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Halotolerant carbonic anhydrase with unusual N-terminal extension from marine *Hydrogenovibrio marinus* as novel biocatalyst for carbon sequestration under high-salt environments

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ABSTRACT

Carbonic anhydrase (CA), an enzyme that catalyzes the carbon dioxide (CO_2) hydration, has been suggested as a potentially powerful agent for CO_2 capture and utilization. For successful application, CA should withstand the harsh environment presented by CO_2-capturing facilities. While there have been intensive efforts to identify and engineer thermostable CAs, other required conditions such as the high salt concentration of CO_2 absorbents have often been ignored. Herein, we expressed, purified, and characterized a novel α-type CA (*hm*CA) possessing an unusual N-terminal extension from the halophilic marine bacterium *Hydrogenovibrio marinus*. We found that the N-terminal extension strongly influenced the enzyme solubility. Recombinant *hm*CA showed catalytic efficiency comparable to other bacterial α-type CAs. *hm*CA was less inhibited by anionic inhibitors showing 1.6- (NO_3^-), 3.1- (NO_2^-), and 3.7-fold (Cl^-) higher inhibition constants than those of mesophilic bovine CA (bCA), suggesting halotolerance. Recombinant *hm*CA was markedly stabilized using most of the alkali metal salts tested, showing 19 °C higher melting temperature at 1 M NaCl compared to bCA that was significantly destabilized. The region of N-terminal extension seemed not to be involved in halotolerance. The remarkable halotolerance may be attributed to the uneven distribution of electrostatic potential and the localized negative charge on the *hm*CA surface. *hm*CA displayed ~29-fold longer half-life than that of bCA at 40 °C in potassium carbonate as a practical absorbent, suggesting that halotolerance should be considered another key characteristic in the development of biocatalysts for CO_2 capture using high-salt-containing CO_2 absorbents.

1. Introduction

Carbonic anhydrase (CA) is an enzyme that reversibly catalyzes the hydration of carbon dioxide (CO_2) [1]. CAs have important physiological roles in various organisms and are classified into seven distinct families (α, β, γ, δ, ζ, η, and ϴ) [2]. CAs show ultrafast catalysis with a k_{cat} of up to $4.4 \times 10^6\ s^{-1}$ [3], making them potentially powerful agents for biomimetic CO_2 capture [4–6]. The major obstacle for their industrial application is the unstable nature of the enzymes, which limits the use of CA under the harsh conditions presented by CO_2-capturing facilities [4,7].

To address this limitation, there have been intensive efforts to identify and engineer stable CAs [4,8–14]. In most of the studies, however, the required properties other than thermal stability have not been clearly defined or have not been considered in the development of biocatalysts for CO_2 capture. In practice, CA is expected to be exposed to CO_2 absorbents consisting of a high concentration of mineral salts such as potassium carbonate (PC, K_2CO_3) [15–17]. Additionally, the reaction of major flue gas contaminants such as SO_x and NO_x with solvents results in anions (*e.g.*, sulfite, sulfate, or nitrate) that may severely inhibit enzyme activity [5,18]. In this regard, tolerance to these mineral salts (that is, halotolerance), along with thermostability, should be one of the most important properties for the successful application of CA to CO_2 capture. Nevertheless, there have been only a few studies on halotolerant CAs, almost exclusively on an α-type CA (*ds*CA) from *Dunaliella salina* [13,19–21]. Thus, expanding the pool of halotolerant CAs might be a key starting point for the design and development of practical biocatalysts for CO_2 capture under high-salt conditions.

Hydrogenovibrio marinus is a Gram-negative marine proteobacterium that exhibits obligate chemolithoautotrophy [22]. Similar to

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Hydrogenovibrio crunogenus (formerly, *Thiomicrospira crunogena*) [23], recent genome sequencing of *H. marinus* has revealed that this bacterium possesses genes encoding α-type CA, β-type CA, γ-type CA, and carboxysomal β-like CA [24], which may play essential roles together with RubisCO in autotrophic CO_2 fixation [25]. Notably, *H. marinus* is a halophile that indispensably requires NaCl (optimally 0.5 M) for its growth [22], suggesting that CAs from *H. marinus* might also be halophilic or halotolerant. In particular, among the four *H. marinus* CAs, α-type CA is the only enzyme predicted to be translocated into the periplasm, placing it in the environment most exposed to external sea salts. Additionally, α-type CAs are the most studied enzymes and the fastest CAs are often found in this family [1,3,8]. Furthermore, α-type CAs show high catalytic bias to the forward (CO_2 hydration) reaction [26–28]. Collectively, these characteristics are expected to make *H. marinus* α-type CA (*hm*CA) suitable for CO_2 capture under high-salt conditions.

In the present work, we expressed, purified, and characterized novel recombinant *hm*CA, with special attention to its halotolerance. Unlike other α-type CAs, except for those from *Hydrogenovibrio* or *Thiomicrospira* species, we found that *hm*CA has an unusual N-terminal extension, which remains to be characterized. Thus, we constructed the N-terminus-truncated derivative of *hm*CA along with full-length *hm*CA and compared them with mesophilic α-type CA (bCA) from *Bos taurus* (bovine) to demonstrate the halotolerance of *hm*CA. Furthermore, we discussed the surface characteristics conferring the halotolerance and evaluated the stability of *hm*CA in the presence of high-salt-containing CO_2 absorbents such as PC and seawater.

2. Results and discussion

2.1. Comparative sequence analysis of hmCA

Sequence alignment was performed on *hm*CA with other α-type CAs from the selected organisms, *Hydrogenovibrio kuenenii* (*hk*CA), *Hydrogenovibrio crunogenus* (*hc*CA), *Sulfurihydrogenibium yellowstonense* (*ssp*CA) and *Bos taurus* (bCA) (Fig. 1). As expected, all of the highly conserved residues found in α-type CAs are also present in *hm*CA, *i.e.*, three zinc-coordinating histidine residues and a proton shuttle residue. Two cysteine residues for intramolecular disulfide bridge also exist in *hm*CA and in other bacterial α-type CAs. The sequence of *hm*CA contains a signal peptide at its N terminus, which is found in all of the examined bacterial α-type CAs, suggesting the periplasmic localization of *hm*CA in the host. The most intriguing feature was the presence of an N-terminal extension of ~50 amino acids (excluding the signal peptide) in *hm*CA. This unusual extension has not been found in any other α-type CAs except for those from *Hydrogenovibrio* or *Thiomicrospira* species, including *H. crunogenus* [18,23,29,30]. Although the expression and characterization of recombinant *hc*CA have been reported, the function of the N-terminal extension has not been separately investigated and remains totally unknown.

2.2. Expression and purification of recombinant CAs

The recombinant *hm*CA protein was produced without the first 29 amino acids (including the proposed 26-amino acid signal peptide) (Fig. 1). The protein was designated wt-*hm*CA. To study the role of the N-terminal extension, the truncated version of *hm*CA (named trnc-*hm*CA) without the N-terminal extension (here, 49 amino acids long) was also constructed. In addition, a mutation of H117A (wt-*hm*CA numbering system) was generated to create the proton shuttle residue mutant [31].

All of the recombinant *hm*CAs were highly produced in *E. coli* BL21(DE3) under the control of the T7*lac* promoter (Fig. 2a). The calculated molecular masses are 34.4 kDa for wt-*hm*CA, 29.4 kDa for trnc-*hm*CA, and 34.3 kDa for H117A mutant-*hm*CA, which correspond well with the band positions in SDS-PAGE. Notably, while wt-*hm*CA and H117A mutant-*hm*CA were produced almost exclusively in soluble forms, the removal of the N-terminal extension from *hm*CA significantly lowered the enzyme solubility; trnc-*hm*CA was predominantly found in the insoluble fraction (Fig. 2a). Even after purification of trnc-*hm*CA from the soluble fraction, most proteins also formed insoluble precipitates when dialyzed against phosphate buffer without NaCl supplementation (data not shown). Thus, it can be deduced that the N-terminal extension confers solubility on *hm*CA. The low solubility of trnc-*hm*CA was alleviated by increasing the ionic strength by the addition of 300 mM NaCl to the dialysis buffer (data not shown). wt-*hm*CA and its H117A mutant were prepared along with bCA (molecular mass of 29.1 kDa) by dialysis against phosphate buffer without NaCl unless otherwise mentioned. All of the recombinant CAs were successfully purified to apparent homogeneity (Fig. 2b). Any free thiol group was not detected on the purified wt-*hm*CA and trnc-*hm*CA by Ellman's assay, implying that two cysteines in *hm*CA were involved in the formation of

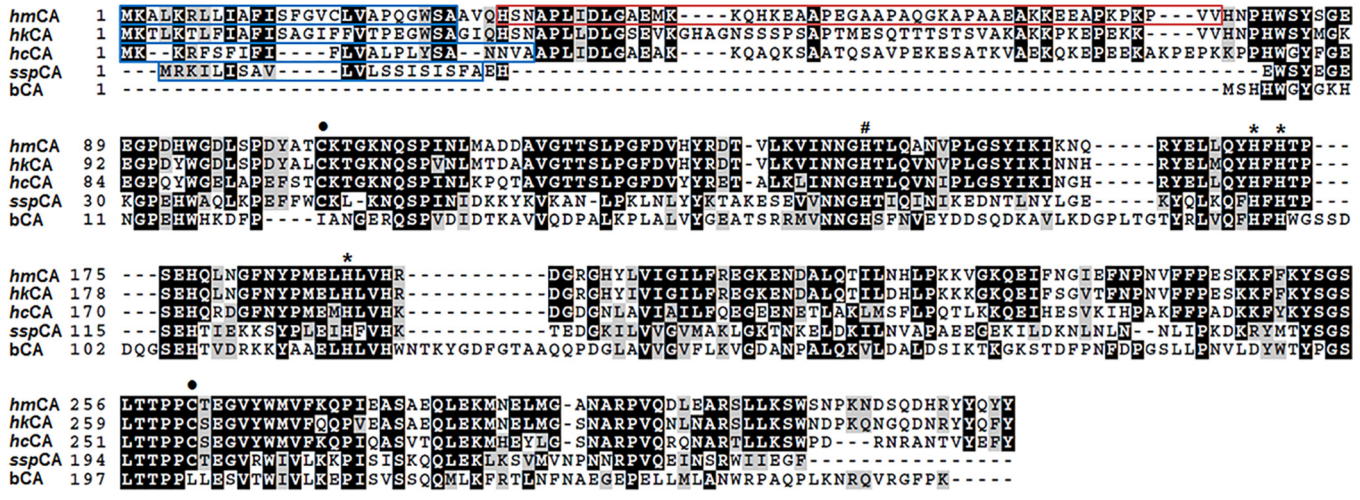

Fig. 1. Multiple alignment of α-type CAs. The sequences are from *H. marinus* (*hm*CA; KDN95606), *H. kuenenii* (*hk*CA; WP_024851558), *H. crunogenus* (*hc*CA; WP_011370966), *S. yellowstonense* (*ssp*CA; ACD66216) and *B. taurus* (bCA; NP_848667). Conserved or similar residues across three or more sequences are shaded in black or gray, respectively. The signal sequences of prokaryotic CAs are enclosed in blue boxes. The N-terminal extension of *hm*CA is shown in the red box. The two cysteine residues for the formation of the intramolecular disulfide bond are indicated by a closed circle. The three zinc-coordinating histidine residues (*) and proton shuttle residue (#) are also marked. The alignment was performed using ClustalX 2.0 and was shaded with Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)