cobalamin. Additionally, the slow dissociation of NaDT into SO$_2^{-}$ radicals (the active reducing species) has been shown to be problematic in mechanistic studies of nitrogenase.

In light of the dependence of H$_{ox}$H and H$_{red}$/H on NaDT, and of NaDT’s reported “non-innocent” behavior, we decided to investigate the effect of NaDT and its oxidation products on [FeFe] hydrogenases. Formation of the H$_{ox}$H state was observed when the [FeFe] hydrogenase from Chlamydomonas reinhardtii (CrHydA1) was treated with oxidized NaDT. Addition of Na$_2$SO$_3$ (the dominant oxidation product of NaDT$^{36}$) to CrHydA1 at low pH reproduced the same effect as oxidized NaDT. Under H$_2$, H$_{red}$/H was also observed. We propose that, at low pH, the dissolved sulfur dioxide (SO$_2$) generated by the protonation of SO$_3^{-}$ binds to the H-cluster. Based on our spectroscopic observations, we hypothesize that this occurs near [4Fe-4S]$_H$ with submicromolar binding affinity as estimated by IR titrations. Based on the ratios of the H$_{ox}$/H$_{red}$ and H$_{ox}$/H$_{red}$/H$_H$H states under H$_2$, binding of SO$_2$ causes the redox potential of [4Fe-4S]$_H$ to increase by ~60 mV. The effect of this on catalysis was investigated via protein film electrochemistry (PFE), showing that binding of SO$_2$ has an inhibitory effect on both H$^+$ reduction and H$_2$ oxidation activity of [FeFe] hydrogenases. Together, these results reveal that the so-called H$_{ox}$H and H$_{red}$/H states are not related to protonation events at the [4Fe-4S]$_H$ subcluster of the H-cluster, but are instead artifacts generated by oxidized NaDT. This result challenges their involvement in the catalytic cycle of [FeFe] hydrogenases. Furthermore, these findings highlight the importance of carefully considering the possible side-reactions of NaDT and its oxidation products when choosing to use this reducing agent with metalloenzymes, particularly iron–sulfur enzymes.

## RESULTS

**Treatment of CrHydA1 with oxidized NaDT causes formation of the HoxH state.** Our investigation on the effect of the oxidation products of NaDT on [FeFe] hydrogenases focused, in the first instance, on CrHydA1, the most well characterized [FeFe] hydrogenase, which contains only the H-cluster. In particular, the enzyme containing the native [2Fe] cofactor with the ADT ligand (CrHydA1$^{ADT}$) was used. Thus, CrHydA1$^{ADT}$ produced in the strict absence of NaDT was treated with a solution of oxidized NaDT (oxNaDT). This solution was prepared by dissolving fresh NaDT in water to a concentration of 1 M (the most effective concentration of NaDT for H$_{ox}$H formation at pH 6$^\pm$) under aerobic conditions and stirring for 2 h under atmospheric oxygen. A decrease in the pH to ~2 and appearance of a yellow precipitate (most likely elemental sulfur) indicated oxidation and degradation of the dithionite anion. The oxNaDT solution was then thoroughly degassed and moved into an anaerobic glovebox before being added to CrHydA1$^{ADT}$, in order to avoid damaging the highly air-sensitive H-cluster.

As shown by the IR spectra of CrHydA1$^{ADT}$ (Figure 2), dilution of the enzyme in the oxNaDT solution results in the appearance of a new set of vibrational signals, slightly shifted to higher energy (<10 cm$^{-1}$) with respect to H$_{ox}$. These new signals are consistent with those reported for H$_{ox}$H$^{10,19,27}$ Even though the pH of the oxNaDT solution was measured to be around 2, the buffer present in the CrHydA1$^{ADT}$ sample (25 mM Tris-HCl, pH 8) will render the pH value after oxNaDT addition slightly higher than this (ca. pH 6). Interestingly, when CrHydA1$^{ADT}$ was treated with a solution oxNaDT whose pH had been corrected to 7, conversion to H$_{ox}$H was not observed (Figure S1), suggesting that formation of this state requires acidic conditions.

The observation that H$_{ox}$H can be formed by treatment with oxidized NaDT challenges the hypothesis that H$_{ox}$H and H$_{red}$/H are protonated versions of the H-cluster. However, it was not clear which component of oxNaDT was interacting with the H-cluster. To better understand the nature of these two states and their role in the catalytic cycle of [FeFe] hydrogenases, we sought to identify the oxidation product(s) of NaDT responsible for their formation.

H$_{ox}$H forms in the presence of sulfite at low pH. The main oxidation and degradation products of NaDT are sulfate.
(SO$_4^{2-}$), thiosulfate (S$_2$O$_3^{2-}$), and sulfite (SO$_3^{2-}$), all of which could potentially interact with the H-cluster of CrHydA1 and cause conversion to the H$_{ox}$H state. Therefore, to identify the NaDT oxidation products responsible for this conversion, we tested these species individually on CrHydA1 at both pH 8 and pH 5. Treatment of CrHydA1 with Na$_2$SO$_4$ and Na$_2$S$_2$O$_3$ at either pH 8 or pH 5 failed to reproduce the H$_{ox}$H state (Figure S2). In contrast, we found that addition of 80 mM of Na$_2$SO$_3$ at pH 5 reproduced the effect of oxNaDT and caused almost full conversion to the H$_{ox}$H state, while at pH 8, demonstrating that both low pH and Na$_2$SO$_3$ are required for H$_{ox}$H formation. Na$_2$SO$_4$, Na$_2$S$_2$O$_3$, and Na$_2$SO$_3$ solutions were pH corrected before use—this is particularly important for Na$_2$SO$_3$, which is a mild base.

In addition to CrHydA1, also the bacterial [FeFe] hydrogenases HydAB from Desulfovibrio desulfuricans (DdHydAB) and HydA1 from Clostridium pasteurianum (CpHydA1) have been reported to form the H$_{ox}$H state at low pH and in the presence of NaDT. These enzymes harbor additional [4Fe-4S] clusters (F-clusters) that form an electron-transfer chain from the protein surface to the H-cluster, and compared to CrHydA1, their active site is deeply buried inside the protein scaffold. When treated with Na$_2$SO$_3$ under acidic conditions, also DdHydAB and CpHydA1 converted to the H$_{ox}$H state (Figure S3), indicating that the interaction of the H-cluster with the oxidation product of NaDT is a generalized phenomenon in [FeFe] hydrogenases.

**A protonated form of sulfite interacts with the H-cluster.** Next, we decided to carry out titrations of CrHydA1 with Na$_2$SO$_3$ at various pH values in order to provide further details on the particular form of Na$_2$SO$_3$ that binds, as well as determining the binding affinity. In order to simplify the titrations, we chose to use a chemical variant of CrHydA1 with a [2Fe]$_H$ analogue containing a propane dithiolate (PDT) bridging ligand instead of ADT (CrHydA1PDT, Figure 1A). Compared to the amine in ADT, the methylene group in PDT cannot be easily protonated. As a result, CrHydA1PDT has very low catalytic activity and the H-cluster cannot assume states with a reduced [2Fe] H (i.e., H$_{ox}$H and H$_{red}$H$^+$) (Figure 1C). This greatly reduces the number of states observable in the IR spectra, simplifying data analysis. The PDT-containing enzyme was previously shown to convert to H$_{ox}$H and H$_{red}$H at low pH in the presence of NaDT. CrHydA1PDT was titrated with increasing amounts of Na$_2$SO$_3$ at five different pH values (Figure 3 and Figures S4–S6). In an anaerobic glovebox with a 100% N$_2$ atmosphere, the H-cluster was in the oxidized state H$_{ox}$ at the beginning of the titration for all the pH values tested. As already observed for native CrHydA1ADT, at pH 8 addition of even a very high concentration of Na$_2$SO$_3$ did not affect the state of the H-cluster, which remained in the H$_{ox}$ state. Conversely, at pH 7, H$_{ox}$H appeared already with less than 250 mM Na$_2$SO$_3$, and complete conversion was observed at around 700 mM. The concentration of Na$_2$SO$_3$ needed in order to observe complete conversion was observed at around 700 mM. The concentration of Na$_2$SO$_3$ needed in order to observe complete...
conversion from Hox to HoxH decreased at pH 6 to about 200 mM and at pH 5 to less than 8 mM. At pH 4, 1 mM Na2SO3 gave essentially complete conversion to HoxH, while 1 mM Na2SO3 at pH 5 gave a roughly equal mixture of Hox and HoxH (Figure S5).

In aqueous solutions SO3$^{2-}$ is in equilibrium with its protonated form bisulfite (HSO3$^{-}$), which in turn can be further protonated to form sulfurous acid (H2SO3), which immediately decomposes to sulfur dioxide (SO2) and water (Figure 3B).51−53 As Figure 3 shows, the lower the pH, the lower the concentration of sulfite needed to convert Hox to HoxH. This, therefore, excludes that SO3$^{2-}$, whose abundance is predicted to greatly decrease when changing the pH from 8 to 6, is responsible for formation of HoxH. Since, as shown in Figure 3A, lowering the pH from 6 to 5, and then to 4 (Figure S5), caused a further reduction in the required concentration of Na2SO3 needed to convert Hox to HoxH, while the fraction of HSO3$^{-}$ should be constant in this range (Figure 3B), HSO3$^{-}$ is also unlikely to be the form of Na2SO3 binding to the H-cluster. In a pH titration of Na2SO3 monitored by IR spectroscopy we observed that the intensity of peaks relative to HSO3$^{-}$ indeed saturated after pH 6.0−5.5, while signals indicative of the presence of SO2 appeared at pH 5 (Figure S6). Therefore, we hypothesize that the species interacting with the H-cluster to form HoxH is SO2. This seems reasonable considering that SO2 is a neutral molecule able to easily diffuse through hydrophobic channels54,55 to reach the H-cluster from the protein surface, while the anions HSO3$^{-}$ and SO3$^{2-}$ will be prevented from entering due to their charge and their large hydration spheres in aqueous solution.56 A similar suggestion was made to explain how S2$^{−}$ reaches the H-cluster as H2S to form the Hinact state.57

At pH 7 and 6, even at high concentration of sulfite, the concentration of dissolved SO2 is expected to be very low. Thus, in order to observe binding to the H-cluster and formation of HoxH, SO2 must have a tight affinity for the enzyme. Figure 3C shows the conversion from Hox to HoxH as a function of the estimated concentration of SO2 at each Na2SO3 addition, at either pH 6 or 7. The population of the two states was monitored from the intensity of the most prominent CO band at 1942 cm$^{-1}$ for Hox and 1946 cm$^{-1}$ for HoxH, in both cases corresponding to the stretch of the terminal CO on Fe6. The titration curves at pH 7 and 6 as a function of the concentration of SO2 overlay nicely, in contrast to those obtained using the estimated concentrations of HSO3$^{-}$ and SO3$^{2-}$ (Figure S6). Fitting the data in Figure 3C to a simple equilibrium model describing one SO2 molecule binding to the hydrogenase (SO2 + E $\rightleftharpoons$ E:SO2) gave an estimated binding affinity of $\sim$500 nM. In our analysis, we considered that the pool of Na2SO3 can act as a buffer system for SO2, replenishing what is consumed to form the enzyme:SO2 complex (E:SO2). For all the data points, the concentration of E:SO2 formed was negligible compared to the total concentration of Na2SO3, so that the concentration of
SO₂ at equilibrium could be assumed to be independent of the formation of E:SO₂ and could be determined only by the pH and the total concentration of Na₂SO₃, an important consideration for such tight binding interactions. To put this in context, CO has been estimated to bind with 100 nM affinity to CrHydA₁DT.⁵⁸

**Addition of sulfite under reducing conditions (H₂ atmosphere) forms H_red′H.** The titration of CrHydA₁DT with sulfite was repeated in the presence of 2% H₂ in the atmosphere of the anaerobic glovebox (Figure 4). Under these conditions, slow reactivity of the CrHydA₁DT with H₂ can lead to reduction of the [4Fe-4S]₄⁻ subcluster, in particular at high pH values. This is due to the potential of the 2H⁻/H₂ couple, which becomes more positive as the pH decreases, while the redox potential of [4Fe-4S]₄⁻ is pH independent.¹² At pH 7, after addition of a small amount of Na₂SO₃, we observed a mixture of the HoxH, H_red and H_red′H states in the IR spectra, plus a new set of signals. These are consistent with the vibrational frequencies of the HoxH state, which Stripp and co-workers reported to form with NaDT at low pH and either under H₂ or at low electrochemical potential.¹⁰,²⁷ Similar to what was observed under N₂, at lower pH the formation of H₂H and H_red′H was observed at lower concentration of Na₂SO₃. In order to estimate the proportion of each state present under each condition, we performed a pseudo-Voigt peak-fitting analysis of the region of the spectrum between ~1955 cm⁻¹ and ~1920 cm⁻¹, containing the most dominant bands for Hox (1942 cm⁻¹, blue), H_red (1935 cm⁻¹, cyan), H_red′H (1946 cm⁻¹, red), and H_red′H (1939 cm⁻¹, purple) (Figures 4B, S8–S10). In Figure 4C, the intensity of these contributions is plotted as a function of the concentration of Na₂SO₃ at pH 7. At low Na₂SO₃ both the HoxH and H_red′H states are observed, but at high concentrations of Na₂SO₃, H_red′H is converted to H_redH. This indicates oxidation of the [4Fe-4S]₄⁻ subcluster by Na₂SO₃. Since the samples were prepared in a closed IR cell and the concentrations of sulfite used are much higher than the dissolved concentration of H₂, oxidation by Na₂SO₃ will slowly deplete the H₂ concentration.

It is tempting to speculate that SO₂ diatomic gas could somehow interact with the H-cluster of [FeFe] hydrogenases. It is tempting to speculate that SO₂ diatomic gas could somehow interact with the H-cluster of [FeFe] hydrogenases. It is tempting to speculate that SO₂ diatomic gas could somehow interact with the H-cluster of [FeFe] hydrogenases. It is tempting to speculate that SO₂ diatomic gas could somehow interact with the H-cluster of [FeFe] hydrogenases. It is tempting to speculate that SO₂ diatomic gas could somehow interact with the H-cluster of [FeFe] hydrogenases. It is tempting to speculate that SO₂ diatomic gas could somehow interact with the H-cluster of [FeFe] hydrogenases. It is tempting to speculate that SO₂ diatomic gas could somehow interact with the H-cluster of [FeFe] hydrogenases. It is tempting to speculate that SO₂ diatomic gas could somehow interact with the H-cluster of [FeFe] hydrogenases.

In order to get further information on the SO₂ binding site, we measured ⁵⁷Fe nuclear resonance vibrational spectroscopy (NRVS). This technique measures Fe-ligand vibrational energies using nuclear excitation of ⁵⁷Fe and has been used extensively to probe ligand binding to the [2Fe]₁ subcluster in [FeFe] hydrogenase.²⁵,²³,²⁸,²⁹ We artificially matured apo-CrHydA₁ samples with a ⁵⁷Fe-labeled diiron subcluster precursor ([²⁵Fe₃ADD]) and measured NRVS in the HoxH and H_red′H states (Figure S5). This enzyme is labeled with ⁵⁷Fe in the [2Fe]₁ subcluster and not in the [4Fe-4S]₄⁻ subcluster, so only it can be calculated that an ~60 mV difference in the redox potentials indicates a K_d of ~60 nM for SO₂ binding to the H_red state, approximately 1 order of magnitude tighter. This also means that low concentrations of Na₂SO₃ have a larger effect in the presence of H₂ (compare Figure 4B with Figure S4B).

The site of SO₂ binding is not the open coordination site on [2Fe]₁. From the previous section, it is clear that SO₂ somehow interacts with the H-cluster of [FeFe] hydrogenases. It is tempting to speculate that SO₂ diffuses through the hydrophobic gas channel leading to the open coordination site on Fe₃. However, we cannot exclude that SO₂ binds elsewhere, and indeed, the change in the redox potential of [4Fe-4S]₄⁻ would suggest that binding near to [4Fe-4S]₄⁻ is more likely. To test whether binding of SO₂ with the H-cluster occurs at the open coordination site on [2Fe]₁, we investigated how its presence can affect the interaction of the enzyme with CO, a competitive inhibitor of [FeFe] hydrogenases that binds to Fe₃. At pH 5 exposure of CrHydA₁DT to 100% CO gas for 10 min in the absence of Na₂SO₃ generates pure H₂H–CO (Figure S12). In the presence of a high concentration of sulfite at pH 5, exposure of CrHydA₁ to CO caused the appearance of new peaks that correspond to neither HoxH–CO nor HoxH, and are similar to the HoxH–CO state described by Stripp and co-workers (Figure S12).²⁷ This suggests that SO₂ does not compete for the same binding site as CO, which is the open coordination site at Fe₃.

In order to get further information on the SO₂ binding site, we measured ⁵⁷Fe nuclear resonance vibrational spectroscopy (NRVS). This technique measures Fe-ligand vibrational energies using nuclear excitation of ⁵⁷Fe and has been used extensively to probe ligand binding to the [2Fe]₁ subcluster in [FeFe] hydrogenase.²⁵,²³,²⁸,²⁹ We artificially matured apo-CrHydA₁ samples with a ⁵⁷Fe-labeled diiron subcluster precursor ([²⁵Fe₃ADD]) and measured NRVS in the HoxH and H_red′H states (Figure S5). This enzyme is labeled with ⁵⁷Fe in the [2Fe]₁ subcluster and not in the [4Fe-4S]₄⁻ subcluster, so only it can be calculated that an ~60 mV difference in the redox potentials indicates a K_d of ~60 nM for SO₂ binding to the H_red state, approximately 1 order of magnitude tighter. This also means that low concentrations of Na₂SO₃ have a larger effect in the presence of H₂ (compare Figure 4B with Figure S4B).

Scheme 1. Thermodynamic Cycle Connecting HoxH, H_red, HoxH/H_red, HoxH/H_red′H⁴

⁴One-electron reduction of Hox and HoxH gives H_red and H_red′H, respectively, with redox potentials of E₁~−350 mV and E₂~−290 mV, respectively. Hox and H_red convert to HoxH and H_red′H, respectively, by binding SO₂. The K_d for SO₂ binding to the HoxH state was measured to be ~500 nM. By consideration of the fact that the Gibbs free energy is a state function, the ΔG associated with the transition from Hox to HoxH is the same regardless of whether we go via HoxH (ΔG₁) or via H_red (ΔG₂), allowing us to calculate the K_d for binding of SO₂ to the H_red state to be ~60 nM.

https://doi.org/10.1021/jacs.1c07322
J. Am. Chem. Soc. 2021, 143, 18159–18171
vibrations involving motion of the [2Fe]H subcluster can be observed. The spectra of Hox and HoxH are very similar but with small shifts of the peaks to lower energy for the HoxH, indicative of decreased electron density on the [2Fe]H subcluster, similar to results observed by Mebs et al. In contrast, ligand binding to Fed on the [2Fe]H subcluster would be expected to have much more dramatic changes, particularly, the generation of additional Fe\(^{2-}\) or Fe–O vibrations.

The results cannot definitively confirm the [4Fe-4S]H subcluster as the point of SO\(_2\) binding, but together with the observation of the HoxH–CO state, they do exclude the open coordination site on Fed as the SO\(_2\) binding site.

**Figure 5.** Comparison of the NRVS spectra of CrHydA1 maturated with an \(^{57}\)Fe-labeled [2Fe]ADT precursor complex in the Hox (red) and HoxH (blue) states measured at 10 K. The regions of the spectra corresponding to Fe-protein, Fe–S, Fe-CN, and Fe-CO vibrations are highlighted in brown, yellow, pink, and blue along the x-axis.

**Figure 6.** Protein film electrochemistry of DdHydAB in the presence of sulfite. DdHydAB was covalently attached to a pyrolytic graphite electrode and cyclic voltammetry (CV) was performed in 20 mM mixed buffer with 100 mM NaCl at pH 5 (A), 6 (B), 7 (C), and 8 (D) under 100% H\(_2\) (1000 mL/min), at 25 °C, with 2000 rpm rotation, and a scan rate of 0.02 V/s. After 3 CVs in the absence of Na\(_2\)SO\(_3\) (only the third trace is shown, black trace), 40 mM Na\(_2\)SO\(_3\) was added (red trace). Only a single CV before and after the addition of Na\(_2\)SO\(_3\) are shown. However, consecutive CVs showed the same shape. After replacing the buffer in the electrochemical cell with Na\(_2\)SO\(_3\)-free buffer, DdHydAB recovered its original activity (blue traces). The red arrow indicates the point of Na\(_2\)SO\(_3\) injection. The black arrows indicate the scan direction of the CV. The dashed horizontal line shows the zero current position, and the dashed vertical line shows the equilibrium 2H\(^+\)/H\(_2\) potential at each pH value. Enlarged versions of A and B are reported in Figure S13.

**SO\(_2\) inhibits catalysis by [FeFe] hydrogenase.** In order to investigate the effect of SO\(_2\)-binding to the H-cluster on catalysis, we performed protein film electrochemistry on the DdHydAB enzyme covalently attached to a pyrolytic graphite electrode. We chose DdHydAB rather than CrHydA1, as the former is, in our hands, much easier to covalently attach to graphite electrode surfaces. As shown in the cyclic voltammograms (CVs) in Figure 6 and in the enlarged version of the CVs reported in Figure S13, a large negative current at low potentials is observed when Na\(_2\)SO\(_3\) is injected into the electrochemical cell under acidic conditions (pH 5 and pH 6, respectively A and B in Figure 6). Controls experiments (bare graphite electrode injecting Na\(_2\)SO\(_3\), Figure S14) suggest that this reduction current is likely due to HSO\(_3\)^− and SO\(_2\) being reduced by the pyrolytic graphite electrode. Comparisons of bare graphite electrodes and DdHydAB-modified electrodes at various pH values are presented in the absence (Figure S15) and presence (Figure S16) of Na\(_2\)SO\(_3\). Unfortunately, this massive reduction current masks the effect of Na\(_2\)SO\(_3\) on the catalytic H\(^+\)-reduction current.

However, as shown in Figure 6A and B (CV at pH 5 and 6 in the presence of Na\(_2\)SO\(_3\), respectively), in the presence of Na\(_2\)SO\(_3\) the catalytic H\(_2\)-oxidation current decreases, suggesting inhibition of the enzyme as a result of the H-cluster somehow interacting with SO\(_2\). The inhibitory effect on the catalytic H\(_2\)-oxidation current is more pronounced at lower pH (the CVs at pH 7 and 8 are reported in Figure 6C and D, respectively), in agreement with the pH-dependent formation of HoxH and Hred\(^\prime\)H observed in the IR measurements. To explore whether the inhibition is reversible and the electrocatalytic H\(_2\)-oxidation current can be recovered, the buffer in
the electrochemical cell was exchanged to a fresh buffer without Na$_2$SO$_3$ during the course of the CVs. Sulfite-exposed DdHydAB recovered 100% of the electrocatalytic H$_2$-oxidation current once Na$_2$SO$_3$ was removed from the electrochemical cell, suggesting that SO$_2$ binding and inhibition are fully reversible (blue trace in Figure 6A and B) and that the enzyme is not irreversibly damaged by SO$_2$.

The massive current at low potential due to direct reduction of HSO$_3^-$ and SO$_2$ species by the electrode makes it difficult to assess the effect of Na$_2$SO$_3$ on the electrocatalytic H$^+$-reduction current. To distinguish the enzymatic contribution from the direct HSO$_3^-$ and SO$_2$ reduction by the electrode, we performed chronoamperometry experiments (the applied potential is held at a specific value while the current is monitored vs time) in the presence and absence of CO (Figure 7). As previously described, the current decrease due to CO addition (as CO binds to open coordination site on Fe$_3$ and inhibits the enzyme) provides a direct measurement of the enzymatic H$^+$ reduction. In the experiment in Figure 7A, performed at pH 5, DdHydAB attached on the pyrolytic graphite electrode was initially exposed to 90% H$_2$/10% N$_2$ at −109 mV vs SHE, where a positive current due to H$_2$ oxidation was observed (as the applied potential is more positive than the thermodynamic potential of the 2H$^+$/H$_2$ couple at this pH, −295 mV vs SHE). Switching to −459 mV gave a small negative current due to H$^+$ reduction (as the applied potential is now more negative than $E_{2H^+/H_2}$ at this pH). Adding Na$_2$SO$_3$ at this potential gave an extremely large negative current, which was unaffected by addition of 10% CO into the gas feed (replacing the 10% N$_2$). This indicates that the large negative current is entirely due to Na$_2$SO$_3$ reduction and that catalytic H$^+$ reduction by DdHydAB is completely inhibited under these conditions. Replacing the buffer with fresh Na$_2$SO$_3$-free buffer decreased the current to the original value observed before addition of Na$_2$SO$_3$. An analogous experiment at pH 6 (Figure 7B) showed a small decrease in the current after addition of CO, as well as experiments at pH 7 and pH 8 (Figure 7C and 7D, respectively), suggesting that at these pH values there is some contribution from the enzymatic H$^+$ reduction current, in agreement with the pH dependent formation of SO$_2$ from...
Control experiments in the complete absence of Na₂SO₃ showed full inhibition of the electrocatalytic H⁺-reduction current by CO, thus demonstrating that in the absence of Na₂SO₃, the reductive current is indeed enzymatic H⁺ reduction (Figure S17). At this stage, it is unclear whether the loss in activity in both directions due to Na₂SO₃ addition is directly related to the increase in the redox potential of [4Fe-4S]H. The higher redox potential of the cluster may disrupt the proton-coupled electronic rearrangement between [4Fe-4S]H and [2Fe]H. These experiments help to understand the discrepancy between reported H⁺ reduction activity solution assays and electrochemistry. While solution assays (where NaDT is used as electron source) indicate a maximum in activity at pH 7, and almost no activity at pH 5, electrochemical measurements show the highest H⁺ reduction activity at pH 5 (Figure S15). Regardless, these data show that, under the conditions where HoxH and HredH form, the enzyme has lower activity, suggesting that these states are not active intermediates of the catalytic cycle of [FeFe] hydrogenases. This is in stark contrast to the suggestion from Stripp and Haumann that a catalytic cycle involving HoxH is actually the faster branch of the cycle compared to that involving the HredH⁻¹ and HoxH⁻¹ states (Figure 1D).

**DISCUSSION**

In this work we have shown that in CrHydA1 the HoxH state forms in the presence of oxidation products of NaDT at low pH, specifically SO₂. SO₂ binding caused formation of HoxH not only with CrHydA1 but also with the bacterial enzymes CpHydA1 and DdHydAB, suggesting this is a common behavior in [FeFe] hydrogenases. Additionally, we have shown that with Na₂SO₃ and in the presence of H₂, the reduced HredH state can also form. The electrochemistry measurements showed loss in electrocatalytic activity when DdHydAB was exposed to Na₂SO₃, especially at low pH, suggesting that HoxH and HredH are less active states and challenging their inclusion in the catalytic cycle. Taken together, these findings suggest that HoxH and HredH are not protonated versions of Hox and Hred, but instead are forms of Hox and Hred in which a product of NaDT oxidation, most likely SO₂, is bound. Thus, we suggest renaming Hox-DT₁ and Hred-DT₁ (for dithionite inhibited) to avoid confusion, and for the rest of the discussion we will name them as such.

This result helps explain previous findings in the literature regarding these states. Originally, Hox-DT₁ and Hred-DT₁ were discovered during NaDT-mediated H⁺ reduction by [FeFe] hydrogenase at low pH. Under these conditions H⁺ reduction rates are high, leading to rapid oxidation of NaDT to generate a mixture of SO₃⁻, HSO₃⁻, and SO₂. At low pH, SO₂ forms due to the protonation equilibria and it can bind to the hydrogenase yielding the Hox-DT₁ and Hred-DT₁ states. It was noticed that the accumulation of Hox-DT₁ was dependent both on pH and on NaDT concentration, both of which will affect the rate of SO₂ accumulation. Furthermore, it was noted that less active forms of the hydrogenase (e.g., with the PDT cofactor) accumulated Hox-DT₁ more slowly. In this case, the accumulation of SO₂ depends on the rate of NaDT oxidation by the catalytic activity of the hydrogenase, and it is well established that the PDT-form of the hydrogenase is catalytically much less active than the native ADT-form. Protonation at [4Fe-4S]H is a critical component in the catalytic cycle proposed in Model 2 (Figure 1D). We recently demonstrated that (in the absence of NaDT) the redox potential of [4Fe-4S]H is pH-independent, challenging the involvement of PCET in the formation of HoxH and the protonation at [4Fe-4S]H. Our current work further challenges protonation at [4Fe-4S]H by showing that the Model 2 key intermediate Hox-DT₁ (HoxH in Figure 1D) is generated by the oxidation products of NaDT. If reduction of [4Fe-4S]H is coupled to protonation then it has to be coupled to protonation in all the steps involving reduction of [4Fe-4S]H. Considering that the hydrogenase enzyme is reversible, with a very low overpotential in either direction, it must be assumed that each step in the catalytic cycle is also reversible and, thus, HoxH should be able to protonate to give Hox-DT. However, incubation of Hox at low pH in the absence of NaDT does not generate Hox-DT₁ (Figure 2), so Hox-DT₁ is clearly not a reversibly protonated form of Hox.

Our results also help to explain the misassignment of the pH dependence of the Hox/Hred transition. It is important to recall that in this study we also observe that the Hox-DT₁/Hred-DT₁ transition is about 60 mV more positive than the HoxH/Hred transition, as also reported by Senger et al. If the conversion of Hox to Hox-DT₁ and Hred to Hred-DT₁ depend on the pH, then we expect that the “apparent” redox potential of both transitions will shift from the intrinsic redox potential of Hox/Hred to the intrinsic redox potential of Hox-DT₁/Hred-DT₁, as the pH is decreased. This is simply a consequence of the redox and protonation equilibria being coupled (see Supporting Information and Figure S18 for further details and a model illustrating this behavior). As we demonstrated that the SO₂ concentration in solution increases with decreasing pH and that SO₂ is responsible for binding to Hox/Hred to generate Hox-DT₁/Hred-DT₁, then this gives us a pH dependent conversion of Hox/Hred to Hox-DT₁/Hred-DT₁, and, therefore, an apparent pH dependence of the redox potential.

A further important finding regarding the [FeFe] hydrogenase is the fact that SO₂, appears to inhibit the H₂ oxidation and H⁺ reduction activity of the enzyme. This may be due to the increased redox potential of [4Fe-4S]H. While we do not yet completely understand this effect, it highlights the importance of the balance of redox potentials between the two parts of the H-cluster in facilitating electronic coupling and efficient catalysis. We previously showed that mutation of a cysteine ligating [4Fe-4S]H to histidine increased the redox potential by ~200 mV. This completely abolished H⁺ reduction activity, while actually enhancing H₂ oxidation at high overpotentials.

The now well-characterized HoxH₁ intermediate can be generated under conditions of high NaDT at low pH. It is not clear yet whether this state is also somehow influenced by the presence of SO₂. However, it has always been intriguing how such an intermediate could be so stable by simply generating it at low pH in the presence of NaDT. Previous explanations have employed Le Chatelier’s principle and the concept of proton pressure. It may indeed be the case that SO₂ binding stabilizes the HoxH₁ state by increasing the redox potential of the [4Fe-4S]H₁ subcluster slowing electron transfer to [2Fe]H to generate H₂. Recent evidence shows that versions of HoxH₁ can be generated from HoxH⁺ and HredH⁻¹.25 The so-called HoxH₁ state is generated from HoxH⁺ and should have a reduced [4Fe-4S]H₁ subcluster analogous to HoxH. Interestingly, the IR bands of HoxH₁ are shifted to higher energy compared with HoxH by a similar amount to Hox-DT₁ vs Hox (Table S2). Careful revaluation of the HoxH₁ state generated with NaDT at low pH is clearly necessary.
In addition to shedding light on the catalytic cycle of [FeFe] hydrogenases, this work reports how NaDT, a compound commonly employed as a reducing agent in metalloenzyme research, is responsible for the generation of artifacts, which were erroneously characterized as catalytically relevant states. To our knowledge, this is the first report of such “non-innocent” behavior of NaDT with [FeFe] hydrogenases, in this case caused by the interaction of one of the NaDT oxidation products with the enzyme. The experimental conditions should thus, be carefully evaluated when NaDT is chosen as the reducing agent with these enzymes. As we have shown, acidic conditions facilitate formation of Hox-DTi, but at a high concentration of sulfitc this state also forms at pH 7. Therefore, particular care must be taken when [FeFe] hydrogenase samples that contain (or contained) NaDT are studied at low pH, or in those cases where NaDT is used as a continuous source of electrons. While this is the first time that NaDT has been shown to interfere with spectroscopic studies of [FeFe] hydrogenases, several previous studies of various other metalloenzymes have reported similar effects. This problematic behavior has been attributed to several factors, from the slow kinetics of NaDT dissociation limiting the catalytic behavior to the unwanted interaction of its oxidation products with the system under study, as we described for [FeFe] hydrogenases. Importantly, the enzymes affected catalyze various reactions and harbor various metallocofactors, suggesting that it is difficult to predict which enzymes will be affected. As such, it is possible that similar effects are still going undetected for other systems. Therefore, the chemistry of NaDT and of its oxidation products should be carefully considered when choosing this compound as a reducing agent for metalloproteins research, and important control experiments should be routinely employed to identify possible side-reactions that can engage with the system under study. In the future it will also be important to evaluate alternative artifacts from those that represent the physiological behavior of the enzyme of interest.

CONCLUSIONS

In this work we have shown that SO₂, an oxidation product of the commonly used nonphysiological reductant sodium dithionite, binds tightly to [FeFe] hydrogenase converting the catalytic intermediate states H₀₄ and H₀₄D into the H₀₄DT and H₀₄₂DT states (previously named H₀₄H and H₀₄H). Thus, our results do not support the notion of protonation of [4Fe-4S]₄H subcluster of the H-cluster, nor that the H₀₄DT state is a critical intermediate in the catalytic cycle. SO₂ most likely binds at or near the [4Fe-4S] subcluster and appears to increase the cluster redox potential. This in turn may explain the observed decrease in catalytic activity. Overall, these results highlight the importance of finely tuned redox potentials for catalytic activity and reversibility. More generally, these results should come as a cautionary note to all who use sodium dithionite in metalloprotein studies without concern for its “non-innocent” effects. Sodium dithionite is routinely used in studies on a wide range of metalloenzymes including nitrogenase, CO dehydrogenase, formate dehydrogenase, and many more. Careful evaluation of results from a range of nonphysiological reductants should help to establish the effects that are artifacts from those that represent the physiological behavior of the enzyme of interest.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c07322.

- Experimental section
- supplementary figures and tables
- supplementary discussion (PDF)

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https://pubs.acs.org/10.1021/jacs.1c07322
Funding
Open access funded by Max Planck Society. All the authors would like to thank the Max Planck Society for funding. J.A.B., M.A.M., and S.D. also acknowledge the Deutsche Forschungsgemeinschaft (DFG) Priority Programme “Iron–Sulfur for Life: Cooperative Function of Iron–Sulfur Centers in Assembly, Biosynthesis, Catalysis and Disease” (SPP 1927) Projects BI 2198/1-1 (J.A.B. and M.A.M.) and DE 1877/1-2 (S.D.). P.R.-M. is supported financially by the European Research Council (ERC-2018-CoG BiocatSusChem 819580, to K.A. Vincent), and acknowledges Linacre College Oxford for her Junior Research Fellowship.

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS
The authors would like to thank Inge Heise and Tabea Mussfeld, for synthesizing the unlabeled diiron cofactors, and Prof. Tom Rauchfuss, for supplying the $^{57}$Fe-labeled diiron cofactor. We acknowledge DESY (Hamburg, Germany), a member of the Helmholtz Association HGF, for the provision of experimental facilities. Parts of this research were carried out at PETRA-III, and we would like to thank Prof. Hans-Christian Hildebrandt, P. Zeber, I. Vibrational spectroscopy reveals the initial steps of biological hydrogen evolution. Chem. Sci. 2016, 7 (11), 6746–6752.

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