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Structural insights into θ -type carbonic anhydrases 3 and 4: Tuning the directionality of CO₂ hydration in a diatom

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Carbonic anhydrase (CA) catalyzes the reversible hydration of carbon dioxide (CO₂) to bicarbonate (HCO₃[−]) and plays an essential role in carbon fixation in marine diatoms. Here we report the structural and functional characterization of a novel CA, θ -CA3, from the diatom *Phaeodactylum tricornutum*, elucidating its physiological role and catalytic mechanism. AlphaFold prediction, sequence alignment, and metal analysis showed that θ -CA3 is a dimeric enzyme, with each monomer composed of two zinc-binding catalytic domains. High-resolution X-ray crystallographic structures of domain 2 of θ -CA3 in the CO₂-bound form revealed the detailed substrate binding pattern in the active site. Site-directed mutagenesis showed that Asp49 and Arg117 in the active site are essential for catalysis. Notably, introducing a negative charge near the active-site entrance resulted in a mutant enzyme with markedly increased activity under acidic pH, suggesting that electrostatic modulation of the active-site environment regulates proton transfer and catalysis. Furthermore, we identified an HCO₃[−] ion at the dimer interface that contributes to enzyme activation. Collectively, our findings provide fundamental structural insight into how the active-site electrostatic charges and metal environment govern the catalytic efficiency of θ -CA3, offering a new perspective on the molecular basis of carbon fixation in diatoms.

Introduction

Establishing an efficient global carbon dioxide (CO₂) cycle is an urgent challenge in achieving a sustainable green society. As long as fossil fuels remain in use, the CO₂ fixation capacity of land plants and marine algae continues to draw attention for its essential role in global carbon regulation. Among these organisms, diatoms are estimated to contribute approximately 20% of global CO₂ fixation, underscoring their critical role in the biosphere's carbon cycle [1,2]. Due to the high salinity and alkalinity of seawater, most dissolved

inorganic carbon (DIC) exists as bicarbonate (HCO₃[−]) ions, while the concentration of molecular CO₂ remains extremely low. To sustain active CO₂ fixation under such limiting conditions, algae have evolved specialized CO₂-concentrating mechanisms (CCMs) [3]. These mechanisms generally involve two main steps. First, DIC—primarily HCO₃[−]—is actively transported into the cell from the external environment or cytosol via specific transporters located in the plasma membrane and chloroplast membranes. In the model

Abbreviations

CA, Carbonic Anhydrase; CCM, Carbon-Concentrating Mechanism; DIC, Dissolved Inorganic Carbon; DLS, Dynamic Light Scattering; RMSD, Root mean square deviation; Rubisco, Ribulose 1,5-bisphosphate carboxylase/oxygenase.

diatom *Phaeodactylum tricornutum*, genes encoding HCO_3^- transporters from the SLC4 as well as Bestrophin (Best) family anion channels have been identified. These transporters are thought to deliver HCO_3^- to the pyrenoid, a sub-chloroplast compartment where ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) condensates [4–6]. In the second step, carbonic anhydrase (CA) rapidly catalyzes the conversion of HCO_3^- to CO_2 through the reaction of $\text{HCO}_3^- + \text{H}^+ \rightarrow \text{CO}_2 + \text{H}_2\text{O}$, thus supplying CO_2 to Rubisco. This reaction is essential for maintaining a high local CO_2 concentration within the pyrenoid and preventing CO_2 leakage.

Carbonic anhydrases are metalloenzymes that catalyze the reversible hydration between CO_2 and HCO_3^- . Based on their amino acid sequence, CAs are categorized into eight distinct classes: α , β , γ , δ , ζ , η , θ , and ι . Although these classes are not phylogenetically related, most CAs possess metal ions (e.g., Zn^{2+}) at their catalytic centers, with the exception of some ι -class members [7–9]. In *P. tricornutum*, α -, β -, γ -, and θ -type CAs have been identified. α -CA is localized to the matrices of the four-layered plastid membranes, whereas β -CA resides in the pyrenoid; both enzymes are thought to prevent CO_2 leakage from their respective compartments. γ -CA is localized in mitochondria, although its physiological role remains unclear [10–12]. Members of the θ -CA family, which was discovered through homology searches using Low CO_2 Inducible Protein B (Lcib) from the green alga *Chlamydomonas reinhardtii*, identifying four novel θ -CAs (θ -CA1–4) on chromosome 1 of *P. tricornutum* [13,14], are thought to be involved in intracellular inorganic carbon flow. θ -CA1, in particular, is embedded in the thylakoid membrane that traverses the pyrenoid, where it facilitates the rapid conversion of HCO_3^- to CO_2 under the acidic luminal conditions, supplying CO_2 to Rubisco. Overexpression of θ -CA1 enhances photosynthesis, whereas suppression impairs photosynthetic performance and cell division, indicating its essential role in carbon fixation [13]. The CA activities of θ -CA1 and θ -CA2 have been demonstrated *in vitro*, and their standard catalytic mechanisms were elucidated via X-ray crystallography [13,15,16].

In contrast, θ -CA3 and θ -CA4 are cytosolic proteins localized between the chloroplast and mitochondria, and between the chloroplast and vacuole, respectively [13]. Their physiological roles and structure–function relationships remain largely unknown. Similar to other CA isoforms, they are hypothesized to participate in inorganic carbon flux regulation within the CCM. In this study, we have explored the structure and function of the uncharacterized θ -CA3 enzyme. Physiological

analyses reveal that both θ -CA3 and θ -CA4 in *P. tricornutum* contribute significantly to photosynthetic efficiency, likely by recapturing CO_2 diffusing from the chloroplast or mitochondria and converting it to HCO_3^- , thereby reducing CO_2 leakage. Furthermore, X-ray crystallographic analysis provides insight into its unique catalytic mechanism and the structural basis for its preferred reaction directionality.

Results

Functional role of θ -CA3 and θ -CA4 in diatom cells

To investigate the intracellular function of *P. tricornutum* θ -CA3 and θ -CA4 in the marine diatom, *P. tricornutum*, we first generated three double-knockout (dKO) strains lacking both θ -CA3 and θ -CA4 and then reintroduced expression of each θ -CA into these dKO lines. dKO strains #45 and #27 had, respectively, a 95-bp insertion with a 7-bp deletion and a 286-bp deletion, resulting in the generation of an early stop codon after amino acids 262/253 and 112/103 in θ -CA3/CA4 (Fig. S1). Conversely, dKO strain #26 had a 204-bp deletion within the gene loci encoding the first Cys-Gly-His-rich (CGHR) domain, a CA domain, in both θ -CA3 and θ -CA4, resulting in the deletion of 68 amino acids (Fig. S1).

When grown in high- CO_2 (1%; HC) conditions, there was no difference between the dKO lines and wild-type (WT) cells, either in maximum photosynthetic rate (P_{max}) or in photosynthetic DIC affinity determined by the half-saturation constant, $K_{0.5}[\text{DIC}]$ (Fig. 1A,B). The $K_{0.5}[\text{DIC}]$ value of ~ 1 mM indicated that the WT and dKO cells all have suppressed CCM under HC conditions. Transferring to atmospheric low- CO_2 (0.04%; LC) conditions had little effect on the P_{max} value for WT and dKO cells (Fig. 1C). In contrast, the $K_{0.5}[\text{DIC}]$ value of LC-grown WT cells was drastically decreased to 20 μM , which was $\sim 2\%$ of that of HC-grown WT cells, indicating full induction of the CCM (Fig. 1D). The LC-grown dKO cells had $K_{0.5}[\text{DIC}]$ values of ~ 0.3 mM (#45), ~ 0.5 mM (#27), and ~ 0.45 mM (#26), which were 30–50% that of HC-WT cells and 15–25-fold higher than that of LC-WT cells (Fig. 1D), clearly indicating that the maximum CCM level of these dKO strains was significantly suppressed in the absence of θ -CA3 and θ -CA4.

Using dKO strain #27 as the host cell, we reintroduced expression of θ -CA3 or θ -CA4 as a C-terminal His $\times 6$ fusion protein (Fig. S1B), obtaining three lines with expression of θ -CA3 and three with expression of θ -CA4. When grown in LC conditions, there was no

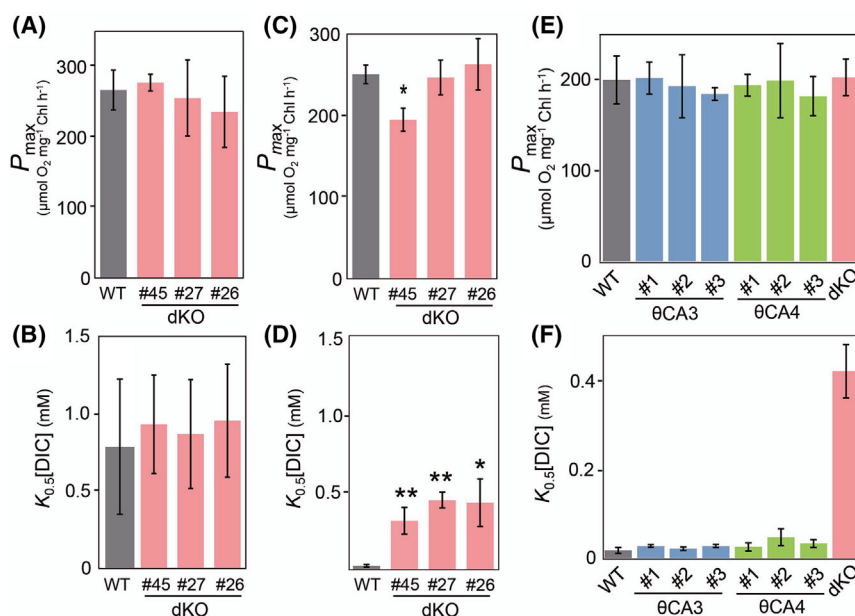


Fig. 1. Photosynthetic parameters of WT cells, θ -CA3/ θ -CA4 dKO cells, and complement cells. (A and B) $P_{\max}$ and $K_{0.5}[\text{DIC}]$ values of HC-grown WT (gray) and dKO (red) cells. (C and D) $P_{\max}$ and $K_{0.5}[\text{DIC}]$ values of LC-grown WT (gray) and dKO (red) cells. (E and F) $P_{\max}$ and $K_{0.5}[\text{DIC}]$ values of LC-grown WT (gray), dKO cells complemented by θ -CA3 (blue), dKO cells complemented by θ -CA4 (green), and dKO (red) cells. Values are shown as mean $\pm$ SD. The number of biologically independent replicates and exact P values are provided in Table S5. Statistical significance was assessed using Student's *t*-test. HC, high CO_2 ; LC, low CO_2 ; WT, wild type; dKO, θ -CA3/ θ -CA4 double knockout; $P_{\max}$, maximum photosynthetic rate; $K_{0.5}[\text{DIC}]$, half-saturation constant for dissolved inorganic carbon.

difference in $P_{\max}$ value relative to LC-grown WT cells (Fig. 1E); in addition, all six lines with complementary expression of θ -CA3 or θ -CA4 had a $K_{0.5}[\text{DIC}]$ value equivalent to that of LC-grown WT cells (Fig. 1F), indicating full phenotypic complementation. These data indicate that θ -CA3 and θ -CA4 have redundant roles and are fully compatible with each other in the operation of the CCM. Hereafter, therefore, we used θ -CA3 for structure–function analyses.

Activity measurement and metal analysis of recombinant θ -CA3

Next, we explored the structure of θ -CA3. AlphaFold2-based structural prediction proposed that θ -CA3 comprises three distinct domains: an N-terminal transmembrane helix domain (residues 1–48), domain 1 (residues 49–296), and domain 2 (residues 297–545) (Figs. 2A and Fig. 2B). In multiple sequence alignment, domains 1 and 2 of θ -CA3 and θ -CA4 showed high amino acid sequence identity with the catalytic domain of θ -CA1 and full-length θ -CA2, which are both known to possess CA activity (Fig. 2C). In addition, the three zinc-coordinating residues (histidine and two cysteines) identified in the structures of θ -CA1 and θ -CA2 were conserved in both θ -CA3 domains, suggesting that

domains 1 and 2 both possess CA activity. To test this theory, we constructed the following three variants lacking the N-terminal transmembrane helix domain: θ -CA3D1 (domain 1 only), θ -CA3D2 (domain 2 only), and θ -CA3D1–2 (domains 1 and 2 in tandem). The three constructs were strongly expressed in *E. coli*, as confirmed by SDS/PAGE (Fig. S2), and the proteins were purified from the soluble fraction. In contrast, full-length θ -CA3 including the N-terminal transmembrane helix domain was not expressed in *E. coli*, probably due to the inhibitory effects of its transmembrane helix on expression.

To determine whether a metal ion is bound by the conserved zinc-coordinating residues in θ -CA3, we used inductively coupled plasma atomic emission spectroscopy (ICP-AES) to quantify the metal content in θ -CA3D1, θ -CA3D2, and θ -CA3D1–2, analyzing for Mn, Fe, Cd, Zn, Cu, and Co (Fig. S3). Each single-domain construct contained approximately one zinc ion per molecule, while the tandem-domain construct contained two zinc ions per molecule; no other metals were detected. These results demonstrate that θ -CA3 comprises two homologous catalytic domains, each coordinating a zinc ion essential for CA activity.

The CO_2 hydration activity of all three constructs was measured at pH 8.3–8.0 by the Wilbur–Anderson

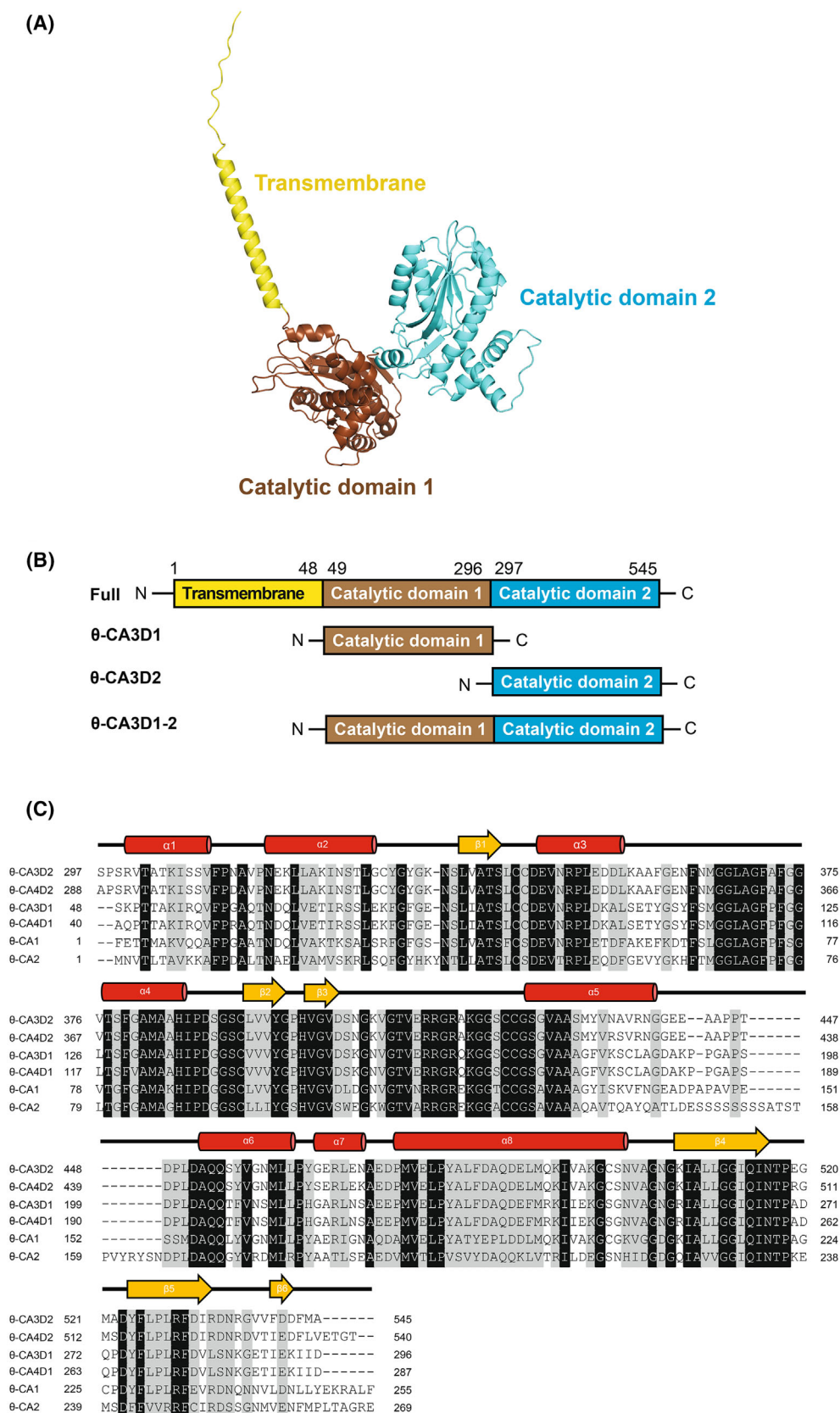


Fig. 2. Domain structure of θ -CA3 and sequence homology among θ -CAs. (A) Structure of θ -CA3 predicted by AlphaFold2. The transmembrane helix is shown in yellow, catalytic domain 1 in brown, and catalytic domain 2 in cyan. (B) Domain structure of θ -CA3 and the three variants θ -CA3D1, θ -CA3D2, and θ -CA3D1–2. (C) Amino acid sequence alignment of the catalytic domains of θ -CA1, θ -CA2, θ -CA3, and θ -CA4. Red cylinders indicate helical regions of θ -CA3D2; yellow arrows indicate β -strand regions of θ -CA3D2; black boxes indicate identical residues; gray boxes indicate similar residues. The amino acid sequences of θ -CA1, θ -CA2, θ -CA3, and θ -CA4 from *Phaeodactylum tricornutum* were obtained from GenBank under accession numbers LC111566, LC111565, LC111568, and LC111567, respectively. Sequence alignment was generated using GENETYX version 13.

(WA) method. All constructs displayed linear activity versus enzyme concentration (0–0.075 mg/mL), confirming the reliability of the assay (Fig. S4). The specific activity was 17.28, 12.78, and 10.22 WAU/mg for θ -CA3D1, θ -CA3D2, and θ -CA3D1–2, respectively. These values are comparable to that of θ -CA1 (~30 WAU/mg) and θ -CA2 (~7 WAU/mg). Notably, the tandem construct showed lower activity than would be expected from the average of its individual domains, suggesting the possibility of interdomain interactions that modulate catalytic efficiency.

X-ray structure of θ -CA3D2

To probe the structure of θ -CA3, we tried to obtain crystals of the three domain variants. Crystallization trials succeeded only for θ -CA3D2, yielding two crystal forms (Forms 1 and 2) of orthorhombic space group $P2_12_12_1$, which diffracted to 1.7 Å and 1.5 Å resolution, respectively (Table S1). In the crystallographic asymmetric unit, both forms contained a homodimer, related by non-crystallographic two-fold symmetry, with a buried interface of ~2100 Å², indicating the stability of the dimer in solution (Fig. 3A, left). Structural superposition with the θ -CA1 dimer yielded an RMSD of ~0.5 Å for C α atoms, confirming the highly conserved main chain structure of each protomer (Fig. S5).

Each protomer adopts a compact α/β fold comprising eight α -helices and six β -strands (Fig. 3A, right). The dimer interface aligns the active sites from both monomers. Zinc ions at the catalytic centers are tetrahedrally coordinated by three residues (Cys47, His103, Cys127) from one monomer and either a water molecule or chloride ion as the fourth ligand, depending on the crystallization condition (Fig. 3B); coordination distances range from 2.1 to 2.3 Å. The active site forms a ~10-Å deep conical pocket at the dimer interface using both hydrophilic and hydrophobic residues. The hydrophilic residues include the three zinc-coordinating residues (Cys47, His103, Cys127); Asp49, Arg117, Ser125, Ser129 from chain A; and

His89' from chain B. The hydrophobic residues include Leu72 from chain A; and Phe67', Thr81', and Ala85' from chain B. There are three ordered water molecules on the hydrophilic side, but none on the hydrophobic side (Fig. 3C). We performed alanine substitution of Asp49 and Arg117, which reduced enzymatic activity to one-tenth and one-eighth of WT levels, respectively, confirming the essential catalytic roles of these two residues (Fig. 3D).

Characteristic portal and binding of CO₂/HCO₃[−] at the θ -CA3D2 active site

To our knowledge, no θ -CA structures with bound CO₂ have been previously reported. In this study, we observed clear electron density for CO₂ in Form 2 crystals of θ -CA3D2 (Fig. 3B). The CO₂ carbon is located 2.6 Å apart from the zinc-bound water, an ideal distance for nucleophilic attack. The CO₂ molecule adopts a slightly bent geometry (~175°), stabilized by both hydrophobic interactions with Phe67, Leu72, and Ala85, and a hydrogen bond between its oxygen and the backbone nitrogen of Ser129 (Fig. 3C).

Comparison with the structure of θ -CA1 revealed differences in electrostatic potential at the active site entrance. While a portal of θ -CA1's active site pocket is positively charged, that of θ -CA3D2 is neutral. We also identified specific substitutions, such as Lys86 in θ -CA1 vs Ala88 in θ -CA3D2 (Fig. 4A). To test the impact of these substitutions, θ -CA3D2 Ala88 was mutated to Lys or Asp and the CO₂ hydration activity was measured.

As compared with WT θ -CA3D2, the A88D mutant showed up to 16-fold higher CO₂ hydration activity at acidic pH (6.6–6.3), despite lower activity at pH 8.3–8.0 (Fig. 4B). Crystal structures of the A88D mutant revealed that it captures both CO₂ as a substrate and HCO₃[−] as a product within the two independent active sites of its dimer (Fig. S6 and Table S1), implying the possibility that reciprocal reactions take place in the dimer.

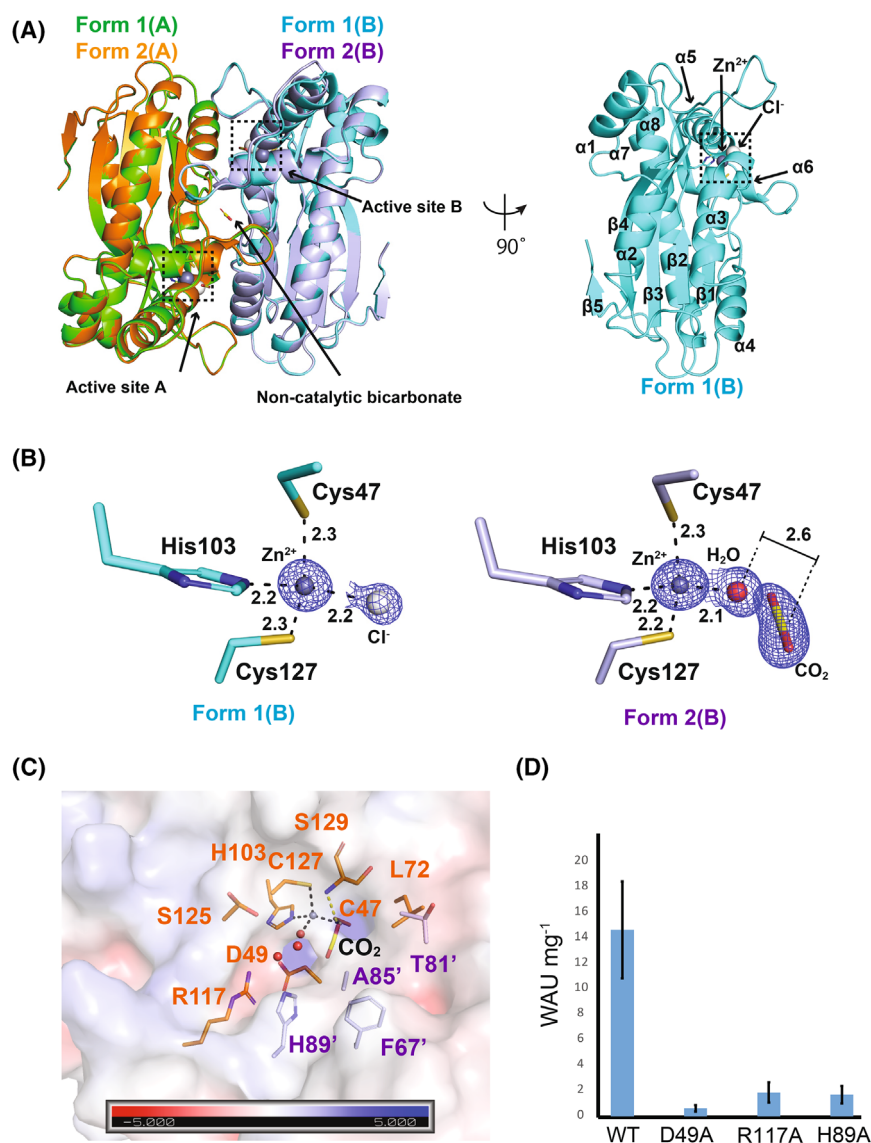


Fig. 3. Crystal structure of θ -CA3D2 and key residues important for its catalytic function. (A) Dimer structure and monomer fold of θ -CA3D2. Chain A of Forms 1 and 2 is shown in green and orange, respectively; chain B is shown in cyan and light blue, respectively. Black dashed box indicates the catalytic center; gray sphere represents the zinc ion; white sphere represents the chloride ion. (B) Active center structure. The omit 2Fo–Fc electron density map is shown for the zinc ion (10σ), chloride ion (4σ), water, and CO₂ (2σ) (blue mesh). Coordination bonds are shown as black dashed lines. (C) Active-site pocket and its electrostatic surface potential. (D) Effect of mutations at active-site residues on CO₂ hydration activity. Values represent the mean $\pm$ standard deviation of 12 (WT enzyme) and 3 (each mutant) measurements. Molecular graphics were generated using PyMOL.

Non-catalytic bicarbonate binding site at the θ -CA3D2 dimer interface

In the WT Form 1 structure, we observed the unexpected electron density of an HCO_3^- ion at the dimer interface, ~ 15 Å from the active site, even though no HCO_3^- had been intentionally included in the crystallization conditions (Fig. 5A). The HCO_3^- ion displayed strong hydrogen-bonding interactions with Gln157 from

both subunits and ordered water molecules. To test the functional role of Gln157, we generated a Q157A mutant. While its overall dimeric state was preserved based on gel filtration and dynamic light scattering (DLS), the mutant had delayed activation kinetics in CO₂ hydration assays, showing a lag phase that was absent in WT (Fig. 5B,C, Fig. S7). Collectively, these results suggest that HCO_3^- binding stabilizes the dimer interface and promotes efficient catalytic regulation.

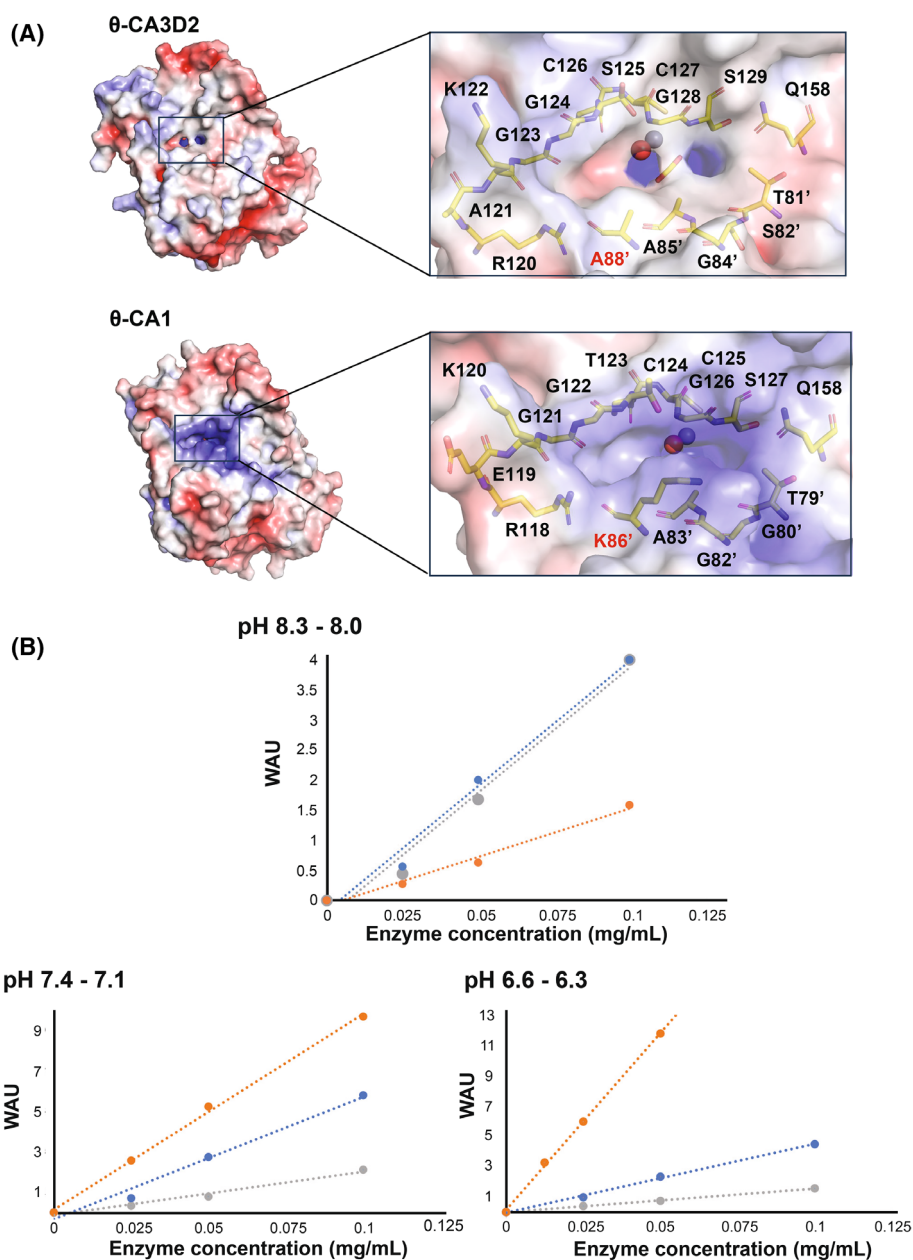


Fig. 4. Comparison of the surface electrostatic potential between θ -CA3D2 and θ -CA1, and its effect on CO_2 hydration activity. (A) Surface electrostatic potentials of the active sites of θ -CA3D2 and θ -CA1. Positive, negative, and neutral charges are shown in blue, red, and white, respectively. Amino acid residues located at the active-site entrance are highlighted in yellow, the zinc ion is shown as a gray sphere, and a zinc-coordinated water molecule as a red sphere. (B) Effect of the A88K and A88D mutation of θ -CA3D2 on CO_2 hydration activity. Gray, orange, and blue lines represent, respectively, WT, A88K mutant, and A88D mutant. Each data point represents a single measurement at the indicated enzyme concentration. Molecular graphics were generated using PyMOL.

Discussion

This study has explored the structure and function of θ -CA3 and θ -CA4, two previously uncharacterized CAs in the marine diatom *P. tricornutum*. Initially, we found that complementary expression of either θ -CA3

or θ -CA4 in dKO cells grown in LC conditions resulted in $P_{\max}$ and $K_{0.5}[\text{DIC}]$ values equivalent to LC-grown WT cells, demonstrating that the roles of θ -CA3 and θ -CA4 are redundant and both proteins can

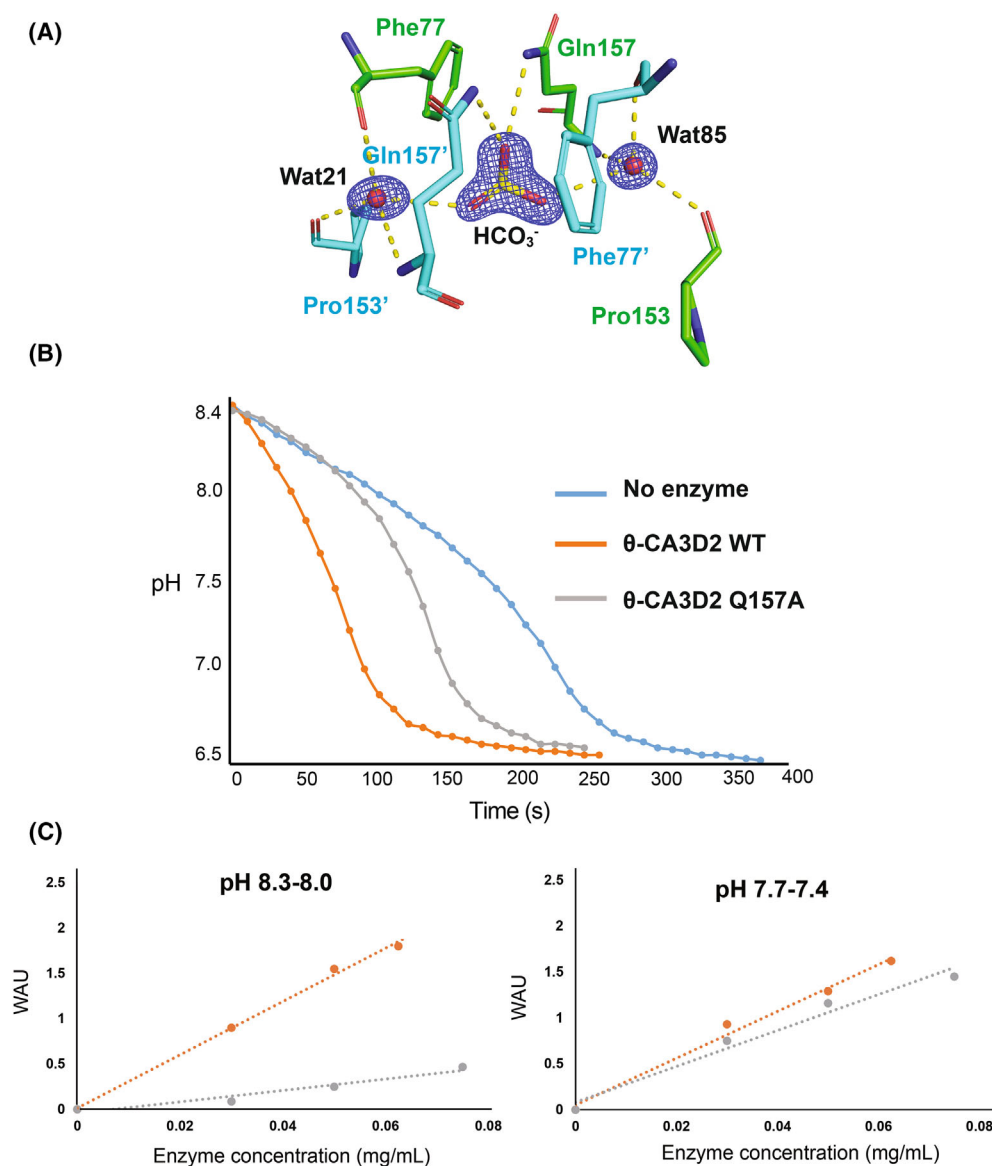


Fig. 5. Structure of the non-catalytic HCO_3^- -binding site in θ -CA3D2 and effect of mutation on its catalytic function. (A) Non-catalytic HCO_3^- -binding site in Form 1 of θ -CA3D2. Residues in chains A and B are shown in green and cyan, respectively. The HCO_3^- ion is shown as a yellow stick model, and the water molecules as red spheres. The omit $2\text{Fo}-\text{Fc}$ electron density map is shown for the HCO_3^- ion (2.0σ) and the water molecule (2.0σ) (blue mesh). Hydrogen bonds are shown as yellow dashed lines. (B) pH time course of the CO_2 hydration reaction. Blue, orange, and gray lines represent, respectively, the non-enzymatic, WT, and Q157A mutant reactions. (C) CO_2 hydration activity at the initial (pH 8.3–8.0) and middle (pH 7.7–7.4) stages of the reaction. Orange and gray lines represent, respectively, WT and Q157A mutant. Each data point represents a single measurement at the indicated enzyme concentration. Molecular graphics were generated using PyMOL.

operate equally in CCM (Fig. 1E,F). Sequence alignment revealed $\sim 95\%$ identity between CA3D1 and CA4D1, and similarly between CA3D2 and CA4D2 (Fig. 2B), but much lower similarity between the respective N-terminal transmembrane helices. The N-terminal α -helix of both θ -CA3 and θ -CA4 (~ 50 and ~ 40 residues, respectively) is predicted to be a

transmembrane helix, which is not observed in θ -CA1 or θ -CA2 (Fig. 2A and Fig. 2B). Thus, differences in the N-terminal transmembrane helix region probably determine the distinct subcellular localizations of θ -CA3 and θ -CA4. Based on their physiological redundancy and high sequence similarity, we focused our structural and biochemical analyses on θ -CA3. We

showed that the two homologous catalytic domains of θ -CA3 each possess CA activity, but the tandem construct of θ -CA3D1–2 has lower CA activity than the single-domain variants, implying that interdomain interactions impose functional regulation (Fig. S5). In support of this, the tandem domain of θ -CA3D1–2 and the mixture of single domains had slightly different elution profiles in gel filtration chromatography (Fig. S8A and Fig. S8B).

To understand the structure–function relationship of this unique CA, we attempted to probe the structure of the three recombinant θ -CA3 variants by X-ray analysis; however, only θ -CA3D2 was successfully crystallized. The resulting X-ray structures demonstrated that the active site of θ -CA3D2 comprises hydrophilic and hydrophobic regions (Fig. 3C) with ordered water molecules present only in the hydrophilic part. This distinctive characteristic of the active site probably serves as a water or CO_2 channel. Indeed, in the crystal structure of the CO_2 -bound form, a CO_2 oxygen forms a hydrogen bond with the backbone nitrogen of Ser129 (Fig. 3B), stabilizing the substrate and enhancing the electrophilic reactivity of its carbon to facilitate nucleophilic attack by a zinc-bound hydroxide ion, a mechanism that has not been experimentally determined before. Asp49 is ideally positioned at ~ 2.6 Å from the zinc-bound water (Fig. 3C) to act as a general base to accept a proton during catalysis. Furthermore, Arg117 forms a salt bridge with Asp49, maintaining the geometry for efficient proton transfer. In support of the essential roles of these residues, their substitution with alanine led to a significant reduction in activity (Fig. 3D). These observations enable us to propose the following catalytic reaction mechanism (Fig. S9). In step 1, zinc-coordinated water is deprotonated by Asp49 to form a hydroxide ion. In step 2, CO_2 is captured in the hydrophobic channel and adopts a slightly bent conformation. In step 3, the zinc-bound hydroxide ion attacks the CO_2 carbon, forming HCO_3^- . In step 4, the resultant HCO_3^- is released as a product via the hydrophilic channel and replaced by another water molecule from the solvent. In this study, the structures corresponding to steps 2 and 4 have been experimentally visualized for the first time.

θ -CA1, which is localized to the pyrenoid-penetrating thylakoid lumen, has a positively charged active-site portal, which probably facilitates HCO_3^- dehydration and CO_2 release to Rubisco [13,15]. In contrast, θ -CA3, which is distributed in the cytoplasmic space between mitochondria and chloroplasts, has a neutral portal, which may favor CO_2 uptake and HCO_3^- release to recapture CO_2 that has not been

used by Rubisco and is leaking from the chloroplast. To understand the structural basis for the reaction directionality of θ -CA3, we compared our structure with that of θ -CA1. Sequence alignments showed that three lysine residues in θ -CA1 (Lys86, Lys137, Lys204) are replaced by neutral residues in θ -CA3 and θ -CA4, altering the electrostatic potential of the active site portal. We propose that this substitution may tune the directionality of each isoform's activity toward its physiological role within the cell.

Interestingly, most CAs exhibit higher CO_2 hydration activity at alkaline pH due to enhanced generation of hydroxide ions [17]; however, the A88D mutant of θ -CA3D2, corresponding to a charge-converting mutant of Lys86 of θ -CA1, displayed the opposite pH preference. Namely, its activity increased dramatically as the pH decreased from 8.3 to 6.3, suggesting an alternative mechanism of facilitated CO_2 supply, HCO_3^- release and proton transfer as follows. The introduced Asp88 residue probably remains negatively charged under experimental conditions, repelling HCO_3^- while allowing neutral CO_2 to access the active site more easily. The electrostatic repulsion of HCO_3^- might also promote release of the product. Due to its exposure at the surface, Asp88's carboxylate may transiently accept protons from the catalytic pocket, rapidly transferring them to the solvent and enhancing the rate of proton transfer under acidic conditions. Our study confirmed that the electrostatic properties of the active site portal are important for CA activity and/or directionality. Notably, the crystal structure of A88D showed the intended change in its surface properties, but unexpectedly revealed the asymmetric binding of substrate and product in the active sites (Fig. S6). The presence of CO_2 and water in one site and HCO_3^- in the other speculates that a reciprocal reaction mechanism may underlie this pH-dependent activation, which warrants further investigation using complementary methods such as rapid kinetic analysis.

Our biochemical and structural data suggest that HCO_3^- binding at the dimer interface may have an additional role (Fig. 5). The Q157A mutant, lacking the HCO_3^- -binding side chain at the dimer interface, showed slightly altered gel filtration behavior but maintained a dimeric state in DLS, indicating that the dimeric form has more structural flexibility in the absence of HCO_3^- (Fig. S10). Binding of HCO_3^- may promote more rigid dimer packing, stabilizing the tighter active site geometry required for efficient catalysis (Fig. 6A). Similar allosteric regulation has been reported for β -type CAs such as HICA (*Haemophilus influenzae*) and ECCA (*Escherichia coli*), which also

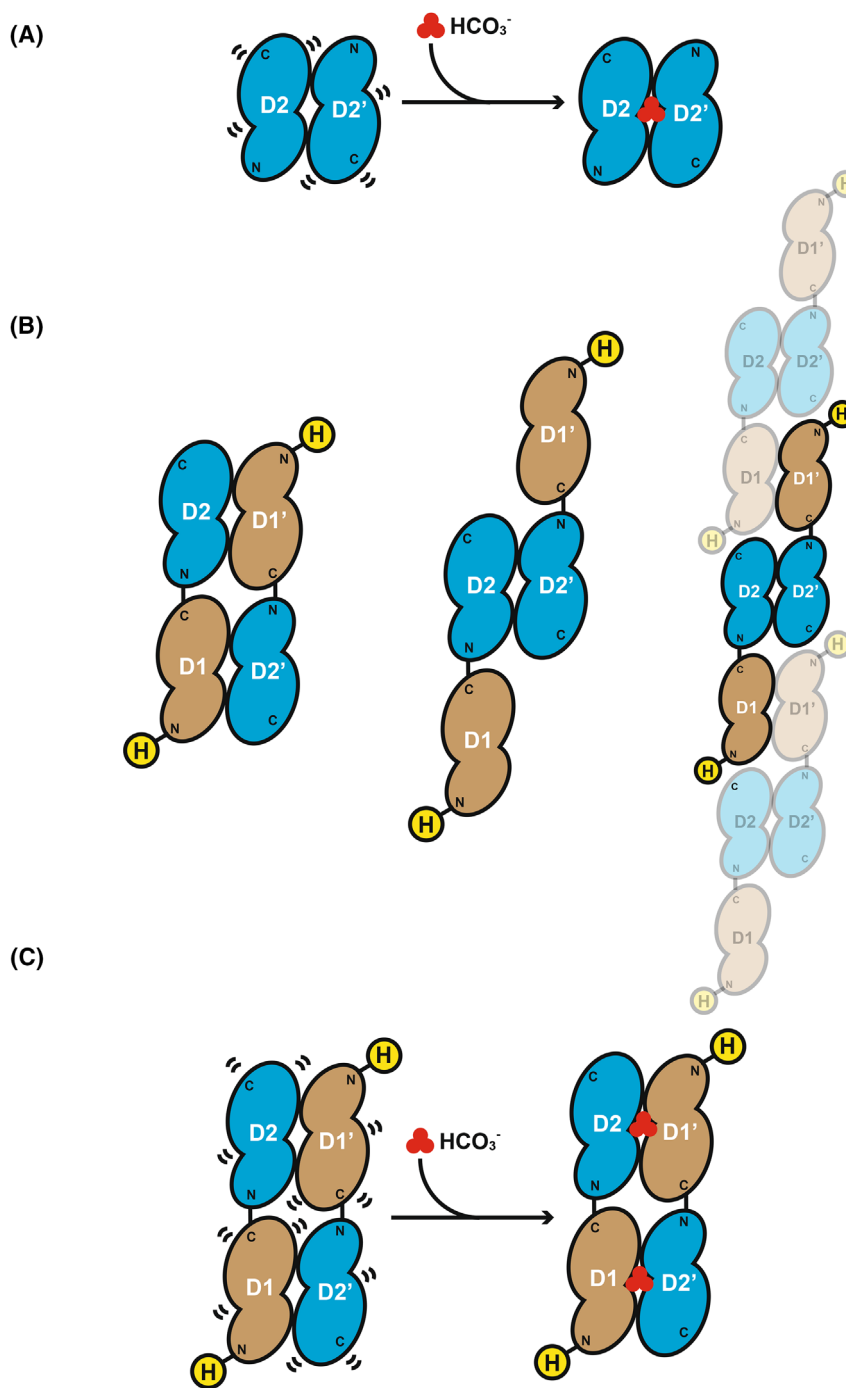


Fig. 6. Proposed regulatory model of θ -CA3. (A) Model of θ -CA3D2 activation by HCO_3^- binding at the dimer interface. (B) Model of the heterodimeric interactions of θ -CA3 in its native state. (C) Model of the activation of θ -CA3 in its native state by HCO_3^- binding at the dimer interface.

have a zinc ion coordinated by two cysteines and one histidine residue, and utilize an aspartate residue as a general base [18]. However, HCO_3^- binding occurs at separate monomeric sites and regulates the activity

negatively in β -CAs, whereas it binds at the dimer interface and regulates the activity positively in θ -CA3D2. This distinct HCO_3^- -dependent regulation suggests that these classes of CA have evolved distinct

regulatory strategies despite having convergent structural features.

The native θ -CA3 protein consists of two tandemly connected catalytic domains (θ -CA3D1 and θ -CA3D2) (Fig. 2A). On gel filtration, θ -CA3D2 formed stable homodimers in solution, whereas θ -CA3D1 tended to form higher-order oligomers, and addition of θ -CA3D2 to θ -CA3D1 in solution promoted dimer formation of θ -CA3D1 rather than the higher-order oligomers, suggesting preferential *in vivo* heterodimeric interactions between θ -CA3D1 and θ -CA3D2. We propose that, in physiological conditions, θ -CA3 exists as a homodimer of two tandem-dimeric catalytic domains, facing θ -CA3D1 to θ -CA3D2' and θ -CA3D2 to θ -CA3D1' (Fig. 6B left). Such an arrangement would ensure proper dimerization, preventing the undesirable polymerization that we observed with isolated θ -CA3D1 domains (Fig. 6B right). Our newly identified HCO_3^- -dependent functional regulation would be able to occur even at the heterodimer interface, because θ -CA3D1 and θ -CA3D2 share high amino acid sequence identity (Fig. 6C).

In conclusion, we have successfully determined the first enzyme–substrate complex structure of θ -CA and demonstrated that θ -CA3 favors the forward $\text{CO}_2 \rightarrow \text{HCO}_3^-$ reaction. Unlike most other CAs with a single catalytic domain, θ -CA3 contains two homologous domains with distinct regulatory features. We have also demonstrated that HCO_3^- binds at the dimer interface, and highlighted the importance of electrostatic tuning at the active site portal for physiological function. Given the high sequence identity and physiological redundancy between θ -CA3 and θ -CA4, similar properties may also apply to θ -CA4. These findings provide new insights into the structure–function relationships and regulatory mechanisms of θ -CA, a key enzyme for efficient CO_2 fixation in diatoms.

Methods

Determination of photosynthetic parameters

Photosynthetic parameters were determined as described previously [13,19,20].

Genome editing of *Pt* θ -CA3/4

Two pairs of single-guide RNA (sgRNA) sequences (designated T1 and T2) were designed to simultaneously target *Pt* θ -CA3 and *Pt* θ -CA4 using ZiFiT Targeter (<http://zifit.partners.org/ZiFiT/>) [21] followed by Cas-Designer (<http://www.rgenome.net/cas-designer/>), which considers both target specificity and microhomology-mediated end joining [22].

For T1, the target sequences were 5'-GACTTCGTCGC AACACAAGG-3' (positions 297–316 in *Pt* θ -CA3 and 288–307 in *Pt* θ -CA4) and 5'-TCCCCTCGATAAGGC TCTGT-3' (322–341 in *Pt* θ -CA3 and 313–332 in *Pt* θ -CA4). For T2, the sequences were 5'-CGCGTCCAGGGGGTCC GAGG-3' (615–634 in *Pt* θ -CA3 and 606–625 in *Pt* θ -CA4) and 5'-AACTCTATGCTCCTTCCCCA-3' (650–669 in *Pt* θ -CA3 and 641–660 in *Pt* θ -CA4). To ensure efficient transcription, an additional G was added to the 5' end of each DNA sequence encoding an sgRNA [23].

The dual-sgRNA vector targeting *Pt* θ -CA3/4 was constructed as described by Nawaly *et al.* (2020) [24] and co-introduced with the Cas9 (D10A) nickase vector into *P. tricornutum* cells via bacterial conjugation, as described previously [25]. Mutant strains were selected on agar medium containing 100 $\mu\text{g mL}^{-1}$ zeocin (InvivoGen) under high- CO_2 (HC) conditions. To ensure monoclonality, each colony was restreaked sometimes, selecting a single colony at each step. Mutations were verified by TIDE analysis and direct sequencing.

Cloning of θ -CA3D1, θ -CA3D2, and θ -CA3D1–2

All expression constructs were generated in a pCold I vector, which was linearized by inverse PCR. All primers used in this study are listed in Tables S2–S4. Insert genes were amplified by PCR using a codon-optimized full-length θ -CA3 gene for *E. coli* expression as the template and PrimeSTAR Max Premix (Takara Bio Inc.) as the polymerase. The amplified fragments were inserted downstream of the His-tag in pCold I using the Gibson Assembly method (NEB) [26].

Site-directed mutagenesis

To obtain mutant variants (D49A, A88D, A88K, H89A, R117A, and Q157A) of θ -CA3D2, site-directed mutagenesis was performed by inverse PCR using primers harboring a single mutation (Table S4), pCold I vector containing the WT θ -CA3D2 gene as a template, and PrimeSTAR Max Premix. The whole θ -CA3D2 gene region was sequenced to confirm integration of the desired substitution in the θ -CA3D2 sequence.

Expression and purification

Recombinant plasmids were transformed into *E. coli* BL21 strain and selected on LB agar medium containing 100 $\mu\text{g mL}^{-1}$ of ampicillin. Single colonies were pre-cultured overnight in 5 mL of LB medium and transferred to 1 L of LB medium. At an OD_{600} of 0.4–0.6, expression was induced by adding IPTG (final concentration 0.5 mM), and cells were incubated at 15 °C for 24 h with shaking. The cells were harvested by centrifugation at 5000 g for 10 min using

a JLA-9.1000 rotor (Beckman Coulter Co, Ltd) and stored at -30°C .

Frozen cells were resuspended in buffer A (50 mM Tris/HCl pH 8.0, 150 mM NaCl) and lysed by sonication using a BRANSON 450 Sonifier. The lysate was clarified by centrifugation at 14000 rpm for 30 min using a HITA-CHI P45AT rotor. The supernatant was loaded onto a 5-mL Ni-NTA agarose column (QIAGEN), washed with 40 mL of buffer B (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 20 mM Imidazole), and eluted in three 5-mL fractions of buffer C (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 200 mM imidazole). Prior to gel filtration, θ -CA3D2 was concentrated, but θ -CA3D1 and θ -CA3D1–2 were not owing to their propensity to aggregate at high concentrations. A 5-mL aliquot of the fractions from Ni-affinity purification was passed through a 0.22 μm -filter and loaded onto a Superdex 200 16/60 gel filtration column equilibrated with buffer D (20 mM Tris–HCl pH 8.0, 150 mM NaCl) with elution at 1 mL/min.

Carbonic anhydrase activity assay

Carbonic anhydrase activity was measured as described by Wilbur and Anderson [27], with some modifications. In brief, 100 μL of purified CA solution was added to 1.4 mL of buffer solution (20 mM barbital [pH 8.4], 20 mM MOPS [pH 7.5], or 20 mM Bis-Tris [pH 6.7]) in an acrylic chamber maintained at 1°C via a water jacket. The CO_2 hydration reaction was initiated by adding 0.5 mL of ice-cold CO_2 -saturated water, and the time required for a pH drop over a defined range was measured. CA activity was calculated using the equation $\text{WAU} = T_0 / T - 1$, where T_0 is the time required for the pH shift in the absence of CA, and T is the time required in the presence of CA. Specific activity (WAU/mg) was calculated by using enzyme concentrations within the linear range of the activity–concentration relationship.

Crystallization of θ -CA3D2

For the WT, after gel filtration, θ -CA3D2 was concentrated to 9 mg/mL using Amicon ultra 10 K (Merck). Crystallization of θ -CA3D2 was performed at 4°C by the sitting-drop vapor diffusion method under condition 1 (0.2 M lithium citrate tribasic tetrahydrate, 20% w/v PEG 3350, pH 8.4) and the hanging-drop vapor diffusion method under condition 2 (8% v/v Tacsimate, pH 6.0, 20% w/v PEG 3350). For X-ray diffraction experiments, a crystal obtained under condition 1 was soaked into a cryoprotectant solution consisting of 0.2 M lithium citrate tribasic tetrahydrate, 22% w/v PEG 3350, pH 8.4, and 22% v/v ethylene glycol; and a crystal obtained under condition 2 was soaked into a cryoprotectant solution containing 8% v/v Tacsimate, pH 6.0, 22% w/v PEG 3350, and 22% v/v ethylene glycol before

being flash-frozen in liquid nitrogen. For the A88D mutant, after gel filtration, the A88D mutant enzyme was concentrated to 6.5 mg/mL using Amicon ultra 10 K (Merck). Crystallization was performed at 4°C using the hanging-drop vapor diffusion method under the condition of 8% v/v TacsimateTM, pH 6.0, and 20% w/v PEG 3350. For X-ray diffraction experiments, the crystal was soaked in a cryoprotectant solution containing 8% v/v TacsimateTM, pH 6.0, 22% w/v PEG 3350, and 22% v/v ethylene glycol before being flash-frozen in liquid nitrogen.

Data collection and structural determination

The diffraction data sets for WT θ -CA3D2 (Forms 1 and 2) and the A88D mutant were collected on BL44XU at SPring-8 (Harima, Hyogo, Japan) using an EIGER X 16 m detector (DECTRIS) at cryogenic temperature (100 K). The Form 1 crystal yielded a data set with 99.6% completeness at 1.7- $\text{\AA}$ resolution from 1800 frames. The Form 2 crystal yielded a 1.5- $\text{\AA}$ resolution data set with 99.6% completeness from 1800 frames. The A88D mutant crystal data set was obtained at 1.64- $\text{\AA}$ resolution with 99.5% completeness, also from 1800 frames.

All diffraction data were processed and scaled using XDS [28]. Resolution cutoffs were determined based on I/σ ($I > 1.0$ and $\text{CC}_{1/2} > 50\%$ in the highest-resolution shell). Initial phases for WT Form 1 were determined by molecular replacement using Phaser [29] in the Phenix suite. Model building was initiated with phenix.autobuild [30], followed by manual building using Coot [31] in the CCP4 suite. Structural refinement was carried out using phenix.refine [32]. The refined Form 1 model was then used as a search model for molecular replacement of Form 2, and the refined Form 2 model was subsequently used as the search model for the A88D mutant. Refinement of the Form 2 and A88D mutant structures was also performed using phenix.refine and Coot. For the A88D mutant, TLS refinement was additionally applied at the subunit level. Molecular graphics were generated using PyMOL.

Inductively coupled plasma atomic emission spectroscopy

The presence of six metals, Mn, Fe, Zn, Cu, Co, and Cd, in purified θ -CA3D1, θ -CA3D2, and θ -CA3D1–2 was analyzed by ICP-AES. To calculate the molar ratio of metals to protein, sulfur was also analyzed. Buffer D from gel filtration chromatography was employed for baseline correction. The molar ratio of a metal to protein was calculated as metal content (mol/mol protein) = (metal concentration (ppm)/atomic weight of metal)/(sulfur concentration (ppm)/atomic weight of sulfur $\times N_{\text{Cys} + \text{Met}}$), where metal and sulfur concentrations are average values from three measurements.

Dynamic light scattering

Purified protein samples in buffer D were filtered through a 0.22- μ m membrane to remove aggregates. DLS measurements were performed at 25 °C using a DynaPro NanoStar instrument. Each sample was measured 10 times, and the autocorrelation functions were analyzed to determine the hydrodynamic radius and polydispersity. Data were analyzed by using DYNAMICS software (version 7.1.0).

Author contributions

GK and YM supervised the study; HN, YM, and GK designed experiments; HN, AO, GS, and HT performed experiments; HN, YM, and GK analyzed data and wrote the manuscript; all authors made manuscript revisions.

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Conflicts of interest

The authors declare no conflicts of interest.

Peer review

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Data availability statement

The structural data that support these findings are openly available in the wwPDB under accession codes 9VZJ, 9W00, and 9W05.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Generation and complementation of θ -CA3/ θ -CA4 double-knockout mutants.

Fig. S2. SDS/PAGE analysis of recombinant θ -CA3 expression.

Fig. S3. Analysis of θ -CA3 for sulfur and metal content by inductively coupled plasma atomic emission spectroscopy (ICP-AES).

Fig. S4. CO_2 hydration activity of θ -CA3D1, θ -CA3D2, and θ -CA3D1–2 at pH 8.3–8.0.

Fig. S5. Superposition of the dimeric structures of θ -CA3D2 and θ -CA1.

Fig. S6. Active-site structure of the A88D mutant.

Fig. S7. pH time course of the CO_2 hydration reaction.

Fig. S8. Size-exclusion chromatography and SDS/PAGE analysis of θ -CA3 homo- and heterodimers.

Fig. S9. Proposed catalytic mechanism of the θ -CA3D2 reaction.

Fig. S10. Structural characterization of wild-type and the Q157A mutant of θ -CA3D2 in solution state.

Table S1. Data collection and refinement statistics.

Table S2. Primers for generating linear pCold I vector.

Table S3. Primers for amplifying θ -CA3D1, θ -CA3D2, θ -CA3D1–2.

Table S4. Primers for site-directed mutagenesis of θ -CA3D2.

Table S5. Statistical values of the photosynthetic parameters shown in Fig. 1.