Investigation of the Ferredoxin’s Influence on the Anaerobic and Aerobic, Enzymatic H₂ Production

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Ferredoxins are metalloproteins that deliver electrons to several redox partners, including [FeFe] hydrogenases that are potentially a component of biological H₂ production technologies. Reduced ferredoxins can also lose electrons to molecular oxygen, which may lower the availability of electrons for cellular or synthetic reactions. Ferredoxins thus play a key role in diverse kinds of redox biochemistry, especially the enzymatic H₂ production catalyzed by [FeFe] hydrogenases. We investigated how the yield of anaerobic and aerobic H₂ production vary among the four different types of ferredoxins that are used to deliver electrons extracted from NADPH within the synthetic, fermentative pathway. We also assessed the electron loss due to O₂ reduction by reduced ferredoxins within the pathway, for which the difference was as high as five-fold. Our findings provide valuable insights for further improving biological H₂ production technologies and can also facilitate elucidation of mechanisms governing interactions between Fe–S cluster(s) and molecular oxygen.

Keywords: ferredoxin, biohydrogen, metalloprotein, redox biochemistry, oxidation

INTRODUCTION

Ferredoxins (Fd) are redox proteins that mediate electron metabolism in numerous kinds of cells across diverse organisms. The [Fe–S] cluster(s) in ferredoxins are responsible for electron receipt and transfer to redox partners. The type and number of [Fe–S] clusters in a ferredoxin can vary, which in turn affect the rate at which electrons are delivered, as well as redox potential of the protein (Guan et al., 2018). For example, Synechocystis sp. PCC 6803 ferredoxin (SynFd) has one [2Fe–2S] cluster while Clostridium pasteurianum ferredoxin (CpFd) harbors two [4Fe–4S] clusters. The midpoint redox potentials for the two are −412 and −387 mV, respectively (Bottin and Lagoutte, 1992; Brereton et al., 1999).

One of ferredoxins’ redox partners is a metalloprotein called hydrogenase. Hydrogenases can receive electrons from reduced ferredoxins (Fd_red) and combine them with protons to produce H₂. The enzymatic activity of hydrogenases, especially the [FeFe] subtype proficient in H₂ production such as the one from the C. pasteurianum (CpI), can be exploited for biological H₂ production (Lu and Koo, 2019); wide deployment of the technology can contribute to reduction of CO₂ emission while supplying H₂. Toward this aim, researchers have successfully developed both fermentative and photosynthetic pathways for reducing ferredoxins, which can subsequently be used for the enzymatic H₂ production (Smith et al., 2011; Yacoby et al., 2011). To date, however, people have tested H₂ production from these pathways only in an anaerobic reactor due to the high O₂ sensitivity of [FeFe] hydrogenases.

O₂ is a byproduct of photosynthesis and also an effective reagent for regenerating adenosine triphosphate (ATP) during fermentation. As such, it is an unavoidable constituent of the
aforementioned biological H₂ production processes; its influence on electron flow and H₂ production need to be understood. Previous work showed that Fd^{red} is capable of reducing O₂ into superoxide and peroxide (Allen, 1975), which is known as the Mehler reaction within the photosynthetic pathways. Hosein and Palmer also reported the Fd oxidation by O₂ and its autocatalytic nature (Hosein and Palmer, 1983). Since Fd^{red} is the source of electrons for the enzymatic H₂ production, presence of O₂ results in a lower H₂ yield via decreasing the amount of electrons delivered to the hydrogenases (Benemann et al., 1973; Koo and Swartz, 2018). O₂ also drives inactivation of the hydrogenases, which is irreversible and fast (within minutes under the atmospheric [O₂]) for most [FeFe] kinds (Lu and Koo, 2019). By fusing ferredoxin to an [FeFe] hydrogenase, Eilenberg et al. (2016) successfully increased the photosynthetic H₂ production in the presence of O₂, proposing that Fd^{red} delayed inactivation of the hydrogenase at the expense of electrons. The authors also confirmed the increase in aerobic H₂ production by fusing different types of ferredoxin and hydrogenase (Koo, 2020).

We developed physiological assays and analytical methods for characterizing O₂ sensitivity of [FeFe] hydrogenases during H₂ production (Koo et al., 2016). Using this method, the authors studied the enzymatic H₂ production in the presence of O₂ at a greater detail to better understand its implications for biological H₂ production and photosynthesis. First, we used isotopically labeled O₂, (¹⁸O₂) to confirm O₂ reduction by Fd^{red}. We then analyzed how the rates of O₂ reduction vary with varying concentrations of reactants, as well as with different types of ferredoxins harboring different kind and number of Fe–S clusters. Lastly, we studied how electron loss from Fd^{red} to O₂ varies among different combinations of ferredoxin NADP⁺ reductases (FNR) and ferredoxins with the goal of identifying the most effective combination in minimizing the electron leakage during the enzymatic H₂ production.

MATERIALS AND METHODS

Protein Expression and Purification

SynFd, CpFd, Zea mays Fd (ZmFd), Anabaena variabilis Fd (AnFd), Synechocystis sp. PCC 6803 FNR (SynFNR), and Rice root FNR (RfFNR) were expressed in vivo in Escherichia coli and purified as described previously (Lu et al., 2015). Cpi was also expressed heterologously in E. coli and purified as reported in the previous work (Koo and Swartz, 2018).

Reagent Preparation

Nicotinamide adenine dinucleotide phosphate (NADPH), dithionite, glucose-6-phosphate (G6P), G6P dehydrogenase (G6PD), superoxide dismutase (SOD) and catalase were purchased from Sigma-Aldrich.

O₂ Reduction and Aerobic H₂ Production Measurements

The O₂ reduction experiments were conducted in 8.4 mL crimp vials where 840 µL reaction mixtures contained the following (unless stated otherwise): 50 mM Tris buffer pH 7.0, 10 mM G6P, 4 units of G6PD, 5.0 mM NADPH, 5.0 or 50 µM FNR, and 5.0 µM of Fd. The reaction mixtures were prepared inside a N₂-only glovebox (CHOA Engineering). Before sealing the vials with rubber septa, magnetic stir bars were added for mixing. After removal from the glovebox, the sealed vials were placed on a stir plate to initiate mixing at 300 rpm. O₂ was introduced to the headspace at t = 0 min (or 10 min when examining various pairs of FNR and Fd) by using a syringe with a 20-gage needle (Daehan Sciences); air or a gas-tight handheld tank filled with O₂ was used as a source. O₂ and H₂ concentrations were measured by sampling 200 µL of the headspace with a valved 23-gage needle (Daehan Sciences), and using gas chromatography (GC 6500, YL Instrument). The H₂ production experiments were done in the same manner with the addition of 10 nM Cpi to the aforementioned reaction mixtures inside the anaerobic glovebox.

¹⁸O₂ Reduction Experiments

The amount of H₂¹⁸O formed by the reaction mixtures (50 mM Tris buffer pH 7.0, 10 mM G6P, 4 units of G6PD, 5.0 mM NADPH, 5.0 or 50 µM FNR, 5.0 µM of Fd, 4.2 U SOD and 4.2 U catalase) were measured as follows. The 8.4 mL crimp vials (Thermofisher Scientific) were first opened using a decapper, and the reaction buffer were immediately put in the 80 °C water bath (Daehan Sciences) for the following hour to terminate any ongoing enzymatic reactions. Next, the reaction buffer was distilled at 120 °C and only the water vapor was collected by condensation. The collected samples were mixed with distilled H₂O at two different ratios (2- and 10-fold dilution) and submitted together with a sample containing only distilled H₂O to the mass spectrometry (National Center for Inter-University Research Facilities, Seoul) for analysis of H₂¹⁸O content.

RESULTS AND DISCUSSION

Confirmation of ¹⁸O₂ Reduction by Fd^{red}

We have previously reported O₂ consumption by SynFd^{red} during fermentative H₂ production where electrons are extracted from NADPH and delivered to hydrogenases via RrFNR and SynFd (Koo et al., 2016). In this study, we replaced the source of O₂ from air to 10 vol% ¹⁸O₂. This allowed us to directly measure accumulation of H₂¹⁸O in the buffer using mass spectrometry. Since Fd^{red} was reported to be capable of only uni- and divalent reduction of O₂ (Allen, 1975), we modified the biochemical reaction as described in Figure 1. Instead of adding the hydrogenase enzyme for the NADPH-driven H₂ production (blue arrows from Fd^{red}), SOD and catalase were added in the absence of the hydrogenase (red arrows from Fd^{red}) to increase the likelihood that any reactive oxygen species (ROS) generated by Fd^{red} would be converted into H₂¹⁸O for isotopic detection.

Four reaction mixtures were prepared, incubated with 10 vol% ¹⁸O₂ for an hour, and analyzed to determine (1) consumption of the headspace ¹⁸O₂ and (2) formation of H₂¹⁸O. The concentrations of reagents were as follows: 10 mM G6P, 4 U G6PD, 5.0 mM NADPH, 5.0 µM RrFNR, 5.0 µM SynFd, 4 U SOD, and 4 U catalase in 50 mM Tris buffer, pH 7.0. Incremental

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addition of reagents in reaction sequence (Figure 1) starting at SynFd clearly confirmed our previous finding (Koo et al., 2016): SynFd\textsubscript{red} is responsible for O\textsubscript{2} consumption (Figure 2). The results also suggested that SynFd\textsubscript{red} is not capable of reducing O\textsubscript{2} fully into H\textsubscript{2}O. This was evident from the observation that a significant amount of H\textsubscript{2}\textsuperscript{18}O was formed only in the presence of both SOD and catalase. The fact that Synechocystis sp. cells have native SOD and catalase is consistent with our interpretation in that these enzymes can rapidly remove harmful O\textsubscript{2} radicals generated by SynFd\textsubscript{red}.

Assuming that SOD do not impact the O\textsubscript{2} reduction activity of SynFd\textsubscript{red}, we expected to see the decrease in headspace O\textsubscript{2} reduce by half (Figure 1, following the red arrows only from Fd\textsuperscript{red}) in the mixture containing only up to SOD. This is indeed what we observed: The loss of headspace O\textsubscript{2} after an hour decreased from roughly 2,900 to 1,600 nmoles upon addition of only SOD, approximately 50% reduction considering experimental error. We also expected to see a further reduction in the amount by which headspace O\textsubscript{2} decreases upon addition of catalase to the mixture containing SOD (Figure 1) since O\textsubscript{2} is regenerated in the presence of both. Indeed, the loss of headspace O\textsubscript{2} after an hour further decreased to roughly 1,000 nmoles. The water molecules containing \textsuperscript{18}O were only formed when both SOD and catalase were added to the reaction mixture. Furthermore, the amount of H\textsubscript{2}\textsuperscript{18}O produced, 1,500 nmoles, is similar to the stoichiometrically expected value of 1,300 nmoles: Each peroxide molecule generated by SOD will be converted into a water molecule by catalase. Based on the headspace O\textsubscript{2} loss, the average turnover number (TON) of O\textsubscript{2} reduction by SynFd was 11 per minute per reduced ferredoxin.

It is important to note that this TON is not physiologically relevant. Fd mostly delivers electrons for NADPH regeneration inside cells while, in our experiments, the SynFd molecules were reduced using the electrons extracted from NADPH to consume O\textsubscript{2}.

**Kinetic Study on O\textsubscript{2} Reduction by Fd\textsuperscript{red}**

We next examined how the rate of electron loss to O\textsubscript{2} varies with respect to O\textsubscript{2} partial pressure in the headspace. As discussed in the previous section, SOD and catalase affect the apparent O\textsubscript{2} consumption by regenerating about half of the putative O\textsubscript{2} radicals back to O\textsubscript{2}. We thus conducted the following experiments in the absence of SOD and catalase. The concentrations of reagents inside the sealed glass vials were as usual except for RfFNR, which was increased by 10-fold to 50 µM RfFNR. The higher [RfFNR] was chosen in order to enhance electron flux to Fd (K\textsubscript{M} of 18 or 39 µM for SynFd and CpFd, respectively) and thereby increase changes in the O\textsubscript{2} peaks for more accurate analysis.
The results summarized in Figure 3 and Supplementary Figure 1 indicated that the O₂ reduction rate, or equivalently the electron loss rate is directly proportional to the headspace [O₂]. A linear relationship between the two was observed for both types of Fd from 1 to 21 vol% O₂ in the headspace of the reactor vial. Within this range, the observed O₂ consumption rate varied from 21 to 255 or 46 to 479 nmoles/min for SynFd and CpFd, respectively. The corresponding specific rate of electron loss to O₂ ranged between 5 and 61 per minute for SynFd (assuming only univalent reduction of O₂) and approximately 1.9-fold higher for CpFd. As expected, increasing the [RrFNR] from 5 to 50 µM increased the specific rate of electron loss, but only by about three-fold.

Based on these observations, we analyzed the kinetic parameters for the reductive O₂ consumption. The following rate law (Eq. 1a) was proposed where the consumption is modeled as a non-enzymatic biochemical reaction between Fd<sup>red</sup> and O₂:

\[
\frac{d [O₂]}{dt} = -k_1[Fd^{red}]^a[O₂]^b
\]  

where [O₂] refers to the concentration of O₂ in the aqueous buffer solution. For the cases where [Fd<sup>red</sup>] is fixed, the rate law further simplifies into the equation only dependent on [O₂] (Eq. 1b). This will be true if the electron flux from FNR to Fd increases as [O₂] increases to maintain [Fd<sup>red</sup>] approximately constant. We observe a linear fit for the plot of δO₂/δt versus [O₂] (Figure 3), which suggests that the O₂ reduction by Fd<sup>red</sup> follows a pseudo first order rate law under the experimental conditions. The rate constant of this kinetic model (eqn. 2, b = 1), namely k₁, is calculated to be 0.0013 and 0.0025 per minute for SynFd and CpFd, respectively.

A multitude of factors may be responsible for the differences in the O₂ reduction activity of the two types of Fd. To begin with, SynFd harbors one [2Fe–2S] cluster while there are two [4Fe–4S] clusters in CpFd (Bertini et al., 1995; Van Den Heuvel et al., 2003). The former is known to be capable of transitioning between [2Fe–2S]<sup>2+</sup> and [2Fe–2S]<sup>3+</sup> while the latter can undergo changes among three states—[4Fe–4S]<sup>3+</sup>, [4Fe–4S]<sup>2+</sup>, and [4Fe–4S]<sup>1+</sup> (Yao et al., 2012). The midpoint redox potentials (RE) are similar: −412 and −387 mV for SynFd and CpFd, respectively (Grzyb et al., 2018). The dissociation constants with RrFNR and CpI are also not significantly different. In this regard, the difference in the range and nature of oxidation states appear to be more important with respect to the Fd’s O₂ reduction activity. The results in the subsequent section suggests that the local environment surrounding the Fe–S clusters also matter.

**Aerobic H₂ Production From Various Pairs of FNR and Fd**

We have previously reported that the rate of O₂ consumption is different when SynFd versus CpFd is used for the NADPH-driven H₂ production pathway (Koo et al., 2016). The rate was approximately twice as fast with CpFd, meaning that more electrons are lost to O₂ reduction instead of being used for H₂ production by the hydrogenase. The choice of CpFd over SynFd would therefore result in a lower NADPH-driven H₂ production efficiency. In this manner, the choice of Fd can impact the efficiency and overall yield of aerobic, biological H₂ production when Fd molecules deliver electrons to the hydrogenase enzyme.

We examined various combinations of Fd and FNR to study how the electron loss to O₂ differs during H₂ production (Figure 1, co-existence of both the blue and red arrow pathways) under 5.0 vol% O₂. Different FNRs were evaluated as well since the electron flux rate through the FNR could influence the steady state [Fd<sup>red</sup>] (Bingham et al., 2012; Lu et al., 2015), which in turn would affect the rate of electron loss to O₂. The concentrations of the reagents were as follows: 10 mM G6P, 1 U G6PD, 5 mM NADPH, 5 µM FNR, 5 µM Fd, and 10 nM WT CpI in 50 mM Tris–HCl buffer pH 7.0. The reaction lasted for an hour, and we analyzed the data with respect to (1) anaerobic FNR TON during H₂ production, (2) relative aerobic H₂ yield, and (3) the overall change in the headspace [O₂].

The results (Table 1) indicated that electrons in Fd<sup>red</sup> are lost to O₂ at a faster rate with a higher (anaerobic) FNR TON within the NADPH-driven H₂ production (r = 0.82). This was expected since a higher FNR TON means a greater electron flux to Fd. In contrast, there was insignificant correlation between the anaerobic FNR TON and the relative aerobic H₂ yield of the reaction (r = −0.25). This confirmed our previous finding from the CpI mutagenesis study where we reported insignificant relationship between the two variables (Koo and Swartz, 2018). This observation suggests that it may be possible to improve both the electron flux and aerobic H₂ yield by engineering FNR and/or Fd. Table 1 also revealed that AnFd is the most efficient in delivering electrons to the hydrogenase in the presence of O₂.
TABLE 1 | The anaerobic FNR TON, the relative aerobic H₂ yield, and the amount of reductive O₂ consumption over 1 hour for various combinations of FNR-Fd under 5.0% O₂.

| Fd  | SynFNR  | ReFNR  | SynFNR  | ReFNR  | SynFNR  | ReFNR  | SynFNR  | ReFNR  |
|-----|---------|--------|---------|--------|---------|--------|---------|--------|
| FNR TON (min⁻¹) | SynFd | 5.2    | 4.4     | 5.0    | 10.5   | 2.7    | 4.9     | 3.8    | 6.0    |
| H₂ production (μmoles) | 0.11  | 0.07   | 0.09    | 0.16   | 0.06   | 0.09   | 0.07    | 0.16   |
| H₂ yield (%) | 8.5    | 6.7    | 6.8     | 6.1    | 8.1    | 7.0    | 7.3     | 10.4   |
| ΔO₂ (μmoles) | 0.9    | 1.8    | 1.8     | 3.3    | 1.0    | 1.5    | 0.7     | 1.0    |
| e⁻ flux to O₂ reduction (%) | 28    | 54     | 51      | 50     | 46     | 48     | 34      | 24     |

The relative aerobic H₂ yield refers to the percent of expected production based on the anaerobic production rate. For example, for the combination of SynFNR and SynFd, the anaerobic FNR TON is 5.2 per minute and the amount of H₂ produced anaerobically over an hour is about 1.3 μmoles; the aerobic H₂ yield of 8.5% means that 0.1 μmoles are produced instead in the presence of 5.0 vol% O₂ in the headspace. For further explanations, please refer to our previous work (Koo et al., 2016).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2021.641305/full#supplementary-material
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