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Mutation of Phe91 to Asn in human carbonic anhydrase I unexpectedly enhanced both catalytic activity and affinity for sulfonamide inhibitors

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ABSTRACT

Site-directed mutagenesis has been used to change one amino acid residue considered non essential (Phe91Asn) to catalysis in carbonic anhydrase (CA, EC 4.2.1.1) isozyme I (hCA I), but which is near the substrate binding pocket of the enzyme. This change led to a steady increase of 16% of the catalytic activity of the mutant hCA I over the wild type enzyme, which is a gain of 50% catalytic efficiency if one compares hCA I and hCA II as catalysts for CO_2 hydration. This effect may be due to the bigger hydrophobic pocket in the mutant enzyme compared to the wild type one, which probably leads to the reorganization of the solvent molecules present in the cavity and to a diverse proton transfer pathway in the mutant over the non mutated enzyme. To our surprise, the mutant CA I was not only a better catalyst for the physiologic reaction, but in many cases also showed higher affinity (2.6–15.9 times) for sulfonamide/sulfamate inhibitors compared to the wild type enzyme. As the residue in position 91 is highly variable among the 13 catalytically active CA isoforms, this study may shed a better understanding of catalysis/inhibition by this superfamily of enzymes.

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1. Introduction

Evolution of the catalytic efficiency of enzymes constitutes a topic of great interest[1–5] as it allows a deeper understanding of enzyme mechanisms, of inhibition/activation processes, which in turn may have important consequences for the design of more efficient such catalysts (i.e., modified enzymes),[5–7] or modulators of enzyme activity (inhibitors, activators) with biotechnological or biomedical applications.[1–10] There are many literature examples of enzymes which have been engineered by evolutionary optimization in order to obtain either a different catalytic activity (i.e., an action on substrates which are diverse of the natural ones)[8,11,12] or an activity which is enhanced (but most of the times it may be decreased) compared to the wild type (wt) enzyme.[7,9,10] Such examples include among others glutathione S-transferase,[3,8,9] triosephosphate isomerase,[4,6] Cu–Zn-superoxide dismutase,[6] transketolase,[7] monolignol 4-*O*-methyltransferase,[11] Kemp eliminase,[12] and the carbonic anhydrases (CAs, EC 4.2.1.1).[13–15] Indeed, CAs, of which 16 isoforms are presently known in mammals,[13] are among the fastest enzymes characterized so far, catalyzing with a high efficiency a very simple reaction, the interconversion of CO_2 to bicarbonate and a proton.[13–15]

CAs are metalloenzymes and the α-CA family, to which the mammalian enzymes belong, has a Zn(II) ion as metal cofactor.[13–16] The α-CA active site is located in a large, cone-shaped cavity which reaches the center of the enzyme. The catalytic Zn(II) ion is located at the bottom of this cavity, being coordinated by three His residues (His94, 96, and 119) and a water molecule/hydroxide ion.[13–16] The Zn^{2+}-bound solvent molecule is also engaged in hydrogen bond interactions with another water molecule (called the deep water) and with the hydroxyl moiety of a Thr residue (Thr199), which in turn is bridged to the carboxylate moiety of a Glu residue (Glu106). These interactions enhance the nucleophilicity of the Zn^{2+}-bound water molecule, and orient the CO_2 substrate in a location favorable for the nucleophilic attack.[13–16] In most CA isoforms, the active site cavity generally consists of two distinct parts, lined predominantly by hydrophobic and hydrophilic amino acid residues, respectively. 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.[13–16] All these amino acid residues involved in catalysis are highly conserved among the different isoforms.[13–16] It has been recently shown by Silverman's group,[15a] by means of X-ray crystallography,

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that the substrate CO_2 binds in a hydrophobic pocket defined by the amino acid residues Val121, Val143, Leu198, Val207, and Trp209 (CA I numbering system), in the CA II–CO_2 adduct. Before this seminal study[15a] has been published, much insight regarding the CA catalytic mechanism has been obtained by mutating amino acid residues within the CA active site.[14,15,17–24] Most of these mutations have been done on the ubiquitous, physiologically dominant isoform CA II and to a lesser extent on the slower isoform CA I[25,26] (which, similarly to CA II is present in high amounts in the blood and gastrointestinal tract).[27] The difference of catalytic activity (for the physiologic reaction, i.e., CO_2 hydration to bicarbonate and H^+) between these two isoforms is rather impressive, with CA I showing around only 33% of the catalytic activity (as TON) of CA II (Table 1), although the two enzymes possess a high sequence homology, and all the catalytically important amino acid residues within the active site being conserved between them.[13–26] There are also important differences among CA I and II, such as for example the diverse number of His residues placed towards the middle and exit of the active site cavity.[28] Indeed, a His residue (His64) is crucial for the CA catalytic cycle,[23] as it acts as a proton shuttle between the Zn(II) bound water molecule and the reaction environment.[13–26] This is the rate-limiting step for the entire catalytic cycle and leads to the formation of the zinc-hydroxide species of the enzyme, which is the nucleophile acting on CO_2 during the catalysis mediated by CAs.[13–26] CA I has however a unique feature; a His residue, deeper within the active site, not far away from Thr199 (and whence the zinc-hydroxide nucleophile) His200, whereas CA II and most other isoforms have a Thr in this position.[16,17,28] Thus, the volume of the CA I active site is considerably smaller compared to that of CA II due to the presence of the bulkier His 200 (instead of Thr200) in this isoform.[16]

As mentioned above, there were many mutagenesis studies reported on CA I and II,[14–26] and the CO_2 hydrase (and sometimes also esterase, with 4-nitrophenol esters as substrates) activity of the mutant enzymes have been compared to those of the wt ones. Most of the time these mutations were done in order to change: (i) the zinc ligands;[20,21] (ii) the proton shuttle residue (His64);[15,17,22,23] (iii) the residues lining the hydrophobic pocket, where CO_2 binds;[15,18,20,21] (iv) when amino acid residues characteristic of one isozyme were changed to those present in another one (e.g., His200 is found in CA I whereas in CA II it is a Thr.[13–26] These two residues have been changed in both isoforms in order to have the residue present in the cognate isoform),[25,26] and (v) in order to change the wt enzyme to mutated enzymes typical of some genetic disorders,[24] such as the CA II deficiency,[29] in which mutation of His107 to Tyr leads to marble brain disease, also known as Guibaud–Vainsel syndrome, which is caused by an autosomal recessive mutation in the hCA II gene.[24,29–31]

Up to now, as far as we know, there were no reports of amino acid residues mutations in CA I which are not directly involved in the catalytic cycle. Thus, we chose a residue which is found near the CO_2 binding pocket, more precisely Phe91, which is not part of the hydrophobic, substrate binding pocket of this enzyme, but is placed in the neighborhood of this pocket. As far as we know, there are no literature data on such a mutant CA isoform possessing a different residue in this position. We report here the construction of the Phe91Asn CA I mutant, its characterization from the kinetic point of view for the CO_2 hydration reaction, as well as inhibition studies with a panel of sulfonamides and one sulfamate, some of which are clinically used drugs.[13] Our main interest was to analyze whether such a 'neutral' mutation, that is, changing of an amino acid residue which is not normally thought to be essential for the catalytic cycle of this enzyme, may lead to a more effective, equally effective or worse catalyst (compared to wt CA I), and how this new protein behaves towards the main class of CA inhibitors (sulfonamides and their bioisosteres).

Table 1
Kinetic properties of wild type (wt) hCA I and II, as well as the mutant hCA I with Asn instead of Phe91

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 I	Phe91Asn	$(2.3 \pm 0.05) \times 10^5$	3.1 ± 0.06	$(7.4 \pm 0.1) \times 10^7$
hCA II	wt	$(1.4 \pm 0.2 \times 10^6$	9.3 ± 0.1	$(1.5 \pm 0.2) \times 10^8$

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

2. Results and discussion

2.1. Cloning and CO_2 hydrase activity of Phe91Asn hCA I

α-CAs are present all over the phylogenetic tree, from *Bacteria* to mammals, and they play a host of physiologic roles in these organisms. For example, these enzymes are involved in crucial physiological processes connected with respiration and transport of CO_2/bicarbonate, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis, and ureagenesis), bone resorption, calcification, tumorigenicity, and many other physiologic or pathologic processes (thoroughly studied in vertebrates), whereas in algae, plants, and some bacteria they play an important role in photosynthesis and other biosynthetic reactions.[13–17] The exact physiological function of CA I, a highly abundant isoform in many tissues, but mostly in the gastrointestinal tract and in the red blood cells, is very much unknown at this moment.[13] This is rather surprising, considering the fact that this enzyme, CA I, together with isoform CA II, which is the physiologically dominant one, show a high degree of sequence homology. Indeed, the metal ion ligands (His94, 96, and 119) as well as other residues involved in catalysis (His64, Glu106, and Thr199 among others), those involved in the binding of substrates and inhibitors, are very much conserved in the two isoforms, as shown in Figure 1. Furthermore, the 3D fold of the two proteins is also very much similar.[16]

As seen from Figure 2, the amino acid residue in position 91 (Phe, evidenced in red in the figure),[32] is found at the center of the enzyme molecule, not directly lining the hydrophobic binding pocket, but very near to it, that is, in contact with Ala121. Indeed, Ala121, Val131, Leu198, and Trp209, form the substrate binding pocket, as shown recently in an X-ray crystal work in which CO_2 (under high pressure) was cocrystallized with hCA II.[15a] This binding site in nearby the zinc ion and its three His residues, which are critical for catalysis. All these residues, together with the proton shuttle residue (His64) are usually conserved in most CA isozymes. However, amino acids nearby the zinc ligands or the substrate binding pocket may vary a lot in various isoforms, which explain the very different catalytic activity of the CAs.[13–18] Indeed, it may be observed that the amino acid in position 91 is Phe in hCA I and Ile in hCA II (Fig. 1). As this amino acid is nearby the CO_2 binding pocket (being in contact with Ala121, Fig. 2), the different nature and dimensions of its side chain may affect the shape and depth of the substrate binding pocket. Another reason why we chose the amino acid in position 91 for the mutagenesis study is that this is one of the most variable ones within the active site of the mammalia α-CAs. Thus, position 91 is Phe in CA I, Ile in CA II, Arg in CA III and XIII, Lys in CA IV, VA, VB and VII, Gln in CA VI, Leu in CA IX and XV, Thr in CA XII, and Ala in CA XIV.[32] It may be observed that 7 different amino acids are present in this position among the 13 CA isoforms found in mammals, and Asn is not among them. Thus, we have engineered by site-directed mutagenesis a mutant hCA I in which Phe91 has been replaced by the less bulky and more polar Asn. Indeed, Asn has a much more polar side

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Wild hCAI    MASPDWGYDDKNGPEQWSKLYPIANGNNQSPVDIKTSETKHDTSLKPISVSYNPATAKEI
Mutant hCAI  MASPDWGYDDKNGPEQWSKLYPIANGNNQSPVDIKTSETKHDTSLKPISVSYNPATAKEI
Wild hCAII   MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRIL

                                                  91  x x          106            x
Wild hCAI    INVGHSFHVNFEDNDNRSVLKGGPFSDSYRLFQFHFHWGSTNEHGSEHTVDGVKYSAELH
Mutant hCAI  INVGHSFHVNFEDNDNRSVLKGGPFSDSYRLNQFHFHWGSTNEHGSEHTVDGVKYSAELH
Wild hCAII    NNGHAFNVEFDDSQDKAVLKGGPLDGTYRLIQFHFHWGSLDGQGSEHTVDKKKYAAELH
            120
Wild hCAI    VAHWNSAKYSSLAEAASKADGLAVIGVLMKVGEANPKLQKVLDALQAIKTKGKRAPFTNF
Mutant hCAI  VAHWNSAKYSSLAEAASKADGLAVIGVLMKVGEANPKLQKVLDALQAIKTKGKRAPFTNF
Wild hCAII   LVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAKPGLQKVVDVLDSIKTKGKSADFTNFD
                                199
Wild hCAI    DPSTLLPSSLDFWTYPGSLTHPPLYESVTWIICKESISVSSEQLAQFRSLLSNVEGDNAV
Mutant hCAI  DPSTLLPSSLDFWTYPGSLTHPPLYESVTWIICKESISVSSEQLAQFRSLLSNVEGDNAV
Wild hCAII   PRGLLPESLDYWTYPGSLTTPPLLECVTWIVLKEPISVSSEQVLKFRKLNFNGEGEPEEL

Wild hCAI    PMQHNNRPTQPLKGRTVRASF
Mutant hCAI  PMQHNNRPTQPLKGRTVRASF
Wild hCAII   MVDNWRPAQPLKNRQIKASFKF
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Figure 1. Amino acid sequence of wt hCA I, mutant hCA I investigated here and wt hCA II. The Zn(II) histidine ligands (His94, His96, and His119), conserved in all mammalian CAs are evidenced by an asterisk above the amino acid, as are also the gate-keeping residues (Thr199 and Glu106) which play an important role in catalysis. The mutated amino acid, in position 91, is evidenced in red.

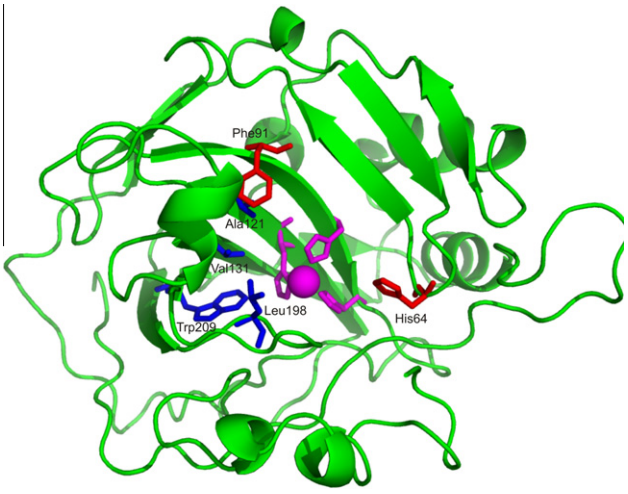


Figure 2. The hCA I protein, in which the amino acid residue in position 91 (in red) has been evidenced, together with the zinc ion and its three His residues (magenta), the proton shuttle residue (His64, in red) as well as the CO_2 binding pocket (Ala121, Val131, Leu198, and Trp209, in blue) The file 2cab[32] has been used for generating the figure. The mutated amino acid, Phe91 is near the substrate binding pocket being probably also involved in the binding of inhibitors.[40] The protein backbone is represented as the green ribbon.

chain compared to Phe, and also a less bulky one. The most similar isozyme to our mutant among the 13 catalytically active ones is CA VI, which has Gln91.[32] The catalytic activity of the mutant hCA I (Phe91Asn) compared to those of the wt hCA I and II, are shown in Table 1.

Data of Table 1 show that both k_{cat} and K_M are different for the mutant enzyme, compared to wt hCA I. Thus, k_{cat} is increased by around 10% for the mutant over the wt enzyme, whereas K_M is decreased from 4.0 mM for the wt hCA I to 3.1 for the mutant one. The overall effect on the k_{cat}/K_M is that the mutant enzyme has around 49.3% activity of that of hCA II, whereas the wt hCA I only 33.3%, as mentioned earlier. Thus, a single mutation of an amino acid not considered essential to catalysis (Phe91) led to a steady increase of 16% of the catalytic activity of hCA I, which is a gain of 50% catalytic efficiency if one compares hCA I and hCA II as catalysts for CO_2 hydration (with the activity of hCA II taken as 100%, of course). We speculate that this effect may be due to the bigger and slightly more polar hydrophobic pocket in the mutant enzyme compared to wt hCA I, which probably leads to the reorganization of the solvent molecules present in the cavity and also to a diverse proton transfer pathway in the mutant enzyme over the wt one. To our surprise, the mutant CA I was a better catalyst for the physiologic reaction, compared to the wt enzyme. These findings thus show that a single, neutral mutation may lead to a better catalyst for CO_2 hydration, even in this family of enzyme with 16 different isoforms, for which all types of catalytic activities were evidenced so far (from highly ineffective isozymes, such as CA III, to medium activity ones (CA I, VA) to highly effective ones, such as CA II and IX, among others).[13,26,30,31] As for the much more complex evolution in biological systems,[33] a single neutral mutation in a protein backbone may be critical for selecting certain amino acid residue in a position of the polypeptide chain, and probably, the accumulation of various such mutations over the time tend to optimize the scaffold for the catalyzed reaction and lead to excellent catalysts such as the perfectly 'evolved' isozymes CA II[23] and IX.[30,31] Obviously, the present finding does not explain why there are so many CA isoforms in mammals and we do not pretend to address such a complex issue with the study of a limited number of mutants.

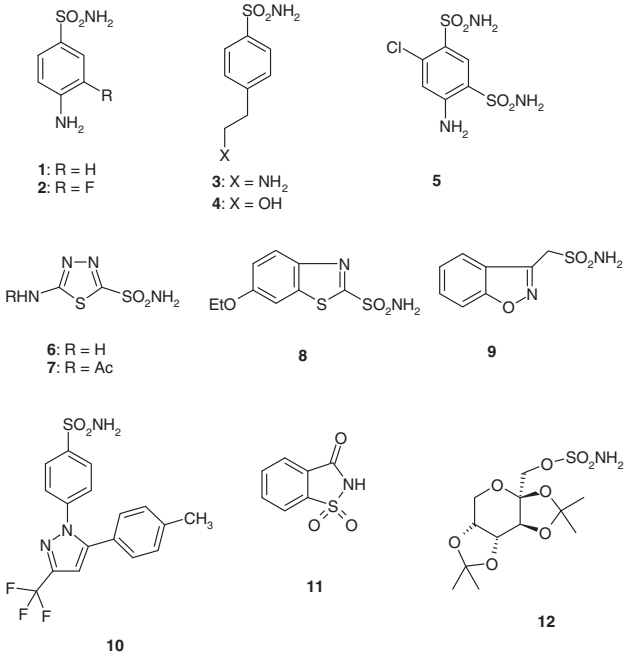
2.2. Inhibition studies of Phe91Asn hCA I

We have investigated the inhibitory activity of a panel of sulfonamides **8** and one sulfamate), of types **1–12**, against the wt hCA I, mutant hCA I as well as wt hCA II (Table 2). It is known that sulfonamides and their bioisosteres (sulfamates, sulfamides, etc.) constitute the main class of CA inhibitors (CAIs), some of which show clinical applications as diuretics, antiglaucoma, antitumor, antiepileptic, and antiobesity agents.[13,16] Indeed, acetazolamide **7** (AAZ), ethoxzolamide **8** (EZA), zonisamide **9** (ZNS), celecoxib **19** (CLX), and topiramate **12** (TPM) are clinically used agents, whereas saccharin **11** (SAC) is a widely used sweetener.[13,16] The simpler sulfonamides **1–6** included in this study are generally used for drug design purposes of CAIs, as they possess scaffolds which can be derivatized easily and lead to compounds with many possible applications as enzyme inhibitors.[13,16]

Table 2
Inhibition of hCA I, II (wt) and mutant hCA I (Phe91Asn) with sulfonamides and sulfamates **1**–**12**

Compound	K_I (nM)[a]		
	hCA I (wt)	hCA I (Phe91Asn)	hCA II
1	25000 ± 76	25400 ± 135	240 ± 12
2	8300 ± 48	8430 ± 62	60 ± 4
3	21000 ± 124	8000 ± 41	160 ± 9
4	21000 ± 189	20000 ± 116	125 ± 10
5	8400 ± 27	4150 ± 73	75 ± 5
6	8600 ± 36	540 ± 27	60 ± 3
7 (AZA)	250 ± 8	263 ± 19	12 ± 1
8 (EZA)	25 ± 1.5	18 ± 0.9	8 ± 0.7
9 (ZNS)	56 ± 4	13 ± 1	35 ± 2
10 (CLX)	50000 ± 248	52300 ± 426	21 ± 1
11 (SAC)	18540 ± 127	7810 ± 115	5959 ± 45
12 (TPM)	250 ± 16	204 ± 13	10 ± 0.5

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



Data of Table 2 show that hCA II is generally much more susceptible to be inhibited by sulfonamides/sulfamates than hCA I, a feature known for decades,[27] but not sufficiently well rationalized so far. Probably the presence of the bulky His200 in hCA I (which is Thr in many other isoforms possessing higher affinity for sulfonamides, including hCA II) may explain this phenomenon. To a certain degree this is also supported by our data, as the replacement of the bulky Phe91 by a less bulky residues, Asn, led to 8 of the 12 investigated inhibitors to show a more effective inhibitory activity against the mutant over wild type CA I. Indeed, compounds 3–**6**, **8**, **9**, 11, and **12** showed inhibition constants lower (and in some cases dramatically lower) against Phe91Asn hCA I compared to their inhibition of wt hCA I. For example, **3** was 2.6 times, **6** was 15.9 times, zonisamide **9** 4.3 times, whereas saccharin **11** 2.4 times more inhibitory for the mutant versus wt hCA I. These compounds showed indeed a varied profile of inhibition, from the very potent inhibitors zonisamide **9** and ethoxzolamide **8** (inhibition constants of 13–18 nM) to the weak ones saccharin, or sulfonamides **3** and **4**, which had K_I-s in the range of 7.81–20.0 μM (Table 2). Four of the 12 compounds on the other hand were quite slightly less inhibitory against the mutant over wt hCA I, and these derivatives were: **1, 2, 7, 8** (acetazolamide), and **10** (celecoxib). In all these cases the inhibition constants against the mutant hCA I were around 10% higher than the corresponding ones against the wt hCA I. It should be also observed that in all cases hCA II was much better inhibited by all these sulfonamides/sulfamates than the two hCA I variants investigated here (Table 2).

Among the inhibitors **1–12** investigated here, there are very few crystallographically characterized adducts with hCA I (but not with hCA II, isoform for which there are several hundred enzyme-inhibitor/activator adducts characterized in detail by this technique).[16] In fact only recently we reported the X-ray crystal structure of the hCA I–topiramate **12** adduct.[39a] The corresponding hCA II–topiramate structure was reported earlier by our group.[39b] For both isoforms the sulfamate moiety of the inhibitor was found coordinated to the Zn(II) ion whereas the derivatized fructopyranose scaffold participated in many interactions with amino acid residues from the enzyme active site.[39] However, one of the great surprises of the hCA I adduct was that a significant conformational rearrangement is necessary in the hCA I active site in order to allow the binding of the inhibitor **12**.[39a] This was not the case with hCA II–**12** adduct, in which the protein backbone was totally unchanged after the binding of the sulfamate.[39b] Indeed, the side chains of residues His200, Trp5, and His67 in the hCA I–**12** adduct were found displaced from their normal position in the uncomplexed enzyme, leading to a massive rearrangement of the entire active site, a situation never observed earlier for other α-CA–inhibitor complexes.[16,39,40] Thus, it is not very realistic to compare hCA II–inhibitor complexes with hCA I–inhibitor complexes due to situations as those evidenced above for the topiramate adduct. On the other hand, in another recent study[40] we have observed that in a hCA II–sulfonamide complex, the residue 91 (which is Ile in hCA II) participates to the binding of inhibitors with longer tails attached to the scaffold, being part of a new hydrophobic pocket in which sulfonamides (and presumably also sulfamates) bind. This new hydrophobic pocket incorporating residue 91 represents a hot spot for designing inhibitors which interact with it, in order to target specifically different CA isozymes, since, as shown earlier, the amino acid in this position is highly variable among the 13 catalytically active mammalian CAs.

3. Conclusions

A single mutation of an amino acid not considered essential to catalysis (Phe91Asn) in carbonic anhydrase I, but which is near the substrate binding pocket of these enzymes, led to a steady increase of 16% of the catalytic activity of the human CA I, which is a gain of 50% catalytic efficiency if one compares hCA I and hCA II as catalysts for CO_2 hydration. This effect may be due to the bigger and more polar hydrophobic pocket in the mutant enzyme compared to the wild type hCA I, which probably leads to the reorganization of the solvent molecules present in the cavity and also to a diverse proton transfer pathway in the mutant enzyme over the non mutated one. To our surprise, the mutant CA I was not only a better catalyst for the physiologic reaction, compared to the wild type enzyme, but in many cases also showed higher affinity (2.6–15.9 times) for sulfonamide/sulfamate inhibitors. As the residue in position 91 is highly variable among the 13 catalytically active CA isoforms, this study may shed a better understanding of catalysis/inhibition by this superfamily of enzymes.

4. Experimental

4.1. Cell line and growth conditions

HL60 (Human promyelocytic leukemia) cell line was kindly provided from Prof. İsmet GÜRHAN (Ege University, Faculty of Bioen-

gineering, Izmir, Turkey). Cells were cultured with RPMI 1640 medium (Biochrom AG) including heat inactivated Final 10% (FCS) Fetal calf serum (HyClone), 1% L-Glutamine (Sigma), 1% Penicillin–Streptomycin solution (Sigma), 5% CO_2 at 37 °C.

4.2. RT-PCR cloning of hCA I and site-directed mutagenesis

Total RNA was prepared from cultured HL60 (Human promyelocytic leukemia) cells (up to 3.5×10^7 pelleted cells) with RNeasy Mini Kit (RNeasy Mini Kit, Qiagen) following the manufacturer's instructions. hCA I gene was amplified from cDNA, by RT-PCR based strategies using the following oligonucleotides; hCA IORF-forward primer (5′-ACATATGATGGCAAGTCCAGACTG-3′) and the reverse primer (5′-AGGATCCTCATTAAAATGAAGCTCTCA-3′) using the following conditions: 94 °C for 15 s, 35 cycles (94 °C for 15 s, 55 °C for 30 s and 68 °C for 1 min) and a final extension of 72 °C for 5 min. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward primer (5′-CCCTTCATTGACCTCAACTACATGG-3′) and the reverse primer (5′-AGTCTTCTGGGTGGCAGTGATGG-3′) were used as a positive control for same conditions. The primers used for hCA I gene amplification were designed from mRNA sequence of the gene from NCBI and were synthesized by Operon. The amplified band containing hCA IORF was cloned into the linearized pGEM-T vector a ligation system from promega with T:A cloning strategy.[34] Recombinant plasmids were transformed into *Escherichia coli* XL1-blue super competent cells [recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacIqZΔM15 Tn10 (Tetr)] and the positive clone was selected based on blue/white assay on X-Gal plates as described in pGEM-T vector (Promega) manual. Automated sequencing of the clone was performed in order to confirm the gene and the integrity of amplified gene. The construct was then excised with BamHI and NdeI restriction enzymes and subcloned into pET21a(+) expression vector. The vectors were transformed into *E. coli* BL21 (DE3) [*E. coli* B F– dcm ompT hsdS(rB–mB–) gal λ(DE3)] competent cells.

Site-directed Mutagenesis was performed on pET21a-hCA I 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 Forward Primer (5′-GAC AGC TAC AGG CTC **AAT** CAG TTT CAT TTT CAC-3′) and Reverse Primer (5′-GTG AAA ATG AAA CTG **AAT** GAG CCT GTA GCT GTC-3′) obtained from Operon USA. Mutant plasmids were transformed into *E. 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 confirmed by Automated Sequence Analysis for the corresponding mutations or the presence of any misincorporations during PCR reactions. The sequencing was performed by the REFGEN Company, Ankara, Turkey.

4.3. Overexpression of recombinant hCA I and mutant Phe91Asn gene product

Recombinant hCA I and mutant Phe91Asn genes were transformed into BL21(DE3) *E. coli* strain containing the T7 promoter region. Overexpression of hCA I and mutant Phe91Asn were induced with 1 mM IPTG (isopropylthiogalactoside) and $ZnCl_2$ (12.5 μM as final concentration), for 5 h at 30 °C. The expression host *E. coli* strain BL21 (DE3) [B F– dcm ompT hsdS(rB–mB–) gal λ(DE3] was transformed with plasmids containing the single mutant and wild type hCA I, inoculated with a single colony from a fresh plate of BL21(DE3)/pET21a in a sterile 50 mL falcon tube, prepared in 10 mL of Luria broth (LB) supplemented with 10 μL of 100 mg/mL ampiciline solution. Both cultures were grown for 16 h at 37 °C with moderate agitation (120 rpm) then 200 mL of LB supplemented with 200 μL of 12.5 mg/mL ampiciline solution was inoculated with 5 mL and cultured overnight. Both cultures were grown at 37 °C with moderate agitation (300 rpm) for 3–5 h and growth of the culture was screened by measuring the optical density at 550 nm. When the cell cultures reached an optical density of 0.6–0.8, expression of the wild type or mutant hCA I was induced with 400 μL of 0.1 M IPTG and 250 μL of 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 I.

4.4. Purification and protein analysis

To purify the wild type and mutant protein, *E. coli* cells were centrifuged at 3000 rpm for 10 min at 4 °C, to pellet the cells. The pellet was washed with 10 mL of buffer (50 mM Tris–Cl, pH 7.6) and resuspended in lysis buffer (20 mM Tris/0.5 mM EDTA/0.5 mM EGTA, pH 8.7) then phenylmetylsulfonyl fluoride (PMSF, 1 mM final concentration) and lysozyme (250 μg/mL final concentrations) solutions were added and the pellet was kept at room temperature for 15 min. Then 1 mL of the 3.0% protamine sulfate solution was added to the cell lysate and centrifuged. The proteins present in the supernatant were collected. Wild and mutant hCA I enzymes were purified by Sepharose 4B-L-Tyrosine specific affinity column chromatography and screened by SDS–PAGE.

4.5. CA activity and inhibition measurements

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity.[35] 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 for the α-CAs) 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 (10 mM) were prepared in distilled-deionized water and dilutions up to 0.01 μM 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. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier,[36,37] and represent the mean from at least three different determinations. Sulfonamides **1–12** were commercially available or reported earlier by these groups.[38]

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