Engineering of *Thermovibrio ammonificans* carbonic anhydrase mutants with increased thermostability

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Abstract

Carbonic anhydrase can be used as an additive to improve the efficiency of carbon capture and utilisation processes, due to its ability to increase the rate of CO_2 absorption into solvents. Successful industrial application requires robust carbonic anhydrases, able to withstand process conditions and to perform consistently over long periods of time. Tolerance of high temperatures, pH and salt concentrations are particularly desirable features. We have previously used molecular dynamics simulations to rationally design four mutants of *Thermovibrio ammonificans* carbonic anhydrase with increased rigidity, and we hypothesized that this will result in an increased thermostability. Herein, we report on the successful recombinant expression and characterization of these mutants. Four of the TaCA variants showed increased stability at 90 ºC during 1 h, compared to wild-type. Two out of the four mutations predicted by the theoretical studies resulted in marked stabilization of the protein, with up to 3-fold higher time of half-life for mutant N140G compared to the wild-type enzyme at 60 ºC. A significantly 50-fold increased ester hydrolysis activity was also observed with the most thermostable variant at 95 ºC compared to 25 ºC, suggesting an increased flexibility of the active site at high temperatures.

1 Introduction

The development of technologies to mitigate CO_2 release from human activities is of utmost importance for the well-being of our planet. Amongst these is the capture of CO_2 from industrial processes, to be further stored or utilised.[1] The removal of CO_2 from flue gases can be done by either absorption or adsorption, with the former employing basic aqueous solvents. In this context, the enzyme carbonic anhydrase (CA; EC 4.2.1.1) has been used to accelerate the hydration of CO_2 during absorption in the liquid phase. Carbonic anhydrase is a ubiquitous metalloenzyme that maintains the balance of CO_2 / HCO_3^- in cells, by the reversible hydration of carbon dioxide into bicarbonate ions. This function is important in processes of carbon fixation, which use metabolic pathways with either CO_2 or bicarbonate as the carbon source.[2] Given their functionality, CAs have been used in carbon capture processes, to increase the efficiency of CO_2 absorption and biomineralisation.[3] One example is the acceleration of the reaction rate during reactive absorption of CO_2 by alkaline solvents such as amines or K_2CO_3 solutions, which results in increased absorber loading capacity and therefore has the potential to improve the energy efficiency of the carbon capture process.[4-6] On the other hand, the application of CAs in large scale industrial flue gas clean-up requires increased protein stability at the process conditions.[7-8] For this reason, many efforts have been directed to engineer carbonic anhydrase with increased tolerance to high temperatures,[9] alkaline conditions[10] and high salt concentrations.[11-12]

To meet the stability requirements, interest was developed in bioprospecting for thermostable CAs that dwell in ocean vents, soil and hot springs.[13] Thermophilic CAs from the α family, discovered over the past decade, have been shown to have some of the highest activities and thermal stabilities known to date (Table 1). In particular, CA from *Thermovibrio ammonificans* (TaCA hereafter) has been identified as a highly robust enzyme, with a half-life of ~ 150 days at

40 °C.[14] The increased thermostability of this enzyme was attributed to the presence of an inter-subunit disulfide bond formed between two cysteines at position 67, which promoted the organization of this protein in a tetrameric structure.[15] Further engineering of TaCA by directed evolution led to a 3-fold improvement in the half-life compared to the wild-type (WT) recombinant TaCA in 1.45 M K_2CO_3 at pH 10 and > 70 °C, using mutations in the *N*-terminal region of the protein.[16] In the same α-CA family, genetic engineering of mesostable CAs has also been used to increase thermostability. For example, the thermostability of α-CA from *Neisseria gonorrhoeae* (NgCA) was 8-fold increased by engineering disulfides at the protein surface, whilst human carbonic anhydrase II (hCAII) was stabilized by either proline substitution, surface loop engineering or disulfide bond design.[9, 17-18]

In a previous theoretical study, we have shown how the high thermal stability of α-CAs was related to the high rigidity of their structures.[19] A systematic investigation of flexible sites within TaCA using molecular dynamics (MD) simulations led to the design of four mutants, which were suggested to stabilize the TaCA structure even further and therefore lead to ultra-thermostability. In the present contribution, we report on the experimental preparation of the previously designed TaCA mutants and on their activities and stabilities at high temperatures. Out of the four rigidifying mutations, two showed improved thermostability parameters compared to WT, confirming the predictions of the MD simulations (Table 1). Additionally, mutant C67G was also investigated, where the inter-subunit disulfide bond was absent, in order to assess the impact of this feature on thermostability.

Table 1. Comparison of activity and thermal stability parameters of thermostable α-carbonic anhydrases.

Entry	α-CA variant	$T_{optimum}$ (°C)[a]	Half-life, $t_{1/2}$ / Temp. (°C)	Specific activity WA unit / mg[b]	Ref.
1	*Sulfurihydrogenibium yellowstonense*, SspCA	90 °C	53 days / 40 °C	7254	[20]
2	*Sulfurihydrogenibium azorense*, SazCA	80 °C	n.r.	30000	[21]
3	*Persephonella marina*, PmCA	n.r.	75 days / 40 °C	4960	[22]
4	*Neisseria gonorrhoeae*, NgCA, N63C-P145C mutant	80 °C	31.4 h / 70 °C		[9]
5	*Thermovibrio ammonificans,* TaCA	95 °C	152 days / 40 °C 77 days / 60 °C	5236[c]	[14-15]
6	*Thermovibrio ammonificans,* TaCA, N140G mutant	95 °C	271 days / 40 °C 203 days / 60 °C	5338	This work

Note: [a] Determined by esterase activity assay with *p*NPA as substrate; [b] Determined by CO_2 hydration at 0 °C; [c] determined at 25 °C; n.r. = not reported.

2 Materials and Methods

2.1 Cloning and site directed mutagenesis

The gene encoding for wild-type *Thermovibrio ammonificans* carbonic anhydrase (WT TaCA) without the *N*-terminal signal peptide sequence was synthesized by Integrated DNA Technologies, USA, with the codon usage optimized for *E. coli* using the GeneOptimizer software. The gene was cloned between the NdeI and XhoI restriction sites of the expression vector pET19b (Novagen inc., Life Science research, Washington, DC, USA), which confers a polyhistidine tag sequence at the *N*-terminus. The resulting 6400 bp recombinant plasmid was named pET19b-TaCA. Mutagenesis was performed using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies), following manufacturer's instructions. The WT TaCA sequence and the primers used for mutagenesis are described in the ESI.

2.2 Protein expression and purification

E. coli BL21(DE3) cells (Invitrogen, USA) were transformed with plasmid pET19b-TaCA (and mutants thereof) and cultured overnight at 37 °C in Luria-Bertani (LB) medium supplemented with 50 µg/mL carbenicillin. Starter cultures (5 mL) were then used to inoculate 500 mL LB containing 50 µg/mL carbenicillin. Cultures were grown in baffled flasks at 37 °C and 200 rpm until the OD_{600} reached 0.6-0.8. Subsequently, $ZnSO_4$ was added (final concentration 0.1 mM) and the expression of TaCA gene was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG; final concentration 0.8 mM). Cultures were incubated at 20 °C and 250 rpm for further 4-16 h. The cells were then harvested at 5000 x *g* at 4 °C for 20 min and stored at -80 °C.

The cell pellet was resuspended in lysis buffer (20 mM Tris-SO_4, 300 mM NaCl, pH 8.3, supplemented with 1 mg/mL lysozyme, cOmplete™ protease inhibitor (Roche) and benzonase® endonuclease (Merck), total volume of 25 mL). After stirring for 1 hour on ice, cells were disrupted by sonication (Soniprep 150; MSE, England) on ice for 4 min and the cell debris was removed by centrifugation (23000 x *g,* 30 min, 4 °C). The clarified cell lysate was then heat-treated at 65 °C for 30 min and centrifuged again (23000 x *g*, 30 min, 4 °C), to remove precipitated denatured proteins. Recombinant TaCA was purified by immobilized metal affinity chromatography (IMAC), using a 1 mL or 5 mL HisTrap column (GE Healthcare Lifesciences), following manufacturer's instructions. The buffers consisted of binding buffer (20 mM Tris-SO_4, 300 mM NaCl, 30 mM imidazole, pH 8.3) and elution buffer (20 mM Tris-SO_4, 300 mM NaCl, 1.5 M imidazole, pH 8.3). All the eluted fractions containing the protein with the appropriate molecular weight (confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE) were collected and the buffer was exchanged against storage buffer (20 mM Tris-SO_4, 300 mM NaCl, pH 8.3), using Sephadex G-25 desalting columns (GE Healthcare, HiTrap Desalting).

Typical yields from 0.5 L lab-scale fermentation were 6 – 7.5 mg TaCA, depending on the variant. Enzyme solutions were routinely stored in storage buffer, at 4 °C for 60 days without any noticeable loss in activity. Purity was routinely assessed by SDS-PAGE followed by Coomassie staining. Protein concentration was determined by the Bradford assay, using bovine serum albumin as standard.

2.3 Enzymatic assays

The CO_2 hydrolysis activity of TaCA was assayed as previously described. A pH meter was used to monitor the pH decrease from approximately 8.3 to 6.3, due to the formation of HCO_3^- and release of H^+.[14-15, 23] Enzymatic assays were performed at 0 °C and all stock solutions were incubated at this temperature prior to the assays. Double deionised water (300 mL) was saturated with CO_2, by bubbling CO_2 through the water for 1 h at 0 °C. The enzyme solution (3.5 µL) at an appropriate concentration (0.6-1.5 mg mL^{-1}, depending on the mutant) was added to 4 mL of assay buffer (20 mM Tris-SO_4, pH 8.3). Once the pH was stabilized at 8.3, the CO_2-saturated water (2 mL) was added to the mixture and the time for the pH to drop from 8.3 to 6.3 was recorded (typical reaction times are shown in ESI). The pH was also measured for the uncatalysed reaction, where the assay buffer was used instead of the enzyme solution. All catalyzed and uncatalysed assays were performed in triplicate. CO_2 hydration activity was expressed in Wilbur-Anderson (WA) units, defined in equation 1. Specific WA units were expressed as WA units per mg of enzyme (calculation showed in the ESI).

$\boldsymbol{WA\ units} = \frac{(t_0 - t)}{t}$ $(equation\ 1)$, where t_0 = time for the uncatalysed reaction to reach pH 6.3 and t = time for the catalysed reaction to reach pH 6.3.

The hydrolysis of *p*-nitrophenyl acetate (*p*-NPA) was assayed by adapting a previously described procedure,[14] in which the increase in absorbance was determined at 348 nm (the isosbestic point of nitrophenol and of the conjugate nitrophenolate ion). In a capped plastic tube, a *p*-NPA stock solution (0.1 mL, 30 mM in acetonitrile) was added to the assay buffer (0.8 mL, potassium phosphate, 50 mM, pH 7) and the reaction was initiated by addition of enzyme (100 µL, 0.2 – 3 mg mL^{-1} stock in storage buffer, depending on the mutant). The same assays were performed for the uncatalysed reactions, where the enzyme solution was replaced with assay buffer. All catalyzed and uncatalysed assays were performed in triplicate. The average reaction rate was determined by end-point measurement of the absorbance after 3 min of incubation at 25 °C (see ESI for the reaction time course for each mutant). The extinction coefficient of *p*-nitrophenol (5000 M^{-1} cm^{-1}) was used to calculate the esterase activity of TaCA variants. The specific esterase activity was calculated by subtracting the uncatalysed rate from the catalysed rate of reaction, and was expressed in µmol *p-NPA* (µmol TaCA)$^{-1}$ min^{-1}.

To determine the temperature effect on esterase activities, the esterase assay was performed as described above in capped plastic tubes, which were incubated at the desired temperature (25 °C, 55 °C, 85 °C or 95 °C). For these assays, the mutants were purified by heat treatment only. Specific activities were calculated by subtracting the uncatalysed rate from the catalyzed rate of reaction, and were expressed in µmol *p*-NPA (mg TaCA)$^{-1}$ min^{-1}.

2.4 Thermostability tests

Thermostability tests were performed by determining residual enzyme activity after incubation at the specified temperatures for different times. The 1 h thermostability tests were performed using purified mutants (after column chromatography). Enzyme solutions were prepared in storage buffer (0.6 mg mL^{-1}), then incubated for 1 h in a water bath with a temperature control at the

desired temperature conditions. They were subsequently cooled for 15 minutes to room temperature, centrifuged to remove precipitated solids and stored at 4 °C until activity measurement (for approx. 30 minutes). Before the assay, protein stocks were diluted to the appropriate concentration.

Short-term (15 min) and long-term (up to 60 days) thermostability tests were performed using partially purified mutants. After heat shock treatment (65 °C, 30 min) and removal of precipitated proteins by centrifugation, the buffer was exchanged into storage buffer (20 mM Tris 300 mM NaCl, pH 8.3). The enzyme solutions (3 mg mL^{-1}) were incubated in capped 15 mL plastic tubes with cap lock clips, for the specified time period inside a water bath at the indicated temperatures. For temperatures above 90 °C, a heating oven was used. They were then cooled and stored at 4 °C until their activities were measured.

Enzyme activities were determined as described above, by CO_2 hydrolysis at 0 °C and by ester hydrolysis at 25 °C. Residual activities were calculated as a percentage, relative to the activity of a non-heated sample of the corresponding TaCA mutant, stored at 4 °C (equation 2). There was no significant loss of protein activity and concentration during the storage for up to 60 days.

$$Residual\ activity\ (\%) = \frac{Activity\ of\ heated\ sample}{Activity\ of\ non-heated\ sample} \times 100 \qquad (equation\ 2)$$

2.5 Temperature cycling tests

TaCA variants were subjected to temperature cycling conditions to evaluate their performance at industrial-like scrubbing operation. The enzyme samples were repeatedly incubated at two different temperatures, 40 and 77 °C, in thermostatic water baths (Julabo TW12). A whole cycle of incubation consisted of 4 min: absorption for 2 min at 40 °C, followed by desorption for 2 min

at 77 °C. The cycles were repeated for one hour and enzyme activities were monitored every five continuous cycles (20 min).

3 Results and Discussion

3.1 Design, expression and catalytic activity of TaCA variants

Previous molecular dynamic simulations showed that TaCA possessed increased flexibility at certain regions.[19] Rigidifying mutations were designed at flexible sites (Figure 1), containing either stabilising amino acids or disulfide bonds. Four mutants with increased overall protein rigidity were further characterized by molecular dynamic simulations and showed a similar flexibility of the active site residues, when compared to WT TaCA: N140G, T175P, A242P and P165C-Q170C. Based on this, we predicted that the newly designed mutants would have a similar activity, but an increased thermal stability when compared to WT TaCA. An additional fifth mutant, C67G, was designed, lacking the inter-subunit disulfide bond, which has previously been suggested to be responsible for the increased thermostability in TaCA.[15]

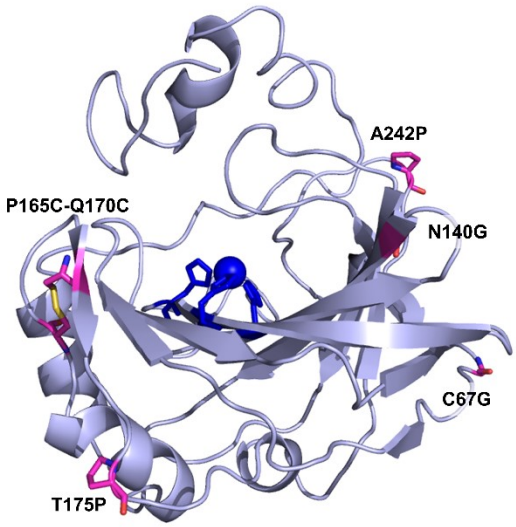

Figure 1. Cartoon model of *Thermovibrio ammonificans* carbonic anhydrase with rigidifying mutation sites. The structure was prepared from pdb file 4C3T and mutations were

introduced using Pymol. The zinc binding site is coloured in blue and the mutation sites are coloured in magenta.

An expression construct containing the TaCA gene was designed, to express TaCA without the 22-residue *N*-terminal signal peptide sequence, which could potentially impact on enzyme activity and stability.[16] For ease of purification and to avoid interference with the tetrameric organization, which was suggested to involve interactions at the *C*-terminal end,[15] we installed an *N*-terminal hexahistidine tag. Successful soluble expression was obtained for WT TaCA and all single mutants (Figure 2A), in yields ranging from 6.5 to 7.5 mg per 0.5 L culture for all mutants. The mutant containing the designed disulfide bond precipitated during freeze-thaw cycling (Figure 2A, lane 6), suggesting a somewhat decreased flexibility compared to the other mutants.

The enzymes were purified by Ni^{2+} affinity chromatography. SDS-PAGE analysis showed bands at ~ 25-30 kDa, attributed to the monomeric forms with a molecular weight of 28.6 kDa (Figure 2B). Bands of dimeric forms were also observed by Western blot analysis for all mutants, albeit with low intensity (see ESI). All these results are in line with previously reported behaviour of this thermostable enzyme expressed in a mesophile.[14]

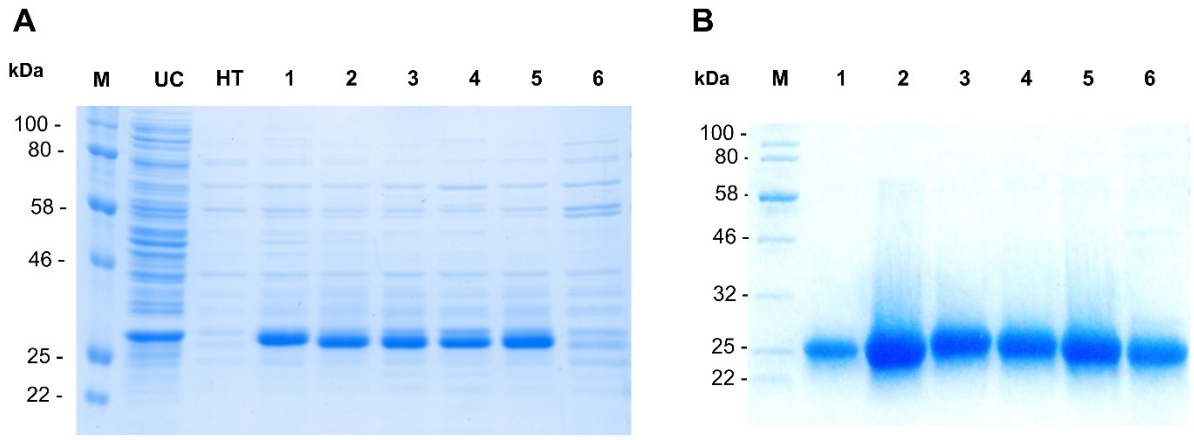

Figure 2. Expression and purification of WT TaCA and mutants. (A) SDS-PAGE of soluble proteins after heat treatment (65 °C for 30 min), followed by storage at -20 °C. (B) SDS-PAGE of proteins purified by affinity chromatography (loaded at 6 µg). Lane: M, molecular weight marker; UC, uninduced control (WT TaCA plasmid) without heat treatment; HT, uninduced

control (WT TaCA plasmid) with heat treatment; 1, WT TaCA; 2, C67G; 3, N140G; 4, T175P; 5, A242P; 6, P165C-Q170C.

The thiol content of TaCA variants WT, C67G and P165C-Q170C was quantified by Ellman's assay, in order to evaluate disulfide bond formation. In the absence of DTT, none of the three proteins showed the presence of free thiol groups, suggesting that all thiols were involved in disulfide bonds, including the C67 residue in WT and the C165 and C170 residues in the corresponding disulfide mutant. When treated with the disulfide reducing agent DTT, the wild-type enzyme showed the expected number of three free thiols per monomer, originating from the native intra-monomer disulfide bond C47-C202, as well as from C67. Removal of one cysteine in mutant C67G resulted in two free thiols, whilst introduction of the *de novo* designed disulfide P165C-Q170C resulted in five free thiols in the DTT-treated proteins, as expected (see ESI).

The specific activities of the purified TaCA WT and mutants were determined using the CO_2 hydrolysis assay at 0 °C and were comparable to previously reported activities for WT TaCA and for bovine carbonic anhydrase bCA (5000-5500 WA units mg^{-1}; Table 2). An exception was the double mutant P165C-Q170C, which had a lower specific activity of 3205 WA units mg^{-1}. The increased rigidity conferred by the *de novo* introduced disulfide bridge, albeit far from the active site, might be responsible for the decrease in protein activity.

Esterase activities of the purified TaCA mutants were also measured at 25 °C, using *p*-NPA as substrate (Table 2). Hydrolysis of activated esters is a promiscuous activity of carbonic anhydrases, which is easier to implement than the CO_2 hydrolysis assay and can also be used as a measure of enzyme activity. As expected, these activities were very low (< 10 % of the esterase activity of bCA) for most TaCA variants, probably due to the rigidity of the structure and to the inability of the ester substrate to bind in a position suitable for catalysis. Interestingly, C67G showed an 8.4-

fold improvement of esterase activity compared to WT. This mutant lacks the inter-subunit disulfide bond between TaCA dimers, which leads to tetramer formation. In a previous study, James *et al.* suggested the low esterase activities of TaCA to be a result of secondary structure stabilization by the ion pair interactions among Arg207, Asp102 and Arg192 residues.[15] These interactions were hypothesized to restrict flexibility of the active site and were used to explain the difference in esterase activity between TaCA and the other thermostable α-CAs (SspCA and SazCA[21]). Our results show that the inter-subunit disulfide bond formed by C67 residues also plays an important role in restricting *p*-NPA substrate binding and hydrolysis.

Table 2. Enzymatic activities of wild-type (WT) and mutant TaCA variants.

TaCA variant	Specific activity for CO_2 hydrolysis (WA units / mg TaCA)[a]	Esterase activity (min^{-1})[b]
WT	5267 ±178	2.0 ±0.1
C67G	5549 ±39	16.6 ±0.7
N140G	5338 ±31	3.6 ±0.6
T175P	5467 ±22	0.8 ±0.06
A242P	4924 ±17	4.8 ±0.2
P165C-Q170C	3205 ±118	0.3 ±0.1
bCA	5267 ±0	61.5 ±3.1

Note: [a] Measured at 0 ^{0}C in Tris-SO_4 pH 8.3; [b] Measured at 25 °C, in potassium phosphate pH 7.5 with 3 mM *p*-NPA; esterase activity is given as (μmol *p*-NPA) (μmol TaCA)$^{-1}$ min^{-1}. Enzymatic activities are reflected as mean ±standard deviation (SD).

3.2 Stability assessment of CA variants

3.2.1 Thermostability after one hour heating

To evaluate the thermostability of TaCA variants, the purified enzymes were heated for 1 h under different temperature conditions and the residual activities for CO_2 hydrolysis were compared with the initial activities of the corresponding TaCA mutant (Figure 3). All TaCA variants showed higher stability than bCA, which was completely inactivated after heating at 80 °C. In our hands, WT TaCA retained more than 70 % activity after heating for 1 h at 80 °C, and lost most activity (30 % residual activity) after incubation at 90 °C. These residual activities were in line with previously reported data for the TaCA enzyme devoid of the *N*-terminal peptide sequence.[14, 16] They were also higher than the residual activities of the enzyme containing the signal peptide (< 20 % residual activity after 1 h at 80 °C),[15] thus confirming that the signal peptide has a destabilizing effect. Most of the designed mutants, except for A242P, displayed higher thermostabilities than WT TaCA under these conditions. Mutant N140G reported the highest thermostability among all variants by retaining 100 % of its activity after 1 h of incubation at 90 °C. Mutant P165C-Q170C was also one of the most thermostable variants, suggesting that the structural stability of the TaCA was not lowered by the presence of the *de novo* introduced disulfide bridge. On the other hand, this mutant was not stable upon freezing at -20 ºC and thawing (see section 3.1 above), perhaps because the excessive rigidity designed in the mutant is detrimental to stability at lower temperatures. Surprisingly, the C67G mutant, lacking the stabilizing intra-monomer disulfide bond, showed an increased thermostability compared to WT after 1 h heating, therefore suggesting that the inter-subunit disulfide might not be responsible for TaCA stabilisation. All TaCA mutants were completely inactivated after 1 h of incubation at 100 °C, which is in line with previously published reports. In our hands, all enzymes demonstrated precipitation after 1h incubation at 100 °C, with N140G and P165C-Q170C showing the least precipitate. This leads us to speculate that the enzyme gets irreversibly thermoinactivated by

aggregation upon incubation at 100 °C for prolonged times. No precipitate was observed at the lower temperatures, which could raise the question of reversible denaturation upon high temperature incubation, with subsequent refolding during the storage time before the assay (~ 45 min cooling time, see Methods). However, variation of cooling and storage times led to very similar results. Therefore, we suggests that the observed stability is not due to refolding during cooling.

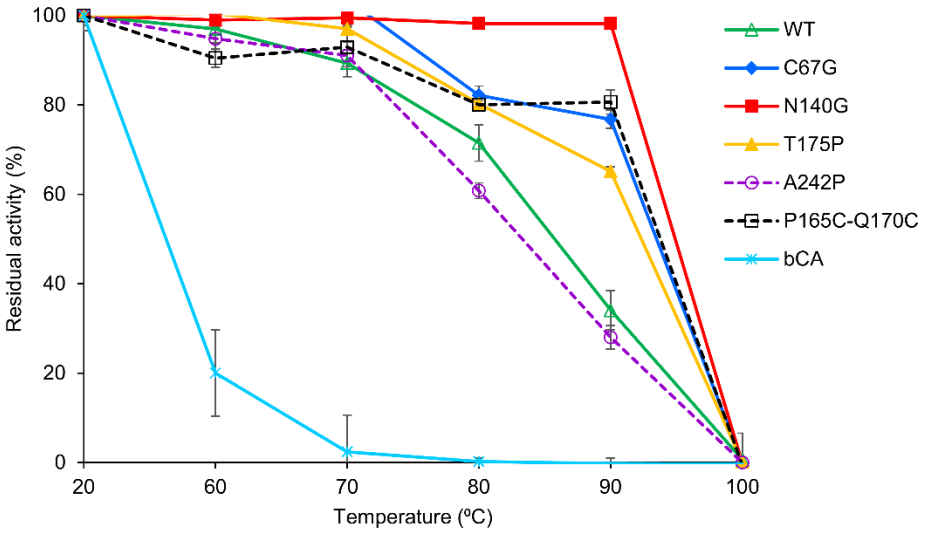

Figure 3. Thermostabilities of TaCA variants. Enzyme solutions (0.6 mg mL^{-1}) were incubated for 1 h at the indicated temperatures, after which the activities were measured by CO_2 hydrolysis assays. Residual activities were calculated as percentages of the initial activities of each CA. Each value represents the average of triplicate assays, and the error bars represent the standard deviations.

3.2.2 Short-term thermostability of TaCA mutants

Next, we assessed whether the recombinant expression of these thermophilic enzymes in a mesophilic host had any effect on their activities. First, WT and mutants were partially purified from cell lysates by a 30 min heat treatment at 65 °C. This treatment was also likely to act as a "thermostimulation" step, inducing conformational changes similar to those occurring during

production under thermophilic conditions.[14] To assess whether the thermostimulation was further affected by the temperature, the partially purified mutants were incubated at different temperatures for 15 minutes and the residual activities were determined for the hydrolysis of both CO_2 and *p*-NPA substrates (Figure 4). The CO_2 hydrolysis activity showed no thermostimulation effect. This might have been because the heat-shock step at 65 °C during purification was enough to induce any conformational changes facilitating the access of the small CO_2 substrate. On the other hand, this short-term thermostability test confirmed that A242P was the least stable mutant, with almost half of its activity lost in the first 15 min of heating at 90 °C. Furthermore, mutants C67G and A242P degraded most rapidly above 90 °C.

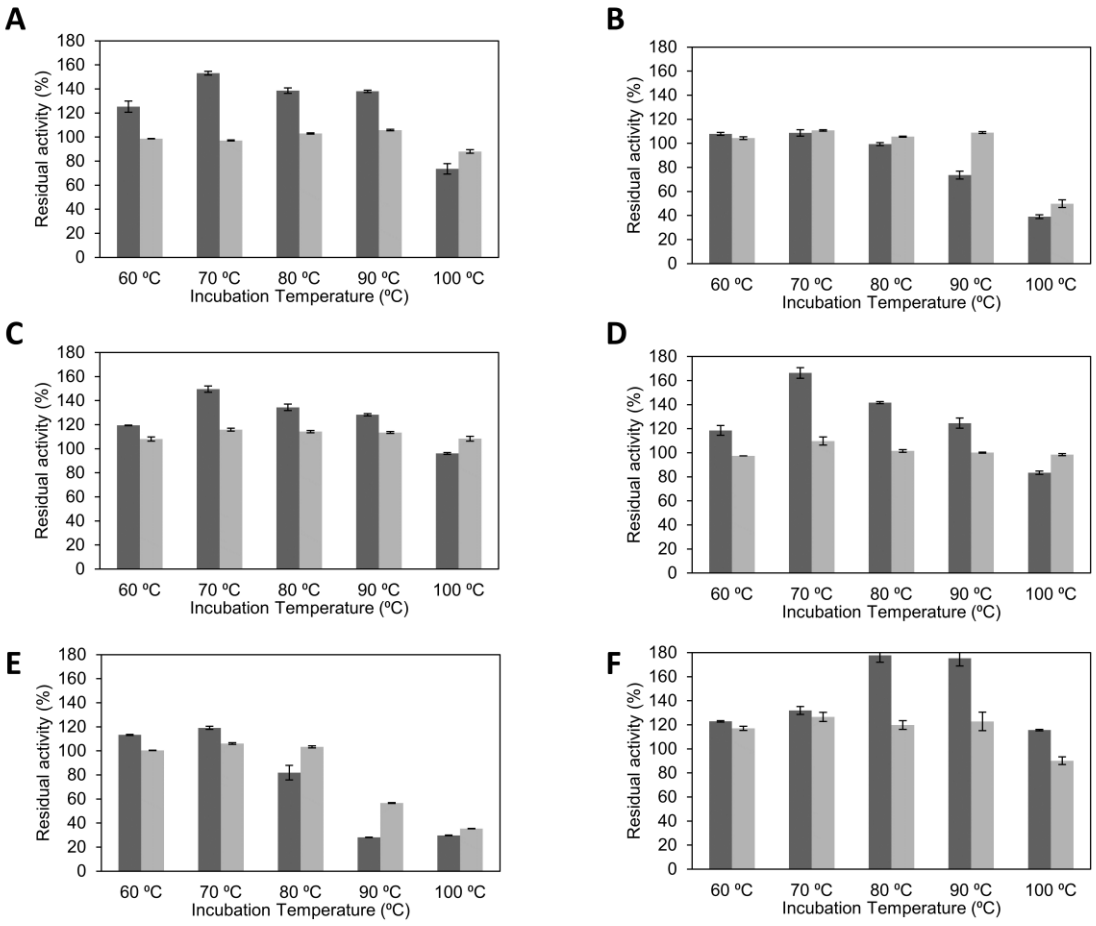

Figure 4. Short-term thermostabilities of TaCA variants. (A) WT, (B) C67G, (C) N140G, (D) T175P, (E) A242P and (F) P165C-Q170C. Enzyme solutions (3 mg mL^{-1}) were incubated for 15 min at the indicated temperatures, and the residual activities were measured by esterase activity at 25 °C (black bars) and CO_2 hydration activity assay at 0 °C (gray bars) assays. Activities of 100% correspond to the untreated samples. Each value represents the average of triplicate assays, and the error bars represent the standard deviations.

In contrast to CO_2 hydrolysis, the esterase activity showed a thermostimulation effect, which was previously observed with WT TaCA and attributed to broadening of the cavity through which the *p*-NPA substrate binds to the active site.[14] With the exception of C67G and A242P, the other TaCA variants showed a similar behaviour to WT, with an increased esterase activity after a 15 min heat-activation step below 100 °C and in particular at higher temperatures (70 – 90 °C). In the case of C67G, it is likely that the cavity is already flexible due to the disruption of tetramer formation, which could also explain the lack of activation after heating.

3.3 Long-term thermostability of TaCA variants

The application of enzymes to post combustion carbon capture requires them to be stable and kinetically active under operating conditions for extended periods of time. Therefore, the stability of WT TaCA and mutants was determined under industrial-like absorption conditions, at 40 and 60 °C during a period of 60 days.[13, 24] Samples were taken at 15 days intervals and their CO_2 hydrolysis activity was determined (Figure 5). The N140G mutant showed the highest long-term thermostability, with 95 % and 90 % residual activities after 60 days incubation at 40 and 60°C, respectively. Mutants T175P and C67G showed similar behaviour to WT TaCA, with > 60 % residual activities after two months of incubation at 60 °C. On the other hand, mutants A242P and P165C-Q170C showed residual activities below 35 % at similar conditions, with the disulfide variant showing a rapid degradation even at 40 °C (Figure 5A). The similarity between WT and C67G in terms of long-term thermostability again suggests that the inter-subunit disulfide bond might not be responsible for the increased stability of this enzyme.

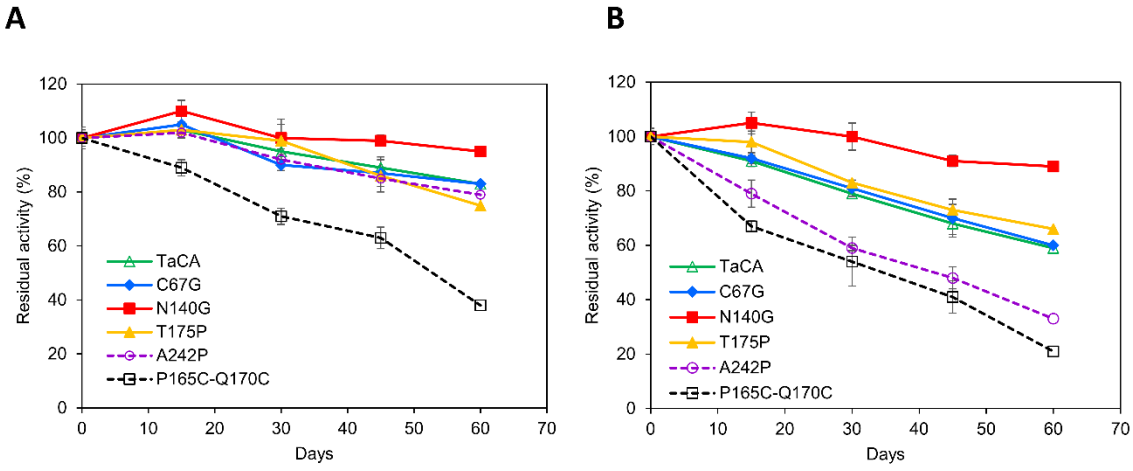

Figure 5. Long-term thermostabilities of TaCA variants at (A) 40 °C and (B) 60 °C. Enzyme solutions (3 mg mL^{-1}) in storage buffer (20 mM Tris 300 mM NaCl, pH 8.3) were incubated at the indicated temperatures, and the residual activities were measured by CO_2 hydration activity assay

at 0 °C. Activities of 100% correspond to the untreated samples (day 0). Each value represents the mean of triplicate experiments, and the error bars represent the standard deviations.

The half-lives of WT TaCA and mutants were estimated by fitting the experimental data to an exponential decay model (Table 3 and ESI). Due to the thermostimulation effect, data values at day 0 were not used for this model fitting. WT TaCA was shown to be a very stable catalyst and exhibited similar half-life values to previously reported data, of 166 days at 40 °C and 77 days at 60 °C.[14] The most thermostable N140G variant showed a half-life of 203 days at 60 °C, which represents a 260 % increase in stability compared to WT TaCA. Overall, stability trends were similar at 40 and 60 °C. Exceptions were mutant A242P, which degraded more rapidly than others when the temperature was increased, and mutant T175P, which was one of the most stable at 60 °C, whilst its performance was relatively poor at 40 °C. This latter result could potentially be explained by a shift in the stability maximum for this mutant towards the higher temperatures, which has previously been suggested as a mechanism to achieve thermostability.[25]

Table 3. Time of half-life ($t_{½}$ in days) of TaCA variants at 40 °C and 60 °C.

Temperature (°C)	WT	C67G	N140G	T175P	A242P	P165C-Q170C
40	166	158	271	118	140	50
60	77	80	203	90	40	31

Note: Times of half-life values were calculated in days, by fitting residual activity experimental data to a first order decay, using a linearized exponential decay equation (see ESI). Time of half-life = time necessary to reach 50 % residual activities (days).

The stability of the newly designed TaCA variants was also tested under temperature cycling conditions, whereby samples were continuously subjected to 4 min temperature cycles between 40 °C and 77 °C, designed to mimic absorption-desorption cycles during carbon capture processes. A cycle was defined as the time of exposure to 40 °C for 2 min (absorption cycle), followed by 77

°C for 2 min (desorption cycle). The total time of the cycling experiment was 60 min, corresponding to 15 cycles. The results showed that WT and mutants C67G, N140G and T175P displayed a similarly stable behaviour, without loss in activity during cycling. Mutants A242P and P165C-Q170C were again the least stable, with 10 % and 20 % activity lost after 1 hour of temperature cycling, respectively (see ESI).

The rapid degradation of mutants A242P and P165C-Q170C at higher temperatures was not predicted by our previous molecular dynamics simulations, which had indicated higher structural rigidity for these mutants. It must be noted that MD simulations were performed on a monomeric protein model, which resulted in a limited understanding of the conformational stabilization of the tetrameric state. Position A242 is situated in a surface loop, in a region of contact between two monomers to form a bacterial dimer. Hence, whilst removal of the hydrophobic amino acid alanine and its replacement with proline might have increased local rigidity as predicted by MD, it might also have disrupted a stabilising interaction between two TaCA monomers, thus leading to the lower overall stability observed experimentally. The low stability of mutant P165C-Q170C during long-term studies was even more surprising, given the increased short-term stability (up to 1 hour) and the decreased activity, which could both be correlated with the predicted monomer rigidity. Here also, changes in conformational space and in solvent exposed surface area between monomeric and tetrameric structures could potentially explain the difference between the model and experimental results. Thus, future work aiming to design thermostability in TaCa should focus on modelling inter-monomer interactions.

3.4 Temperature effect on the activity of TaCA mutants

The effect of temperature on the activity of the mutants was determined by measuring ester hydrolysis activities at four different temperatures: 25 °C, 55 °C, 85 °C and 95 °C (Figure 6). Carbon dioxide hydrolysis at different temperatures could not be tested, due to the difficulty to control the CO_2 concentration in the aqueous buffer at these temperatures. As expected, the non-thermostable bCA showed an increase in esterase activity at 55 °C, compared to 25 °C, but became inactivated at the higher temperatures. On the other hand, all TaCA variants exhibited a continuous increase in esterase activity up to 95 °C, which was the maximal temperature that could be tested in the aqueous system. The N140G TaCA mutant showed the highest increase in esterase activity, with a 53-fold increase observed at 95 °C compared to 25 °C. The T175P mutant showed a similar behaviour to N140G up to 85 °C, after which the effect of temperature increase on esterase activity was less pronounced. The other four TaCA variants (WT and mutants C67G, A242P and P165C-Q170C) showed 14 to 23-fold increased activity at 95 °C compared to those at 25 °C, which was similar to previously reported WT behaviour.[14]

The activity increase of TaCA variants at higher temperature is likely due to an additional, different mechanism from the thermostimulation observed after 15 minutes incubation at high temperatures, which provided and increase in activity of maximum 1.7-fold at 90 °C (Figure 4, section 3.2.2). Whilst thermostimulation during the 3 min activity measurement cannot be excluded, we suggest that the highly increased esterase activities at high temperature are mainly due to an increased access of the (organic) substrate to the active site. We would expect a similar behaviour with CO_2 as a hydrolysis substrate, which would result in a highly increased CO_2 absorption efficiency for some of the designed mutants compared to wild-type, under high temperature conditions. With the exception of mutants A242P and P165C-Q170C, the specific

activities of the TaCA variants at 95 °C were all higher than the specific optimum activity of bCA at 55 °C (2.9 μmol *p*NCA (mg CA)$^{-1}$ min^{-1}). The highest activity was shown by mutant C67G (10 μmol *p*NCA (mg CA)$^{-1}$ min^{-1}), which was also the most active of the TaCA mutants during the esterase assay at 25 °C (Figure 6B). As discussed in section 3.1 above, the absence of the inter-monomer disulfide bond in this mutant is probably responsible for the increased flexibility and therefore enhanced esterase activity compared to the other mutants.

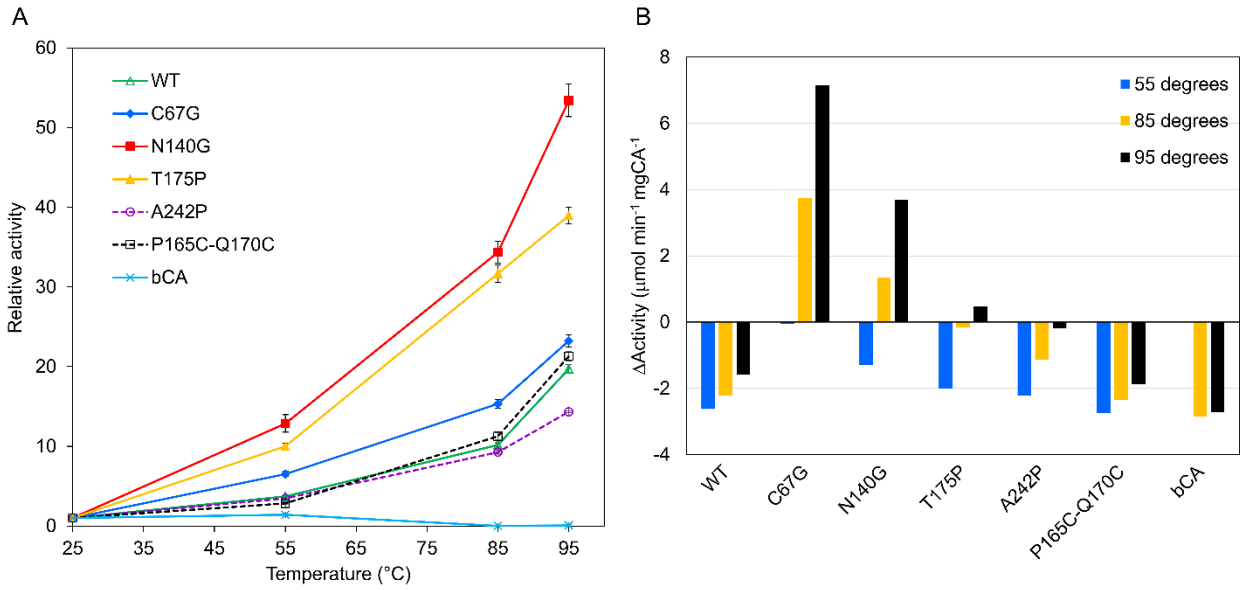

Figure 6. Effect of temperature on the esterase activities of TaCA variants. (A) Relative activities calculated as the ratio between activity at the tested temperature and activity at 25 °C; (B) Difference between absolute activity of mutants at different temperatures and activity of bCA at 55 °C (ΔActivity). Enzymatic assays were performed in phosphate buffer (50 mM, pH 7) with 3 mM *p*-NPA (see ESI for specific activities at 25 °C). Each value represents the mean of triplicate experiments, and the error bars represent the standard deviations.

4 Conclusions

The development of carbonic anhydrases with very high thermal stability is one important requirement for their implementation in post combustion carbon capture processes. We have previously used molecular dynamics simulations to rationally design stabilized mutants of α-

carbonic anhydrase from *Thermovibro ammonificans.* Here, we produced these mutants in *E. coli,* and we evaluated their catalytic activities and thermostability parameters using CO_2 hydrolysis and esterase assays. Remarkably, the N140G mutant maintained 100 % residual activity after 1 h heating at 90 °C, whereas the wild-type enzyme was only 30 % active after exposure to the same conditions. From the four mutants suggested by the theoretical studies, two (N140G and T175P) exhibited times of half-life which were up to 3-fold higher than wild-type at 60 °C. The ability of TaCA to act as an esterase was increased after short-time heating at high temperatures, and this behaviour was attributed to a thermostimulation effect. Furthermore, ester hydrolysis activities of mutants N140G and T175P at 95 °C were increased more than 50 and 35 times, respectively, indicating a potentially similar increase of CO_2 hydrolysis rates at high temperatures. Interestingly, mutant C67G displayed a comparable behaviour to wild-type at high temperatures, suggesting that the inter-monomer disulfide bond might not be responsible for increased thermostability. Further efforts are currently underway in our group towards further characterisation of the beneficial mutations and of their combination, as well as towards the study of their effect on CO_2 capture under different conditions, such as alkaline solvents.

Supporting information. The Electronic Supporting Information contains details of the genetic material, of protein expression and characterisation, of long-term thermostability data and of enzymatic assay data.

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5 References

1. Cuéllar-Franca, R. M.; Azapagic, A., Carbon capture, storage and utilisation technologies: A critical analysis and comparison of their life cycle environmental impacts. *J. CO2 Util.* **2015,** *9*, 82-102.
2. Shi, J.; Jiang, Y.; Jiang, Z.; Wang, X.; Wang, X.; Zhang, S.; Han, P.; Yang, C., Enzymatic conversion of carbon dioxide. *Chem. Soc. Rev.* **2015,** *44* (17), 5981-6000.
3. Kanth, B. K.; Lee, J.; Pack, S. P., Carbonic anhydrase: Its biocatalytic mechanisms and functional properties for efficient CO2 capture process development. *Eng. Life Sci.* **2013,** *13* (5), 422-431.
4. Leimbrink, M.; Sandkämper, S.; Wardhaugh, L.; Maher, D.; Green, P.; Puxty, G.; Conway, W.; Bennett, R.; Botma, H.; Feron, P.; Górak, A.; Skiborowski, M., Energy-efficient solvent regeneration in enzymatic reactive absorption for carbon dioxide capture. *Appl. Energy* **2017,** *208*, 263-276.
5. Sahoo, P. C.; Kumar, M.; Singh, A.; Singh, M. P.; Puri, S. K., Biocatalyzed Accelerated Post-combustion CO2 Capture and Stripping in Monoethanolamine. *Energy & Fuels* **2017,** *31* (10), 11007-11012.
6. Hu, G.; Smith, K. H.; Nicholas, N. J.; Yong, J.; Kentish, S. E.; Stevens, G. W., Enzymatic carbon dioxide capture using a thermally stable carbonic anhydrase as a promoter in potassium carbonate solvents. *Chem. Eng. J.* **2017,** *307*, 49-55.
7. Bose, H.; Satyanarayana, T., Microbial Carbonic Anhydrases in Biomimetic Carbon Sequestration for Mitigating Global Warming: Prospects and Perspectives. *Front. Microbiol.* **2017,** *8*, 1615.
8. Sahoo, P. C.; Kumar, M.; Puri, S. K.; Ramakumar, S. S. V., Enzyme inspired complexes for industrial CO2 capture: Opportunities and challenges. *J. CO2 Util.* **2018,** *24*, 419-429.
9. Jo, B. H.; Park, T. Y.; Park, H. J.; Yeon, Y. J.; Yoo, Y. J.; Cha, H. J., Engineering de novo disulfide bond in bacterial [Alpha]-type carbonic anhydrase for thermostable carbon sequestration. *Sci. Rep.* **2016,** *6*, 29322.
10. Alvizo, O.; Nguyen, L. J.; Savile, C. K.; Bresson, J. A.; Lakhapatri, S. L.; Solis, E. O. P.; Fox, R. J.; Broering, J. M.; Benoit, M. R.; Zimmerman, S. A.; Novick, S. J.; Liang, J.; Lalonde, J. J., Directed

Evolution of an Ultrastable Carbonic Anhydrase for Highly Efficient Carbon Capture from Flue Gas. *Proc. Natl. Acad. Sci.* **2014,** *111* (46), 16436-16441.
11. Warden, A. C.; Williams, M.; Peat, T. S.; Seabrook, S. A.; Newman, J.; Dojchinov, G.; Haritos, V. S., Rational engineering of a mesohalophilic carbonic anhydrase to an extreme halotolerant biocatalyst. *Nat. Commun.* **2015,** *6*, 10278.
12. Jo, B. H.; Im, S.-K.; Cha, H. J., Halotolerant carbonic anhydrase with unusual N-terminal extension from marine Hydrogenovibrio marinus as novel biocatalyst for carbon sequestration under high-salt environments. *J. CO2 Util.* **2018,** *26*, 415-424.
13. Di Fiore, A.; Alterio, V.; Monti, S.; De Simone, G.; Ambrosio, K., Thermostable Carbonic Anhydrases in Biotechnological Applications. *Int. J. Mol. Sci.* **2015,** *16* (7), 15456-15480.
14. Jo, B. H.; Seo, J. H.; Cha, H. J., Bacterial Extremo-α-Carbonic Anhydrases from Deep-Sea Hydrothermal Vents as Potential Biocatalysts for CO_2 Sequestration. *J. Mol. Catal. B: Enzym.* **2014,** *109*, 31-39.
15. James, P.; Isupov, M. N.; Sayer, C.; Saneei, V.; Berg, S.; Lioliou, M.; Kotlar, H. K.; Littlechild, J. A., The structure of a tetrameric [alpha]-carbonic anhydrase from *Thermovibrio ammonificans* reveals a core formed around intermolecular disulfides that contribute to its thermostability. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2014,** *70* (10), 2607-2618.
16. Voyer, N.; Daigle, R.; Madore, É.; Fradette, S. Variants of *Thermovibrio Ammonificans* carbonic anhydrase and CO_2 capture methods using *Thermovibrio Ammonificans* carbonic anhydrase variants. Int. Pat. Appl. WO2017035667 A1, Mar 09, 2017.
17. Boone, C. D.; Habibzadegan, A.; Tu, C.; Silverman, D. N.; McKenna, R., Structural and catalytic characterization of a thermally stable and acid-stable variant of human carbonic anhydrase II containing an engineered disulfide bond. *Acta Crystallogr D Biol Crystallogr* **2013,** *69* (Pt 8), 1414-1422.
18. Boone, C. D.; Rasi, V.; Tu, C.; McKenna, R., Structural and catalytic effects of proline substitution and surface loop deletion in the extended active site of human carbonic anhydrase II. *FEBS J.* **2015,** *282* (8), 1445-1457.
19. Parra-Cruz, R.; Jäger, C. M.; Lau, P. L.; Gomes, R. L.; Pordea, A., Rational Design of Thermostable Carbonic Anhydrase Mutants Using Molecular Dynamics Simulations. *J. Phys. Chem. B* **2018,** *122* (36), 8526-8536.
20. Capasso, C.; De Luca, V.; Carginale, V.; Cannio, R.; Rossi, M., Biochemical Properties of a Novel and Highly Thermostable Bacterial α-Carbonic Anhydrase from *Sulfurihydrogenibium yellowstonense* YO3AOP1. *J. Enzyme Inhib. Med. Chem.* **2012,** *27* (6), 892-897.
21. Luca, V. D.; Vullo, D.; Scozzafava, A.; Carginale, V.; Rossi, M.; Supuran, C. T.; Capasso, C., An α-Carbonic Anhydrase from the Thermophilic Bacterium *Sulphurihydrogenibium azorense* is the Fastest Enzyme Known for the CO_2 Hydration Reaction. *Bioorg. Med. Chem.* **2013,** *21* (6), 1465-1469.
22. Kanth, B. K.; Jun, S.-Y.; Kumari, S.; Pack, S. P., Highly Thermostable Carbonic Anhydrase from *Persephonella marina* EX-H1: Its Expression and Characterization for CO_2-Sequestration Applications. *Process Biochem.* **2014,** *49* (12), 2114-2121.
23. Wilbur, K. M.; Anderson, N. G., Electrometric and colorimetric determination of carbonic anhydrase. *J. Biol. Chem.* **1948,** *176* (1), 147-154.
24. Savile, C. K.; Lalonde, J. J., Biotechnology for the Acceleration of Carbon dioxide Capture and Sequestration. *Curr. Opin. Biotechnol.* **2011,** *22* (6), 818-823.

25. Coleman, R. G.; Sharp, K. A., Shape and evolution of thermostable protein structure. *Proteins* **2010,** *78* (2), 420-433.