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Carbonic anhydrase inhibitors. Inhibition of the human cytosolic isozyme VII with aromatic and heterocyclic sulfonamides

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Abstract—The inhibition of a newly cloned human carbonic anhydrase (CA, EC 4.2.1.1), isozyme VII (hCA VII), has been investigated with a series of aromatic and heterocyclic sulfonamides, including some of the clinically used derivatives (acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, brinzolamide and benzolamide), as well as the sulfamate antiepileptic drug topiramate. Inhibition data for the the other physiologically relevant cytosolic isoforms hCA I, hCA II and mCA XIII are also provided for comparison. hCA VII shows a high catalytic activity for the CO_2 hydration reaction, with a k_{cat} of 9.5×10^5 s^{-1} and k_{cat}/K_m of 8.3×10^7 M^{-1} s^{-1} at pH 7.5 and 20 °C. A very interesting inhibition profile against hCA VII with this series of 32 sulfonamides/sulfamates was observed. hCA VII shows high affinity for all the investigated compounds, with inhibition constants in the range of 0.45–210 nM. Topiramate, ethoxzolamide and benzolamide showed subnanomolar hCA VII inhibitory activity, whereas acetazolamide, methazolamide, dorzolamide and brinzolamide showed K_I-s in the range of 2.1–3.5 nM. Dichlorophenamide was slightly less active (K_I of 26.5 nM). A number of heterocyclic or bicyclic aromatic sulfonamides also showed excellent hCA VII inhibitory properties (K_I-s in the range of 4.3–7.0 nM) whereas many monosubstituted or disubstituted benzenesulfonamides were less active (K_I-s in the range of 45–89 nM). The least active hCA VII inhibitors were some substituted benzene-1,3-disulfonamides as well as some halogenated sulfanilamides (K_I-s in the range of 100–210 nM). The inhibition profile of hCA VII is rather different of that of the other cytosolic isozymes, providing thus a possibility for the design of more selective, hCA VII-specific inhibitors. In addition, these data furnish further evidence that hCA VII is the isozyme responsible for the anticonvulsant/antiepileptic activity of sulfonamides and sulfamates.

1. Introduction

Vertebrates, humans included, express a multitude of isozymes of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1), which play a host of physiological roles in processes where the three chemical entities acting as substrates/reaction products of the CAs (i.e., CO_2, bicarbonate and H^+ ions) are involved. Such processes include respiration and gas exchange, pH homeostasis, renal excretion of anions and urine formation, detoxification of metabolic waste products, production of electrolytes in many tissues/organs (aqueous humor in the eye, cerebrospinal fluid (CSF) in the brain, bicarbonate secretion in the kidney, pancreas and other organs, HCl production in the stomach, etc.), biosynthetic reactions, bone resorption, cell proliferation and differentiation, cell adhesion, modulation of neuronal transmission, etc.[1–6] In humans, 15 isozymes are presently known, twelve of which are catalytically active (CAs I–Va, CA Vb, CAVI, CA VII, CA IX and CAs XII–XIV), whereas the CA-related proteins (CARPs VIII, X and XI) are devoid of catalytic activity due to the fact that one or more histidine zinc ligand are replaced by other amino acid residues.[7] Among the active isozymes, five show a cytosolic subcellular localization: CAs I–III, CA VII and CA XIII.[1–5,8] Whereas CA III is a very low activity enzyme, abundant in the skeletal muscles and adipocytes (also poorly inhibited by

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Table 1. Kinetic parameters for CO_2 hydration reaction catalyzed by the cytosolic α-CA isozymes I–III, VII and XIII, at pH 7.5, and their inhibition data with acetazolamide **AAZ** (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), a clinically used compound

Isozyme	Activity level	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	K_I (acetazolamide) (nM)
hCA I*	Moderate	2×10^5	5×10^7	250
hCA II*	Very high	1.4×10^6	1.5×10^8	12
hCA III**	Low	1.0×10^4	3×10^5	300,000
mCA VII**	High	9.4×10^5	7.6×10^7	16*
mCA XIII*	Moderate	8.3×10^4	4.3×10^7	17

h = human; m = murine isozyme.

* From Ref. 8a.

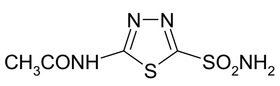
** From Ref. 11.

sulfonamides),[1–5] the other four are quite active (Table 1). Some isozymes (i.e., CA II) were known for decades to be physiologically important ones,[1–5] others (such as CA I) have physiological functions little understood at the moment,[1–5] whereas for CA XIII, an important role in pH regulation in the reproductive tract of both females and males has only recently been recognized.[8]

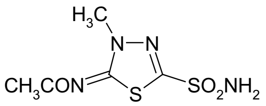
CA VII appears to be the less studied and understood among the cytosolic CAs. The CA VII was isolated from a human genomic library in 1991 by Montgomery et al.,[9] and found to be located on chromosome 16. The gene is approximately 10 kb long and contains seven exons and six introns found at positions identical to those determined for the previously identified CA I, CA II and CA III genes. The derived amino acid sequence of the CA VII gene is of 263 amino acids showing 50%, 56%, and 49% identity with hCA I, hCA II and hCA III, respectively (these were the cytosolic isozymes known in 1991).[9] An initial purification of the mouse isozyme, mCA VII, by Tashian's group led to the conclusion that this is a low activity CA,[10] but subsequent work of the same group,[11] with a better purification protocol led to the thorough purification and characterization of mCA VII (in fact a chimeric protein, with the first 9 amino acids of human origin, and the remaining 253 of murine origin). This was found to be catalytically highly active (k_{cat}/K_m for CO_2 hydration described by two ionizations at pK_a 6.2 and 7.5, with a maximum approaching $8 \times 10^7\ M^{-1}\,s^{-1}$) and inhibitable by sulfonamides such as the clinically used acetazolamide and ethoxzolamide (in the low nanomolar range—Table 1).[11] From these data it is apparent that CA VII is strikingly similar to the physiologically most relevant isozyme, CA II, which shows a comparable high k_{cat}/K_m for CO_2 hydration of $1.5 \times 10^8\ M^{-1}\,s^{-1}$, and is also very sensitive to sulfonamide CA inhibitors (CAIs)—Table 1.[1–5]

From the data in Table 1, it is obvious that CA VII is the second most active cytosolic isozyme after CA II (showing approximately 70% of the hCA II activity), and it seems to be susceptible to inhibition by the clinically used sulfonamide CAI (although only two such derivatives have been investigated with the murine chimeric isozyme).[11] CA VII has been shown to be highly expressed in the brain, by in situ hybridization.[12,13] Very recently, one of our groups[13] showed that the capability to generate stimulation-induced synchronous action potential discharges emerges abruptly in the rat hippocampus at approximately postnatal day 12. This was directly attributable to a steep developmental up-regulation of CA VII in pyramidal neurons.[13] These results pointed to a crucial role for the developmental expression of the isozyme VII activity in shaping long-term plasticity and promoting epileptogenesis.

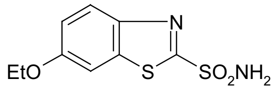
The human isozyme hCA VII has not been cloned and characterized. Here we present such a study, as well as the first investigation of sulfonamide inhibition with this



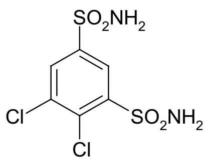
AAZ



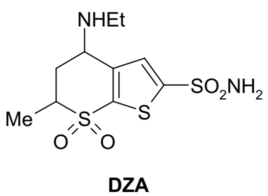
MZA



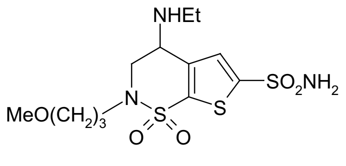
EZA



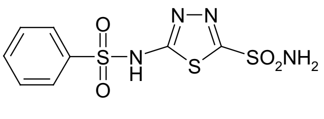
DCP



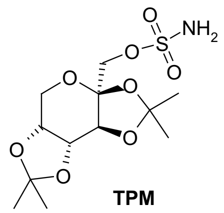
DZA



BRZ



BZA



TPM

isozyme. Sulfonamides, such as, acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA** and dichlorophenamide **DCP**, have been clinically used for more than 40 years as systemic CAIs, whereas two additional drugs dorzolamide **DZA** and the structurally-related brinzolamide **BRZ** are topically acting antiglaucoma CAIs used since 1995.[1–5] Topiramate, **TPM**, an antiepileptic with a complicated mechanism of action,[14] and benzolamide, **BZA**, an orphan drug,[15] belonging to the CAI family have also been included in this study together with a large number of other aromatic and heterocyclic sulfonamide derivatives of types **1**–**24**.

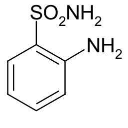
2. Chemistry

Sulfonamides investigated for the inhibition of the cytosolic isozyme hCA VII, of types **1**–**24** are shown below. Compounds **1**, **2**, **4**–**6**, **11**–**12**, **18**–**20** and **23** are commercially available, whereas **3**,[16] **7**–**10**,[17] **13**–**17**[18] and **21**,[19] **22**[19] and **24**[16] were prepared as reported earlier by this group. The eight clinically used compounds **AAZ**–**TPM** (commercially available from Sigma–Aldrich, Merck, Alcon or Johnson & Jonson) were also assayed, since no such data are available in the literature.

3. CA inhibition data

We have cloned hCA VII from a cDNA library previously described,[9] and purified large amounts of this protein for screening studies in order to detect potent inhibitors.[20] Similarly to the chimeric, murine protein purified earlier (mCA VII),[11] hCA VII shows a high catalytic activity for the CO_2 hydration reaction, with a k_{cat} of $9.5 \times 10^5\ s^{-1}$ and k_{cat}/K_m of $8.3 \times 10^7\ M^{-1}\ s^{-1}$ at pH 7.5 and 20 °C.[21] The esterase activity of this isozyme is quite low (data not shown), similarly to that of mCA VII,[11] and no detailed studies have been performed for determining the kinetic parameters of this reaction.

Inhibition data against four cytosolic CA isozymes, that is, CA I, II, VII and XIII, with a series of sulfonamides of types **1**–**24** and the clinically used inhibitors **AAZ**–**TPM**, for the CO_2 hydration reaction catalyzed by these isozymes, are shown in Table 2. Data for isozymes I, II and XIII have previously been published,[2–4,8b] and are presented here since they are useful when discussing the inhibition profile of the new isozyme, hCA VII. CA III inhibition data were not included, as this isozyme has a rather low affinity for sulfonamide inhibitors.[2–4]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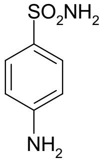
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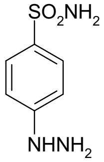
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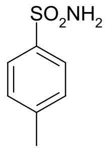
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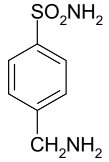
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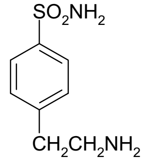
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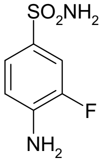
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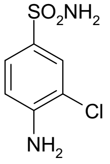
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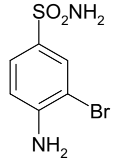
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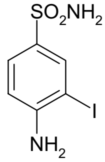
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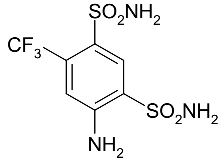
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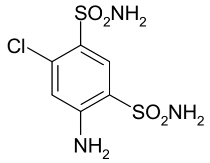
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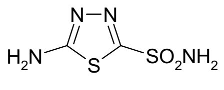
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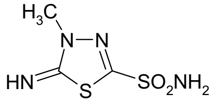
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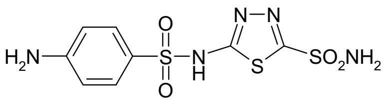
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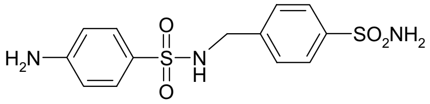
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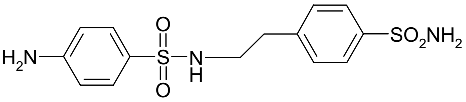
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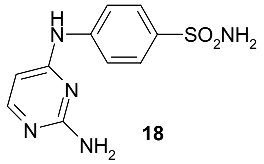

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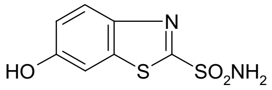

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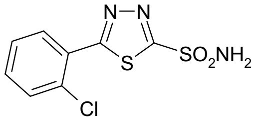

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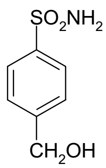

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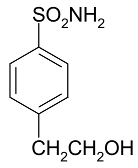

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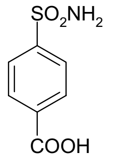

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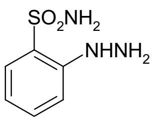

24

The following should be noted regarding hCA VII inhibition data with the set of 32 compounds investigated here: (i) hCA VII, similarly with hCA II, is a ‘sulfonamide-avid’ isozyme, being inhibited substantially (K_I-s in the range of 0.45–210 nM) by all the investigated compounds **1**–**24** and **AAZ**–**TPM** (hCA II shows inhibition constants in the range of 2–320 nM for this set of sulfonamides/sulfamates). Thus, hCA VII is the most sensitive to these classes of CAIs among all CA isozymes investigated up to now. mCA XIII is also a sulfonamide inhibitable isozyme (K_I-s in the range of 1.9–56 nM for a series of 14 sulfonamides investigated—see Table 2) but this newly purified isozyme was available in quite limited amounts, and this is the reason why only this small number of sulfonamides has been tested in a previous work from this and Parkkila’s laboratories.[8] Isozyme hCA I on the other hand is much less sensitive to sulfonamide/sulfamate CAIs, showing K_I-s in the range of 6–78,500 nM for the 32 derivatives included in the study; (ii) the weakest hCA VII inhibitors in this series of compounds were derivatives **8** and **10**–**12**, which showed K_I-s in the range of 100–210 nM. All these derivatives, which are either halogenated sulfanilamides or benzene-1,3-disulfonamide derivatives act much better as hCA II and mCA XIII than as hCA VII inhibitors, but are quite weak hCA I inhibitors; (iii) another group of derivatives, including **1**–**7**, **9** and **21**–**24**, are better hCA VII inhibitors as compared to the previously discussed compounds, showing inhibition constants in the range of 45–89 nM. SAR is relatively straightforward here as all these compounds are simple benzenesulfonamide derivatives, mainly *para*-substituted, although several such derivatives are *ortho*-substituted (**1** and **24**) or possess two substituents (e.g., the halogenated sulfanilamides **7** and **9**). It is also interesting to note that sulfonamides **1**–**6** and **22**–**24** are much better hCA VII than hCA II inhibitors, whereas in the case of **7**, **9** and **21**, the reverse is true (only **21** is a good hCA I inhibitor, all other discussed compounds act as rather weak inhibitors of this isozyme). On the other hand, those of these derivatives, which have been tested for the inhibition of mCA XIII showed a better activity towards this isozyme (Table 1); (iv) the clinically used compound dichlorophenamide **DCP** showed an even increased affinity for hCA VII, with an inhibition constant of 26.5 nM, rather similar to that determined against mCA XIII. **DCP** is on the other hand a weaker hCA II inhibitor (K_I of 38 nM) and a much weaker hCA I inhibitor (K_I of 1200 nM). Thus this rather simple aromatic disulfonamide has an

Table 2. CA I, II, VII and XIII inhibition data with sulfonamides **1**–**24** and clinically used inhibitors. Data for hCA I and II are from Ref. 2 whereas mCA XIII data are from Ref. 8b

Inhibitor	K_I* (nM)			
	hCA I[a]	hCA II[a]	hCA VII[a]	mCA XIII[b]
1	45,400	295	45	43
2	25,000	240	70	32
3	28,000	300	89	Nt
4	78,500	320	88	Nt
5	25,000	170	75	41
6	21,000	160	80	43
7	8300	60	75	50
8	9800	110	120	56
9	6500	40	61	54
10	6000	70	150	50
11	5800	63	100	Nt
12	8400	75	210	Nt
13	8600	60	5.2	Nt
14	9300	19	4.3	Nt
15	6	2	7.0	Nt
16	164	46	5.6	2.0
17	185	50	6.5	1.9
18	109	33	6.8	Nt
19	95	30	4.0	Nt
20	690	12	5.4	Nt
21	55	8	60	Nt
22	21,000	125	66	Nt
23	23,000	133	52	Nt
24	24,000	125	68	Nt
AAZ	250	12	2.5	17
MZA	50	14	2.1	19
EZA	25	8	0.78	Nt
DCP	1200	38	26.5	23
DZA	50,000	9	3.5	18
BRZ	—	3	2.8	Nt
TPM	250	5	0.87	Nt
BZA	15	9	0.45	Nt

Nt = not tested.

[a] Human recombinant isozymes.

[b] Murine recombinant isozyme, CO_2 hydrase assay method.[21]

* Errors in the range of 5–10% of the shown data, from three different assays.

hCA VII inhibition profile intermediate between that of the simple aromatic compounds discussed earlier (of types **1**–**7**, **9** and **21**–**24**) and the more complicated derivatives belonging to the heterocyclic or polycyclic class, which will be discussed shortly; (v) very potent hCA VII inhibitory properties were observed for derivatives **13**–**20**, **AAZ**, **MZA**, **EZA**, **DZA**, **BRZ**, **TPM** and **BZA**, which showed low nanomolar or sub-nanomolar K_I-s for this isozyme, in the range of 0.45–7.0 nM (Table 1). Thus, among these very active hCA VII inhibitors there are some heterocyclic derivatives, belonging to the well-known ring systems present in the clinically used CAIs, of the 1,3,4-thiadiazole-2-sulfonamide type (in **13**, **15**, **20**, **AAZ** and **BZA**), the corresponding thiadiazolines (in **14** and **MZA**), the benzothiazole-2-sulfonamide type (in **19** and **EZA**) or the thienothiopyran/thiazine type of derivatives (**DZA** and **BRZ**). Other such potent hCA VII inhibitors incorporate the sulfanilyl-sulfonamide scaffold (such as **15**–**17**) known also for a long time to induce efficient CA inhibitory activity towards many isozyme,[1,18] or belong to the sulfamate type of CAIs (i.e., topiramate **TPM**). Derivatives **13**, **14**, **16**–**20**, as well as the clinically used compounds, are much better hCA VII than hCA II/I inhibitors, whereas **15** (aminobenzolamide) is a better hCA II than hCA VII inhibitor. Compounds **16** and **17** are better mCA XIII than hCA VII inhibitors, whereas **AAZ**, **MZA** and **DZA** are just the reverse, inhibiting more potently isozyme VII than isozyme XIII.

At this point, considering the very high activity as hCA VII inhibitors of a lot of derivatives (either clinically used, such as **AAZ**, **MZA**, **EZA** or **TPM**, or useful in neurophysiological experiments, such as **BZA**) known to possess anticonvulsant and antiepileptic activity,[22,23] this work strengthens the previous conclusion of Ruusuvuori et al.[13] that hCA VII is the main target of the antiepileptic sulfonamides/sulfamates, and allows the possibility for future drug design for obtaining compounds with a different inhibition and isozyme selectivity profiles. It should be mentioned that up to now hCA II has been considered the main isozyme responsible for the CNS effects of the sulfonamide/sulfamate inhibitors.[3]

4. Conclusions

We have cloned, purified and characterized a new CA isozyme, hCA VII, which similarly to the previously reported (chimeric) murine isozyme, shows high catalytic activity for the physiological reaction (k_{cat} of 9.5×10^5 s^{-1} and k_{cat}/K_m of 8.3×10^7 $M^{-1} s^{-1}$ at pH 7.5 and 20 °C), and low catalytic activity as esterase. A total of 32 sulfonamides/sulfamates have been investigated as possible hCA VII inhibitors, and their inhibition profile was compared to that of the other cytosolic, catalytically active isozymes, hCA I, II and mCA XIII. hCA VII shows high affinity for all the investigated compounds, with inhibition constants in the range of 0.45–210 nM. Topiramate, ethoxzolamide, and benzolamide showed subnanomolar hCA VII inhibitory activity, whereas acetazolamide, methazolamide, dorzolamide and brinzolamide showed K_I-s in the range of 2.1–3.5 nM. Dichlorophenamide was slightly less active (K_I of 26.5 nM). A number of heterocyclic or bicyclic aromatic sulfonamides also showed excellent hCA VII inhibitory properties (K_I-s in the range of 4.3–7.0 nM) whereas many substituted or disubstituted benzenesulfonamides were less active (K_I-s in the range of 45–89 nM). The least active hCA VII inhibitors were some substituted benzene-1,3-disulfonamides as well as some halogenated sulfanilamides (K_I-s in the range of 100–210 nM). The inhibition profile of hCA VII is rather different of that of the other cytosolic isozymes, thus providing a possibility for the design of more selective, hCA VII-specific inhibitors. These data furnish further evidence that hCA VII is the isozyme responsible for the anticonvulsant/antiepileptic activity of sulfonamides and sulfamates.

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20. CA VII-pGEX-2TL constructs. To produce mCA VII or hCA VII, DNA fragments covering the initiation codon and termination codon were cloned by PCR from mouse brain cDNA.[9] The mouse sense primer was 5′-CCT GAA TTC ATG ACC GGC CAC CAC TGTT-3′, which has an Eco RI site at the 5′ end. The antisense primer was 5′-CCT AAG CTT TGG GGA GGT GGG AAG TTCA-3′. This primer has a Hind III site at the 5′ end. The corresponding primers for human cDNA were: 5′-ACC GAA TTC ATG ACC GGC CAC CAC GGCT-3′ and 5′-CCC AAG CTT TCA GGC CCG GAA GGA GGC-3′. PCR was performed using Sigma REDAccuTaq LA DNA polymerase mixture. The PCR mixtures were run one cycle at 98 °C for 1 min, followed by 2 cycles of 94 °C for 10 s, 55 °C for 30 s, 68 °C for 90 s, 30 cycles of 94 °C for 10 s, 65 °C for 30 s, 68 °C for 90 s, and finally 1 cycle of 68 °C for 5 min. The PCR products were cleaved with the corresponding restriction enzymes, purified and cloned into a modified pGEX-2TL vector (kindly supplied by Dr. Johan Peränen) using T4-ligase (Promega). The constructs were then transfected into *E. coli* strain BL21 for production of the CA VII protein, similarly to the procedure already described for mCA VII.[11]
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