Bioorganic & Medicinal Chemistry 20 (2012) 2208–2213

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Mutation of active site residues Asn67 to Ile, Gln92 to Val and Leu204 to Ser in human carbonic anhydrase II: Influences on the catalytic activity and affinity for inhibitors

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ARTICLE INFO

Article history:
Received 4 January 2012
Revised 6 February 2012
Accepted 8 February 2012
Available online 16 February 2012

Keywords:
Carbonic anhydrase
Site directed mutagenesis
Active site
Sulfonamide
Sulfamate
Enzyme inhibitor
Inhibition mechanism

ABSTRACT

Site-directed mutagenesis has been used to change three amino acid residues involved in the binding of inhibitors (Asn67Ile; Gln92Val and Leu204Ser) within the active site of human carbonic anhydrase (CA, EC 4.2.1.1) II (hCA II). Residues 67, 92 and 204 were changed from hydrophobic to hydrophilic ones, and vice versa. The Asn67Ile and Leu204Ser mutants showed similar k_{cat}/K_M values compared to the wild type (wt) enzyme, whereas the Gln92Val mutant was around 30% less active as a catalyst for CO_2 hydration to bicarbonate compared to the wt protein. Affinity for sulfonamides/sulfamates was decreased in all three mutants compared to wt hCA II. The effect was stronger for the Asn67Ile mutant (the closest residue to the zinc ion), followed by the Gln92Val mutant (residue situated in the middle of the active site) and weakest for the Leu204Ser mutant, an amino acid situated far away from the catalytic metal ion, at the entrance of the cavity. This study shows that small perturbations within the active site architecture have influences on the catalytic efficiency but dramatically change affinity for inhibitors among the CA enzymes, especially when the mutated amino acid residues are nearby the catalytic metal ion.

1. Introduction

Site directed mutagenesis has widely been used to unravel the catalytic and inhibition mechanisms of the mammalian carbonic anhydrase (CAs, EC 4.2.1.1).[1–9] Most such studies have been performed on the abundant red blood cell isoforms hCA I and II (h = human isoform),[1,2,4–6] and activity of the mutant enzymes has been compared to those of the wild type (wt) ones. Most such mutations were done in order to change: (i) the three residues acting as zinc ligands, His94, 96 and 119[5]; (ii) the proton shuttle residue (His64)[1–3]; (iii) the residues lining the hydrophobic pocket, where the substrate CO_2 binds[1a,4,5]; (iv) amino acid residues characteristic of one isoform were changed to those present in another one (e.g., His200 found in CA I was changed to Thr, found in CA II) etc[1–6]; and, (v) in order to change the wt enzyme to mutated enzymes typical of some genetic disorders,[7] such as the CA II deficiency,[10] in which the mutation His107Tyr leads to marble brain disease, also known as Guibaud–Vainsel syndrome, which is caused by an autosomal recessive mutation in the hCA II gene.[7,9,10]

In fact, the CAs are enzymes involved in many physiological and pathological processes, such as respiration and transport of CO_2 and bicarbonate between metabolizing tissues and lungs; pH and CO_2 homeostasis; electrolyte secretion in various tissues and organs; biosynthetic reactions (such as gluconeogenesis, lipogenesis and ureagenesis); bone resorption; calcification; and tumorigenicity.[9,10] Therefore, many of them are valuable therapeutic targets.[9] Several clinically used drugs belonging to the sulfonamide, sulfamate or sulfamide classes possess significant CA inhibitory properties.[9] As specific isoforms are responsible for different biological responses, the diverse inhibition profiles of the various isozymes explains the different actual and potential clinical applications of the CA inhibitors (CAIs), which range from diuretics and antiglaucoma agents, to anticancer, anti-obesity and anti-epileptic drugs.[9–13] However, a crucial problem in CAIs design is related to the high number of isoforms (16 are presently known in mammals), their diffuse localization in many tissues and organs, and the lack of isozyme selectivity of the presently available inhibitors of the sulfonamide/sulfamate type.[9]

The reported X-ray crystal structures of various hCA isoforms in adducts with sulfonamides, sulfamates and other classes of inhibitors,[14–16] has provided a detailed understanding of the molecular

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doi:10.1016/j.bmc.2012.02.029

interactions between inhibitor and enzyme. This led to the rational drug design of inhibitors with reduced side effects and selectivity for the target isoform.[13,14] The active site of the mammalian CAs has a very particular shape, with half of it being predominantly hydrophobic and the opposite side being hydrophilic.[9,14–16] Residues in position 121, 131, 141, 143, 198 and 209 confine the hydrophobic region, while those in position 62, 64, 67, 92 and 200 identify the hydrophilic one.[1–9,14–16] The CA isoforms with therapeutic/medicinal chemistry applications have several non-conserved, variable such residues (both in the hydrophobic and hydrophilic halves of the active site), which may be used for drug design purposes in order to increase affinity for the target isoform and reduce affinity for the offtarget one(s).[9–16] Thus, changing the polarity of one or more such residues to the opposite type (i.e., hydrophobic residue changed to hydrophilic, and vice versa), may lead to a better understanding of the processes governing the design of isoform-selective CAIs. Indeed, many of the therapeutically relevant isoforms, such as CA II, VII, IX, XII; etc., have such 'substitutions' of hydrophilic versus hydrophobic (or vice versa) amino acids within the active site, which were shown to be involve in the binding of inhibitors.[9–16]

The aim of the present study was to redesign the active site of hCA II by mutations that modulate the shape of the hydrophobic/hydrophilic pockets and to investigate how these modifications influence the catalytic efficiency and the binding of sulfonamide/sulfamate inhibitors. Here, we report the catalytic properties of three hCA II mutants (Asn67Ile, Gln92Val and Leu204Ser) and the inhibition of these enzymes with a series of sulfonamides/sulfamates, some of which are clinically used drugs.

2. Results and discussion

Hydrophilic residues 67 and 92 within the hCA II active site are known to be involved in the binding of many inhibitors of the sulfonamide/coumarin type, such as ethoxzolamide **EZA**, topiramate **TPM** and its sulfamide analog, zonisamide **ZNS**, sulpiride, valdecoxibe, celecoxib **CLX**, etc., only to cite a few of the clinically used CAIs.[17–22] We have thus replaced them by two hydrophobic residues, producing the mutant enzymes Asn67Ile, Gln92Val. The hydrophobic residue Leu204 in hCA II is also involved in the binding of inhibitors with longer tails, such as benzolamide derivatives, ureido-substituted benzenesulfonamides as well as benzenesulfonamides with long polyfluorinated alkyl chains,[19–21] being situated in the proximity of Leu198 and Pro202 (which define the hydrophobic region on one side, and the hydrophilic one on the opposite side, respectively).[14] Thus, we changed the nature of this residue by site directed mutagenesis to a hydrophilic, more compact one, producing the Leu204Ser mutant hCA II. We tested in this way the hypothesis whether reversing the nature of residues at positions 67, 92 and 204 (from hydrophilic to hydrophobic for the first two, and vice versa for the last residue) would make the mutants significantly different regarding their catalytic efficiency and binding of sulfonamide/sulfamate inhibitors, compared to the wild type hCA II. The position of these amino acid residues in the polypeptide chain of hCA II is shown schematically in Figure 1, where other residues important for catalysis, such as the zinc binding ones (His 94, 96 and 119) and the proton shuttle (His64), are also indicated.

Data of Table 1 shows that similar to hCA II, the three mutant enzymes investigated here are also highly effective as catalysts for the hydration of CO_2 to bicarbonate, with k_{cat} values in the range of 1.0–1.5 $\times$ 10^6 s^{-1} and K_M values in the range of 9.0–9.3 mM. Considering the error range of the stopped-flow method used for the assay,[23] which is in the range of 5–10% of the reported values, it may be considered that the wt and mutant hCA II investigated here have the same K_M for CO_2 as substrate. However, the Gln92Val mutant showed a k_{cat}/K_M value of around 30% lower compared to that of the wt enzyme, which is a significantly lower value for such an active isoform as hCA II (and considering the error range mentioned above). Thus, the substitution of the hydrophilic Gln by a hydrophobic Val residue diminished the efficacy of the mutant by around 28% (Table 1), this effect being due to the lower k_{cat} of the mutant over the wt enzyme. This is probably due to the fact that the hydrogen bond network involved in the transfer of the proton from the zinc-coordinated water to the environment is perturbed in the mutant enzyme, as shown by Silverman's and McKenna's groups for a rather large number of hCA II mutants.[1–3,6] The remaining mutants (Asn67Ile and Leu204Ser) had slightly higher k_{cat}/K_M values compared to the wt enzyme, by a factor of 103% for the first one and of 107% for the second one. However, these variations are

```
Wild type  1  MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRIL 60
Asn67/Ile  1  MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRIL 60
Gln92/Val  1  MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRIL 60
Leu204/Ser 1  MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRIL 60
hCAI       1 MASPDWGYDDKNGPEQWSKLYPIANGNNQSPVDIKTSETKHDTSLKPISVSYNPATAKEII 61
                    *                          * *                       *

Wild type  61 NNGHAFNVEFDDSQDKAVLKGGPLDGTYRLIQFHFHWGSLDGQGSEHTVDKKKYAAELHL 120
Asn67/Ile  61 NNGHAFIVEFDDSQDKAVLKGGPLDGTYRLIQFHFHWGSLDGQGSEHTVDKKKYAAELHL 120
Gln92/Val  61 NNGHAFNVEFDDSQDKAVLKGGPLDGTYRLIVFHFHWGSLDGQGSEHTVDKKKYAAELHL 120
Leu204/Ser 61 NNGHAFNVEFDDSQDKAVLKGGPLDGTYRLIQFHFHWGSLDGQGSEHTVDKKKYAAELHL 120
hCAI       62 NVGHSFHVNFEDNDNRSVLKGGPFSDSYRLFQFHFHWGSTNEHGSEHTVDGVKYSAELHV 121

Wild type  121 VHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAKPGLQKVVDVLDSIKTKGKSADFTNFDP 180
Asn67/Ile  121 VHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAKPGLQKVVDVLDSIKTKGKSADFTNFDP 180
Gln92/Val  121 VHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAKPGLQKVVDVLDSIKTKGKSADFTNFDP 180
Leu204/Ser 121 VHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAKPGLQKVVDVLDSIKTKGKSADFTNFDP 180
hCAI       122 AHWNSAKYSSLAEAASKADGLAVIGVLMKVGEANPKLQKVLDALQAIKTKGKRAPFTNFD 181

Wild type  181 RGLLPESLDYWTYPGSLTTPPLLECVTWIVLKEPISVSSEQVLKFRKLNFNGEGEPEELM 240
Asn67/Ile  181 RGLLPESLDYWTYPGSLTTPPLLECVTWIVLKEPISVSSEQVLKFRKLNFNGEGEPEELM 240
Gln92Val   181 RGLLPESLDYWTYPGSLTTPPLLECVTWIVLKEPISVSSEQVLKFRKLNFNGEGEPEELM 240
Leu204/Ser 181 RGLLPESLDYWTYPGSLTTPPLSECVTWIVLKEPISVSSEQVLKFRKLNFNGEGEPEELM 240
hCAI       182 PSTLLPSSLDFWTYPGSLTHPPLYESVTWIICKESISVSSEQLAQFRSLLSNVEGDNAVP 241

Wild type  241 VDNWRPAQPLKNRQIKASFK*  260
Asn67/Ile  241 VDNWRPAQPLKNRQIKASFK*  260
Gln92Val   241 VDNWRPAQPLKNRQIKASFK*  260
Leu204/Ser 241 VDNWRPAQPLKNRQIKASFK*  260
hCAI       242 MQHNNRPTQPLKGRTVRASF*  261
```

Figure 1. Amino acid sequence of hCAI, hCA II (wild type) and of the three mutants investigated here. The mutated residues are shown in red for the Asn67Ile mutant, in sky blue for the Gln92Val mutant and in green for the Leu204Ser mutant. The proton shuttle (His64, deep blue) and the three Zn(II) ligands, His 94, 96 and 119, in magenta, are indicated by an asterisk above the corresponding letter in the sequence.

Table 1
Kinetic properties of wild type (wt) hCA I and II, as well as the mutant hCA II enzymes Asn67Ile, Gln92Val, Leu204Ser for the CO2 hydration reaction by a stopped-flow assay, at 20 °C and pH 7.5[23]

Isoenzyme[a]	Protein details	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})
hCA I	wt	$(2.0 \pm 0.1) \times 10^5$	4.0 ± 0.2	$(5.0 \pm 0.3) \times 10^7$
hCA II	wt	$(1.4 \pm 0.2) \times 10^6$	9.3 ± 0.1	$(1.50 \pm 0.2) \times 10^8$
hCA II	Asn67Ile	$(1.4 \pm 0.3) \times 10^6$	9.0 ± 0.1	$(1.55 \pm 0.2) \times 10^8$
hCA II	Gln92Val	$(1.0 \pm 0.1) \times 10^6$	9.2 ± 0.2	$(1.08 \pm 0.2) \times 10^8$
hCA II	Leu204Ser	$(1.5 \pm 0.2) \times 10^6$	9.3 ± 0.1	$(1.61 \pm 0.2) \times 10^8$

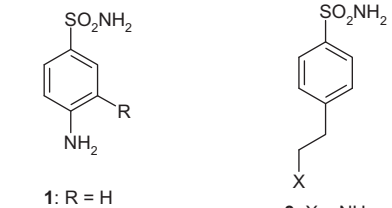
[a] All data represent the mean ± standard error, from 3 different measurements.

within the error range of the assay method, and one can consider that these mutations did not perturb significantly the catalytic efficacy of these enzymes for the CO_2 hydration reaction.

A small library of 11 sulfonamides and 1 sulfamate, compounds **1–12**,[9] have been investigated as inhibitors of the wt and three mutant hCA II enzymes (Table 2), in order to assess how the changes of the amino acid residues in positions 67, 92 and 204 influence the inhibitory properties. Among the investigated compounds, derivatives **1–6** are simple aromatic/heterocyclic derivatives widely employed in drug design studies of CAIs,[23,24] whereas compounds **7–12** are clinically used drugs as antiglaucoma (acetazolamide **7** and ethoxzolamide **8**),[9] antiepileptic (zonisamide **9** and topiramate **12**) or analgesic agents (celecoxib **10**). Saccharin **11**, the only secondary sulfonamide among the investigated compounds, is a sweetener for which the interesting CA inhibitory properties were only recently reported.[25]

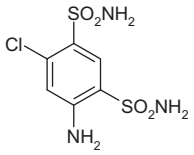
Data of Table 2 shows that all compounds **1–12** act as CAIs against the mutant hCA II enzymes investigated here, but with less efficacy as compared to the wt enzyme. Indeed, except saccharin **11** which is a weak hCA II inhibitor, sulfonamides **1–10** and sulfamate **12** are effective wt hCA II inhibitors with inhibition constants in the range of 8–240 nM, depending on the scaffold present in their molecules. However, with the exception of saccharin against the Asn67Ile and Leu204Ser mutants, these compounds were less effective CAIs of the mutant enzymes compared to the wt one. For example, against the Asn67Ile mutant, the inhibition constants of derivatives **1–12** ranged between 54.1–5180 nM, against the

Table 2
Inhibition of hCA II (wt) and its mutants with sulfonamides and sulfamates **1–12**, by a stopped-flow CO_2 hydrase assay at 20 °C[23]

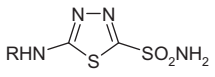


1: R = H
2: R = F

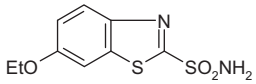
3: X = NH_2
4: X = OH



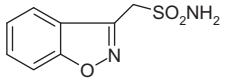
5



6: R = H
7: R = Ac



8



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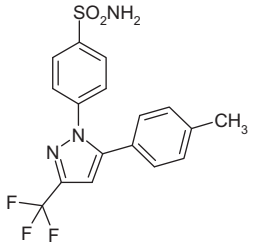
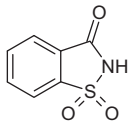
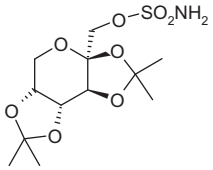


10



11



12

Compound	K_I (nM)[a]			
	hCA II	Asn67Ile	Gln92Val	Leu204Ser
1	240 ± 12	363 ± 14	296 ± 21	251 ± 24
2	60 ± 4	392 ± 21	340 ± 29	121 ± 11
3	160 ± 9	312 ± 25	201 ± 18	234 ± 19
4	125 ± 10	424 ± 30	247 ± 22	193 ± 15
5	75 ± 5	330 ± 23	172 ± 13	98 ± 9.1
6	60 ± 3	409 ± 28	91 ± 8.0	80 ± 6.3
7 (AZA)	12 ± 1	54.1 ± 2.1	29.7 ± 3.0	26.1 ± 2.2
8 (EZA)	8 ± 0.7	178 ± 14	69.7 ± 5.4	25.1 ± 1.8
9 (ZNS)	35 ± 2	303 ± 16	68.1 ± 6.4	57.3 ± 4.2
10 (CLX)	21 ± 1	421 ± 19	76.8 ± 7.1	369 ± 18
11 (SAC)	5959 ± 45	5180 ± 37	7470 ± 65	4780 ± 39
12 (TPM)	10 ± 0.5	243 ± 11	47.8 ± 4.2	30.7 ± 2.4

[a] All data represent the mean ± standard error, from three different measurements.

Gln92Val mutant between 29.7–7470 nM and against the Leu204-Ser mutant between 25.1–4780 nM (Table 2). It may be observed that these compounds showed the highest decrease of inhibitory power (compared to the wt enzyme) against the Asn67Ile mutant, followed by the Gln92Ile one whereas the smallest loss of inhibition was observed against the Leu204Ser mutant enzyme (except for compounds **3** and **10**). Strikingly, the loss of inhibitory power is directly correlated with the distance between the mutated amino acid residue with respect to the catalytically critical metal ion. Indeed, Asn67 is the residue which is closest to the Zn(II), followed by Gln92 which is in the middle of the active site cavity, whereas Leu204 is situated towards the entrance of the active site, being the most distant one to zinc among the three mutated amino acid residues investigated here. The explanation of the effect may be due to the fact that the deeper one amino acid is within the active site, more sterical hindrance will encounter (as the active site is conically shaped, restricting gradually towards its bottom)[14,15] and any structural change there will produce significant perturbation in the van der Waals contacts and hydrogen bonds/polar interactions in which that residue is involved. On the other hand, if the changed residue is situated towards the more opened part of the active site (e.g., its entrance) where the sterical hindrance is less important, probably even a change of polarity, from hydrophobic to hydrophilic (as the Leu204Ser mutation investigated here) will have less consequences for the binding of inhibitors, since the available free space will allow for a reorganization of the amino acid side chains in such a way as to accommodate the substitution of one amino acid and the bound inhibitor without significant loss of binding energy. This is the situation which we observed here for all inhibitors except saccharin **11**. For example, acetazolamide **7** showed a 4.50 times loss of inhibitory activity against the Asn67Ile mutant (compared always to wt hCA II), a 2.47 times loss for the Gln92Val mutant and only a 2.17 times loss for the Leu204Ser mutant. For topiramate **12**, these values of the loss of inhibitory power were of 24.3 times for the Asn67Ile mutant, of 4.78 times for the Gln92Val mutant and of 3.07 times for the Leu204Ser mutant, respectively (Table 2). The same situation has been observed also for the less efficient inhibitors of type **1**–**6**, although the differences are not always as high as for topiramate and acetazolamide discussed above. Saccharin **11** is a special case as this is the only compound investigated here which showed an irregular trend in the inhibition of the wt and mutant hCA II enzymes obtained in the present study. Indeed, only the Gln92Val mutant showed a weaker inhibition with saccharin compared to the wt enzyme, whereas the other two mutants had a slightly enhanced affinity for this inhibitor. This may be due to the fact that saccharin binds in a very particular manner to hCA II, as shown by high resolution X-ray crystallography.[25] This is the only example of secondary sulfonamide coordinated to the Zn(II) ion by means of the deprotonated $CON^{-}SO_2$ moiety.[26] In addition, one oxygen atom of the SO_2 moiety makes a hydrogen bond with the OH of Thr199, a residue conserved in all mammalian CAs, but no other significant contacts of the aromatic scaffold of saccharin with the hCA II active site were observed in the X-ray crystal structure.[25] This inhibitor binds very deep within the active site, nearby the Zn(II) ion and does not interact with the residues situated in the middle of the active site (Asn67, Gln92) or at its entrance (Leu204). This may explain the inhibition pattern observed for saccharin against two of the mutant hCA II enzymes, although it does not explain why for the Gln92Val mutant this compound showed a loss of inhibitory activity.

3. Conclusions

Three amino acid residues involved in the binding of inhibitors (Asn67, Gln92 and Leu204) within the active site of hCA II, a highly abundant and physiologically significant isoform, were mutated to residues of opposite polarity. The Asn67Ile and Leu204Ser mutants showed similar k_{cat}/K_M values compared to the wild type (wt) enzyme, whereas the Gln92Val mutant was about 30% less active as a catalyst for CO_2 hydration to bicarbonate compared to the wt protein. The affinity for the main class of inhibitors, the sulfonamides/sulfamates was also investigated for the mutant enzymes. Benzenesulfonamides, benzene-1,3-disulfonamides, acetazolamide, ethoxzolamide, zonisamide, celecoxib, saccharin and topiramate generally showed decreased affinity for the three mutants compared to wt hCA II. The effect was stronger for the Asn67Ile mutant (the closest residue to the zinc ion), followed by the Gln92Val mutant (situated in the middle of the active site) and was weaker for the Leu204Ser mutant, an amino acid situated far away from the catalytic metal ion, at the entrance of the cavity. This study thus showed that small perturbations in the active site architecture have influences on the catalytic efficiency but may dramatically change the affinity for inhibitors among the CA enzymes, especially when the mutated amino acid residues are nearby the catalytic metal ion.

4. Experimental

4.1. Chemicals

Compounds **1–12** are either commercially available (from Sigma–Aldrich, Milan, Italy) or were reported earlier by this group.[23–25]

4.2. Site directed mutagenesis, cloning and purification of the mutant hCA II enzymes

Site-directed Mutagenesis was performed on the double-stranded form of the expression plasmid pET31, which was a gift from Prof. David N. Silverman (University of Florida, Gainesville, USA) using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA) according to the instructions. PCR based site-directed mutagenesis was carried out using the mutant oligonucleotides Asn67/Ile: Forward 5′-C CTC AAC AAT GGT CAT GCT TTC **ATC** GTG GAG TTT-3′, Reverse 5′-AAA CTC CAC **GAT** GAA AGC ATG ACC ATT GTT GAG G-3′ Gln92/Val: Forward 5′-G GAT GGC ACT TAC AGA TTG ATT **GTC** TTT CAC TTT CA-3′, Reverse 5′-TG AAA GTG AAA **GAC** AAT CAA TCT GTA AGT GCC ATC C-3′ and Leu204/Ser: Forward 5′-C CCT CCT CTT **TCC** GAA TGT GTG ACC T-3′, Reverse 5′-A GGT CAC ACA TTC **GGA** AAG AGG AGG G-3′ obtained from the Genomic Company MWG-BIOTECH AG. Mutant plasmids were transformed into *Escherichia coli* XL1-Blue (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacIqZΔM15 Tn10 (Tetr)]) cells and the sequences of the entire coding region were verified by automated sequence analysis for the corresponding mutations or the presence of any misincorporations during the PCR reactions. The sequencing was performed by the LARK Company, UK. The expression host *E. coli* strain BL21 (DE3) [B F–dcm ompT hsdS(rB–mB–) gal λ(DE3] was transformed with plasmids representing each single mutant and wild-type hCA II. Inoculates with a single colony from a fresh plate of BL21(DE3)/pET31hCA II and mutant hCA II were prepared in a sterile 50 mL falcon tube, prepared in 10 mL of Luria Broth supplemented with 10 μL 100 mg/mL ampicillin solution. Cultures were grown at 37 °C with moderate agitation (120 rpm). Overnight cultures (5 mL) were inoculated in a sterile 500 mL flask with 200 mL of Luria Broth supplemented with 200 μL of 12.5 mg/ml ampiciline solution. When the cell cultures reached an optical density of 0.6–0.8 (at 550 nm), expression of the wild-type or mutant hCA II was induced with 400 μL 0.1 M IPTG (isopropylthiogalactoside) and 250 μL 5 mM $ZnCl_2$ and incubation was continued for 4–5 h at 30 °C. Cells were harvested by

centrifugation at 3000 rpm and frozen at −20 °C prior to purification of the recombinant native or mutant hCA II enzymes. To purify the protein, *E. coli* cells were collected by centrifugation at 3000 rpm for 10 min at 4 °C. The pellet was washed with buffer (50 mM Tris–HCl, pH 7.6) and resuspended in the lysis buffer (20 mM Tris/0.5 mM EDTA/ 0.5 mM EGTA/pH 8.7). Hundred microliter of 100 mM PMSF (1 mM final concentration) and 250 μl of a 10 mg/ml solution of lysozyme were added and the pellet was thawed at room temperature. After 30 min, 1 mL of the 3.0% protamine sulfate solution was added to the cell lysate and centrifuged. Before purification of proteins, cell lysates were dialyzed to affinity equilibration buffer for 3 h at 4 °C. Every 30 min, the affinity equilibration buffer was changed. Wild type and mutant carbonic anhydrases were purified from *E. coli* lysate by affinity chromatography as reported earlier.[27]

4.3. CA inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity.[23] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–I complex or for the eventual active site mediated hydrolysis of the inhibitor. The kinetic parameters were obtained using Lineweaver–Burke curves whereas the inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng–Prusoff equation,[28] and represent the mean from at least three different determinations.

Acknowledgments

We are grateful to Professor David N. Silverman (Univ. of Florida, Gainesville, USA) for the gift of the wt hCA II plasmid. This research was financed in part by a 7th FP EU project (Metoxia) and by the Balikesir University Research Projects under Grant no 2006/35.

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