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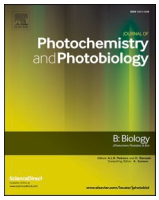

Rapid oxygen isotopic exchange between bicarbonate and water during photosynthesis

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ABSTRACT

Whether rapid oxygen isotopic exchange between bicarbonate and water occurs in photosynthesis is the key to determine the source of oxygen by classic ^{18}O-labeled photosynthetic oxygen evolution experiments. Here we show that both *Microcystis aeruginosa* and *Chlamydomonas reinhardtii* utilize a significant proportion (>16%) of added bicarbonate as a carbon source for photosynthesis. However, oxygen isotopic signal in added bicarbonate cannot be traced in the oxygen in organic matter synthesized by these photosynthetic organisms. This contradicts the current photosynthesis theory, which states that photosynthetic oxygen evolution comes only from water, and oxygen in photosynthetic organic matter comes only from carbon dioxide. We conclude that the photosynthetic organisms undergo rapid exchange of oxygen isotope between bicarbonate and water during photosynthesis. At the same time, this study also provides isotopic evidence for a new mechanism that half of the oxygen in photosynthetic oxygen evolution comes from bicarbonate photolysis and half comes from water photolysis, which provides a new explanation for the bicarbonate effect, and suggests that the Kok-Joliot cycle of photosynthetic oxygen evolution, must be modified to include a molecule of bicarbonate in addition to one molecule of water which in turn must be incorporated into the cycle instead of two water molecules. Furthermore, this study provides a theoretical basis for constructing a newer artificial photosynthetic reactor coupling light reactions with the dark reactions.

1. Introduction

Photosynthesis is the most crucial biochemical process on Earth, in which the reactions resulting in oxygen evolution are key steps, providing a continuous supply of oxygen for the biological world. The accepted theory of photosynthesis is that the oxygen released during photosynthesis comes directly from water, while the oxygen in the organic compounds ultimately synthesized through photosynthesis comes from carbon dioxide (Fig. 1). The earliest experiment to test if the oxygen evolved directly from water during photosynthesis was conducted by Ruben et al [1]; when they suspended *Chlorella vulgaris* in a reaction system for measuring photosynthetic oxygen evolution containing oxygen-18 water (0.85% H_2 ^{18}O) with normal oxygen-16 potassium bicarbonate and carbonate, the *Chlorella vulgaris* cells produced $^{18}O_2$, under illumination. However, when they suspended *Chlorella vulgaris* in a reaction system of photosynthetic oxygen evolution containing oxygen-16 water, but oxygen-labeled (O^{18}) potassium bicarbonate and carbonate, *Chlorella vulgaris* cells produced only unlabeled oxygen-16 under illumination. Therefore, these authors concluded that the released oxygen was derived only from water and not from the added bicarbonate in the reaction system. However, this conclusion assumes that there is no or very little exchange of oxygen isotopes between bicarbonate and water during photosynthetic oxygen release in algae [1].

Ruben et al.'s experiments were conducted before the enzyme carbonic anhydrase (CA), which can rapidly catalyze the conversion between carbon dioxide and bicarbonate, was known to be present in plants [2]. After the discovery of this enzyme, it was realized that rapid isotopic exchange between water and bicarbonate under the catalysis of CA occurs in plants [3]. Therefore, to minimize the possible influence of endogenous inorganic carbon and CA on the possible source of oxygen for photosynthetic oxygen evolution, Stemler and Radmer [4] did a $HC^{18}O_3^-$ labeling experiment, using chloroplast thylakoids depleted of both endogenous inorganic carbon and CA, to determine whether water

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or bicarbonate serves as the direct source of O_2. Their experimental results showed that compared to the photosynthetic oxygen evolution in the reaction system,without the addition of $HC^{18}O_3^-$, the oxygen evolved by the HCO_3^--depleted chloroplast thylakoids under illumination was five times higher when $HC^{18}O_3^-$ was added, and almost all of the oxygen evolved were not labeled. They deduced that the oxygen produced by photosynthesis can come only from water in the reaction system, not bicarbonate in the context of no 'noise' from internal inorganic carbon sources and no rapid exchange of oxygen isotopes between water and bicarbonate in the absence of CA catalysis [4].

Due to the continuous transformation of dissolved inorganic carbon in water as well as isotope fractionation resulting from different inorganic carbon utilization pathways of plants, the amount of transformation and isotope fractionation values must vary significantly under different environmental conditions. Therefore, a bidirectional carbon isotope tracer culture technology was developed to solve the problem of the isotope exchange and the signal interference in the process of inorganic carbon transformation instead of using the single isotope labeling technology, and to quantitatively obtain the utilization of inorganic carbon by plants [5,6]. Similar to the utilization of inorganic carbon, we may try to use bidirectional oxygen isotope tracer culture technology to obtain the assimilation of inorganic oxygen in plants.

Determining the source of oxygen in the above classic ^{18}O-labeled photosynthetic oxygen evolution experiments hinges on whether rapid oxygen isotopic exchange between bicarbonate and water takes place during photosynthes [1,4].Both Ruben et al.[1]and Stemler and Radmer [4] argued that their experiments have eliminated the possibility of such exchange occurring. Actually, CA is widely distributed in cell membranes, mitochondria, chloroplasts, and other organelles, and each organelle has multiple CA isoforms [7]. In particular, there are two types of CA in chloroplasts: one is a tightly bound CA on the thylakoid membrane, and the other is a soluble CA located in the stroma of the chloroplasts [8–11], which poses difficulties in completely eliminating CA activity and in preventing isotopic exchange between water and bicarbonate. In addition, it is well established that photosystem II has CA activity [12–15], which also makes it impossible to artificially control the rapid isotopic exchange between water and bicarbonate. Therefore, in the process of photosynthetic oxygen evolution, the presence or the absence of the rapid exchange of oxygen isotopes between water and bicarbonate becomes the key to know whether there is the possibility of involvement of bicarbonate in oxygen evolution in photosystem II.

Stable isotope techniques have been widely used in tracing geological and biological processes [16]; however, single isotope labeling experiments can only provide information on the isotopic exchange of a single chemical process, but not on the isotopic exchange in the complex photosynthetic process, which involves three substrate elements: carbon, hydrogen, and oxygen. Therefore, we decided to use the carbon and oxygen dual-element isotope tracing to study the oxygen evolving steps in algae. According to the current theory of photosynthesis, carbon and oxygen in carbon dioxide, which can be transformed from bicarbonate, is assimilated and incorporated into the synthesized organic matter (Fig. 1). If there is no exchange of isotopes, then the carbon and oxygen isotope signals in bicarbonate will be reflected in the photosynthesized organic matter. If there is a rapid exchange of oxygen isotopes between bicarbonate and water in photosynthesis, then the oxygen isotopic signal in bicarbonate will be greatly weakened or even disappear in the photosynthesized organic matter. Thus, in isotopic labeling experiments, the carbon and oxygen isotopic signal of the photosynthesized organic

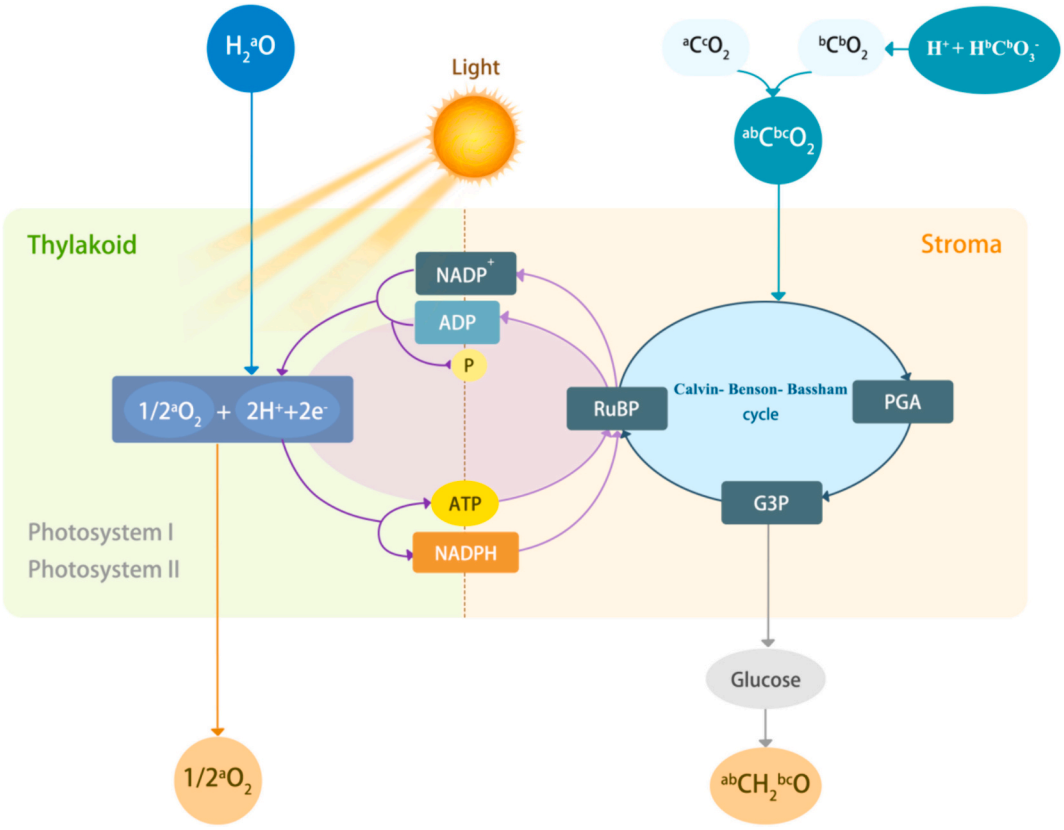

Fig. 1. Putative process of the incorporation of carbon and oxygen into organic matter without a rapid exchange of oxygen isotopes between bicarbonate and water according to the current theory of photosynthesis (^{a}O is from oxygen in water, ^{c}O is from oxygen in carbon dioxide in the atmosphere, ^{b}O is from oxygen in exogenous bicarbonate, ^{a}C is from carbon in carbon dioxide in the atmosphere, ^{b}C is from carbon in exogenous bicarbonate, ^{ab}C indicates that $\frac{a}{a+b}$ of carbon is from carbon in carbon dioxide in the atmosphere [$^aCO_2$] and $\frac{b}{a+b}$ of carbon is from carbon in exogenous bicarbonate [$HC^bO_3^-$]; ^{bc}O indicates that oxygen has $\frac{b}{b+c}$ of oxygen from oxygen in exogenous bicarbonate [$HC^bO_3^-$], and $\frac{c}{b+c}$ of oxygen from oxygen in carbon dioxide in the atmosphere[C^cO_2]).

matter at same time is the key to determine whether there is a rapid exchange of oxygen isotopes between bicarbonate and water in photosynthesis.

Algae have different bicarbonate use modes. The extracellular CA-mediated bicarbonate dehydration and the direct bicarbonate transport via anion transport channels are two of the most important utilization modes of bicarbonate [17,18]. Acetazolamide (AZ) is an inhibitor of extracellular CA and disodium 4,4′-diisocyanate 2,2′-styrene sulfonate (DIDS) is an anion channel blocker, both of which are known to significantly decrease inorganic carbon utilization in plants [19]. Our present study aims to demonstrate the existence of the rapid oxygen isotope exchange between water and bicarbonate during photosynthesis. *Chlamydomonas reinhardtii,* with extracellular CA activity, and *Microcystis aeruginosa,* without extracellular CA activity, were cultured under several treatment regimes with and without inhibitor/blocker (including extracellular CA inhibitor and anion channel blocker); the carbon and oxygen bidirectional stable isotope labeling culture technology was used to study the utilization of carbon and oxygen in added bicarbonate and changes in stable carbon and oxygen isotope composition of biomass. In addition, our current study also aims to provide isotopic evidence for a new mechanism, where half of the oxygen in photosynthetic oxygen evolution comes from bicarbonate photolysis and the other half comes from water photolysis [20].

2. Results

When identical quantities of microalgae are simultaneously cultured in two medium supplemented separately with distinct stable isotope-labeled $NaHCO_3$, if it is observed that there exists no significant difference in biomass but rather substantial variations in the isotopic composition of microalgae, the bidirectional stable isotope tracing technique can be employed. Chl-*a* was used to represent the biomass of microalgae. There was no significant difference in the biomass of identical algal species separately grown in media with Label 1 labeled-$NaHCO_3$ ($\delta^{13}C$, −19.02‰; $\delta^{18}O$, −19.27 ‰) and Label 2 labeled-$NaHCO_3$ ($\delta^{13}C$, 2.21‰; $\delta^{18}O$, −2.67‰) in the same treatment group, which is a prerequisite for calculating the proportion of carbon and oxygen in the added bicarbonate used by microalgae (Fig. 2).

The stable carbon isotopic composition of the microalgae cultured in media added with Label 1 labeled-$NaHCO_3$ and Label 2 labeled-$NaHCO_3$ from the same treatment group showed significant differences (Fig. 3A, B). However, there was no significant difference in the stable oxygen isotopic composition of the microalgae between those cultured in the media with Label 1 labeled-$NaHCO_3$ and Label 2 labeled-$NaHCO_3$ from the same treatment group (Fig. 3 C, D).

Compared to the DIDS treatment group, the proportion of carbon in the added bicarbonate used by *Microcystis aeruginosa* in the control group and AZ treatment group was higher, exceeding 30%. Compared to the control group, the proportion of carbon in the added bicarbonate used by *Chlamydomonas reinhardtii* in the DIDS treatment group and AZ treatment group was also higher, surpassing 25% (Fig. 2 C). However, we could not observe that the oxygen in the added bicarbonate was utilized by both algal species since there was no significant difference in the oxygen isotopic composition of identical algal species separately grown in media with Label 1 and Label 2 labeled-$NaHCO_3$ in the same treatment group.

3. Discussion

3.1. Utilization of carbon and oxygen in added bicarbonate by microalgae

It has long been known that photosynthetic organisms can use bicarbonate as a carbon source for photosynthesis [21,22]; further, there are well-established mathematical models for the use of bicarbonate for this purpose [5]. Our present study shows that the two species, used here, have different proportions of utilization of added bicarbonate

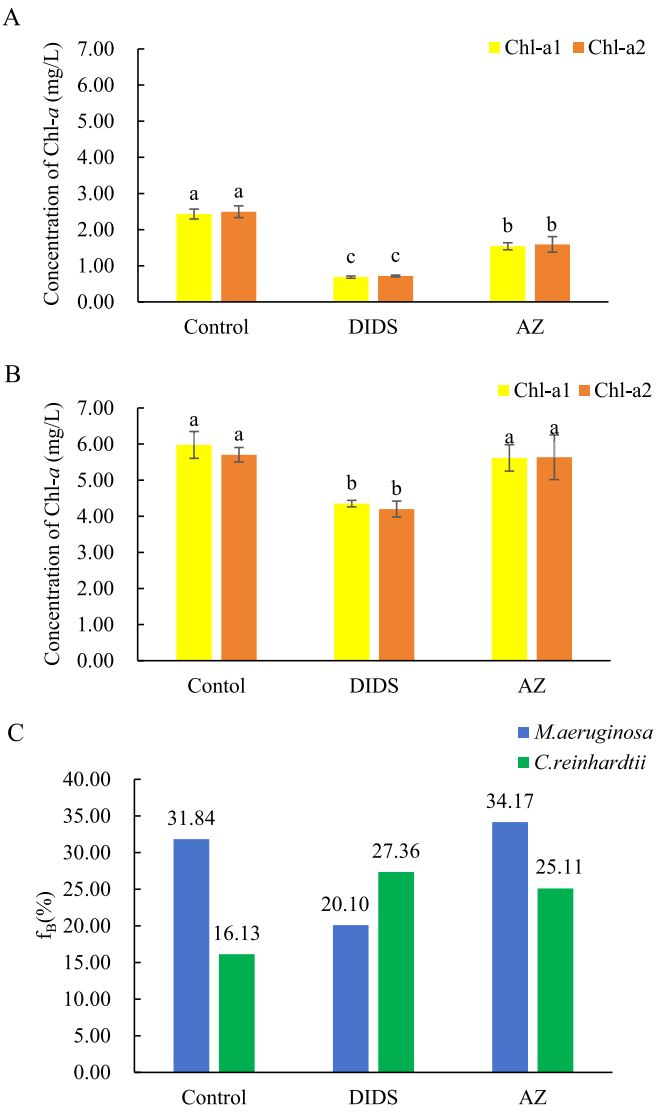

Fig. 2. Concentration of chlorophyll *a* (Chl-*a*) of *Microcystis aeruginosa* (A) and *Chlamydomonas reinhardtii* (B) grown in media with two isotope labeled-$NaHCO_3$ and the proportion of carbon in added bicarbonate used by *Microcystis aeruginosa* and *Chlamydomonas reinhardtii* (C) under three treatments (Chl-a1, Chl-a2 indicates the concentration of Chl-*a* of the microalgae cultured in the media with Label 1 labeled-$NaHCO_3$ and Label 2 labeled-$NaHCO_3$ respectively; Control, DIDS and AZ represents the control (i.e.,without any addition), DIDS treated (with 0.5 mmol/L DIDS), and AZ treated (with 1 mmol/L acetazolamide), respectively; the same letter on the column indicates the no significant difference between same treatment with different isotope labeled-$NaHCO_3$ (Duncan's multiple range tests, $P < 0.05$); data in the column (blue and green) indicate the proportion of carbon in added bicarbonate used by microalgae (f_B (%))). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

under different treatments (Fig. 3). The activities of extracellular CA and anion transport channel protein in microalgae determine the utilization, in the cells, of added bicarbonate. In this study, the proportion of added bicarbonate use by *Microcystis aeruginosa* in the control group, DIDS treatment group, and AZ treatment group was 31.8%, 20.1%, and 34.2%, respectively, while that by *Chlamydomonas reinhardtii,* it was 16.1%, 27.4%, and 25.1%, respectively. Compared with *Chlamydomonas reinhardtii* in natural conditions, the higher proportion of added bicarbonate use by *Microcystis aeruginosa* may be related to the fact that it lacks extracellular CA [23]. Therefore, we concluded that a significant proportion of the carbon sources used by these photosynthetic

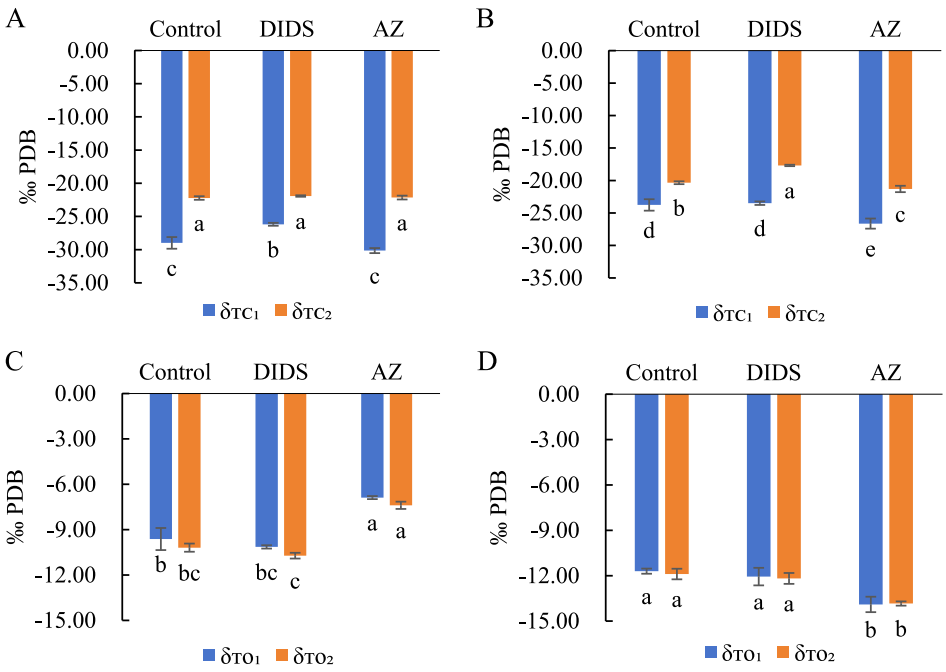

Fig. 3. Stable carbon (A, B) and oxygen (C, D) isotope abundance of organic matter in newborn *Microcystis aeruginosa* (A, C) and *Chlamydomonas reinhardtii* (B, D) (Control, DIDS and AZ represents the control (i.e.,without any addition), DIDS treated (with 0.5 mmol/L DIDS), and AZ treated (with 1 mmol/L acetazolamide), respectively; δ_{TC1}, δ_{TC2} designates the carbon isotopic abundance of the microalgae cultured in the media with Label 1 labeled-$NaHCO_3$ and Label 2 labeled-$NaHCO_3$ respectively; δ_{TO1}, δ_{TO2} designates the oxygen isotopic abundance of the microalgae cultured in the media with Label 1 labeled-$NaHCO_3$ and Label 2 labeled-$NaHCO_3$ respectively. Before treatment, the initial carbon isotope abundance of *Microcystis aeruginosa* and *Chlamydomonas reinhardtii* is −21.19 ‰ and − 17.39 ‰, respectively; the initial oxygen isotope abundance of *Microcystis aeruginosa* and *Chlamydomonas reinhardtii* is −12.20‰ and − 13.34‰., respectively. Data is presented as the mean ± standard error (M ± SE). The same letter for each sample indicates no significant difference; the different letter on the column represents the significant difference, and, the same letter on the column represents the no significant difference between the same treatment with different isotope labeled-$NaHCO_3$ (Duncan's multiple range tests, $P < 0.05$)).

organisms do come from exogenous bicarbonates, regardless of culture conditions.

The current theory is that photosynthetic oxygen evolves only from water, and oxygen in organic matter comes only from oxygen-containing inorganic carbon (carbon dioxide or bicarbonate) (see Fig. 1). However, our current study shows that the oxygen from exogenous bicarbonate seems to be not utilized by both algal species. The oxygen in organic matter produced by photosynthesis does not seem to come from oxygen-containing inorganic carbon, which is clearly contrary to the current theory of photosynthetic oxygen evolution, and may be explained by the rapid isotopic exchange between oxygen in water and oxygen in inorganic carbon during photosynthesis, as well as the direct substrate of photosynthetic oxygen evolution being water and bicarbonate.

In photosynthetic organisms, carbon isotopic exchange frequently occurs between carbon dioxide and bicarbonate, and under normal physiological conditions, this exchange occurs slowly [24,25]. On the other hand, oxygen isotopic exchange frequently occurs between water, bicarbonate, carbonate ion, and carbon dioxide. These oxygen isotopic exchanges occur solely through the reversible hydration of carbon dioxide. Although the reversible hydration of carbon dioxide in natural water is slow, it occurs extremely quickly under the action of CA and photosystem II that has a similar function to CA [26], so that the isotopic exchange of oxygen occurs very rapidly in oxygen-evolving organisms.

Both *Microcystis aeruginosa* and *Chlamydomonas reinhardii* have a wide variety of other CA isozymes, although we have added extracellular CA-specific inhibitor in this study to inhibit extracellular CA activity; therefore, there must be a rapid oxygen isotopic exchange between the bicarbonate ions and water in this study. Since the concentration of bicarbonate used in this study, reported here, was 4 mM, which is <0.008% (55.6 M) of the water concentration in the system, the oxygen in the added bicarbonate was completely exchanged with the oxygen in the water, and the oxygen isotopic signal in added bicarbonate was not traced in the organic matter formed by photosynthetic inorganic carbon fixation in this study. We conclude, from our current study, that the photosynthetic organisms undergo rapid oxygen isotopic exchange between water and bicarbonate during photosynthesis, resulting in almost all the oxygen in the bicarbonate being replaced by oxygen from water.

3.2. *Water photolysis versus bicarbonate photolysis or both*

In the past, ^{18}O-labeled photosynthetic oxygen evolution experiments seem to have led to the conclusion that the oxygen released during photosynthesis comes from water, not oxygen-containing inorganic carbon [1,4]. However, the premise for this conclusion had been that the added bicarbonate in the photosynthetic oxygen evolution system does not undergo oxygen isotopic exchange with water. In our opinion, such a premise does not exist. Although the experimental conditions are usually controlled as much as possible, CA in the reaction system cannot be completely removed, nor can the photosystem II. Similar to this study, there must have been a rapid oxygen isotopic exchange between water and exogenous bicarbonate, and the oxygen in the exogenous bicarbonate entirely replaced by the oxygen in the water in those earlier ^{18}O-labeled photosynthetic oxygen evolution experiments [1,4]. Therefore, the oxygen released during photosynthesis in these earlier ^{18}O-labeled photosynthetic oxygen evolution experiments are not ruled out; it can also originate from bicarbonate photolysis. This study provides a new interpretation for the earlier ^{18}O-labeled photosynthetic oxygen evolution experiments [1,4] as well as critical isotope evidence for bicarbonate to serve as the direct substrate of bicarbonate in photosynthetic oxygen evolution, further revealing the details of this important biochemical process [20,26].

3.3. The process of incorporating carbon and oxygen isotopes into organic matter

Wu [20] has proposed a new mechanism that bicarbonate photolysis and water hydrolysis account for equal amount of oxygen evolution in photosynthesis; this was done by analyzing the characteristics of thylakoid CA and photosystem II as well as the overall equation of photosynthesis ($CO_2 + 2H_2O(H_2O + H^+ + HCO_3^-) + \text{light} \xrightarrow{\text{chlorophyll}} C(H_2O) + O_2$). The validity of the new mechanism has been verified by the analysis of the Dole effect and the carbon-water balance in nature [20]. The present study shows that carbon from the exogenous bicarbonate can be directly incorporated into the synthesized organic matter, but the oxygen in these organic molecules is not directly incorporated from the exogenous bicarbonate (Fig. 2, and Fig. 3). Therefore, the current theory of photosynthesis (including photosynthetic oxygen evolution) (see Fig. 1) cannot explain the results presented here, but the new mechanism proposed by Wu [20] can fully explain the data presented in this paper (see Fig. 4). Photosynthetic oxygen evolution leads to the production of carbon dioxide, which enters the Calvin-Benson-Bassham cycle for inorganic carbon assimilation, and the oxygen incorporated into the synthesized organic matter is almost completely replaced by oxygen exchanged with water. Our current study provides isotopic evidence to support Wu's mechanism [20].

Wu's mechanism (see Fig. 4) indeed explains part of the bicarbonate effect in photosystem II, and some puzzles in the Kok-Joliot cycle [27] for oxygen evolution in photosynthesis. Whether the stimulation of oxygen evolution by bicarbonate (bicarbonate effect) occurs on the electron acceptor side, the electron donor side, or both of photosystem II has been studied by many in the past (see review [28,29]). There is clearly an effect on the electron acceptor as well as on the electron donor side of photosystem II (For effects on the electron acceptor side, see e.g., References [30–35]. For effects on the electron donor side, see e.g., References [36–49]. For effects on both the electron acceptor and the donor side, see e.g., Reference [50]). Obviously, photosynthetic oxygen evolution accompanied by the release of carbon dioxide must involve the electron acceptor side as well as the electron donor side of photosystem II. We deduced that bicarbonate undergoes photolysis on the electron donor side of photosystem II, yielding two electrons and releasing a molecule of carbon dioxide, which in an alkaline environment forms a bicarbonate bridge (electron transfer bridge), located on the Q_A-Fe-Q_B structure of the electron acceptor side of photosystem II. Two electrons produced from water photolysis pass through this "bridge". When another molecule of carbon dioxide and other two electrons produced from a new reaction of bicarbonate photolysis approach the "bridge", the bridge breaks and the bicarbonate on the bridge immediately loses electrons and undergoes carbon dioxide conversion. The lost electrons enter the electron transport chain, and the converted carbon dioxide

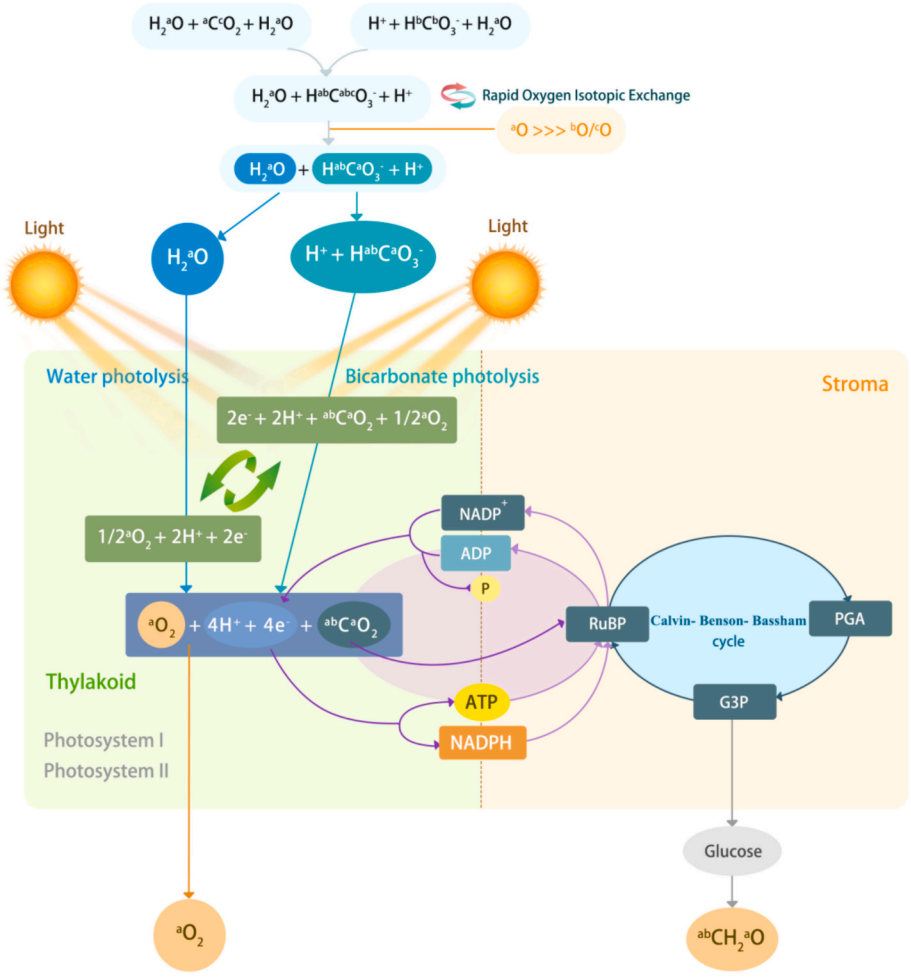

Fig. 4. Putative process of carbon and oxygen isotopic incorporation into organic matter (^aO denotes the oxygen in water, ^cO represents the oxygen in carbon dioxide from the atmosphere, ^bO indicates the oxygen in added bicarbonate, ^aC denotes the carbon in carbon dioxide from the atmosphere, ^bC represents the carbon in added bicarbonate, ^{ab}C indicates that $\frac{a}{a+b}$ of carbon is from that in carbon dioxide in the atmosphere and $\frac{b}{a+b}$ of carbon is from that in added bicarbonate; ^{abc}O indicates that oxygen in bicarbonate has $\frac{a}{a+b+c}$ of oxygen from water, $\frac{b}{a+b+c}$ of oxygen from oxygen in added bicarbonate and $\frac{c}{a+b+c}$ of oxygen from carbon dioxide in the atmosphere, CH_2O denotes carbohydrates, the amount of ^aO is much greater than that of ^bO or ^cO).

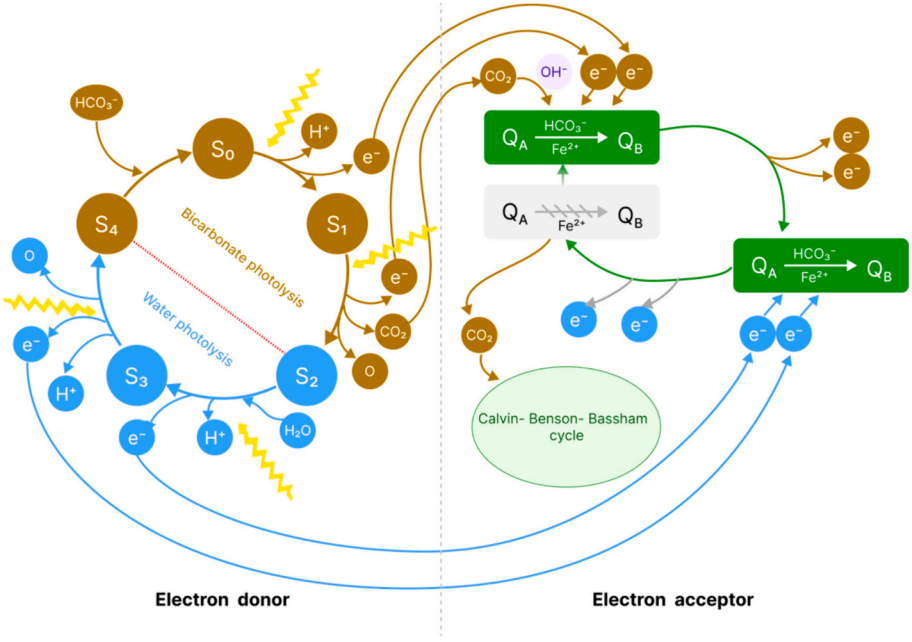

Fig. 5. The putative action locations in bicarbonate effects on the electron acceptor side and electron donor side of photosystem II and the new connotation of the Kok-Joliot cycle based on the Wu's mechanism. In the electron donor side, $S_4 \rightarrow S_0 \rightarrow S_1 \rightarrow S_2$ transition state of the Kok-Joliot cycle suffers from bicarbonate photolysis, a bicarbonate molecule instead of a water molecule is incorporating into $S_4 \rightarrow S_0$, then pass through $S_1 \rightarrow S_2$, 2 electrons are generated, CO_2 is released, and the electrons and CO_2 reach the Q_A-Fe-Q_B structure on the electron acceptor side, an electron transfer bridge is formed in the physiological environment, and then the electrons produced by bicarbonate photolysis and water photolysis pass through the electron transfer bridge in turn. Then the CO_2 is released into the Calvin-Benson-Bassham cycle, at the meantime, the electron transfer bridge is broken. However, the electron transfer bridge is formed again, and a new round of electron transfer and CO_2 release and transport is carried out when a new bicarbonate photolysis occurs.

flows towards the stroma for assimilation, while a new "bridge" is built. This process repeats during photosynthetic oxygen evolution (Fig. 5).

At present, it is generally believed that two water molecules are incorporated into the Kok-Joliot cycle during photosynthetic oxygen evolution, specifically into S_2-S_3 and S_4-S_0 transition state. However, there is an energy barrier problem in the $S_3 \rightarrow S_4 \rightarrow S_0$ transition state of the Kok-Joliot cycle in photosynthetic oxygen-evolving center and the S_4 state has not been directly observed [51-53]. Bicarbonate photolysis has a lower free energy than that of water photolysis [54]. If bicarbonate is incorporated into the $S_4 \rightarrow S_0$ transition state of the Kok-Joliot cycle during photosynthetic oxygen evolution instead of water, the energy barrier from $S_3 \rightarrow S_4 \rightarrow S_0$ transition state of the Kok-Joliot cycle is greatly reduced or even nonexistent, and the reaction rate of this step is greatly accelerated, making it difficult to capture the S_4 state. Therefore, equivalent amounts of bicarbonate photolysis and water photolysis during photosynthetic oxygen evolution can better explain the bicarbonate effect and the Kok-Joliot cycle in photosynthetic oxygen evolution (Fig. 5).

3.4. Conclusions and outlook

Dual-element bidirectional stable isotope labeling culture technology has facilitated the study of the utilization of added inorganic carbon and oxygen by photosynthetic organisms. A considerable proportion of carbon in microalgal organic matter comes from added inorganic carbon, while the oxygen in organic matter seems to be unrelated to the oxygen in added inorganic substances. From the currently obtained results we can confirm that the photosynthetic organisms indeed undergo rapid isotopic exchange between bicarbonate and water oxygen isotopes during photosynthesis, and the oxygen incorporated into organic matter is almost that exchanged with oxygen in water. In some classic ^{18}O-labeled photosynthetic oxygen evolution experiments, the oxygen evolved during photosynthesis can be explained as coming from either bicarbonate photolysis or water photolysis. The new mechanism, presented here, using bicarbonate photolysis, in addition to water photolysis, accounting for half of the photosynthetic oxygen evolution, can well explain the bicarbonate effect and the Kok-Joliot cycle of photosynthetic oxygen evolution. This study provides isotopic evidence for this mechanism. In the future, we can confirm the occurrence of bicarbonate photolysis by studying the crystal structure of photosystem II in normal dynamic physiological environment; and carry out dual-element bidirectional stable isotope labeling culture experiments for other photosynthetic organisms to obtain isotopic evidence for bicarbonate photolysis and water photolysis under different scenarios; and provide a theoretical basis for constructing artificial photosynthetic reactors that couple light reaction to dark reaction.

4. Materials and methods

4.1. Materials and culture procedure

Two typical model single-celled species were selected: a green alga *Chlamydomonas reinhardtii*, and a cyanobacterium *Microcystis aeruginosa*. They were obtained from the Institute of Hydrobiology, Chinese Academy of Sciences and cultured in a greenhouse at the Institute of Geochemistry, Chinese Academy of Sciences. They were cultured axenically in a modified BG-11 medium [55], at 25.0 °C ± 1.0 °C under a light intensity of 50 μmol photons $m^{-2} \cdot s^{-1}$, with a 12/12 h day/night cycle. The cells of the two microalgae were transferred to 250 mL Erlenmeyer flasks with aseptically sealed membranes. Two samples of sodium bicarbonate with different isotopic abundance of carbon and oxygen were added into the culture medium at a concentration of 4 mmol/L. The abundance of stable carbon isotope ($\delta^{13}C$) was −19.02‰ and that of the stable oxygen isotope ($\delta^{18}O$) -19.27 ‰ in what we have called Label 1 labeled-$NaHCO_3$. The abundance of stable carbon isotope ($\delta^{13}C$) was 2.21‰ and that of stable oxygen isotope ($\delta^{18}O$) -2.67‰ in Label 2 labeled-$NaHCO_3$. The experimental culture was divided into three groups: the control (i.e.,without any addition); DIDS treated (with

0.5 mmol/L DIDS); and AZ treated (with 1 mmol/L acetazolamide). All the treatments were conducted using 4–5 replicates for seven days. And during incubation, the positions of the experimental samples were randomly changed several times to eliminate the influence of subtle differences in the culture chamber environment.

4.2. Determination of chlorophyll in microalgae

Chlorophyll *a* (Chl-*a*) content is often used to characterize the biomass of planktonic algae in water [56]. Chl-*a* was determined as follows: 5 ml of the sample suspension was taken in a centrifuge tube, centrifuged at 1600 *g* for 15 min, the supernatant was discarded, and then 5 ml of 95% ethanol was added, subsequently, the sample was placed in the centrifuge tube in a refrigerator at 2–4 °C in darkness and soaked for 24 h, after that, the sample in the tube was centrifuged at 1600 *g* for 15 min; the supernatant was placed in a cuvette, and the absorbance was measured at 630 nm, 647 nm, 664 nm, and 750 nm by a spectrophotometer (LabTech UVPower). Finally, Chl *a* concentration was calculated according to the following equation:

$$[\mathrm{Chl}-a] = [11.85(D_{664}-D_{750}) - 1.54(D_{647}-D_{750}) - 0.08(D_{630}-D_{750})]V_E/(Vs\delta) \quad (1)$$

where, D is the optical density (absorbance) of the extract at the wavelengths, in nm, listed as subscripts; V_E is the constant volume of the extraction solution in the centrifuge tube (mL); Vs is the volume of the water sample(L); and δ (cuvette) is pathlength (cm); and [Chl-*a*] is the concentration of chlorophyll *a* in the sample (μg/L).

4.3. Determination of the $\delta^{13}C$ and $\delta^{18}O$

The treated microalgae were collected by centrifugation at 1600 *g* for 15 min. Then, 1 mol/L HCl was added to soak the samples to remove inorganic carbon; this step was repeated three times for 12 h. The microalgal samples were washed three times with ultra-pure water, and then dried thoroughly with a freeze-dryer.

The stable carbon isotope abundance of the organic matter ($\delta^{13}C$) was determined using a MAT253 gas stable isotope mass spectrometer. The carbon isotope standard using Pee Dee Belemnite (PDB), is expressed as:

$$\delta^{13}C\ (‰) = \left[\left(R_{sample}/R_{standard}\right) - 1\right]1000 \quad (2)$$

where, R_{sample} is the ratio of ^{13}C to ^{12}C in the sample; and, $R_{standard}$ is the proportion of ^{13}C to ^{12}C in the standard.

The oxygen isotope abundance in both *Chlamydomonas* and *Microcystis* was determined using continuous flow mass spectrometer (IRMS). Similarly, the oxygen isotope abundance of microalgae ($\delta^{18}O$) was expressed by the Eq. 3.

$$\delta^{18}O\ (‰) = \left[\left(P_{sample}/P_{standard}\right) - 1\right]1000 \quad (3)$$

where, P_{sample} is the ratio of ^{18}O to ^{16}O in the sample; and, $P_{standard}$ is the proportion of ^{18}O to ^{16}O in the standard (Pee Dee Belemnite).

The harvested samples at the end of the treatment included both the inoculated and the newly- grown microalgae. When compared to the original, the $\delta^{13}C$ values of the latter reflect accurately the effect of treatment. To appropriately represent all the experimental data, we used an isotope mixing model to determine the $\delta^{13}C$ values of the 'new born' samples . The measured $\delta^{13}C$ values for each treatment were calibrated as follows:

$$(N_0/N) \times \delta^{13}CN_0 + (1 - N_0/N) \times \delta^{13}CN_A = \delta^{13}CN \quad (4)$$

where, N_0 and N are the values of the biomass (represented by chlorophyll content Chl-a) at the inoculation time, and after harvest, respectively; $\delta^{13}CN_0$ and $\delta^{13}CN$ are the measured $\delta^{13}C$ values at inoculation and after harvest, respectively; and $\delta^{13}CN_A$ is the calibrated $\delta^{13}C$ value for each treatment, i.e., the $\delta^{13}C$ value for the "newborn". The $\delta^{13}C$ values used in the subsequent parts of this study are the calibrated $\delta^{13}C$ values under each treatment [5]. Similarly, the $\delta^{18}O$ values used in the subsequent parts of this study are the calibrated $\delta^{18}O$ values under each treatment condition.

4.4. The utilization proportion of carbon and oxygen in the added bicarbonate

The stable carbon isotopic composition of *Chlamydomonas* and *Microcystis*, used in this study, can reflect their utilization of different inorganic carbons in the environment [57]. Generally, microalgae can utilize two sources of inorganic carbon, one from the atmosphere (CO_2), and the other from the water body (dissolved inorganic carbon, DIC). And for each inorganic carbon source, microalgae have two forms of inorganic carbon utilization pathways, CO_2 and HCO_3^-. In the experiments, presented here, both *Microcystis aeruginosa* and *Chlamydomonas reinhardtii* are able to utilize two inorganic carbon sources, CO_2 from the atmosphere and HCO_3^- from the water [5,58]. Furthermore, the microalgae, used here, are shown to have two pathways for utilizing inorganic carbon, namely CO_2 and HCO_3^-, for each source of inorganic carbon [5]. We note that there is a stable carbon isotopic fractionation of approximately 9‰ between CO_2 and HCO_3^- utilizing pathways in inorganic carbon metabolism in many microalgae [58–60]. Ultimately, $\delta^{13}C$ of microalgae can be calculated using the following equations:

$$\delta_{TA} = (1 - f_{bai})\delta_a + f_{bai}(\delta_a + 9‰) \quad (5)$$

$$\delta_{TB} = (1 - f_{bbi})\delta_{ai} + f_{bbi}(\delta_{ai} + 9‰) \quad (6)$$

where, δ_{TA} characterizes the $\delta^{13}C$ value of microalgae resulting from microalgae utilizing inorganic carbon from atmospheric sources (including CO_2 and HCO_3^- converted from CO_2); δ_{TB} denotes the $\delta^{13}C$ value when microalgae utilize inorganic carbon from the added HCO_3^- (including HCO_3^- and CO_2 converted by HCO_3^-); f_{bai} signifies the proportion of bicarbonate use by microalgae through bicarbonate use pathway where bicarbonate originates from the transformation of atmospheric CO_2 that is dissolved in water; Conversely, (1-f_{bai}) denotes the proportion of CO_2 utilization by microalgae derived from the atmosphere through the CO_2 use pathway. Similarly, f_{bbi} indicates the proportion of added bicarbonate utilization by microalgae via the bicarbonate use pathway; (1-f_{bbi}) refers to the share of CO_2 utilization by microalgae through the CO_2 use pathway, where CO_2 is obtained from the conversion of bicarbonate added to the culture media.

Given the equality of the share of each inorganic carbon utilization pathway under identical treatment for the same microalgae, whether they use inorganic carbon from the atmosphere or the added inorganic carbon, f_{bai} is equal to f_{bbi}. δ_a represents the $\delta^{13}C$ value of microalgae when entirely utilizing carbon dioxide from the atmosphere and exclusively engaging in the CO_2 utilization pathway. Similarly, (δa + 9‰) signifies the $\delta^{13}C$ value when microalgae fully use carbon dioxide from the atmosphere and exclusively engage in the bicarbonate utilization pathway. Additionally, δ_{ai} denotes the $\delta^{13}C$ value when microalgae use the added inorganic carbon source and exclusively follow the CO_2 utilization pathway, while (δ_{ai} + 9‰) refers to the $\delta^{13}C$ value when microalgae use the same added inorganic carbon source and exclusively perform the bicarbonate utilization pathway.

Microalgae present in natural water possess the ability to utilize both atmospheric inorganic carbon sources and intrinsic inorganic carbon sources found in the water itself. Each of these carbon sources can be utilized through two pathways: CO_2 and HCO_3^-. Therefore, we propose the implementation of the following two end-member isotope mixing model:

$$\delta_{Ti} = (1-f_{Bi})\delta_{TA} + f_{Bi}\delta_{TB} = (1-f_{Bi})[(1-f_{bi})\delta_a + f_{bi}(\delta_a + 9‰)] + f_{Bi}[(1-f_{bi})f_{ai} + f_{bi}(\delta_{ai} + 9‰)](i = 1,2) \quad (7)$$

In Eq. 7, δ_{Ti} represents the $\delta^{13}C$ value associated with microalgae that were cultured using sodium bicarbonate with a known $\delta^{13}C$ value, whereas, f_{Bi} is for the proportion of added inorganic carbon utilized by microalgae in relation to the overall carbon source, and while ($1\text{-}f_{Bi}$) signifies the proportion of atmospheric carbon dioxide in the overall inorganic carbon source used by microalgae ($1\text{-}f_{bi}$).

For the microalgae cultured under the same conditions, as above, the Eq. 7 can be expressed as follows:

$$\delta_{T1} = (1-f_{B1})\delta_{TA} + f_{B1}\delta_{TB} = (1-f_{B1})[(1-f_{b1})\delta_a + f_{b1}(\delta_a + 9‰)] + f_{B1}[(1-f_{b1})f_{a1} + f_{b1}(\delta_{a1} + 9‰)] \quad (8)$$

and

$$\delta_{T2} = (1-f_{B2})\delta_{TA} + f_{B2}\delta_{TB} = (1-f_{B2})[(1-f_{b2})\delta_a + f_{b2}(\delta_a + 9‰)] + f_{B2}[(1-f_{b2})f_{a2} + f_{b2}(\delta_{a2} + 9‰)] \quad (9)$$

In the eqs. 8 and 9, δ_{T1} represents the $\delta^{13}C$ value of microalgae cultured in the medium, where the first label of sodium bicarbonate (Label 1-labeled sodium bicarbonate) was added with a known $\delta^{13}C$ value; δ_{T2} designates the $\delta^{13}C$ value of microalgae cultured in the medium that had the second label of sodium bicarbonate (Label 2-labeled sodium bicarbonate) with a known $\delta^{13}C$ value in it; f_{B1} signifies the proportion of Label 1-labeled sodium bicarbonate utilized by microalgae in the total carbon source; f_{B2} designates the proportion of Label 2-labeled sodium bicarbonate utilized by microalgae in the total carbon source; f_{b1} denotes the proportion of HCO_3^- pathways utilized by microalgae cultured in the medium with Label 1-labeled sodium bicarbonate; f_{b2} indicates the proportion of HCO_3^- pathways utilized by microalgae cultured in the medium with Label 2-labeled sodium bicarbonate; δ_{a1} represents the $\delta^{13}C$ value of microalgae cultured in a medium containing Label 1-labeled sodium bicarbonate, utilizing the added bicarbonate and undergoing the CO_2 pathway; δ_{a2} designates the $\delta^{13}C$ value of microalgae cultured in a medium containing Label 2-labeled sodium bicarbonate, utilizing the added bicarbonate and \undergoing the CO_2 pathway; (δ_{a1} + 9‰) signifies the $\delta^{13}C$ value of microalgae cultured in a medium containing Label 1-labeled sodium bicarbonate, utilizing the added bicarbonate and completely undergoing the HCO_3^- pathway; (δ_{a2} + 9‰) indicates the $\delta^{13}C$ value of microalgae cultured in a medium containing Label 1-labeled sodium bicarbonate, utilizing the added bicarbonate and completely undergoing the HCO_3^- pathway.

The same chemical substances have the same chemical properties when their concentration is the same and their isotopic compositions (within the range of natural abundance) are slighter different. Thus, regardless of which labeled sodium bicarbonate was added, the same microalgae exercised the same proportion of the bicarbonate pathway under the same culture conditions as well as using the same ratio of added sodium bicarbonate as the carbon source, using the bidirectional isotope tracing culture technology used here, i.e. $f_{b1} = f_{b2} = f_b$; $f_{B1} = f_{B2} = f_B$ [5,6]. Building upon these principles, Eq. 8 is subtracted from Eq. 9, and subsequently, we arrive at Eq. 10 through the simplification of equation.

$$f_B = (\delta_{T1} - \delta_{T2})/(\delta_{a1} - \delta_{a2}) \quad (10)$$

In Eq. 10, obtaining δ_{a1} and δ_{a2} poses great challenges, yet δ_{a1} can be derived as the sum of the $\delta^{13}C$ of Label 1-labeled sodium bicarbonate (δ_{C1}) and the carbon isotope fractionation value (Δ_1) when microalgae utilize inorganic carbon through the CO_2 use pathway. Similarly, δ_{a2} is the sum of the $\delta^{13}C$ of Label 2-labeled sodium bicarbonate (δ_{C2}) and the carbon isotope fractionation value (Δ_2) under identical conditions. Since the culture and growth conditions of microalgae are identical, Δ_1 and Δ_2 are precisely equal, denoted as $\Delta_1 = \Delta_2 = \Delta$. Consequently, the difference between δ_{a1} and δ_{a2} can be converted into the discrepancy between δ_{C1} and δ_{C2}. This conversion leads to a further expression of eq. 10 as depicted in equation 11.

$$f_B = (\delta_{T1} - \delta_{T2})/(\delta_{C1} - \delta_{C2}) \times 100\% \quad (11)$$

where, δ_{C1} is the $\delta^{13}C$ value of Label 1-labeled sodium bicarbonate and δ_{C2} is the $\delta^{13}C$ value of Label 2-labeled sodium bicarbonate.

The obtaining for the proportion of oxygen in the added bicarbonate used by microalgae is similar to that of carbon in the added bicarbonate. Therefore, the proportion of oxygen in added bicarbonate used by microalgae can be expressed as:

$$f_O = [(\delta_{TO1} - \delta_{TO2})/(\delta_{O1} - \delta_{O2})] \times 100\% \quad (12)$$

where, δ_{TO1} represents the $\delta^{18}O$ value of microalgae cultured in a medium with Label 1-labeled sodium bicarbonate, δ_{TO2} designates the $\delta^{18}O$ value of microalgae cultured in a medium Label 2-labeled sodium bicarbonate. Here, δ_{O1} signifies the $\delta^{18}O$ value of Label 1-labeled sodium bicarbonate, while δ_{O2} indicates the $\delta^{18}O$ value of Label 2-labeled sodium bicarbonate.

CRediT authorship contribution statement

Shaogang Guo: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Yanyou Wu:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Conceptualization. **Mohamed Aboueldahab:** Writing – review & editing.

Declaration of competing interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphotobiol.2024.112924.

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