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Review

Diversity of CO_2 Concentrating Mechanisms in Macroalgae Photosynthesis: A Case Study of *Ulva* sp.

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Abstract: Many algae respond to the CO_2 limitation in seawater by inducing a CO_2 concentrating mechanism (CCM) to obtain sufficient inorganic carbon to meet their photosynthetic needs, and *Ulva* sp. is a model population suitable for studying the ecological adaptability of macroalgae. As the dominant species of green tide disaster, *Ulva* sp. often faces strong inorganic carbon restriction due to its rapid growth and high population density and must have evolved a variety of carbon acquisition strategies, such as CCM, to overcome these limitations. This paper briefly summarizes the position and function of the important components of CCM (inorganic carbon transporters, carbonic anhydrase, Rubisco, and pyrenoid) and introduces several indexes suitable for evaluating the relative function of CCMs in macroalgae from the aspects of affinity between photosynthesis and Rubisco for CO_2, and carbonic anhydrase inhibitor. The methods of judging the carbon sequestration pathway of *Ulva* sp., the CCM responses of diversity under different carbon sources, and the related genes that may be involved in the operation of CCMs were summarized. This work could provide a reference for revealing the CCMs of macroalgae and lay a foundation for further research on the inorganic carbon utilization strategy of the *Ulva* sp.

Keywords: CO_2 concentrating mechanism; *Ulva* sp.; green tide; inorganic carbon transporters; carbonic anhydrase; Rubisco; pyrenoid

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1. Introduction

Algae can obtain CO_2, HCO_3^-, and CO_3^{2-} types of inorganic carbon from the ocean, but only CO_2 can easily pass through the cell membrane [1]. CO_2 is the basic substrate of algae photosynthesis. Although seawater is rich in dissolved inorganic carbon (the concentration is as high as 2.2 mM, approximately 170 times that of the atmosphere, and HCO_3^- accounts for about 90% thereof), free CO_2 accounts for less than 1% (approximately 13 μM) and only 1/10,000 as much CO_2 diffuses in seawater as it does in the atmosphere [2]. The manner of obtaining free CO_2 in seawater may be limited by low diffusion coefficient and surface boundary layer range [3]. Calvin cycle is the main way of carbon sequestration in seaweed, while ribulose-1,5-diphosphate carboxylase/oxygenase (Rubisco) is the key enzyme of carbon fixation, which requires inorganic carbon in the form of CO_2. However,

the need for CO_2 by seaweed photosynthesis is generally higher than the CO_2 concentration in natural seawater [4]. For example, the half-saturation constant K_m (DIC concentration when photosynthesis reaches half of the maximum rate) of *Ulva prolifera* to CO_2 is about 250 μM [5], while the CO_2 concentration in natural seawater is only 5–25 μM. The concentration of CO_2 in the environment can not meet the needs of Rubisco, so it must rely on the CO_2 concentrating mechanisms (CCMs) to increase the intracellular CO_2 concentration to maintain normal photosynthesis. Therefore, CCMs evolved in most algae, including improved carbonic anhydrase (CA) activity, active absorption of HCO_3^-, and external acidification region [6,7]. In general, CO_2 concentrating mechanisms refer to the sequentially acting of inorganic carbon transporters and CA in different cellular regions to change the availability of photosynthetic carbon so that CO_2 accumulates in large quantities at the active site of Rubisco, which is beneficial for the enzyme to play the role of carboxylase and inhibit its oxidase activity.

Ulva sp. is a kind of seaweed with wide temperature, wide salt, strong stress resistance, and reproductive ability. Because of its ability to tolerate stress conditions, it has become a popular model organism for studying the biological resistance mechanism of photosynthesis [8]. Some seaweeds of *Ulva* sp., such as *Enteromorpha* (at present, it belongs to the genus *Ulva*, including the main population of green tide, *U. prolifera*, *U. linza*, *U. compressa*, *U. flexuosa*, etc., Figure 1), can rapidly accumulate biomass under suitable environmental conditions, causing macroalgal bloom disasters known as green tides, with serious economic and ecological consequences [9]. In the early stage of the green tide, the rapid proliferation of *Enteromorpha* can remove a large amount of DIC from the seawater, and the free-floating algae form an algal pad with a thickness of 0.5 m. It is difficult for the algae exposed to the atmosphere to obtain DIC from the sea to support photosynthesis, so using CO_2 in the atmosphere as a carbon source leads to a decrease in the sea surface CO_2 partial pressure (pCO_2) [10,11]. It can be said that *Ulva* sp. has a high potential for adaptation to the lack of inorganic carbon. Scholars at home and abroad have done a lot of research on its origin, ecological habits, distribution characteristics, and stress resistance mechanism [8], but the research on its carbon utilization strategy is relatively scarce. Xu and Gao [12] measured the relationship between the photosynthetic oxygen evolution and the concentration of dissolved inorganic carbon (DIC) (P-C curve) in the green algae *U. prolifera*, which was used to reflect the relationship between photosynthesis and inorganic carbon affinity. P-C curve showed that *U. prolifera* grown under high CO_2 condition had higher $K_{1/2}$DIC or $K_{1/2}CO_2$. The decrease of photosynthetic affinity to DIC and/or CO_2 indicated that CCM or CCM activity was decreased. *U. prolifera* grown under the condition of low CO_2 showed higher DIC affinity. In other words, when the inorganic carbon is insufficient, *U. prolifera* will activate the CCM to obtain enough CO_2 for photosynthesis. In addition, as technology advances, scientists are trying to record the overall pattern of photosynthetic carbon capture through the distribution of $^{13}C/^{12}C$ in plant tissues. Liu et al. [4] demonstrated that through the corresponding changes of $\delta^{13}C$ tissue, the relationship between the activities of C_3 enzyme (Rubisco), C_4 enzymes (PEP-Case, PEPCKase, and PPDKase), and CA enzymes (extra- and intra-cellular CA) and photosynthate was analyzed, and the participation of C_3 and C_4 photosynthetic pathways and a CA mechanism (i.e., CCM) in *U. prolifera* was determined. The results show that C_3 and C_4 pathways and the HCO_3^- mechanism supported by CA coexist in *U. prolifera*, which is the first report on the existence of a CCM in *Ulva* sp. [4]. Understanding the CCMs of *Ulva* sp. is of great significance for studying the CCMs of macroalgae and predicting the development trend of green tide under long-term environmental CO_2 increase. Therefore, this study summarized the organization, evaluation, and operational mechanism of CCM from *Ulva* sp. to provide a theoretical foundation for subsequent research.

Figure 1. *U. prolifera*, the dominant species of the Yellow Sea green tide.

2. Composition of CCMs

Equal concentrations of CO_2 and O_2 in surface waters around 500 million years ago may have led to the emergence of algal CCMs [13]. Eukaryotic algal CCMs play an important role in global productivity, molecular phylogeny, and diversity. More than four-fifths of oceans' primary productivity is promoted by some form of CCM [14,15]. The CCM in *Ulva* sp. has three main components: (i) inorganic carbon transporters of plasma and chloroplast membranes; (ii) CAs located in key positions; and (iii) chloroplast microcompartments (pyrenoid) in which large amounts of Rubisco are accumulated [16]. Below, the roles and connections of inorganic carbon transporters, carbonic anhydrase, Rubisco, and pyrenoid in CCM are summarized.

2.1. Inorganic Carbon Transporters

In order to better understand CCM, it is very important to study related proteins, especially the possible existence of plasma membrane and chloroplast membrane inorganic carbon transporters. CO_2 and HCO_3^- are the main sources of inorganic carbon in water. Active CCM leads to rapid uptake of inorganic carbon by plasma membrane [17]. HLA3 (high light activated 3) and LCI1 (low CO_2 induced protein 1) HCO_3^- transporters have been confirmed to be involved in the active absorption of CO_2 and HCO_3^- on the plasma membrane of *Chlamydomonas reinhardtii* [18]. HLA3 has the structural characteristics of hydrophilicity and transmembrane region of ABC transporter and can play a role in the form of HCO_3^- transporter on the plasma membrane under low CO_2 conditions. LCI1 can not only enhance the absorption of HCO_3^-, but also proved to be necessary for active absorption of CO_2 at low CO_2 levels [19,20]. Wang and Spalding [21] reported that at very low CO_2 concentrations, the active transport of HCO_3^- on the chloroplast envelope of *C. reinhardtii* requires LCIA, and LCIB may play a parallel role with HLA3 or LCIA in CO_2 or HCO_3^- uptake pathways. So far, HCO_3^- transporters working on the plasma membrane

and chloroplast envelope of *C. reinhardtii* have been identified, but not on the thylakoid membrane. Previous studies have found that three Bestrophin genes (BST1, BST2, and BST3) located on the thylakoid membrane controlled by the main regulator 'CIA5' are necessary to transport HCO_3^- accumulated in the chloroplast matrix to the thylakoid cavity [22]. CIA5 is essential for inducing the expression of several CCM genes encoding inorganic carbon transporters HLA3 and LCI1 [23]. At present, there is a lack of systematic and in-depth study on the inorganic carbon transporters of *Ulva* species. Gao [24] found the expression of low-CO_2-inducible proteins, low-CO_2-inducible membrane proteins, and CIA5 in some ESTs obtained from the transcriptome sequencing of *U. prolifera*. Rautenberger [25] found four hypothetical HLA3 ABC transporters in the ESTs of *U. prolifera*. Several low CO_2 inducible proteins, a chloroplast Ci transporter (LCIB), several ABC transporters, and a nuclear transcriptional regulator (CIA5, LCR1) of the CCM element were also found in the *U. linza* transcription group [26].

2.2. Carbonic Anhydrase

CA is an enzyme that catalyzes the exchange of CO_2 and HCO_3^- and other reactions (hydration of small CO_2-like molecules, such as COS, and CS_2 to form H_2S, CO_2, or some hydrolysis reactions) in solution [27]. Without CA, the transformation reaction between CO_2 and HCO_3^- will occur; however, the mutual transformation is slow. In nature, CA has several families, denoted by the Greek letters α to θ. All CA active sites have a "bipolar" cavity, half of which are only aligned with hydrophobic amino acid residues, while the other half are aligned with hydrophilic amino acid residues. The hydrophobic part can capture CO_2 molecules, while the hydrophilic part is involved in the release of ions (HCO_3^- and H^+) produced by CO_2 hydration from the cavity. CA is widespread in photosynthetic autotrophs and plays a crucial role in the CCM that promotes the transport of CO_2 to Rubisco. In addition, CA has a similar effect in all types of algae, although the types of CA are different. For example, α-CA and θ-CA catalyze the conversion of bicarbonate to CO_2 in green alga *C. reinhardtii* and diatom *Phaeodactylum tricornutum*, respectively [28]. Different subtypes of CA in plants and eukaryotic algae have also been described [27,29–31]. One of the most well-known functions of CA in algae is to catalyze the conversion between CO_2 and HCO_3^-. The carboxysome CA in cyanobacteria has this effect, and the CA forms include β-CA [27,32], ε-CA [27,33], and γ-CA [27,34], etc. CA with the above functions also exists in the chloroplast thylakoid lumen of eukaryotic algae, including α-CA, CAH3 [35], CAH1, and θ-CA, etc. In addition, the protein complex LCIB/C, which is associated with the chloroplast pyrenoid, was found in the matrix, and the θ-CA-like protein may recapture CO_2 that leaked from the pyrenoid [21]. In algal CCM, another function of CA is to promote the entry of external CO_2 from the environment. These CA located in the cell wall or periplasmic space may be able to sense the level of CO_2 in the environment, maintain the chemical balance among Ci types in the periplasm, and provide sufficient Ci for plasma membrane HCO_3^- transporters or active transport of CO_2. Similarly, various CAs have been found on the cell walls of different algae, such as α-CA in green algae [30,36–38]. However, studies of CA in green macroalgae are few and far between. In *Ulva* sp., Zhang et al. [26] found a possible α-CA in *U. linza*, De Clerck et al. [39] annotated 9 CA homologous genes in the *U. mutabilis* genome. Wang et al. [40] cloned and identified a *U. prolifera* α-CA gene sensitive to environmental changes, and then Wang et al. [41] found two γ-CA genes highly expressed at low pH (pH7.5).

2.3. Rubisco and Pyrenoid

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is present in all oxygenic photosynthetic organisms, participates in the effect of CO_2 on green plants and photoautotrophic bacteria, and is an important enzyme in the photosynthetic C_3 reaction because it catalyzes the first step of photosynthesis by integrating CO_2 in the air into ribulose-1-diphosphate (RuBP). It catalyzes the carboxylation of RuBP and then cleavage to form two PGA (3-phosphate-glyceric acid) molecules. Rubisco is thus the main gateway of inorganic

carbon into the biosphere and has a profound impact on the global scale [42]. Although this enzyme is one of the most abundant proteins on earth, it is very inefficient [43]. Through the sequential action of inorganic carbon transporters and carbonic anhydrase in different cellular regions, CO_2 is actively accumulated around Rubisco [44]. Compared to ordinary enzymes, Rubisco can catalyze both carboxylation and oxygenation. It has a bifunctional property and performs either carboxylase or oxygenase activity depending on the availability of the substrate, for which both CO_2 and O_2 can be used [43,45,46]. O_2 and CO_2 are competitive inhibitors of the carboxylase and oxygenase reactions, respectively. Rubisco, therefore, lies at the intersection of two opposite but interlocking cycles of photosynthetic carbon reduction (photosynthesis) and photosynthetic carbon oxidation (photorespiration) (Figure 2). When the concentration ratio of CO_2:O_2 is high, Rubisco catalyzes carboxylation, which catalyzes the first key carbon fixation reaction in the Calvin cycle in photosynthesis, converting free CO_2 in the atmosphere into energy storage molecules in organisms. When the concentration ratio of CO_2:O_2 is low Rubisco promotes the catalyzation of oxygenation and participates in the photorespiratory cycle [45]. Since Rubisco tends to participate in oxygenase reactions and the catalytic conversion rate of active sites is low, organisms have evolved various strategies to optimize Rubisco's performance when adapting to carbon limitations in a specific environment. There are mainly two strategies developed synergistically. On the one hand, Rubisco forms with different catalytic efficiencies have evolved. On the other hand, the underlying structure evolved to ensure that Rubisco received high concentrations of CO_2 substrate, often referred to as the CCM [3], for example, C_3 plants capture CO_2 from the surrounding atmosphere by passive diffusion based on Rubisco, which has developed a high affinity for CO_2; C_4 plants that evolved from C_3 ancestors added structural and biochemical specializations which allows them to concentrate CO_2 around the Rubisco active site [47].

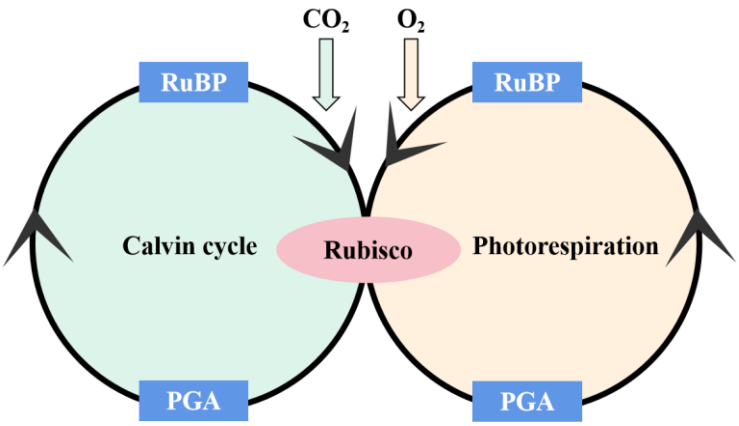

Figure 2. Schematic diagram of Rubisco functions. RuBP is ribulose 1, 5-diphosphate, and PGA is 3-phosphoglyceric acid.

Pyrenoid is an evolutionary adaptation that enables algae to fix inorganic carbon from CO_2-limited environments more efficiently. This contemporary definition was first reported in 1782, with an unannotated dot mark in a painting of *Conferva jugalis* (now Spirogyra) in the Flora Danica, one of the earliest records of pyrenoids [48]. The word pyrenoid comes from the Greek $\pi\upsilon\rho\eta\nu$ (*pyren*, kernel), created by Schmitz in 1882 in a monograph on algal chloroplasts [48]. Rubisco promotes about 10^{14} kg of carbon into the biosphere each year [49,50]. However, in many plants, the rate at which Rubisco fixes CO_2 at atmospheric CO_2 levels is less than 1/3 of its maximum rate, which restricts the growth of many crops. To overcome this limitation, numerous photosynthetic organisms comprising algae increased the CO_2 fixation rate of Rubisco by providing it with concentrated CO_2. In algae, this CCM occurs in a phase-separated organelle called the pyrenoid. Pyrenoid-based CO_2 concentrating mechanisms (PCCMs) mediate approximately 1/3 of the global CO_2 sequestration [50]. Nevertheless, not all algae have pyrenoid or CCMs. The appearance of

CCM is not completely consistent with the existence of pyrenoids, which is not the only component of CCM. Kevekordes et al. [51] showed that the occurrence of CCM was not completely related to the presence of pyrenoids in *Caulerpa*.

Pyrenoids are permeable microchambers that contain tightly packed Rubisco that are present in the chloroplast stroma of many algae and operate CCM [47,52]. CCM increases the concentration of CO_2 near Rubisco using an inorganic carbon pump, the synergy of one or more CA, and the packaging of Rubisco into pyrenoid [53]. Pyrenoids are found in almost all algae except Chrysophyta [53] and are relatively rare in algae with thallus but have been observed in *Ulva*, *Monostroma*, and *Porphyra* [54,55]. Some algae lacking pyrenoid increase the acquisition of CO_2 by acidifying the extracellular environment, effluxing active protons, or exporting CA to periplasm [56]. Pyrenoids are not necessary for CCM. However, the CCM is more effective when pyrenoids are present [57]. For example, the accumulation of Ci in *C. reinhardtii* is 5–10 times higher than that of closely related species without pyrenoids, such as *Chloromonas* [58]. Therefore, evolutionary adaptation must take place in pyrenoids to improve the performance of the CCM. In many cases, pyrenoids and chloroplasts do not necessarily occur together. For green macroalgae, *Ulvales* are present in both, while in *Caulerpales*, some *Codium* and *Caulerpa* sp. have multiple chloroplasts but lack pyrenoid [51]. Most green algae contain pyrenoids, the thylakoid extends into the pyrenoid and starch grains are attached to the pyrenoid surface. The chloroplast of *Ulvales* is layered with thylakoid lumen and pyrenoid [47]. He et al. [59] reported that electron microscope photos of most *U. clathrata* showed that there were multiple pyrenoids in their chloroplasts, their morphological and structure were the same, and the pyrenoids were located in chloroplasts, oval- or bean-shaped. The pyrenoids were surrounded by starch sheaths and were connected to the thylakoid of the chloroplast through longitudinal channels extending to both ends. When cells move from a high CO_2 concentration environment to a low CO_2 concentration environment, the ultrastructure of the pyrenoid changes greatly, and the starch sheath surrounding the pyrenoid can be rapidly formed. However, Villarejo et al. [60] have shown that the starch sheath is closely related to CCM but is not involved in the process. He et al. [59] utilized metallographic immunomolecular localization and found that Rubisco gold-labeled granules of *U. clathrata* were mainly distributed in the pyrenoid and starch sheath chloroplasts and were rarely distributed in the chloroplast thylakoids and stroma. The molecular localization of Rubisco activase demonstrated that it was mainly distributed in the pyrenoid and starch sheath. These results indicated that the pyrenoid (and starch sheath) of *U. clathrata* was the same as that of unicellular green algae and had a photosynthetic function. In addition, Cai et al. [61] found that CO_2 affects the distribution of Rubisco in *U. clathrata*. When CO_2 concentration increased, Rubisco tended to diffuse into the chloroplast matrix, and when cultured with a low concentration of CO_2 or without CO_2, Rubisco was continuously concentrated in the pyrenoids.

3. Indicators to Measure CCMs in Macroalgae

According to the different initial photosynthetic products of carbon assimilation in photosynthesis, terrestrial plants were divided into three types: C_3, C_4, and CAM plants. The majority of the plants are C_3 plants, and C_4 and CAM plants developed from the C_3 plants. The C_3 pathway is the most common and primitive pathway among the three types of photosynthetic carbon metabolism pathways, and its initial photosynthetic product is 3-phosphoglycerate (PGA) [62]. Compared with C_3 plants, C_4 plants increase the CO_2 concentration around Rubisco through a biochemical CCM [63,64]. Terrestrial C_4 plants generally have Kranz anatomy, which is necessary for most terrestrial plants to perform the C_4 photosynthetic pathway [65]. The Kranz anatomy is composed of mesophyll cells and their adjacent vascular bundle sheath cells, which can effectively prevent CO_2 from escaping outward [66], thereby accumulating the CO_2 concentration at the active site of Rubisco [67]. Algae are a diverse group of aquatic organisms and do not have the Kranz anatomy of terrestrial plants. In order to adapt to the low CO_2 environment of

seawater, most algae have evolved a CCM to ensure the supply of CO_2 for photosynthesis. Although algae do not have Kranz anatomy like terrestrial C_4 plants, they still possess the photosynthetic properties of the C_4 pathway [68]. An example is the use of pyruvate orthophosphate dikinase (PPDK) to catalyze the formation of phosphoenolpyruvate (PEP), the initial molecule receptor for fixed CO_2.

To assess the diversity of the CCM functions and activities in different algae, reliable metrics to measure and quantify the relative functions of CCM need to be established. As reported by Badger et al. [47], we screened out several indicators that may be suitable for measuring the CCM of macroalgae were demonstrated.

3.1. CO_2 Affinity of Photosynthesis versus Rubisco

Rubisco's low affinity for external CO_2 drove the evolution of CCMs. Therefore, one of the most useful indicators of whether a photosynthetic organism has a CCM is to compare the affinity of photosynthesis for external CO_2 with the affinity of Rubisco for CO_2. This approach was originally applied to higher plants, and by measuring the affinity K_m (Rubisco CO_2) of Rubisco extracted from photosynthetic organisms to substrate CO_2, it was inferred that C_3 species might require a CCM [69]. However, due to the limitation of extraction technology, the affinity determination of the photosynthetic organism Rubisco lacked accuracy. With the improvement of extraction and analysis techniques, this method has become widely used to evaluate algal CCMs. In general, the researchers measured the photosynthetic oxygen evolution rate under different inorganic carbon concentrations and used the data analysis software to fit the inorganic carbon response curve (P-C curve) by using Michaelis–Menten equation, where K_m is the substrate concentration (DIC concentration) when the photosynthetic rate is half of the maximum, from which we can infer the affinity of algae photosynthesis to CO_2 K_m (photosynthetic CO_2). Since marine algae may experience more persistent CO_2 limitations than fresh- and brackish-water autotrophs, it can be assumed that the type of water environment determines the effectiveness of CCM. Comparing the in vivo photosynthesis and the in vitro carboxylation of Rubisco under ambient oxygen levels can show its ability to concentrate CO_2 exceeds the level of environmental CO_2 [70]. For example, *C. reinhardtii*'s ratio of K_m (photosynthetic CO_2) to K_m (Rubisco CO_2) is approximately 1:30, and that of *Ulva* sp. is about 5–10:68 [1,71,72]. Both apparently rely on CCM to improve the efficiency of Rubisco, whereas other algae with close specific gravity may not use CCM.

3.2. Effects of CA Inhibitors

Because CA is central in all chloroplast CCM models, determining the effect of CA inhibitors is useful in studying the functional aspects of algal CCM. This is especially true for chloroplast-penetrating inhibitors, such as EZA (Ethoxzolamide), which are likely to inhibit most forms of internal CA. EZA is often used to obtain low inorganic carbon affinity for photosynthesis of algae. If the algae showed low inorganic carbon affinity under the action of EZA, it is proved that there is intracellular CA-mediated CCM in the algae, but no effect may not be conclusive evidence of CCM deletion. For CA inhibitors that cannot penetrate cell membranes, such as AZA (Acetazolamide), only apply to inhibit the activity of external CA [47], the effect on the function of chloroplast CCM is less easily explained. By comparing the relative effects of EZA and AZA, the relative contributions of internal and external CA forms to photosynthetic Ci absorption can be determined (Figure 3). For example, in the study of Gao et al. [73], after adding AZA the net photosynthetic rate of *U. linza* decreased, and the inhibition rate was noted to be 26.26%. Compared with AZA, EZA demonstrated a higher inhibition rate of photosynthesis (75.19%), which indicated that the internal CA contributed more to the absorption of photosynthesis. Xu et al. [74] found that *U. linza* and *U. prolifera* have obvious extracellular and intracellular CA activity by using different CA inhibitors. However, extracellular CA enzyme activity itself accelerates the mutual conversion between HCO_3^- and CO_2 but cannot affect the CO_2 equilibrium concentration, and CO_2 mainly enters the cell through passive diffusion. This indicates that

HCO_3^--utilization in this manner does not function well at high pH (pH > 9.4) because the equilibrium concentration of CO_2 is low. It is obvious that in addition to the extracellular CA catalytic utilization of HCO_3^-, there must be another means of using HCO_3^- in *U. prolifera*.

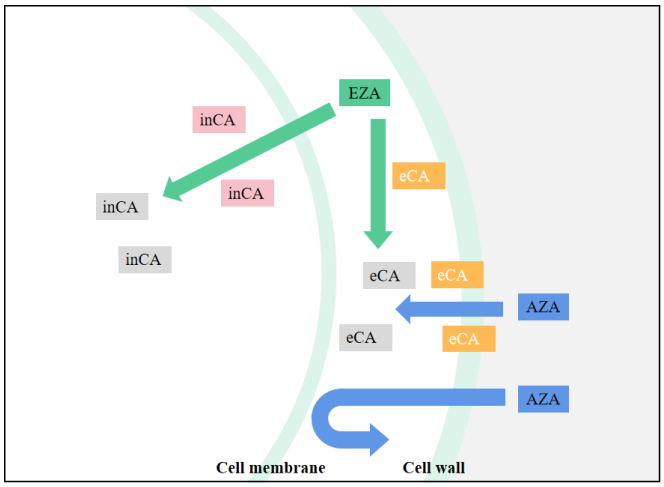

Figure 3. Schematic diagram of CA inhibitor actions. inCA means intracellular CA, eCA means extracellular CA, and when the CA icon is gray, CA is suppressed.

3.3. Using HCO_3^- as Photosynthetic Substrate

The function of HCO_3^- in photosynthesis has been perceived as a property of algae [75]. The utilization of HCO_3^- by algae depends on the involvement of external CA and plasma membrane transporters [76–78]. The use of HCO_3^- is strongly correlated with the presence of a CCM. However, some macroalgae are not only capable of utilizing HCO_3^- as a carbon source, some have demonstrated the use of CO_2 in specific situations. Through the detection of $\delta^{13}C$ isotopes in *Gracilaria* and *Ulva*, it was found that when the concentration of environmental CO_2 was high, there was a physiological transition from using HCO_3^- almost exclusively to predominantly using CO_2. At the current seawater pCO_2, many macroalgae use HCO_3^- rather than dissolved CO_2 and utilize CA to convert HCO_3^- to CO_2 for use by Rubisco [79–82]. For example, Mercado et al. [83] found that under the current seawater CO_2 level, the chlorophyll plants *U. rigida* and *U. compressa* cannot obtain sufficient CO_2 through diffusion absorption alone; therefore, a CCM must be used to obtain HCO_3^-. However, macroalgae may downregulate their CCM, reduce HCO_3^- use, and become dependent on CO_2 as the main carbon source when CO_2 concentrations are high [1,84–86]. Consequently, when using these indicators to evaluate the CCM of *Ulva* sp., there may be a decrease in the use of HCO_3^- in certain circumstances, where there is still CCM activity but with a certain degree of downregulation.

3.4. Changes in Affinity to External Ci Depends on Growth Ci Conditions

When there is Ci limitation in the external environment, the affinity of the Ci transporter for external CO_2 and HCO_3^- increases, and the intracellular and extracellular CA activity also increases. The two work together to increase the affinity of microalgae for external Ci by more than 10 times [76]. This inducible change appears to be strong evidence that cellular infrastructure is involved in supplying CO_2 to Rubisco in chloroplasts. These inducible CCM are not limited to microalgae but have been observed in many macroalgae as well, including *Ulva* [1], *Gracilaria* [87], *Porphyra* [88] and *Fucus* [89]. That is, some algae may have an inducible CCM to meet their environmental needs when facing external Ci periodic constraints, while other algae may not have such a flexible arrangement.

4. Operation of *Ulva* sp. CCM

To determine the type of photosynthetic carbon metabolism of seaweed, it is usually investigated from two aspects: a. The flow direction of C element in photosynthetic metabo-

lites during the carbon assimilation process; b. The activity of photosynthetic enzymes in the carbon assimilation pathway. The former uses ^{14}C isotope tracing technology to focus on the radioactivity intensity of key metabolites (3-phosphoglyceric acid, malic acid, oxaloacetic acid, aspartic acid, etc.) after supplying ^{14}C to seaweed. If the initial fixed product is 3-phosphoglyceric acid (3-PGA), it may be the C_3 pathway. If the initial fixed products are malic acid, oxaloacetate, and aspartic acid, it may be the C_4 pathway. The latter can focus on the activity differences of Rubisco carboxylase and PEPC enzymes, the key enzymes of the C_3 and C_4 pathways, from an enzymatic perspective.

Enteromorpha is the dominant species of green tide, which is a large-scale algal bloom disaster. Its thick floating pad is bound to be strongly restricted by inorganic carbon (Figure 4). Thus, it must have evolved a variety of carbon acquisition strategies, such as CCM, to overcome these limitations. Karekar and Joshi [90] found in *U. lactuca* and *U. tubulosa* that ^{14}C appeared not only in 3-PGA but also in aspartic acid after feeding for 10 s, while considerable ^{14}C labeled malic acid appeared in *U. lactuca* after 30 s. Therefore, it is considered that there are both C_3 and C_4 pathways in *Ulva* sp. *Ulva* sp. has weak photorespiration and a low CO_2 compensation point, demonstrating characteristics of C_4 plants in gas exchange [91]. However, Beer et al. [92] examined an *Ulva* sp. alga, the ^{14}C isotope tracer results showed that approximately 90% of the radioactive ^{14}C appeared in 3-phosphoglyceric acid (3-PGA), and the Rubisco carboxylase activity was about 10-times that of the PEPC activity. At the same time, due to the efficient uptake of HCO_3^-, algae may transport high concentrations of CO_2 to chloroplasts, which inhibits the activity of Rubisco oxygenase. This hypothetical inorganic carbon enrichment mechanism was confirmed in the green algae *U. compressa* and *U. fasciata*. However, after a few seconds, about 12% of the radioactive ^{14}C was present in aspartic acid, indicating that *Ulva* sp. could use other metabolic pathways besides C_3 pathway [93]. Xu et al. [94] and Liu et al. [4] reported that the C_3 and C_4 pathways and CCM coexist in *U. prolifera*. Based on the above research, we listed all *Ulva* sp. known to have CCMs (Table 1) and attempted to summarize the CCMs of *Ulva* sp.

Figure 4. Thick floating pad in 2018 Qingdao green tide.

Ulva mainly uses HCO_3^- as a carbon source in normal seawater. The CCMs have three pathways to utilize HCO_3^- (Figure 5): (i) Extracellular CA bound to the cell wall promotes the dehydration of HCO_3^-, forming CO_2 in the cell membrane. (ii) When the pH of the environment is high, the low concentration of CO_2 in the cell membrane induces the DIDS-sensitive membrane anion exchanger to transport HCO_3^- into the cell and, under the action of intracellular CA, dehydrated to form CO_2. (iii) The proton pump located in the cell membrane utilizes the energy generated by the hydrolysis of ATP to pump H^+ out of the cell membrane, forming an acidic region in the cell wall, and promoting the dehydration of HCO_3^- to form CO_2, which enters the cell membrane.

Table 1. Species of *Ulva* known to have CO_2 concentrating mechanisms (CCMs).

Species	References
Ulva australis	[95]
U. pertusa	[96,97]
U. lactuca	[98]
U. linza	[68,99,100]
U. prolifera	[4,74,100–102]
U. rigida	[1,103,104]
U. compressa	[100]
U. pulchra	[1]
U. reticulata	[1]

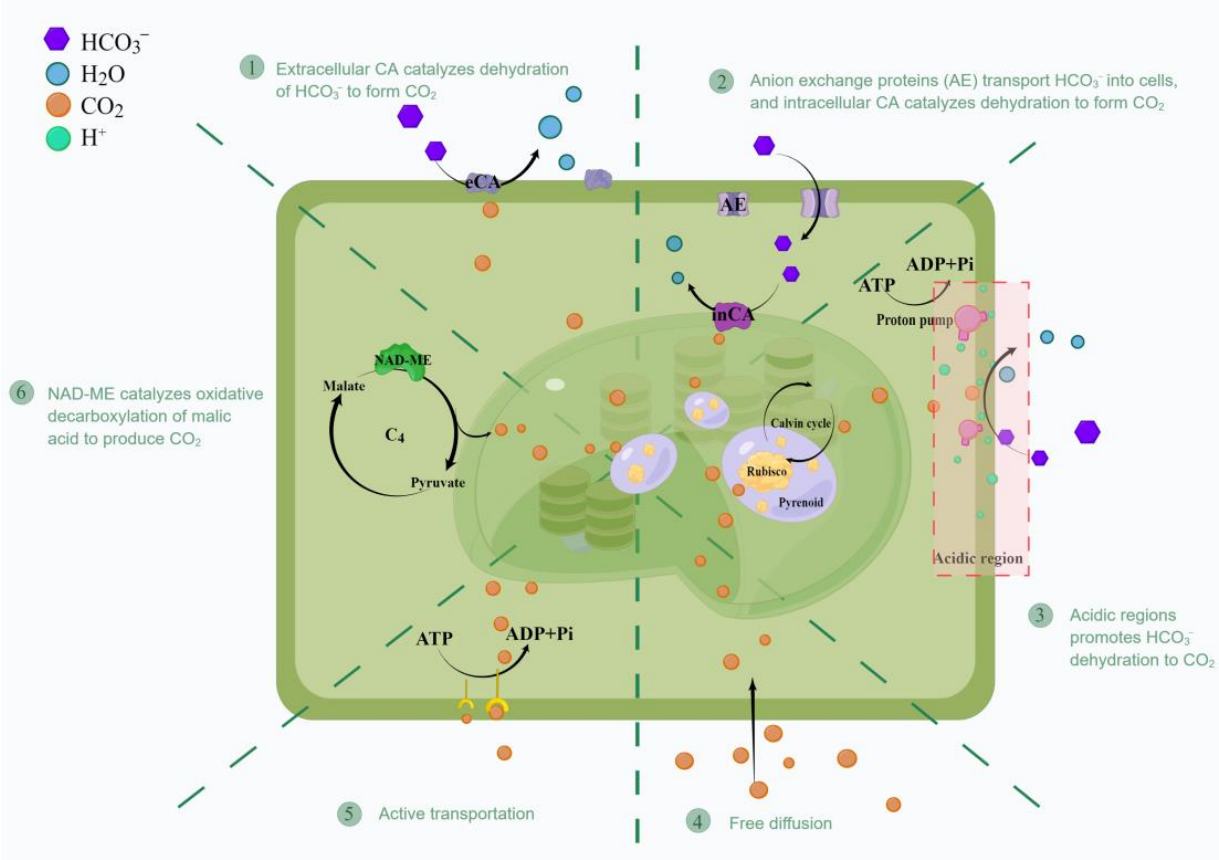

Figure 5. Model diagrams of different methods of CO_2 concentrating mechanism (By Figdraw). Different colors are used to represent different molecules/ions: purple: HCO_3^-, blue: H_2O, orange: CO_2, green: H^+. ① Extracellular CA bound to the cell wall promotes the dehydration of HCO_3^-, forming CO_2 in the cell membrane. ② When the pH of the environment is high, the low concentration of CO_2 in the cell membrane induces the DIDS-sensitive membrane anion exchanger to transport HCO_3^- into the cell and, under the action of intracellular CA, dehydrated to form CO_2. ③ The proton pump located in the cell membrane utilizes the energy generated by the hydrolysis of ATP to pump H^+ out of the cell membrane, forming an acidic region in the cell wall, and promoting the dehydration of HCO_3^- to form CO_2, which enters the cell membrane. When the environmental pH and the content of HCO_3^- were low, but the concentration of CO_2 was high, *Ulva* sp. preferred to use CO_2 as a carbon source, and CO_2 was transported into the cells through ④ passive diffusion or ⑤ possible CO_2 transporters. ⑥ NAD-ME catalyzes the oxidative decarboxylation of malic acid to produce CO_2, which is added to the Calvin cycle.

In the work of Björk et al. [105], the existence of pathway (i) in the green algae (*U. rigida* C. Ag.) was demonstrated. They believe that for *U. rigida* C. Ag., the main form of

inorganic carbon entering the cell through the plasma membrane is CO_2, while HCO_3^- is dehydrated on the cell wall surface or in the cell wall under the catalysis of external CA to form CO_2, and then enters the cell. External CA-mediated dehydration preceded Ci absorption. CA-mediated HCO_3^- uptake is a conventional form of CCM in macroalgae, and when the environment changes, macroalgae appear to have inducible CCMs similar to microalgae.

Drechsler and Beer [106] and Drechsler et al. [107] reported the mechanism of direct absorption of HCO_3^- in *Ulva* sp. (pathway (ii)). Its function is similar to the anion transport in red blood cells mediated by the anion exchange protein AE1. In subsequent studies, it was found that growing algae in high pH conditions induces an AE1-like system. In addition, AE1 plays a crucial role in the electron neutralization exchange of HCO_3^- and inorganic carbon in red blood cells, which is a basic process of clearing respiratory CO_2 through blood flow to the lungs [108]. Due to the functional similarity between the putative HCO_3^--transporter of *Ulva* sp. and the anion exchange of erythrocytes, we found that there are structural similarities between them. This functional similarity, combined with the current findings at the structural level, suggests that similar HCO_3^--transport systems exist in the cells of two different organisms: mammalian and green macroalgae [109]. That is, the uptake of HCO_3^- by *Ulva* sp. is mediated by a protein that has a function similar to that of an anion exchanger on mammalian cell membranes, especially erythrocyte cell membranes. The evidence for the absorption of HCO_3^- by *Ulva* sp. (without an external CA) is that the photosynthetic rate observed under pH 8.2 was higher than that predicted by the extracellular CO_2 supply, which indicates that Ci must be absorbed in the form of ions. Once inside the cell, the lower internal pH and presence of an internal CA promote the dehydration of HCO_3^-, thus providing Rubisco with sufficient CO_2 to meet the measured high photosynthetic rate [89].

Gao et al. [73] treated *Ulva* sp. with Tris buffer and found that it had an inhibitory effect on photosynthesis, demonstrating and reporting for the first time that the *Ulva* cell wall has an acidic region as one of the CCM pathways (pathway (iii)). Acidic regions have previously been found in other macrophytes, such as *Ruppia cirrhosa* [110], *Zostera marina*, *Z. noltii* [111,112], and *Laminaria saccharina* [113]. Acidic regions are created by expelling protons out of the plasma membrane, making the CO_2 concentration in these regions higher than its level in the medium, which can facilitate the rapid diffusion of CO_2 into the cell through the membrane itself or the protein pores [113,114]. The acidic region located in the cell wall of *Ulva* sp. promotes the dehydration of external HCO_3^-, and the increase of extracellular CO_2 concentration is conducive to the entry of CO_2 into the cell membrane to participate in follow-up reaction, that is, CCM of *Ulva* sp. can absorb exogenous Ci under the cooperation of acidic region and external CA.

Pathway (i) seems to be ubiquitous in *Ulva* sp., in this mechanism HCO_3^- is dehydrated outside the cell with the external CA, and then the formed CO_2 is absorbed into the cell. Photosynthesis that is dependent on this mechanism is completely and selectively inhibited by CA inhibitors that do not pass through the plasma membrane (e.g., dextran-bound sulfonamide) or pass very slowly through the plasma membrane (e.g., acetazolamide), but is not sensitive to DIDS. Due to the efficient utilization of CA enzymes, this method of using HCO_3^- has a high proportion in normal seawater at pH 8.1. With the increased pH, the pathway (i) is gradually suppressed. Correspondingly, algae have evolved an inducible CCM similar to those described by several green microalgae to utilize HCO_3^- under high pH conditions. For a few green macroalgae, the photosynthesis induced by high pH could be inhibited by anion exchange protein inhibitors (DIDS). The basic mechanism is insensitive to AZA. With the support of this mechanism, photosynthesis under high pH conditions is faster than spontaneous dehydration of HCO_3^-. Therefore, the direct uptake of HCO_3^- through the plasma membrane is mediated by the anion exchangers process [115].

In addition, some studies have found that although *Ulva* sp. has high expression of key enzymes in the C_4 pathway under stress [68], *Ulva* sp.'s PEPCK may be involved

in other biological processes (such as gluconeogenesis or providing PEP for antibacterial intermediate metabolites) or indirectly affect CCM (by affecting amino acid metabolism and transport, indirectly affect the carbon sequestration process), and it is more likely to use NAD-ME C_4 type CCM to tolerate stress caused by CO_2 deficiency [8,116].

5. CCM Gene of *Ulva* sp.

Many aquatic organisms, especially green algae and cyanobacteria, have evolved CCMs to increase the concentration of intracellular CO_2 to compensate for the low affinity of Rubisco to CO_2. For example, *Synechocystis* PCC6803, *Synechococcus* 7942, and *C. reinhardtii* are model organisms for studying CCMs. The proteins involved in the algae CCM mainly include various CA, transcriptional regulators, low CO_2-inducible membrane proteins, low CO_2-inducible proteins, various carrier proteins, chloroplast proton extrusion proteins, plasma membrane-type H^+-ATPase (proton pump) and various key enzymes involved in the carbon fixation process (Table 2). We used SequenceServer 2.0.0.rc8 [117] to perform BLASTP alignment of the collected predicted CCM proteins using the *U. prolifera* genome data. When $e < 10^{-5}$, the aligned sequences were considered to have high homology (Table 2).

Table 2. Copy number of proteins that may be involved in algal CCM found in the *U. prolifera* genome database.

Protein	NCBI Accession Number	Copy Number in *U. prolifera genome*	References
alpha carbonic anhydrases	CAH1 [BAA14232]	5	[24,25,118]
	CAH2 [CAA38360.1]	5	
	CAH3 [EDP00852.1]	5	
beta carbonic anhydrases	CAH6 [AAR82947.1]	0	
	CAH8 [ABS87675.1]	0	
gamma carbonic anhydrase	CAG2 [XP_001701594]	3	
carboxysomal-located carbonic anhydrase	ccaA/icfA [P27134.1]	0	[119]
phosphoribosyl aminoimidazole carboxylase	purK [AAB05791]	1	
NADH dehydrogenase	ndhB [CAA46161.1]	0	
nuclear transcriptional regulators of CCM elements	CIA5 [AAG37909.1]	2	[24,25,118,120]
	CIA5 [AF317732_1]	2	
	LCR1 [BAD13492.1]	1	
low-CO_2-inducible proteins	LCIA [BAD16681.1]	1	
	LCIB [BAD16682.1]	3	
	LCIB [EDP04243.1]	3	
	LCIC [BAD16683.1]	3	
	Lci2 [AAC31958.1]	1	
low-CO_2-inducible membrane protein	[KAF5834422.1]	1	
	LCIA [XP_001703387.1]	0	
chloroplast carrier protein 1	CCP1 [EDP04147.1]	27	[25,118]
chloroplast proton extrusion protein	CemA [XP_001696592]	1	
pyruvate orthophosphate dikinase	PPDK [JN222388.1]	4	[68]
	PPDK [JN936854.1]	2	
ribulose-1, 5-biphosphate carboxylase	RuBPCase [AAR19268.1]	2	

Table 2. *Cont.*

Protein	NCBI Accession Number	Copy Number in *U. prolifera genome*	References
high and medium affinity HCO_3^- transporters	SbtA [UOW71290.1]	0	[24,121]
	BicA [Q14SY0.1]	1	
putative ABC transporter/high light-activated 3	MRP1/HLA3 [AAL35383.1]	26	[25,118,122]
	HLA3 [XP_001700040.1]	26	
plasma membrane-type H^+-ATPase	[AQM50087.1]	12	[73,123,124]
	[P19456.2]	7	
bestrophin-like protein	BSTs [NP_191691.2]	7	[22]
proton gradient regulation 5	PGR5 [OAP09444.1]	1	[44]
proton gradient regulation like protein	PGRL1 [XP_001692513.1]	1	
flavodiiron protein B	FlvB [AMJ52190.1]	2	

6. Conclusions

The study of the mechanism of photosynthetic carbon sequestration is the basis for understanding the photosynthetic physiology, ecological adaptation, and biomass accumulation of macroalgae, and *Ulva* sp. is a model population suitable for the study of inorganic carbon utilization of macroalgae. The green tide formed by the outbreak of *Ulva* sp. can occur all over the world, which shows the great potential of *Ulva* sp. to adapt to the limitation of inorganic carbon. *Ulva* sp. may form an effective adaptation strategy to survive and develop under the inorganic carbon limitation caused by high population density, which may contain diverse and flexible CCMs. So far, the CCMs of *Ulva* sp. have been reported to include: under normal seawater pH, extracellular CA, and acidic region act synergistically; when the green tide breaks out, a large number of algae cover the sea surface, resulting in a sharp increase in pH, which will induce the anion exchange protein AE to transport HCO_3^- into the cell. In the future ocean acidification environment, higher CO_2 concentration in the environment will inhibit the extracellular CA and anion exchange protein AE pathways and mainly rely on the acidic region and CO_2 diffusion or transport pathways; under high light stress, extracellular CA pathway and C_4 pathway cooperate to supplement C_3 cycle; in addition, *Ulva* sp. also uses NAD- ME C_4 type CCM to tolerate stress caused by lack of CO_2.

The study of *Ulva* sp. CCM is helpful in revealing the adaptation strategies of macroalgae to inorganic carbon limitation, but there are still some unsolved problems. The function and expression of specific genes of CCMs, the physiological function of various transporter proteins of CCMs, and the changes in cell level, expression level, and metabolite level in the response process of CCMs are all urgent problems to be solved. With the continuous advancement of science and technology, the method of multiomics has been applied to the analysis of various metabolic mechanisms of algae. By comparing the differences of *Ulva* sp. in CCM using physiology and multiomics, it may be possible to make some progress in elucidating how *U. prolifera* gains a competitive advantage and explaining the internal reasons for its success in the Yellow Sea green tide outbreak.

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Abbreviations

CCM: CO_2 concentrating mechanism; DIC, dissolved inorganic carbon; CA, carbonic anhydrase; PEPC, phosphoenolpyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; ME, malic enzyme; NAD, nicotinamide adenine dinucleotide.

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