A Stepwise NaHSO₃ Addition Mode Greatly Improves H₂ Photoproduction in Chlamydomonas reinhardtii

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NaHSO₃ addition greatly increases the yield of H₂ photoproduction in a unicellular green alga Chlamydomonas reinhardtii through removing O₂ and activating hydrogenase but significantly impairs the activity of PSII, an electron source for H₂ photoproduction. Here, a stepwise addition mode of total 13 mM NaHSO₃, an optimal concentration for H₂ photoproduction of C. reinhardtii identified in a previous one step addition method, significantly improved H₂ photoproduction. Such improvement was believed to be the result of increased residual PSII activity in an anaerobic background, but was at least independent of two alternative electron sinks for H₂ photoproduction, cyclic electron transport around PSI and CO₂ assimilation. Based on the above results, we propose that increased residual PSII activity in an anaerobic environment is an efficient strategy to enhance H₂ photoproduction in C. reinhardtii, and the stepwise NaHSO₃ addition mode is a case study in the strategy.

INTRODUCTION

With the increasing awareness of fossil fuel depletion and global warming, efforts have been undertaken to develop clean and sustainable energy sources (McKendry, 2002). Molecular hydrogen (H₂) is one of the potential future energy sources (Hansel and Lindblad, 1998; Momirlan and Veziroglu, 2002). C. reinhardtii, a unicellular green alga, has been recognized as an ideal system for sustainable H₂ photoproduction under anaerobic conditions; however, this alga cannot efficiently and continuously produce H₂ in an aerobic environment because its H₂ase is extremely sensitive to O₂ (Ghirardi et al., 1997). To activate H₂ase for sustainable and efficient H₂ photoproduction in C. reinhardtii, therefore, numerous strategies have been extensively developed mainly through engineering O₂ tolerance in H₂ase (Flynn et al., 2002; Liebgott et al., 2011; Wu et al., 2011) or decreasing O₂ content around H₂ase (Melis et al., 2000; Kruse et al., 2005; Surzycki et al., 2007; Wu et al., 2007; Wu et al., 2010; Xu et al., 2011; Jurado-Oller et al., 2015; Xiong et al., 2015; Shu et al., 2018).

Abbreviations: AA, antimycin A; CET, cyclic electron transport around PSI; Chl, chlorophyll; C. reinhardtii, Chlamydomonas reinhardtii; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DO, dissolved oxygen; Fₘ/Fₚ, maximal quantum yield of PSII; GA, glycolaldehyde; H₂ase, hydrogenase; Lin, lincomycin; MV, methyl viologen; one step method, a one step addition method of NaHSO₃; stepwise mode, a stepwise addition mode of NaHSO₃; TAP, Tris-acetate-phosphate.
Meanwhile, our studies demonstrate that NaHSO$_3$ addition strategy is capable of decreased the O$_2$ content around H$_2$ase, thereby activating the enzyme activity and promoting H$_2$ photoproduction (Wang et al., 2010; Ma et al., 2011; Wei et al., 2017). This strategy can result in an approximately 10-fold or 200-fold increase in H$_2$ photoproduction in the nitrogen-fixing cyanobacterium Anabaena sp. strain PCC 7120 (Wang et al., 2010) or the unicellular green alga C. reinhardtii (Ma et al., 2011; Wei et al., 2017), respectively. Despite these increases, this yield is still not sufficient to meet the requirements of industrial applications. Thus, extensive optimization of this NaHSO$_3$ addition strategy is necessary to increase H$_2$ photoproduction in C. reinhardtii further.

The yield of H$_2$ photoproduction caused by sulfur deprivation is also not sufficient to meet the requirements of industrial applications. Under sulfur deprivation conditions, therefore, many strategies have been developed to improve the yield of H$_2$ photoproduction in C. reinhardtii via metabolic and genetic engineering (for review, see Esquível et al., 2011; Dubini and Ghirardi, 2015). Among them, increased residual photosystem II (PSII) activity was found to play a vital role in efficient H$_2$ photoproduction (Zhang et al., 2002; Kosourova et al., 2005; Kim et al., 2010; Volgusheva et al., 2013; Grewe et al., 2014; Steinbeck et al., 2015; Chen et al., 2016), since the PSII activity is significantly impaired by sulfur deprivation (Melis et al., 2000). Similarly, in the NaHSO$_3$ addition background, the PSII activity is also significantly impaired (Wang et al., 2010). To test whether increased residual PSII activity in the NaHSO$_3$ addition background can also enhance H$_2$ photoproduction, we monitored the accumulated H$_2$ level and residual PSII activity in the stepwise mode of total 13 mM NaHSO$_3$, an optimal concentration for H$_2$ production of C. reinhardtii identified in a previous one step addition method (Ma et al., 2011). We also measured the content of dissolved O$_2$ and activities of two alternative electron sinks for H$_2$ photoproduction, CET and CO$_2$ assimilation, in the stepwise NaHSO$_3$ addition mode. Our results demonstrate that the stepwise NaHSO$_3$ addition mode evidently enhances the yield of H$_2$ photoproduction in C. reinhardtii; such enhancement is mostly the result of increased residual PSII activity in an anaerobic environment, but is at least independent of two alternative electron sinks for H$_2$ photoproduction.

**MATERIALS AND METHODS**

**Culture Conditions**

Cells of C. reinhardtii (CC-503 strain) were cultured at 25°C in TAP medium (Harris, 1989). The medium was buffered with Tris–HCl (20 mM; pH 7.3), bubbled with air under continuous illumination with cool-white fluorescent lamps (40 μmol photons m$^{-2}$s$^{-1}$), and inoculated with approximately 8.1 × 10$^4$ cells mL$^{-1}$ of C. reinhardtii (inoculum size, 1%).

**Sample Preparation and NaHSO$_3$ Addition**

Cells of C. reinhardtii were continuously illuminated by growth light of 40 μmol photons m$^{-2}$s$^{-1}$ and were cultured in 0.5 L of TAP medium for 2 days with bubble aeration ($A_{750}$ = 0.8–1.0), after which a fixed volume of cells containing 300 μg of Chl was transferred to 60 mL serum bottles (30 mL head space and 30 mL cells) with rubber seals. After cells were statically pre-cultured under continuous illumination of 200 μmol photons m$^{-2}$s$^{-1}$ for 36 h, total 13 mM of NaHSO$_3$ was directly or step by step added to the serum bottles, as indicated in Figures 1, 2, Supplementary Figure S1, and described in Table 1, with Lin of 5 mM (final concentration) or AA of 10 μM (final concentration) or GA of 2 mM (final concentration) or DCMU of 20 μM (final concentration) or not. Subsequently, the

![FIGURE 1](https://example.com/figure1.png)

FIGURE 1 | A stepwise NaHSO$_3$ addition mode significantly increases (A) H$_2$ photoproduction, and (B) in vivo and (C) in vitro H$_2$ase activity in C. reinhardtii. After cells were statically pre-cultured under continuous illumination of 200 μmol photons m$^{-2}$s$^{-1}$ for 36 h, total 13 mM of NaHSO$_3$ was directly or step by step added to the serum bottles, as indicated by arrows 1–3 and described in Table 1. Values are means ± SD (n = 5).
cells were still illuminated at 200 μmol photons m⁻²s⁻¹ or were incubated in the dark to induce the production of H₂.

**Monitoring H₂ Photoproduction**

At predetermined time intervals, 200 μL of gas samples were withdrawn from the bottles using a gas-tight syringe and injected into a gas chromatograph (Agilent 7890A; Agilent Technologies Inc., United States) with a thermal conductivity detector for determining the concentrations of H₂, O₂, and N₂ simultaneously. The column of the gas chromatograph was a molecular sieve column (type 5Å; 2 m × 1/8 mm), and argon was used as the carrier gas.

**H₂ase Activity Assay**

*In vivo* and *in vitro* H₂ase activity was monitored as described earlier (Ma et al., 2011; Wei et al., 2013, 2017) with some modifications. In brief, 1 mL cell suspension samples upon exposure to 200 μmol photons m⁻²s⁻¹ were anaerobically withdrawn from the 60 mL serum bottles at designated time points (see Figures 1B,C) and then injected into 10 mL glass vials. To measure *in vivo* H₂ase activity, the cell suspension samples were immediately purged with argon gas for 1 min to eliminate the inhibitory effect of O₂ on the H₂ase activity. The cell suspension samples were then placed in a 25°C water bath for 1 h and shaken continuously (150 rpm) whilst exposed to a constant light of 200 μmol photons m⁻²s⁻¹. To measure *in vitro* H₂ase activity, we used vials containing 1 mL of 10 mM oxidized MV prepared in O₂-free 50 mM Tris buffer (for pH 7.1–9.0) and 0.2% (w/v) Triton X-100. The reaction was started when MV was reduced by the addition of 100 μL of 100 mM anaerobic sodium dithionite in 0.03 N NaOH. This assay was performed at 37°C in the dark for 20 min. We determined the amount of H₂ produced in the headspace of the glass vial by gas chromatography, and the rate of H₂ production was calculated on the basis of the total Chl content in the glass vial, unless otherwise indicated.

**Dissolved Oxygen Measurement**

Dissolved oxygen was monitored as described earlier (Wei et al., 2017). In brief, a DO meter (Orion Star A213, Thermo Scientific, United States) was used to monitor the DO attenuation process after the addition of NaHSO₃ to the cell suspension cultures of *C. reinhardtii*. The DO meter was corrected before each measurement. The DO meter probe was placed in the middle of the cell suspension cultures and the data were recorded at several designated time points.

**Chl Fluorescence and P700 Analysis**

The yields of Chl fluorescence at a steady-state of electron transport were measured at room temperature using a Dual-PAM-100 monitoring system (Walz, Effeltrich, Germany) equipped with an ED-101US/MD unit (Schreiber et al., 1986; Ma et al., 2008; Wei et al., 2013, 2017). Minimal fluorescence at open PSII centers in the dark-adapted state (F₀) was excited by a weak measuring light (650 nm) at a photon flux density of 0.05–0.15 μmol photons m⁻²s⁻¹. A saturating pulse of red light (600 ms, 10,000 μmol photons m⁻²s⁻¹) was applied to determine the maximal fluorescence at closed PSII centers in the dark-adapted state (Fₘ). Fₘ/F₀ was evaluated as (Fₘ−F₀)/F₀ (Kitajima and Butler, 1975; Wei et al., 2013, 2017).

The reduction of P700⁺ in darkness was measured with the aforementioned Dual-PAM-100 fluorometer by monitoring absorbance changes at 830 nm and using 875 nm as a reference. Cells were kept in the dark for 2 min, and 10 μM of DCMU was added to the cell suspension cultures prior to the measurement. The P700 was oxidized by far-red light with a maximum at 720 nm from a light-emitting diode lamp for 30 s, and the subsequent re-reduction of P700⁺ in the dark was monitored and its half-time was calculated.

**Oxygen Evolution Activity**

Oxygen production in intact *C. reinhardtii* cells by photosynthesis was determined at 25°C by monitoring the changes in O₂ levels with a Clark-type oxygen electrode (Hansatech Instruments, King’s Lynn, United Kingdom). Prior to the measurements, 10 mM of NaHCO₃ was added to the

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**TABLE 1 |** A table schematically represents one step method and stepwise mode of total 15 mM NaHSO₃.

| NaHSO₃ addition | Arrow 1 | Arrow 2 | Arrow 3 | Total |
|-----------------|-------|-------|-------|------|
| One step method | 13    | 0     | 0     | 13   |
| Stepwise mode   | 9     | 2     | 2     | 13   |

Arrows 1–3 shown in Figures 1–5 and both arrows have a 24 h interval.
cell suspension cultures. The intensity of light used for the measurements was 1,000 μmol photons m⁻²s⁻¹.

RESULTS

A Stepwise NaHSO₃ Addition Mode Significantly Increases the Yield of H₂ Photoproduction in C. reinhardtii

To test whether a stepwise NaHSO₃ addition mode can enhance the yield of H₂ photoproduction, we monitored accumulated H₂ amounts in the one step addition method and stepwise mode of total 13 mM NaHSO₃ (hereafter one step method and stepwise mode, respectively; see Table 1), an optimal concentration for H₂ photoproduction of C. reinhardtii identified in a previous one step method (Ma et al., 2011). Compared to the one step method, stepwise mode evidently enhanced the yield of H₂ photoproduction (Figure 1A and Table 2). The H₂ level in stepwise mode was approximately 1.5 times greater than that in one step method and, approximately 350 times greater than that in untreated cells (Figure 1A and Table 2). This was confirmed by the results of in vivo (Figure 1B) and in vitro (Figure 1C) H₂ase activity. We therefore conclude that the stepwise mode considerably improves H₂ photoproduction in the green alga C. reinhardtii.

The Stepwise Mode Can Also Establish an Anaerobic Environment

To elucidate the mechanism underlying the increase in the H₂ yield under stepwise mode, we monitored the dissolved O₂ (DO) content in the cell suspension cultures of C. reinhardtii. The results indicated that addition of total 13 mM NaHSO₃ to the cell suspension cultures in both the one step method and stepwise mode can similarly create an anaerobic environment (Figure 2), although the stepwise mode was slightly slow to generate an anaerobic environment when compared to the one step method (insert in Figure 2). It is worthy of note that, when an initial concentration of NaHSO₃ in stepwise mode was less than or equal to 7 mM, the cell suspension cultures did not enter or maintain an anaerobic environment, which evidently suppressed the increase of H₂ photoproduction in the stepwise mode (data not shown). Based on the above results, we propose that the stepwise mode is necessary to operate in an anaerobic environment as an efficient strategy for H₂ photoproduction in C. reinhardtii.

The Stepwise Mode Maintains a Relatively High Residual Activity of Electron Source for H₂ Photoproduction

To understand why the stepwise mode can increase the yield of H₂ photoproduction, we measured the activity of PSII, an electron source for H₂ photoproduction. After the addition of total 13 mM NaHSO₃ to the cell suspension cultures, the residual activity of PSII was much higher in stepwise mode than that in one step method, as evaluated by the F₆/F₃ values (Figure 3). This was supported by the results that the stepwise mode maintained a slightly high DO content at an efficient H₂ production stage when compared to the one step method (Figure 2). This implies that the relatively high PSII activity under anaerobic conditions is an important reason for improved H₂ photoproduction in the stepwise mode.

The Stepwise Mode Slightly Enhances the Activities of Two Alternative Electron Sinks for H₂ Photoproduction

We also measured the activities of CET and CO₂ assimilation, two alternative electron sinks for H₂ photoproduction. The rates of CET and CO₂ assimilation were slightly faster in the stepwise mode than those in the one step method, as estimated by the rate of re-reduction of P700⁺ (Figure 4), and photosynthetic production of O₂ with NaHCO₃ as an artificial electron acceptor (Figure 5), respectively. It appears plausible that at least the two alternative electron sinks for H₂ photoproduction do not contribute to enhance the photoproduction of H₂ in the stepwise mode. If this possibility is true, an increase in H₂ photoproduction caused by impaired the activity of either CET or CO₂ assimilation will be higher in the stepwise mode than that in the one step method. The results shown in Figure 6 support our hypothesis that the increase in H₂ photoproduction...
FIGURE 4 | A stepwise addition mode slightly alleviates the inhibitory effects of NaHSO$_3$ on cyclic electron transport around PSi in C. reinhardtii. The rate of cyclic electron transport around PSi was judged by half-time of P700$^+$ dark reduction. Values are means ± SD (n = 5).

FIGURE 5 | A stepwise addition mode slightly alleviates the inhibitory effects of NaHSO$_3$ on CO$_2$ assimilation in C. reinhardtii. Activity of CO$_2$ assimilation was assessed by photosynthetic production of O$_2$ with NaHCO$_3$ as an artificial electron acceptor. Values are means ± SD (n = 5).

was slightly higher in the stepwise mode than that in the one step method in the presence of either AA that specifically inhibits the CET activity (Tagawa et al., 1963) or GA that disrupts the Calvin–Benson cycle activity via inhibiting the phosphoribulokinase (Rühle et al., 2008). Taking all these results together, we may conclude that in the anaerobic background, increased residual PSII activity can significantly enhance the yield of H$_2$ photoproduction in C. reinhardtii.

If this conclusion is true, impaired PSII activity in an anaerobic environment is an efficient strategy to improve H$_2$ photoproduction in C. reinhardtii and the stepwise NaHSO$_3$ addition mode is a case study in this strategy.

DISCUSSION

Whether NaHSO$_3$ addition promotes photosynthesis or H$_2$ photoproduction depends on its concentrations: NaHSO$_3$ in a low amount improves photosynthesis (Wang et al., 2003) but in a moderate amount can enhance H$_2$ photoproduction (Wang et al., 2010; Ma et al., 2011). Wang et al. (2003) demonstrate that a low amount (100 μM) of NaHSO$_3$ increases cyclic photophosphorylation and consequently improves photosynthesis via optimizing the ATP/NADPH ratio. By contrast, Wei et al. (2017) demonstrate that a moderate amount (13 mM) of NaHSO$_3$ can remove O$_2$ efficiently through the reaction of bisulfite with superoxide anion produced at the acceptor side of PSI, especially under sufficient light conditions, consequently activates H$_2$ase and promotes H$_2$ photoproduction. The results of this study indicate that a moderate amount of NaHSO$_3$ under a stepwise addition mode can quickly establish an anaerobic environment (Figure 2) and significantly improves H$_2$ photoproduction in a unicellular green alga C. reinhardtii (Table 1 and Figure 1). Such improvement is at least independent of two alternative electron sinks for H$_2$ photoproduction, CET (Figures 4, 6), and CO$_2$ assimilation (Figures 5, 6) and, most likely the result of maintained a relatively high electron source for H$_2$ photoproduction, PSII activity (Figures 3, 6).

Under a photon flux density of 200 μmol photons m$^{-2}$s$^{-1}$, the Mehler reaction is usually considered to also be an important alternative electron sink for H$_2$ photoproduction. However, we found that the Mehler reaction is almost absent in NaHSO$_3$ addition strategy, regardless of either one step method or stepwise mode (data not shown), possibly because addition of NaHSO$_3$ to...
the cell suspension cultures quickly results in entering of cells to an anaerobic environment (less than 800 s) (Figure 2). Therefore, the improvement of H2 photoproduction in C. reinhardtii by a moderate amount of NaHSO3 under a stepwise addition mode is also independent of a third alternative electron sink for H2 photoproduction. Mehler reaction, and consolidating the above mentioned possibility that such improvement is the result of increased residual PSII activity, an electron source for H2 photoproduction.

Based on different sources of electrons to H2ase, three pathways for H2 production have been identified in C. reinhardtii. Their sources of electrons to H2ase come from water photolysis via PSII (Melis et al., 2000; Kosourov et al., 2003), NADPH through type II NAD(P)H dehydrogenase (Baltz et al., 2014) and the fermentative degradation of endogenous compounds (Gieller and Gibbs, 1985), respectively. We observed that production of H2 under photon flux densities of 200 µmol photons m−2 s−1 by NaHSO3 addition was almost completely suppressed in cells incubated in the dark (Supplementary Figures S1A,B) or treated with DCMU (Supplementary Figures S1A,C). A quick establishment of anaerobic environment by NaHSO3 addition (Figure 2) suppresses the acetate uptake (Jurado-Oller et al., 2015) and impairs the mitochondrial respiratory electron transport chain function. Taking all these results together, we propose that in the NaHSO3 addition strategy, the source of electrons for H2 production predominantly, if not totally, comes from water photolysis via PSII, regardless of either the one step method or the stepwise mode.

The results of this study further indicate that the stepwise mode increased the maximum accumulated H2 levels, produced a higher maximum velocity of H2 photoproduction, and prolonged the time length of H2 photoproduction when compared to the one step method (Table 2). We thus propose that the stepwise mode developed in this study is an efficient and sustained strategy for improving H2 photoproduction in the green alga C. reinhardtii.

Although a moderate amount of NaHSO3 can remove efficiently O2, the activity of PSII, an electron source for H2 photoproduction, is also significantly impaired (Wang et al., 2010; Ma et al., 2011; Wei et al., 2017). The results of this study observe that in the anaerobic background, a stepwise mode maintains a relatively high PSII activity (Figure 3) and consequently promotes H2 photoproduction (Figure 1). The cause and effect of PSII activity and H2 photoproduction is also present in the sulfur-deprived strategy (Zhang et al., 2002; Kosourov et al., 2005; Kim et al., 2010; Volgusheva et al., 2013; Grewe et al., 2014; Steinbeck et al., 2015; Chen et al., 2016) but the reasons why H2 photoproduction is terminated in sulfur deprivation and NaHSO3 addition strategies are distinctly different. It is known that H2 photoproduction is terminated in the sulfur deprivation strategy because of cell death (Nguyen et al., 2008) and in the NaHSO3 addition strategy because of conversion of too much bisulfite to sulfate (Wei et al., 2017). It is worthy of note that the relationship between H2 production and biomass accumulation in sulfur deprivation and NaHSO3 addition strategies is also distinctly different. Regardless of either one step method or stepwise mode, the simultaneous production of H2 and biomass is present in NaHSO3 addition strategy, as observed in mixotrophic nutrient-replete cultures under low light conditions (Jurado-Oller et al., 2015), but is absent in sulfur deprivation strategy. Therefore, it appears reasonable that improved PSII activity in the NaHSO3 background is considered to be a better strategy to meet future application requirements in comparison with that in the sulfur-deprived background.

**AUTHOR CONTRIBUTIONS**

WM designed and supervised the experiments. XL, BF, and ZR performed the experiments and analyzed the data. LW and WM analyzed and interpreted the data and wrote the article.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01532/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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