Characterization of [FeFe] Hydrogenase O₂ Sensitivity Using a New, Physiological Approach* 

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[FeFe] hydrogenases catalyze rapid H₂ production but are highly O₂-sensitive. Developing O₂-tolerant enzymes is needed for sustainable H₂ production technologies, but the lack of a quantitative and predictive assay for O₂ tolerance has impeded progress. We describe a new approach to provide quantitative assessment of O₂ sensitivity by using an assay employing ferredoxin NADP⁺ reductase (FNR) to transfer electrons from NADPH to hydrogenase via ferredoxins (Fd). Hydrogenase inactivation is measured during H₂ production in an O₂-containing environment. An alternative assay uses dithionite (DTH) to provide reduced Fd. This second assay measures the remaining hydrogenase activity in periodic samples taken from the NADPH-driven reaction solutions. The second assay validates the more convenient NADPH-driven assay, which better mimics physiological conditions. During development of the NADPH-driven assay and while characterizing the Clostridium pasteurianum (Cp) [FeFe] hydrogenase, CpI, we detected significant rates of direct electron loss from reduced Fd to O₂. However, this loss does not interfere with measurement of first order hydrogenase inactivation, providing rate constants insensitive to initial hydrogenase concentration. We show increased activity and O₂ tolerance for a protein fusion between Cp ferredoxin (CpFd) and CpI mediated by a 15-amino acid linker but not for a longer linker. We suggest that this precise, solution phase assay for [FeFe] hydrogenase O₂ sensitivity and the insights we provide constitute an important advance toward the discovery of the O₂-tolerant [FeFe] hydrogenases required for photosynthetic, biological H₂ production.

Hydrogenases catalyze the reversible formation of H₂ from two protons and two electrons. They are classified as [FeFe], [NiFe], or [Fe]-only with respect to the metal atoms included in the active site. Although [NiFe] hydrogenases are generally biased toward oxidation of H₂, [FeFe] hydrogenases have a catalytic bias toward H₂ formation. The most prolific enzymes require overpotentials lower than that for platinum, one of the best metal catalysts reported to date (1). Consequently, [FeFe] hydrogenases offer significant potential for biological production of H₂ (2–6).

We have approached such applications with a particular focus on the [FeFe] hydrogenase from Clostridium pasteurianum. It is a 63.8-kDa protein with one of the highest reported H₂ production-specific activities (2, 7). However, it is also a complex enzyme with three accessory [4Fe-4S] clusters and one [2Fe-2S] cluster that deliver electrons to or from the active site consisting of an [FeFe] sub-cluster bridged to a [4Fe-4S] cluster by a cysteinyl thiol. Much work has been done during the past decade to enable the heterologous expression, maturation, and purification of the active form of CpI² in Escherichia coli (8, 9).

Based on these advances, we are now working on two distinct routes of biological H₂ production using this enzyme: fermentative (2, 10) and photosynthetic (11).

Unfortunately, [FeFe] hydrogenases are highly sensitive to O₂ (12–14), imposing a significant technical barrier for oxygenic photosynthetic H₂ production. In fact, the half-life of CpI in air-saturated buffer is estimated to be 2–3 min (13, 15, 16). Although the exact mechanism of inactivation remains to be elucidated, previous research has provided insights and hypotheses. Strip et al. (17) proposed that O₂ first binds to the distal Fe atom of the [FeFe] sub-cluster of the active site. Reactive oxygen species are then formed and cause dissociation of the neighboring [4Fe-4S] sub-cluster. Cohen et al. (18) suggested that O₂ molecules migrate to the active site through channels within the enzyme. Goldet et al. (13) then developed a kinetic model that consists of two rate-limiting steps: O₂ first diffuses to the active site and then permanently destroys activity. More recently, Swanson et al. (16) reported that the extent of inactivation depends on the initial redox state of the hydrogenase and that the inactivation process results in the loss of the [FeFe] sub-cluster from the active site. Despite these insights, an inability to quantitatively and conveniently assess O₂ sensitivity in a biological context has limited progress in the discovery of O₂-tolerant [FeFe] hydrogenases.

Most studies on the oxygenic inactivation of [FeFe] hydrogenases to date have relied on the experimental technique known as protein film voltammetry (PFV). In this technique, the enzyme is adsorbed on the surface of an electrode and the activity is determined by measuring enzyme-dependent currents.

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The abbreviations used are: CpI, Clostridium pasteurianum [FeFe] hydrogenase; CpFd, Clostridium pasteurianum ferredoxin; FNR, ferredoxin-NADPH-reductase; Fd, ferredoxin; Fdred, reduced ferredoxin; G6P, glucose-6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; SynFd, Synechocystis sp. PCC 6803 ferredoxin; SynFdred, reduced SynFd; RrFNR, Oroxyza sativa (rice root) FNR; PFV, protein film voltammetry; DTH, dithionite; TON, turnover numbers; vol %, percentage by volume.
generated by applying either negative or positive potential bias (12, 19, 20). Although this technique allows precise measurements, it also has drawbacks (21). Most importantly, the technique employs an unnatural electron supply mechanism, and O\textsubscript{2} reduction at the electrode complicates interpretation. Concern has also been expressed that individual hydrogenase molecules may adsorb to the electrode in different orientations, thereby providing different electron transfer distances between the electrode and conducting Fe-S clusters in the enzyme (22). As a result, the findings from PFV experiments may not accurately predict in vivo hydrogenase O\textsubscript{2} sensitivity.

In this study, we describe an alternative approach to assessing O\textsubscript{2} tolerance of hydrogenases that more closely mimics physiological H\textsubscript{2} production as well as our envisioned processes for biological H\textsubscript{2} production. This approach allows us to assess two critical parameters connected to O\textsubscript{2} tolerance: the inactivation rate constant for hydrogenase activity in the presence of O\textsubscript{2}, and the residual hydrogenase activity after O\textsubscript{2} exposure. In assessing both, we use the natural electron source for hydrogenases, a solution phase reduced Fd; the difference lies in how the oxidized Fd is reduced (Fig. 1). The first assay (Fig. 1a) uses a reaction sequence developed as an integral part of our fermentative H\textsubscript{2} production process (2). It delivers electrons from NADPH to Cpl via FNR and Fd. This assay assesses the inactivation of Cpl while it produces H\textsubscript{2} in the presence of O\textsubscript{2}. The second assay (Fig. 1b) uses a sequence in which electrons are delivered from DTH to hydrogenase via Fd. In this assay, the strong reducing power of DTH (E\textsubscript{o} = -0.66 V versus normal hydrogen electrode at pH 7) consumes any remaining O\textsubscript{2} and apparently maintains most of the Fd in the reduced state (Fd\textsuperscript{red}), allowing an assessment of the full residual activity of hydrogenase after O\textsubscript{2} exposure.

Using these assays, we studied the kinetics of inactivation with varying concentrations of O\textsubscript{2} and Cpl. Next, we evaluated the influence of other reagents. Noting that the O\textsubscript{2} concentration decreases over the course of the NADPH-driven assay, we confirmed a significant rate of electron loss directly from the reduced Fd to O\textsubscript{2}. Nonetheless, our approach provides convenient assessment of the O\textsubscript{2} tolerance of [FeFe] hydrogenases and engineered mutants. As an example, we show that a Cpl-Fd fusion protein with a higher hydrogenase activity is also less sensitive to O\textsubscript{2} inactivation.

Results

Influence of Cpl and O\textsubscript{2} Concentration on the Kinetics of Inactivation—Due to our interest in photosynthetic H\textsubscript{2} production, we conducted our initial experiments with the Fd from Synechocystis sp. PCC 6803 (SynFd). Oryza sativa (rice root) FNR (RfFNR) was chosen over the FNR native to SynFd or Cpl because it results in significantly faster H\textsubscript{2} production within the NADPH-driven assay (11). With these choices, we first evaluated the kinetics of Cpl inactivation caused by different concentrations of O\textsubscript{2}. The H\textsubscript{2} accumulation rate appeared to decrease immediately upon the introduction of O\textsubscript{2} and then further diminished over the course of an hour (Fig. 2). As expected, higher concentrations of O\textsubscript{2} were more deleterious but, surprisingly, no additional H\textsubscript{2} production was detectable after introducing 10 vol % O\textsubscript{2} or higher. These reaction mixtures were well mixed to encourage O\textsubscript{2} transfer into the liquid phase, and 10 vol % O\textsubscript{2} in the headspace is expected to produce a dissolved O\textsubscript{2} concentration of roughly 120 \textmu M.

Goldet et al. (13) used PFV and an exponential decay model to evaluate the O\textsubscript{2} sensitivity of [FeFe] hydrogenases based on changes in current over time after O\textsubscript{2} addition. At constant [O\textsubscript{2}], this implies that the rate of activity loss can be expressed as a first order inactivation characterized by a rate constant, \( k_{\text{inact,O}_2} \):\n
\[
\frac{dH_2}{dt} = -k_{\text{inact,O}_2} \frac{dH_2}{dt} \quad \text{[O}_2\text{]} 
\]

(Eq. 1)

Fig. 2b indicates that an attempt to fit the full data set to this model was unsuccessful. The decline in hydrogenase activity measured immediately after O\textsubscript{2} addition was consistently lower than predicted. We show later that the immediate drop in H\textsubscript{2} production rate is caused by direct electron loss from SynFd\textsuperscript{red} to O\textsubscript{2}. However, this loss remains relatively constant, and the subsequent decreases in H\textsubscript{2} production rate primarily reflect Cpl inactivation. The same pattern, immediate increase in current upon O\textsubscript{2} introduction and gradual decrease afterward, was observed with PFV as well (13). In our assay, using only the rates after O\textsubscript{2} addition provides data sets that indicate the expected exponential decay curves (Fig. 2c) with \( k_{\text{inact,O}_2} \) ranging from 0.025 to 0.099 min\textsuperscript{-1} for 1.0 to 5.0 vol % O\textsubscript{2} (Table 1).

To further test the assay, we next varied the initial concentration of Cpl from 1 to 100 nM while adding 5.0 vol % O\textsubscript{2} after 10 min (Fig. 3). First order inactivation will only be valid if the inactivation rate constant does not vary as a function of active hydrogenase concentration. In all cases, significant H\textsubscript{2} production activity was retained for only 30 min after the introduction of 5.0 vol % O\textsubscript{2} (Fig. 3a), limiting analysis to that time period. The magnitude of electron loss to O\textsubscript{2} also precluded analysis with 1 nM Cpl. Nonetheless, the available H\textsubscript{2} production rate measurements (Fig. 3b) indicate first order inactivation with consistent inactivation rate constants (0.097 \pm 0.005 min\textsuperscript{-1}) within the accuracy of our assay over a 20-fold range of Cpl concentrations. For convenience, the first slope after O\textsubscript{2} addition is assumed to approximate the rate after 5 min of exposure, whereas the rates at other time points are the average of the preceding and subsequent slopes.

Influence of Other Reagents on the Kinetics—We next examined the influence of the concentrations of the two potentially rate-limiting electron transfer proteins, FNR and Fd, used for NADPH-driven H\textsubscript{2} production (Fig. 4). Increasing

FIGURE 1. Two biochemical reaction sequences for H\textsubscript{2} evolution from Cpl. (a) driven by NADPH, Fd\textsuperscript{red}, oxidized ferredoxin; PGL, 6-phosphoglucono-6-lactone; b, driven by DTH.
[FNR] slowed inactivation by 5.0 vol % O₂ (Fig. 4a). An increase from 5 to 50 μM increased the anaerobic H₂ production rate by about 10% and decreased the inactivation rate constant, $k_{\text{inact,O}_2}$, from 0.102 ± 0.004 to 0.089 ± 0.008 min⁻¹. This observation suggests that increasing the flux of electrons to the hydrogenase may also increase O₂ tolerance. Note that the anaerobic CpI turnover numbers (TON) in these experiments were about 4.5 s⁻¹ relative to a $k_{\text{cat}}$ of about 400 s⁻¹ with DTH as the electron source. This factor will be discussed in more detail later.

Increasing [Fd] from 5 to 50 μM had a similar impact on the inactivation kinetics (Fig. 4b). $k_{\text{inact,O}_2}$ decreased from 0.097 ± 0.006 to 0.082 ± 0.008 min⁻¹, whereas the CpI TON stayed the same. More significant changes were observed when [Fd] was decreased from 5 to 1 μM. The anaerobic CpI TON decreased to 78% of the level with 5 μM Fd, and after O₂ addition, the H₂ production rate immediately dropped by 87% for 1 μM versus 68% for the higher concentrations. $k_{\text{inact,O}_2}$ also increased to 0.118 ± 0.010 min⁻¹. The larger $k_{\text{inact,O}_2}$ is in agreement with our aforementioned conjecture about the influence of electron flux on the hydrogenase inactivation rate.

**TABLE 1**

Inactivation rate constants for CpI exposed to varying O₂ concentrations

| [O₂] vol % | $k_{\text{inact,O}_2}$ min⁻¹ |
|-----------|-------------------------------|
| 1.0       | 0.025 ± 0.003                  |
| 2.0       | 0.041 ± 0.006                  |
| 3.0       | 0.053 ± 0.010                  |
| 4.0       | 0.075 ± 0.013                  |
| 5.0       | 0.099 ± 0.012                  |

Electron Loss from Reduced Fd to O₂—As mentioned previously, several observations suggested that O₂ addition to the reactor headspace lowered the rate of electron supply to the hydrogenase. As shown in Fig. 5a, O₂ addition to the NADPH-driven assay initiates significant O₂ consumption. Although the O₂ tolerance mechanism for some [NiFe] hydrogenases (reduction of O₂ to H₂O) (23) suggests that the hydrogenase could deplete O₂, this was not apparent in our experiments. Incremental omission of reagents starting at the end of the reaction sequence clearly indicated that the reduced Fd was responsible. Omitting the electron source avoided O₂ depletion as expected. A 1974 study indicates that Clostridial ferredoxins are capable of reducing O₂ to form reactive oxygen species (24).

The study described here was conducted with CpFd because we reasoned that it would interact more favorably with CpI, its native partner. CpFd contains two [4Fe-4S] clusters, whereas SynFd used in our initial experiments has only one [2Fe-2S] cluster. SynFd transferred electrons to O₂ at about 50% of the rate observed for CpFd over an hour within the NADPH-driven assay in the absence of CpI (Fig. 5b). Adding catalase and superoxide dismutase to the assay reduced the rate of O₂ depletion consistent with the hypothesis that anionic O₂ radicals are being formed (data not shown), but a full investigation is beyond the scope of this study.

Next, we monitored the time course of simultaneous H₂ production and O₂ depletion with and without the presence of CpI...
These experiments were done with larger vials (8.4 ml) to decrease the impact of head space sampling (represented by the O₂ dilution trace). O₂ consumption by reduced CpFd was significantly slower when CpI was present, further suggesting that this hydrogenase does not reduce O₂ to H₂O. The hydrogenase activity appeared to reduce the rate of O₂ depletion, most likely by competing for electrons, and the O₂ depletion rate increased as the hydrogenase was inactivated. It is interesting, however, that O₂ depletion appeared to be somewhat slower in the presence of CpI even after H₂ production nearly ceased.

With these new insights, it is now important to review the observations shown in Figs. 2–4. In most cases, the immediate decrease in H₂ production rate after O₂ addition appears to be caused by electron loss directly from Fdred to O₂. This is particularly obvious in Fig. 3a because the slope decreases consistently by ~3 nmol/min, although the hydrogenase concentration varies by 100-fold. Fig. 2a indicates that the rate of electron loss from Fdred increases with increasing [O₂]. The immediate reduction in H₂ production rate after O₂ addition increases from 1.5 to 5.0 nmol/min as [O₂] increases from 1.0 to 5.0 vol %.

After the addition of 10% vol % O₂ and higher, it appears that the rate of electron loss from Fdred to O₂ is so high that no additional H₂ production can be produced.

Fig. 4b also indicates an immediate and consistent H₂ production rate decrease of 3 nmol/min, although [Fd] was increased 10-fold relative to the concentration used in the typical NADPH-driven assay (5 μM). This observation suggests that the FNR activity is rate-limiting such that [SynFdred] remains relatively constant and is primarily controlled by oxygen reactivity.

From the results shown in Fig. 4, the anaerobic TON with 50 nM CpI is about 4.5 s⁻¹¹, which can be compared with a kcat of about 400 s⁻¹ when SynFd is reduced by DTH, implying that in the NADPH-driven reactions, [SynFdred] is far below the Kₘ of about 18 μM. [SynFdred] is estimated at about 0.2 μM or only 4% of the 5.0 μM Fd in a typical assay. This is consistent with the interpretation that FNR is rate-limiting in the NADPH-driven assay because G6P and G6PD will maintain a stable NADPH supply. The diversion of ~60% of the electron flux to O₂ will tend to stabilize [SynFdred] at this low level, and this will also stabilize the rate of electron loss to O₂. Consequently, the continuous decreases in H₂ production rate in the presence of O₂ will primarily reflect decreases in active...
times $k_{cat}$, most likely the former quantity. Thus, exponential decay in $H_2$ production rate after $O_2$ addition appears to be an appropriate indicator of [FeFe] hydrogenase $O_2$ sensitivity.

**Validating the NADPH-driven Assay with the DTH-driven Assay**—To provide additional evidence that the previously described assay is correctly assessing $O_2$ tolerance, we implemented an alternative assay that assesses residual hydrogenase activity at multiple time points. Because reductive reactivation of $O_2$-exposed [FeFe] hydrogenase has been reported (14, 16), we evaluated hydrogenase activity using an assay in which CpI received electrons from DTH via CpFd to maximize the available redox potential. Periodic samples were taken from the NADPH-driven assay, $O_2$ was removed, and CpI activity was measured using the DTH-driven reaction sequence. Unlike in the NADPH-driven reaction sequence, DTH maintains CpFd primarily in the reduced state so that the higher effective reducing potential might reactivate partially modified CpI. In addition, we show that the anaerobic rate of $H_2$ production from the DTH-driven assay is linearly proportional to active [CpI], at least between 0.1 and 20 nm (Fig. 6a). The changes in $H_2$ production rate from this assay, therefore, indicate changes in residual CpI activity.

We also used CpFd in this alternate assay because it supports a higher CpI TON (1700 versus 400 s$^{-1}$ from SynFd) (10) and might be more likely to reactivate the hydrogenase. As shown in Fig. 6b, we obtained the same inactivation rate constant ($k_{inact,O2}$ = 0.099 min$^{-1}$) as we did from the much more convenient NADPH-driven assay. In our opinion, this confirms that the time course of changes in aerobic $H_2$ production rates observed in the NADPH-driven assay (Figs. 2 and 3) represents oxygenic inactivation of CpI. All $O_2$-exposed CpI samples produced $H_2$ at constant rates, suggesting that enzyme reactivation did not occur. Interestingly, we also obtained the same inactivation rate constant after incubating CpI with $O_2$ (without an electron supply) and assessing residual activity with the anaerobic DTH-driven assay (data not shown).

**$O_2$ Tolerance of CpI-Fd Fusion Proteins Versus CpI**—Previous studies have described [FeFe] hydrogenase-ferredoxin fusions that appear to increase $H_2$ production rates and reduce electron loss to competing pathways (25, 26). We hypothesized that such fusion proteins might also have different $O_2$ sensitivities. We therefore evaluated two fusion proteins that differ in the number and type of amino acids that link CpI to CpFd. In the first fusion protein, CpI-15aa-CpFd, the linker extended the CpI C terminus and consisted of 15 amino acids with three repeats of four glycines followed by a serine. In the second construction, CpI-55aa-CpFd, the linker was 55 amino acids long (details on the linker design can be found in Table 2) and was designed to be long enough so that the C-terminal extension could allow CpFd to approach its putative binding pocket on CpI.

Only the fusion protein with the shorter linker exhibited a significant enhancement in $O_2$ tolerance (Fig. 7) with a $k_{inact,O2}$ of 0.079 ± 0.009 min$^{-1}$ as compared with about 0.092 ± 0.010 min$^{-1}$ for CpI and the longer fusion protein, a decrease in sensitivity of about 13%. These studies were conducted with 50 μM RrFNR, 10 nm CpI or fusion protein, and 5.0 vol % $O_2$. Error bars represent S.D.
the NADPH-driven assay to allow a valid comparison between the wild-type (WT) CpI and the fusion proteins. Interestingly, CpI-15aa-CpFd also catalyzed H₂ production significantly faster (1.7-fold), presumably by supplying electrons to the active site at a faster rate (Fig. 7a). Previously, we speculated that higher electron flux to CpI might improve O₂ tolerance based on observations with higher [FNR] and lower [SynFd]. Here we observe the same correlation.

**Discussion**

In this study, we report the development of a convenient assay for assessing [FeFe] hydrogenase O₂ sensitivity. Reducing equivalents for H₂ production are supplied from NADPH via FNR and Fd as a more physiological solution phase alternative to PFV. During assay development, we discovered a significant rate of electron loss directly from reduced Fd to O₂. This occurred

**TABLE 2**

| Primers used in making CpI-CpFd fusion proteins | Mutants and primers |
|-----------------------------------------------|----------------------|
| Cpl-15aa-CpFd | 5'-GAA ATC CTG CAC TTT AAA TAT AAA AAA AAA GGA GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT TCA GCT TAT AAA ATT GCG GAT AGC T-3' |
| Backward | 5'-CCG GTT CAG GAA TAA TGA GTC GAC AGC TTG CGG CCG TCG CAC TCG AGC ACC ACC AC-3' |
| Cpl-55aa-CpFd | 5'-CCT GCA CTT TAA TTA TGA TAA ATC AGC CGG GTC-3' |
| 1’a,b | 5'-TTT CCC TTA CCT TTC GCA CTT CCC GCT GAT CCC GCC GAC CGG GCT GAT TTA TCA TAT TTA-3' |
| 2’c | 5'-AAC TGG GAA AGG AGG AAA AGG TGG CAG TGC GGG GAG CGC AGG CAG CGC AAA GGG CAA-3' |
| 3’a | 5'-TGC CTT TTT TTT GAA ACT GCG GAT GGC TCC ACG CAG ACC CTC TCT TCT TCT TGG CCC TTC-3' |
| 4’c | 5'-ATC CGC AGT TTG AAA AAA GCA AGG GGA AGG GGA AGG GTA AAG GCG CCT CCG CGG CTT CGT GTA-3' |

a These primers were assembled into a single oligonucleotide using a single PCR procedure.

b This primer provides an overlapping region with the plasmid containing the CpI gene to enable Gibson assembly.

c This primer provides an overlapping region with the plasmid containing the CpFd gene to enable Gibson assembly.

![FIGURE 6. Changes in anaerobic H₂ production activity of CpI due to O₂ exposure. a, the linear relationship between active [CpI] and H₂ production rate as indicated by the DTH-driven assay. The red line is a linear fit to the data; the H₂ production rate for each [CpI] was calculated from a time course of H₂ accumulation measured by GC with a linear fit to the first three time points (10-min intervals). b, time course of decrease in the anaerobic H₂ production activity of CpI by caused by 5.0 vol % O₂. Error bars represent S.D.]

![FIGURE 7. O₂ tolerance of the CpI-CpFd fusion proteins. a, time course of NADPH-driven H₂ production with 5.0 vol % O₂ introduced to the headspace at t = 10 min. b, changes in H₂ production rates after exposure to 5.0 vol % O₂. Error bars represent S.D.]

"[FeFe] Hydrogenase O₂ Sensitivity Assay"
with both SynFd and CpFd, although they differ significantly in molecular weight and the type of [Fe-S] clusters. Although the gradual decrease in \([O_2]\) has the potential to lower the \(k_{\text{inact,O2}}\) over time, the excellent agreement of the rate data with the first order model suggests that this effect is not significant in the assay we offer.

The assay was also validated using the DTH-driven activity assay that assesses periodic samples from the NADPH-driven assay. We obtained the same inactivation rate constant using the anaerobic DTH-driven assay (Fig. 6), which is performed with an electron source of higher redox potential. This validation would not have been possible if the hydrogen accumulation rates shown in Figs. 2 and 3 had been dominated by Fd\textsuperscript{red} oxidation by O\(_2\) rather than Cpl inactivation.

By using only the H\(_2\) production rates after O\(_2\) addition, the data from the NADPH-driven assay consistently indicate first order inactivation with rate constants insensitive to changes in initial [FeFe] hydrogenase concentrations. To further demonstrate the utility of the new assay, we show that a Cpl-Fd fusion protein has a decreased inactivation rate constant relative to Cpl possibly by improving the rate of electron supply to the enzyme.

Biological photosynthetic H\(_2\) production holds great promises for sustainable production of H\(_2\) as an important fuel and industrial chemical. However, a major barrier has been the lack of a production enzyme that tolerates the O\(_2\) produced as the unavoidable side product of photosynthesis. We believe that this convenient and potentially predictive assay for O\(_2\) sensitivity will provide an important tool for the discovery of O\(_2\)-tolerant hydrogenases. We also suggest that similar assay formats may be useful in assessing the functional properties of other redox-sensitive metallo-enzymes.

**Experimental Procedures**

**Protein Preparation**—RrFNR, SynFd, CpFd, and Cpl were expressed in vivo in E. coli and purified as described previously (2, 10, 15). Two different fusion proteins in which Cpl is linked to CpFd were designed and tested for effects on O\(_2\) tolerance. The gene for the Cpl-15aa-CpFd fusion (with a 15-amino acid linker composed of three repeats of Gly-Gly-Gly-Gly-Ser) was synthesized via assembly PCR and cloned into the pET21b vector with Gibson assembly. The gene for the other fusion protein, Cpl-55aa-CpFd (with a 55-amino acid linker), was synthesized in the following two steps. First, the variation of the pET21b vector that contains the genomic sequence of the WT Cpl was opened using NdeI and ScaI. Then, Gibson assembly was used to extend the gene to encode the linker and CpFd (Table 2). The same expression and purification system used for obtaining WT Cpl was employed in harvesting both fusion proteins (8). SDS-PAGE was used to verify the sizes of fusion proteins. The Bradford protein assay and methyl viologen fusion proteins (8). SDS-PAGE was used to verify the sizes of fusion proteins. The Bradford protein assay and methyl viologen assay were used to determine the total and active protein concentrations (8, 9).

**Investigation of the Oxygenic Inactivation of Cpl Using Gas Chromatography (GC)**—NADPH was purchased from Sigma-Aldrich, and DTH, G6P, and G6PD were purchased from Santa Cruz Biotechnology Inc. The solutions for both assays were prepared inside a N\(_2\)-only glovebox (Vacuum Atmosphere Co.).

Each reagent (unless otherwise stated) was added in the order indicated to the following final concentrations: 50 mM Tris-HCl buffer, pH 7.0, 10 mM G6P, 2.0 units of G6PD, 5.0 mM NADPH, 5.0 \(\mu\)M RrFNR, 5.0 \(\mu\)M SynFd, and the Cpl concentration indicated. 200- or 840-\(\mu\)l reaction volumes were prepared in 2.0-ml target screw thread vials (National Scientific) or 8.4-ml crimp vials (Fisher Scientific), respectively. The 8.4-ml vials were used only in investigating the kinetics of O\(_2\) consumption. Before sealing the vials with rubber septa, magnetic stir bars were added for mixing. 500 \(\mu\)M DTH was prepared separately in a new vial and added outside the glovebox by using a gas-tight syringe with a 25-gauge needle (Hamilton Co.).

After removal from the glovebox, the sealed vials were placed on a stir plate to initiate mixing at 235 rpm. Because all reagents for the NADPH-driven assay had been added inside the glovebox, some H\(_2\) production took place before making the first time point measurement (denoted by \(t = 0\) min); the typical amounts ranged from 5 to 15 nmol, which we subtracted when presenting data. A gas-tight syringe with a 23-gauge needle (Hamilton Co.) was used to sample 100 or 200 \(\mu\)l of the headspace every 10 min. H\(_2\) and O\(_2\) concentrations were determined using a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard) with a ShinCarbon ST 100/120 mesh column (Restek). All experiments were done in triplicate, and error bars in the figures represent S.D.

O\(_2\) was introduced to the headspace of the vials by injecting varying volumes of air and removing the same volume afterward (maintaining a constant headspace pressure before and after the introduction of O\(_2\)). In the experiments with 5.0 \(\%\) O\(_2\), for example, the samples were prepared inside the N\(_2\)-only glovebox, and 0.58 ml of air was typically injected after 10 min of anaerobic catalysis.

**Measuring the Full Residual Activity after O\(_2\) Exposure**—Glass vials containing the NADPH-driven assay solution with 10 \(\mu\)M Cpl (and 5.0 \(\mu\)M CpFd replacing 5.0 \(\mu\)M SynFd) were incubated with 5.0 \(\%\) O\(_2\) for 0, 10, 20, and 30 min. Next, the vials were purged with 100% N\(_2\) for 2.5 min at a flow rate of 630 ml/min. This removed the O\(_2\) previously introduced as well as accumulated H\(_2\). H\(_2\) production was stimulated by adding 6.0 \(\mu\)l of 500 mM DTH to a final concentration of 15 mM. H\(_2\) concentration in the headspace was measured at three time points to determine the rate of DTH-driven H\(_2\) production as an indication of residual Cpl activity. All experiments were done in duplicate on two separate occasions, and error bars in the figures represent S.D.

**Measuring O\(_2\) Consumption by Reduced Fd**—The experiments were conducted in 8.4-ml crimp vials where 840-\(\mu\)-l reaction mixtures contained the following: 50 mM Tris buffer, pH 7.0, 10 mM G6P, 4 units of G6PD, 5.0 mM NADPH, 5.0 \(\mu\)M RrFNR, and 5.0 \(\mu\)M of either SynFd or CpFd. 5.0 \(\%\) O\(_2\) was introduced to the headspace for both mixtures at \(t = 0\) min. O\(_2\) concentrations were measured at \(t = 0, 5, 10, 20, 40,\) and 60 min. The average consumption rate over an hour was obtained by calculating the time course of changes in [O\(_2\)] in the presence of the reaction mixture and subtracting the changes (caused by dilution) measured in a vial with only water. All experiments were done in duplicate on two separate occasions, and error bars in the figures represent S.D.
**[FeFe] Hydrogenase O2 Sensitivity Assay**

**Author Contributions**—J. R. S. and J. K. conceived of the approach, and J. K. conducted most of the experiments described. M. R. designed and produced the fusion proteins. J. R. S. and J. K. analyzed and interpreted the data with contributions from S. S., M. R., and K. M.

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