^{13}C saturation transfer effect of bicarbonate-carbon dioxide exchange catalyzed by extracellular carbonic anhydrase in *Chlamydomonas reinhardtii*

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Abstract
Acetate assimilation in *C. reinhardtii* leads to bicarbonate and CO_2^{aq} formation in heterotrophic growth condition. Bicarbonate and CO_2^{aq} thus formed under this condition remain in equilibrium with the action of carbonic anhydrases. Carbonic anhydrase catalyzes reversible hydration of carbon dioxide and dehydration of bicarbonate. In this article we report that the rapid exchange catalyzed by extracellular carbonic anhydrase causes a large magnetization (saturation) transfer effect on the ^{13}C signal of bicarbonate at 161.01 ppm when the resonance of the carbon dioxide (aq) at 125.48 ppm is irradiated with RF pulses. In *C. reinhardtii* extracellular space the unidirectional, pseudo first-order rate constant of this exchange in the dehydration direction was determined to be 0.011 ± 0.005 sec^{-1}. The presence of highly specific carbonic anhydrase inhibitor acetazolamide, was also shown to drastically attenuate the observed ^{13}C magnetization transfer effect of the carbon dioxide–bicarbonate exchange in *C. reinhardtii*. We have demonstrated the utility of ^{13}C saturation transfer for determining the exchange rate between bicarbonate and carbon dioxide catalyzed by extracellular carbonic anhydrase in *C. reinhardtii* extracellular space.This study for the first time reports the dehydration rate of bicarbonate to CO_2 in live *C. reinhardtii* cells.

Keywords: acetate metabolism, bicarbonate, carbonic anhydrase, carbon-13, carbon dioxide, magnetic resonance spectroscopy, magnetization transfer

Introduction

Carbonic anhydrase (CA, EC 4.2.1.1) is a ubiquitous, monomeric zinc metalloenzyme, majority of it in *Chlamydomonas reinhardtii* is located in the periplasmic space [1,2]. Carbonic anhydrase is well-characterized protein synthesized by *C. reinhardtii* in response to low CO_2 levels [3]. In cultures of the cell wall-less mutant of *C. reinhardtii*, *cw-15*, between 80% and 90% of the carbonic anhydrase activity is released into the growth medium [1]. After separation of the cells from the medium, other have shown carbonic anhydrase presence (by concentrating the culture medium) and identify a polypeptide with an MW of 37,000 Da [1]. It has been found that the catalytically active CA catalyzes the reversible hydration of carbon dioxide [4]:

$$CO_2 + H_2O \xrightarrow{CA} H^+ + HCO3^-$$

The acid-base balance is tightly controlled in plant cells. Thus, formed extracellular HCO_3^- act as a buffer, which resists changes in pH through inter-conversion with CO_2 in the reaction catalysed by CA. In a case where medium possesses HCO_3- and CO_2 pH could be determined, as their concentration ratio can be used to calculate pH from the Henderson-Hasselbalch equation [5], assuming the pKa is known *in vivo* to be 6.17 [6],

$$pH = pKa + \log_{10}([HCO_3^-]/[CO_2])$$

The biosynthesis of this extracellular CA may provide a model system for examining the events required for the synthesis and secretion of polypeptides and the regulatory mechanisms

involved in their adaptation to a low CO_2 atmosphere. *Chlamydomonas reinhartdii* can be grown photosynthetically, heterotrophically on acetate or mixotrophically, a combination of the two acetate and CO_2 [7]. As such, it can work aerobically and anaerobically. When grown on a diurnal cycle, *C. reinhartdii* undergoes photosynthesis during the day, fixing CO_2 and storing energy in the form of starch and lipids [8]. At night, *C. reinhartdii* switches its metabolism to degrade the stored starch into acetate [9] which can then be used either aerobically or anaerobically through the ATP dependent production of acetyl-CoA.

This study is based on the observation of *C. reinhardtii* cells to produce bicarbonate and CO_2 in heterotrophic growth conditions. Complete acetate assimilation led to the maximum formation of bicarbonate and CO_2 in heterotrophic growth conditions [10]. *C. reinhardtii* can use both CO_2 and HCO_3- as the inorganic sources of carbon, relative availability of which to cells is determined by pH of the solution.

To this end, we studied the rapid exchange catalyzed by extracellular carbonic anhydrases in *C. reinhardtii* that causes a large magnetization transfer effect on the ^{13}C signal of bicarbonate at 161.01 ppm when the resonance of the undetectable carbon dioxide at 125.48 ppm is irradiated with RF pulses. In *cw15* strain of *C. reinhardtii* the unidirectional, pseudo first-order rate constant of this exchange in the dehydration direction was determined by ^{13}C-NMR saturation transfer experiment. Although the line broadening has been used to measure the exchange rate using NOE/exchange NMR spectroscopy earlier [11].

The current study is useful for carbon concentration mechanisms study in *C. reinhardtii* when inorganic carbon (Ci; CO_2 and/or HCO_3-)) is limited. By active Ci uptake systems, internal Ci levels are increased and then carbonic anhydrase supplies sufficient CO_2 to ribulose 1, 5-bisphosphate carboxylase/oxygenase by the dehydration of accumulated bicarbonate. This study for the first time reports the dehydration rate of bicarbonate to CO_2 in live *C. reinhardtii* cells.

Results and Discussion

C. reinhardtii is a metabolically very active microorganism that can be grown phototrophically, heterotrophically as well as mixotrophically [12]. It has been found that *C. reinhardtii* cells have abundant carbonic anhydrase activity. In cultures of the cell wall-less mutant of *C. reinhardtii*, *cw-15*, between 80% and 90% of the carbonic anhydrase activity is released into the growth medium [1]. The ability to release CA activity into the medium upon treatment of wild type *C. reinhardtii* with autolysin, indicates that the majority of CA is localized in the periplasmic space or cell wall [13].

Metabolic assimilation of [1, 2-^{13}C]-acetate during the heterotrophic growth conditions as illustrated in Fig. 1. The complete uptake of acetate resulted in concomitant showing up of two metabolites in the extracellular space in about twenty four hours of incubation, whose intensity increased as a function of incubation time. In the extracellular space, $^{13}CO_2^{aq}$ and bicarbonate observed at 125.48 and 161.01 ppm could be easily assigned as arising from $^{13}CO_2^{aq}$ and bicarbonate, respectively, by comparing the observed chemical shifts with respective standard values [14]. Detection of $^{13}CO_2^{aq}$ in TAP medium (pH 6.8) could be due to the metabolic processes mediated by enzymes like carbonic anhydrases in the cell and certainly not due to the chemical conversion of bicarbonate to CO_2^{aq}, which occurs only at an acidic pH below 6.4.

Under heterotrophic growth conditions, pH of the medium perturbs by the action of carbonic anhydrases. The pH at different time points were compared with the pH determined using an extracellular pH probe and with ^{13}C-NMR experiments. The agreement between the pH determined using these two methods fit well (Fig. 2).

Under these conditions bicarbonate and CO_2 remain in equilibrium because of action of extracellular CA. Even in the absence of carbonic

anhydrase the exchange is rapid; the rate constant describing flux between bicarbonate and CO_2 was measured *in vitro* using magnetization transfer methods as 0.003 s^{-1} (data not shown), The ^{13}C magnetization (saturation) transfer effect of the uncatalyzed exchange between carbon dioxide and bicarbonate was investigated using a phantom sample containing 50 mM $NaH^{13}CO_3$ and no CA (pH =6.8, 25°C), and this exchange is further increased by the enzyme. Exchange between the two molecules and its dependence on extracellular carbonic anhydrase activity was demonstrated by the loss of polarization of $H^{13}CO_3^-$ following selective saturation of the $^{13}CO_2$ resonance at 125.48 ppm (Fig. 3A). In the absence of saturation, the decay of the $H^{13}CO_3$ resonance will be dominated by its T1. However, in the presence of saturation of the $^{13}CO_2$ resonance, the $H^{13}CO_3$ signal will decay with a time constant given by $1/(1/T_1+k)$, where k is the rate constant for the conversion released by the cells. The T_1s measured *in vivo* were 6.4 ± 0.69 s for $H^{13}CO_3$ and 12.6 ± 0.43 s for $^{13}CO_2$ (Supporting material S1). Inhibition of carbonic anhydrase with acetazolamide abolished this decrease of the $H^{13}CO_3$ signal, demonstrating the importance of the enzyme for the exchange of polarization between the two molecules (Fig. 3B). Figure 3A shows the observed ^{13}C saturation transfer effect due to exchange between carbon dioxide and bicarbonate in the presence of extracellular CA. In the control spectrum (upper trace), both bicarbonate (161.01 ppm) and carbon dioxide (125.48 ppm) signals were detected. The bicarbonate signal was slightly reduced upon RF saturation of carbon dioxide (middle trace). The difference spectrum revealed a 8.4 % decrease caused by the catalyzed exchange between carbon dioxide and bicarbonate in the medium. The T_1 of bicarbonate without saturating carbon dioxide in the phantom, which is essentially the same as the T_1 of bicarbonate without exchange because of the dominant presence of bicarbonate, was determined to be 11.4 s. Carbonic anhydrase catalysed pseudo first-order rate constant of the dehydration flux ($k_{dehydration}$) in the cells medium was calculated to be 0.011 ± .005 sec^{-1}.

NMR measurement of the reversible exchange between ^{13}C-labeled carbon dioxide and bicarbonate is significantly faster than the turnover rate of carbon dioxide or bicarbonate. This "amplification" is of significant advantage for *in vivo* detection of the ^{13}C magnetization transfer effect catalyzed by CA. It produces a large CA-catalyzed ^{13}C magnetization transfer effect quantifiable in extracellular space as demonstrated in this study. It sensitizes the ^{13}C magnetization transfer effect to alterations in the catalytic environment, which is affected by, for example, ion substitution [15], the action of sulfonamide inhibitors as shown by the current study. On the other hand, it also makes the ^{13}C saturation transfer effect insensitive to the proton transfer step and therefore to the turnover rate of bicarbonate from carbon dioxide or to that of carbon dioxide from bicarbonate catalyzed by CA.

Acetazolaminde is a highly specific sulfonamide inhibitor of CA. As demonstrated by the results in Fig. 3B, administration of acetazolamide causes a large reduction in the ^{13}C magnetization transfer effect, consistent with its expected strong inhibitory effect on CA [13]. Consistent with the previous observations, significant residual magnetization transfer effect was detected after administration of acetazolamide [16]. The methodology differs mainly by the fact that in this case the study was conducted on cells and in the other on mice. Thus it is interesting to compare and discuss the two rates of dehydration that differ by a factor of 50, the rate of dehydration obtained in cells in the presence of CA being very similar to that obtained in rat brains in absence of CA..

It is because addition of 20 mM acetate to the culture medium resulted in reduction of CA activity. The effect of acetate addition parallels the effect of increasing extracellular CO_2 concentration at 24 hrs in heterotrophic growth is correlated with a significant reduction in CA activity. This reduction in activity is a consequence of lower levels of the CA protein reported earlier [17].

For better visualization, the $k_{dehydration}$ values determined in extracellular environment and cells treated with acetazolamide are plotted in Figure 3C. The acetate metabolism in heterotrophy resulted in near complete uptake of 20 mM acetate by the cells from the medium within 24 hrs, an accumulation of two predominant metabolites, namely bicarbonate and CO_2^{aq}. The CO_2^{aq} formed through the TCA cycle in the mitochondria gets converted to bicarbonate by multiple carbonic anhydrases present in *C. reinhardtii* cell. For NMR to detect CO_2 level so prominently in heterotrophic cells, CO_2^{aq} level might have reached close to millimolar concentrations in the cells.

Bicarbonate and CO_2^{aq} as well as bicarbonate metabolism got further metabolized into TAG during mixotrophy and the same to a lesser extent in heterotrophy.

It have been found that *C. reinhardtii* cells have abundant carbonic anhydrase activity. In cultures of the cell wall-less mutant of *C. reinhardtii*, *cw-15*, between 80% and 90% of the carbonic anhydrase activity is released into the growth medium [1].

Since carbonic anhydrase would act to maintain a rapid equilibrium between HCO_3^- and dissolved CO_2, the location of this enzyme within the periplasmic space would be particularly useful if the velocity of active transport of C, (whether HCO_3^- or CO_2 were the transported species) into the cell exceeds the uncatalyzed equilibration. CA in the periplasmic space would also act as a 'trap' for CO_2 which is passively diffusing out of the algal cell. By rapidly hydrating this CO_2, it would be immediately available for active transport back into the alga (if HCO_3^- were the transported species). This could help to establish a low CO_2 compensation point. The location of CA in the periplasmic space or cell wall would also be advantageous to *C. reinhardtii* when the aqueous environment is limited to a thin layer surrounding the organism, as would be the case for soil organisms (*C. reinhardtii* does grow in such environments). Rapid equilibration between CO_2 and HCO_3^- would allow for efficient utilization of inorganic carbons for fixing into starch and lipids.

Conclusion

We have demonstrated the utility of ^{13}C saturation transfer for determining the exchange rate between bicarbonate and carbon dioxide catalyzed by extracellular carbonic anhydrase in *C. reinhardtii*. This exchange is sufficiently fast to lead to a large and quantifiable ^{13}C magnetization transfer effect. This study for the first time measures the dehydration rate of bicarbonate to CO_2 in *C. reinhardtii*.

Materials and methods

Growth conditions for NMR measurements

C. *reinhardtii* [*cw15* strain] cells were grown at 25 °C in Tris acetate phosphate (pH 7.5, TAP) medium [18]. The cells were exposed to a light flux of 150 µmol. photons m^{-2} s^{-1} from the bottom of a shaker, set at 200 rpm. The cells were grown in unlabeled TAP in a continuous light growth phase till late log phase (~4 to 5 million cells per ml) growth, following which the cells were washed thrice in the TP medium (TAP without acetate). Four million cells per ml were suspended in 20 mM [1, 2-^{13}C]-acetate (99% ^{13}C, Cambridge Isotope Laboratories, Cat no. CLM-440-1), in TP medium incubated in dark conditions for twenty four hours, during which aliquots were withdrawn at various time intervals for NMR measurements. For inhibitor experiments, the cells were treated as above in the presence of acetazolamide (Conc.10 mM, Sigma Aldrich) solubilized in dimethyl sulfoxide.

In vivo ^{13}C saturation NMR measurements

^{13}C-saturation transfer is acquired in ^{13}C channel; saturation of the selected ^{13}C-spin of CO_2 is performed using continuous RF wave irradiation. When a control spectrum is acquired, saturating pulse is placed at an equal spectral distance from the observed ^{13}C spin of bicarbonate but on the opposite side of the saturated $^{13}CO_2$. Control irradiation at 195.85 p.p.m. and saturating irradiation centred on the $^{13}CO2$ resonance at 125.48 p.p.m. were used (Fig. 3A, B). Saturation was performed using a spectrally selective 1 s pulse with a nominal B1 field of 100 Hz.

NMR experiments

The cell suspension under incubation under dark or light conditions in 20 mM [1, 2-^{13}C]-acetate in TP medium was aliquoted at different time points (as specified below) and transferred to a 5 mm NMR tube. The recording of 1D ^{13}C-NMR spectra was carried out at 25 °C on a Bruker Avance 800 MHz NMR spectrometer equipped with a cryogenically cooled 5 mm triple-resonance probe, with ^{1}H carrier placed on H_2O resonance (4.68 ppm) and ^{13}C- carrier at 100 ppm. Following the addition of [1, 2-^{13}C]-acetate a series of proton decoupled 1D-^{13}C-spectra were recorded under identical experimental conditions and with the same set of acquisition parameters for samples retrieved from the culture at different time points of acetate assimilation, as mentioned in the results. The pulse program 'zgdc' was used for this purpose with a ^{13}C-pulse width of 12 µs corresponding to a 90^{o} flip angle, an acquisition time of 0.164 s, a relaxation delay of 50 s, 16384 acquisition data points, and 8 scans. Proton decoupling was 'on' during ^{13}C-NMR detection. All 1D data were processed with the same processing parameters and were analyzed by the standard protocol provided by the spectrometer vendor. In the case of doublet signatures, the area under both the components was integrated and added up. All chemical shifts were referenced with an addition of standard dioxane ^{13}C-signal observed at 67.4 ppm. All the data were processed with a 10 Hz line-broadening parameter. All NMR spectra are corroborated by at least three independent biological experiments. Representative spectra are shown for only one but quantitation was based on data from multiple repeats. The spectra from the same experiment are shown in the form of a standard *"stacked plot"*, so that the equivalent peaks appear non-overlapping and shifted for better visualization.

Using ^{13}C-NMR chemical shifts the pH at each time points was calculated from the relative concentrations of $H^{13}CO_3^-$ and $^{13}CO_2$, using Henderson-Hasselbalch equation and assuming a pKa of 6.17.

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Competing interests

The author declares that he has no competing interests.

Authors' contributions

HS designed, performed the experiments and analyzed the results.

Table

Hours of incubation	Bicarbonate (Conc. mM)	CO_2 (Conc. mM)	pH determination using Henderson-Hasselbalch equation $pH=pKa + \log_{10}([HCO_3^-]/[CO_2])$	pH determination by pH electrode
0	0	0	7.5	7.5
6	3	0.5	7.1	7.2
12	6	2	6.8	6.9
18	9.5	3	6.8	6.8
24	15	4	6.8	6.8

1.

Figure legends

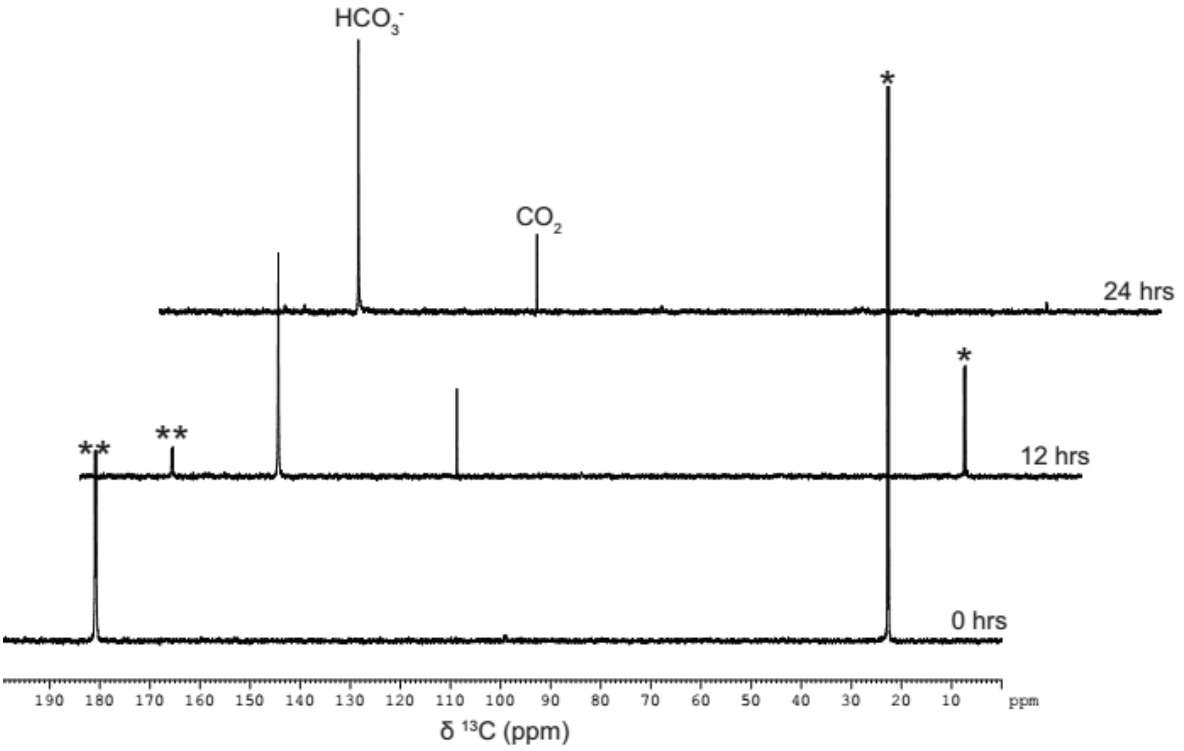

Figure 1.

Assimilation kinetics of acetate by *C. reinhardtii*, as studied by recording proton decoupled 1D ^{13}C-NMR spectra, during heterotrophic growth at different time points after adding the [1, 2-^{13}C]-acetate to the TP growth medium. The spectra from the same experiment are stacked for representation. Peaks at 125.48 and 161.01 ppm show CO_2aq and bicarbonate, respectively. The signs * and ** represent methyl and carboxylic-^{13}C resonances of [1, 2-^{13}C]-acetic acid, respectively.

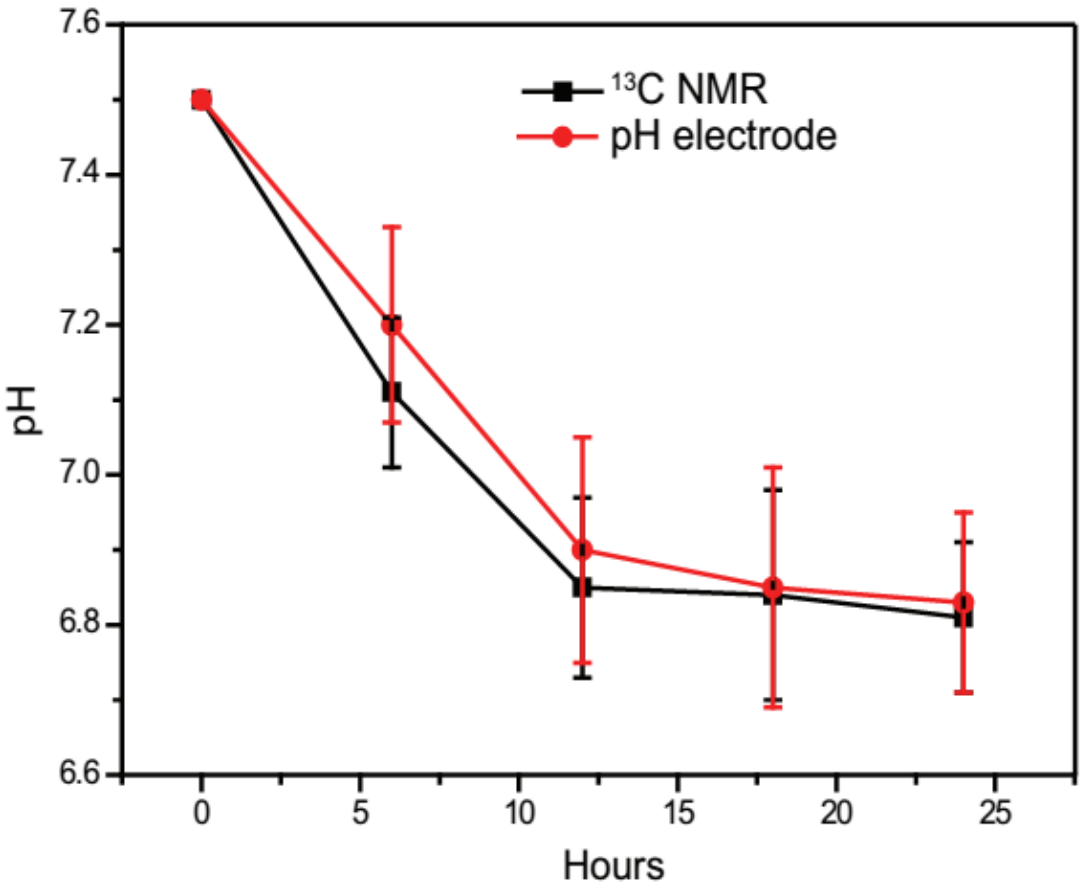

Figure 2.
Correlation of the calculated pHs measured using a pH electrode and pH determined from measuring the concentration of bicarbonate and CO_2 by ^{13}C NMR chemical shifts (see table 1).

Figure 3A

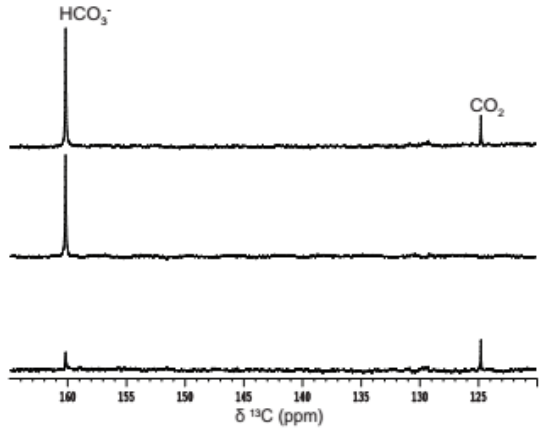

Figure 3C

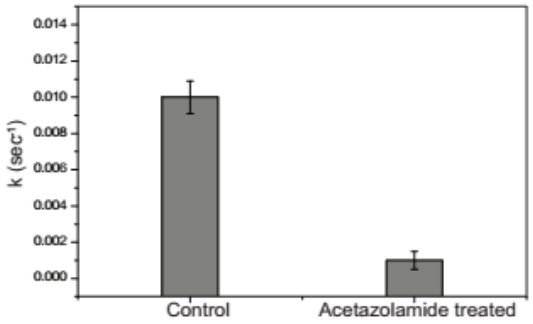

Figure 3B

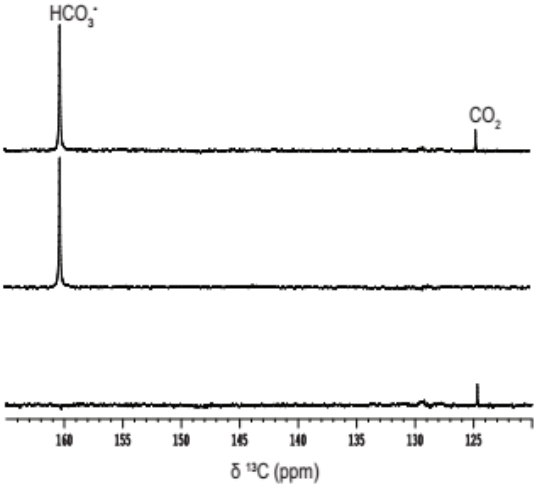

Figure 3.

A) ^{13}C saturation transfer effect due to exchange between carbon dioxide and bicarbonate catalysis by extracellular CA (pH=6.8, 25°C). Upper trace: the control spectrum without RF saturation of carbon dioxide. Both the bicarbonate signal at 161 ppm and the carbon dioxide signal at 124.67 ppm were observed. Middle trace: ^{13}C RF saturation pulse was centered on the resonance frequency of carbon dioxide. Lower trace: difference spectrum. NS=128. LB= 10 Hz. The change in the intensity of the bicarbonate signal due to the 13C saturation transfer effect was determined to be 8.1%.

B) ^{13}C saturation transfer effect due to exchange between carbon dioxide and bicarbonate catalysis by extracellular CA in presence of acetazolamide (pH=6.8, 25°C). Upper trace: the control spectrum without RF saturation of carbon dioxide. Both the bicarbonate signal at 161 ppm and the carbon dioxide signal at 124.67 ppm were observed. Middle trace: ^{13}C RF saturation pulse was centered on the resonance frequency of carbon dioxide. Lower trace: difference spectrum.

C) Pseudo first-order unidirectional dehydration rate constant $k_{dehydration}$ determined in extracellular environment and cells treated with acetazolamide.

Supporting material

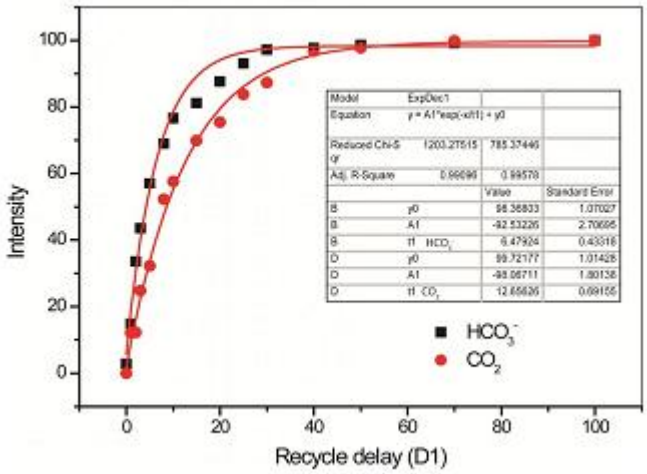

Figure S1. T_1 determination for HCO_3^- and CO_2 in extracellular space of *C. reinhardtii*.

References

1. Coleman JR, Berry JA, Togasaki RK, Grossman AR (1984) Identification of extracellular carbonic anhydrase of Chlamydomonas reinhardtii. Plant physiology 76: 472-477. http://dx.doi.org/10.1104/pp.76.2.472 PMid:16663867 PMCid:PMC1064313
2. Kimpel DL, Togasaki RK, Miyachi S (1983) Carbonic anhydrase in Chlamydomonas reinhardtii I. Localization. Plant and cell physiology 24: 255-259. http://dx.doi.org/10.1093/pcp/24.2.255
3. Coleman JR, Grossman AR (1984) Biosynthesis of carbonic anhydrase in Chlamydomonas reinhardtii during adaptation to low CO2. Proceedings of the National Academy of Sciences of the United States of America 81: 6049. http://dx.doi.org/10.1073/pnas.81.19.6049
4. Lindskog S (1997) Structure and mechanism of carbonic anhydrase. Pharmacology & therapeutics 74:1-20.http://dx.doi.org/10.1016/S0163-7258(96)00198-2
5. Po HN, Senozan N (2001) The Henderson-Hasselbalch equation: its history and limitations. Journal oChemicalEducation78:1499. http://dx.doi.org/10.1021/ed078p1499
6. Gallagher, Ferdia A., Mikko I. Kettunen, Sam E. Day, De-En Hu, Jan Henrik Ardenkjær-Larsen, Pernille R. Jensen, Magnus Karlsson, Klaes Golman, Mathilde H. Lerche, and Kevin M. Brindle (2008) Magnetic resonance imaging of pH in vivo using hyperpolarized 13C-labelled bicarbonate. Nature 453: 940-943. http://dx.doi.org/10.1038/nature07017 PMid:18509335
7. Harris EH (2001) Chlamydomonas as a model organism. Annual review of plant biology 52: 363-406. http://dx.doi.org/10.1146/annurev.arplant.52.1.363 PMid:11337403
8. Therien JB (2013) Insights into key barriers in the implementation of renewable biofuel technologies: MONTANA STATE UNIVERSITY Bozeman.
9. Siaut M, Cuiné S, Cagnon C, Fessler B, Nguyen M, et al. (2011) Oil accumulation in the model green alga Chlamydomonas reinhardtii: characterization, variability between common laboratory strains and relationship with starch reserves. BMC

biotechnology 11: 7. http://dx.doi.org/10.1186/1472-6750-11-7 PMid:21255402 PMCid:PMC3036615

10. Cakmak T, Angun P, Ozkan AD, Cakmak Z, Olmez TT, et al. (2012) Nitrogen and sulfur deprivation differentiate lipid accumulation targets of Chlamydomonas reinhardtii. Bioengineered 3: 343-346. http://dx.doi.org/10.4161/bioe.21427 PMid:22892589 PMCid:PMC3489711
11. Berkowitz BA, Balaban RS (1989) [16] Two-dimensional nuclear magnetic resonance studies of enzyme kinetics and metabolites in vivo. Methods in enzymology 176: 330-341. http://dx.doi.org/10.1016/0076-6879(89)76018-3
12. Harris EH (2009) The Chlamydomonas sourcebook: introduction to Chlamydomonas and its laboratory use: Academic Press.
13. Goodson C, Roth R, Wang ZT, Goodenough U (2011) Structural correlates of cytoplasmic and chloroplast lipid body synthesis in Chlamydomonas reinhardtii and stimulation of lipid body production with acetate boost. Eukaryotic cell 10: 1592-1606. http://dx.doi.org/10.1128/EC.05242-11 PMid:22037181 PMCid:PMC3232719
14. Singh H, Shukla MR, Chary KV, Rao BJ (2014) Acetate and Bicarbonate Assimilation and Metabolite Formation in Chlamydomonas reinhardtii: A 13C-NMR Study. PloS one 9: e106457.http://dx.doi.org/10.1371/journal.pone.0106457
15. Li Y, Han D, Hu G, Sommerfeld M, Hu Q (2010) Inhibition of starch synthesis results in overproduction of lipids in Chlamydomonas reinhardtii. Biotechnology and bioengineering 107: 258-268. http://dx.doi.org/10.1002/bit.22807 PMid:20506159
16. Yang J, Singh S, Shen J (2008) 13C saturation transfer effect of carbon dioxide–bicarbonate exchange catalyzed by carbonic anhydrase in vivo. Magnetic Resonance in Medicine 59: 492-498. http://dx.doi.org/10.1002/mrm.21501 PMid:18224701
17. Fett JP, Coleman JR (1994) Regulation of periplasmic carbonic anhydrase expression in Chlamydomonas reinhardtii by acetate and pH. Plant physiology 106: 103-108. PMid:12232308 PMCid:PMC159504
18. Harris EH, Stern DB, Witman G (2009) The chlamydomonas sourcebook.