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Carbonic anhydrase activators: The first activation study of the human secretory isoform VI with amino acids and amines

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Abstract—The secretory isozyme of human carbonic anhydrase (hCA, EC 4.2.1.1), hCA VI, has been cloned, expressed, and purified in a bacterial expression system. The kinetic parameters for the CO_2 hydration reaction proved hCA VI to possess a k_{cat} of 3.4×10^5 s^{-1} and k_{cat}/K_M of 4.9×10^7 M^{-1} s^{-1} (at pH 7.5 and 20 °C). hCA VI has a significant catalytic activity for the physiological reaction, of the same order of magnitude as the ubiquitous isoform CA I or the transmembrane, tumor-associated isozyme CA IX. A series of amino acids and amines were shown to act as CA VI activators, with variable efficacies. L-His, L-Trp, and dopamine showed weak CA VI activating effects (K_As in the range of 21–42 μM), whereas D-His, D-Phe, L-DOPA, L-Trp, serotonin, and some pyridyl-alkylamines were better activators, with K_As in the range of 13–19 μM. The best CA VI activators were L-Phe, D-DOPA, L-Tyr, 4-amino-L-Phe, and histamine, with K_As in the range of 1.23–9.31 μM. All these activators enhance k_{cat}, having no effect on K_M, participating thus in the rate determining step in the catalytic cycle, the proton transfer reactions between the enzyme active site and the environment.

1. Introduction

Carbonic anhydrase (CA, EC 4.2.1.1) isozyme VI (CA VI) is the only secreted enzyme among the 16 isoforms described up to now in mammals.[1–3] Indeed, this family of metalloenzymes is widespread all over the phylogenetic tree, being fundamental to a variety of physiological processes.[4] By catalyzing the interconversion between carbon dioxide and bicarbonate, with generation of a proton, CAs operate on three very simple molecules/ions involved in a variety of critical life processes. Among them, the most important ones are pH regulation, respiration, secretion of electrolytes, biosynthesis of some important biomolecules such as urea, glucose, lipids, and pyrimidines, excretion of acid and salts, carcinogenesis, signaling, etc.[1–7] Different isozymes among the 16 presently known participate in such processes.[1–7] Indeed, these isoforms present a very diverse cellular localization, catalytic activity, and susceptibility to be inhibited/activated by various endogenous or exogenous regulators of activity.[1–7] In humans only 15 of the 16 mammalian isoforms are present, due to the fact that CA XV is encoded by a pseudogene in all primates investigated so far.[8] Among these remaining 15 isoforms, 12 possess catalytic activity for the CO_2 hydration reaction, whereas CA VIII, X, and XI are devoid of this activity, as these proteins lack one or more histidine residues coordinating the catalytically critical Zn(II) ion within the enzyme active site.[9] Indeed, CA I–III, VII, and XIII are soluble, cytosolic isozymes, CA IV and XV are extracellular, membrane-anchored enzymes by means of glycosylphosphatidyl-inositol (GPI) tails, CA VA and VB are mitochondrial enzymes, whereas CA IX, XII, and XIV are transmembrane proteins with the active site situated outside the cell.[1–8] CA VI, the only secreted CA isoform, has been initially identified in sheep saliva and parotid glands by Fernley's group in 1979.[10,11] CA VI was thereafter shown to be secreted in saliva, tears, and milk of all mammals,[10–14] where it may play important physiological roles, some of which are little understood at this moment. More recently, the enzyme was also evidenced in enamel

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organs in the rodent teeth,[15] but it is not well understood whether the enzyme is secreted into the enamel layers or onto the intercellular spaces of the enamel organ.

There are quite conflicting, rather old literature data[11,12] regarding the catalytic activity of CA VI, and virtually no inhibition/activation study of this enzyme. Here we present the first study regarding the DNA cloning, purification, enzymatic characterization, and activation data with a series of amino acids/amines of the human secreted isoform hCA VI.

2. hCA VI amino acid sequence and catalytic activity

We obtained a cDNA clone encoding the full-length of the open reading frame of hCA VI. Further, the enzyme was successfully produced as a GST-hCA VI fusion protein by using the GST-fusion protein method.[16–18] To date, three cDNA clones covering the open reading frame of hCA VI have been deposited in the GenBank (the Accession Nos. NM_001215, M57892, and AF128411). The cDNA sequence of the clone obtained herein was completely identical to that of the clone NM_001215. The other two clones showed different amino acid usage at three sites, that is, residues Ser:Gly at position 70 (numbering is based on CA I sequence), Ser:Thr at position 130, and Asn:Lys at position 253. Considering the significance of these amino acids in the CA active site (mainly those in position 70 and 130 which are adjacent to two critical residues involved in the binding of inhibitors/activators,[20–23] i.e., amino acids 69 and 131), these substitutions probably represent normal polymorphisms.

The amino acid sequence of the CA domain which was deduced from the cDNA sequence of our hCA VI clone was aligned with that of two cytosolic CA isozymes, hCA I and hCA II (Fig. 1). In Figure 1, the 36 residues that were previously shown to form the active site cavity in all α-CAs[23–25] are indicated by a mixture of asterisk, plus sign, and 'z' above the hCA I sequence. Among these active site residues, 25 amino acids are conserved between hCA VI and hCA II, the isozyme showing the strongest CA activity in the α-CA family (Table 1). It is important to note that hCA VI inherits all the amino acid residues which are critical in the CA catalytic cycle: (i) the three zinc ligands, His94, 96, and 119; (ii) the 'gate-keeping' residues Thr199 and Glu106, which orient the substrate in the right position to be attacked by the zinc-bound hydroxide ion; and (iii) His64, the proton shuttle residue, which transfers protons from the zinc bound water molecule toward the external medium, leading to the generation of the active form of the enzyme with hydroxide as the fourth zinc ligand.[1–4] These findings suggest that hCA VI has a catalytic activity comparable to that of isozymes I and II.[1–4]

Along with the amino acid residues conserved among the α-CA isozymes, hCA VI has a number of characteristic residues. The most notable residue is Thr65 which is nearby the conserved proton shuttle residue of all α-CAs, His64. This amino acid is a Ser65 in CA I and an Ala65 in CA II. As compared to Ala residue, Ser/Thr residues are bulkier as to interfere with the flip movement of His64, resulting in the decreased catalytic activity of CA I and VI as compared to CA II. It is of note that residues 69 and 131, which are an Ile and Tyr in CA VI, respectively, were shown to be involved in the binding of inhibitors/activators in several other isozymes.[23–26]

The kinetic parameters for the CO_2 hydration reaction catalyzed by this newly purified recombinant isozyme, hCA VI, as well as for other isoforms which are targets for the drug design (such as hCA I, II, VA, VB, IX, and XII), are shown in Table 1. As shown above, literature data regarding the catalytic activity of hCA VI are rather conflicting,[11,12] with Murakami and Sly[12] reporting hCA VI to have about 1% of the catalytic activity of hCA II, whereas Fernley[11] showed the secreted isoform to be much more active, with a catalytic activity of around 25–30% that of the perfectly evolved catalyst which is hCA II.[1] It should be mentioned that both

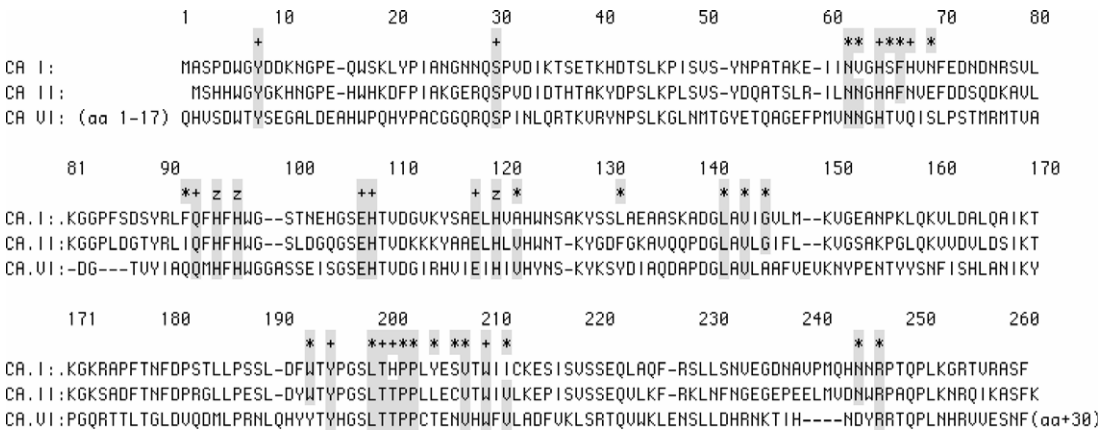


Figure 1. Sequence alignment of the CA domain of human isozymes hCA I, II, and VI. The residues indicated by a mixture of asterisk, plus sign, and 'z' above the hCA I sequence are those forming the active site. Seventeen residues known to participate in a network of hydrogen bonds crucial for the binding of inhibitors/activators are indicated by plus; 'z' indicates the three zinc liganded histidines. Among these residues, conserved amino acids are indicated by a closed box.

Table 1. Kinetic parameters for the CO_2 hydration reaction catalyzed by the cytosolic α-hCA isozymes I and II, the mitochondrial isozymes VA and VB, the secreted isoform hCA VI (full length), and the transmembrane isozymes hCA IX (catalytic domain), and hCA XII (catalytic domain), at 20 °C and pH 7.5, and their inhibition data with acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), a clinically used drug

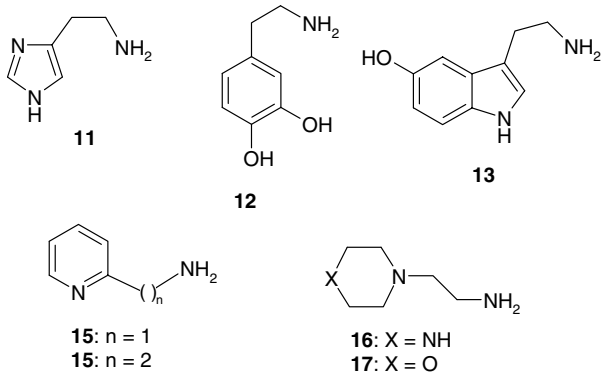
Isozyme	Activity level	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_I (acetazolamide) (nM)
hCA I	Moderate	2.0×10^5	5.0×10^7	250
hCA II	Very high	1.4×10^6	1.5×10^8	12
hCA VA	Low	2.9×10^5	2.9×10^7	63
hCA VB	High	9.5×10^5	9.8×10^7	54
hCA VI	Moderate	3.4×10^5	4.9×10^7	11
hCA IX	High	3.8×10^5	5.5×10^7	25
hCA XII	Moderate	4.2×10^5	3.5×10^7	5.7

groups used hCA VI purified from saliva by sulfonamide column chromatography. The catalytic activity of our recombinant isoform, which is practically free of any contaminant protein, showed hCA VI to have indeed a lower catalytic activity as compared to hCA II, but of the same order of magnitude as that of the highly abundant, cytosolic isoform hCA I (Table 1). Indeed, both isoforms have very similar k_{cat} and almost an identical k_{cat}/K_m, close to 5.0×10^7 M^{-1} s^{-1} (at pH 7.5 and 20 °C, see Table 1). This is indeed around 33% the catalytic activity (as turnover number) of hCA II. Thus, hCA VI is less effective than hCA II or hCA VB, as a catalyst for CO_2 hydration to bicarbonate, but it has a comparable activity with that of two highly investigated and important isozymes, such as CA I (cytosolic isoform) and IX (transmembrane, tumor-associated isozyme). Furthermore, hCA VI is also more active than isozymes VA or XII, which were previously shown to be druggable targets.[1–4] It may be also observed from data of Table 1 that all these isozymes are susceptible to inhibition by acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), **AAZ**, the clinically used sulfonamide CA inhibitor *par excellence*.[1]

3. hCA VI activation studies

A multitude of physiologically relevant compounds such as amino acids, oligopeptides or small proteins, as well as many biogenic amines (histamine, serotonin, and catecholamines among others), were shown to efficiently activate the catalytic activity of several CA isozymes, such as CA I, II, and IV.[7,27,28] Activation of the cytosolic, ubiquitous isoforms CA I and II was then shown to constitute a possible therapy for the enhancement of synaptic efficacy, which may represent a conceptually new approach in the treatment of Alzheimer's disease, aging, and some other disease conditions characterized by an eventual loss of memory functions.[29,30] However, unlike CA inhibitors, widely used clinically for the treatment or prevention of a multitude of diseases,[1–4] CA activators (CAAs) have been much less investigated.[7] Only recently, by means of electronic spectroscopy, X-ray crystallography, and kinetic measurements, it has been proved that CAAs bind within the enzyme active cavity (in the case of the physiologically most important isoforms, hCA I and hCA II) at a site distinct from the inhibitor or substrate binding-sites, participating thereafter in the rate-determining step of the catalytic cycle, the proton transfer reaction between the active site and the environment.[27,28,31–33] As previous[7,27,28,31–33] data on the activation of the cytosolic isoforms CA I and II clearly showed that a CAA must possess specific steric and electronic requirements for good activity, that is, it must fit within the restricted active site cavity of the enzyme, but should interact favorably with amino acid residues present in the activator binding pocket, and second, it should possess a moiety able to participate in proton transfer processes, better if with a pK_a in the range of 6.0–8.0 U, we decided to investigate some amino acid and amine derivatives for their interaction with the secretory form hCA VI.

L-/D-Amino acids **1**–**10** and amines **1**–**17** investigated as hCA VI activators were commercially available from Sigma–Aldrich (Milan, Italy) and were used without further purification.



Kinetic experiments for the physiological reaction (carbon dioxide hydration to bicarbonate and a proton) (Table 2) showed that as for hCA I and II,[7,30–33] activators of the amino acid or amine type enhance k_{cat} of the enzyme, with no effect on K_M. Indeed, as observed from data of Table 2, L- or D-Phe (compounds **3** and **4**) at a concentration of 10 μM produced a notable enhancement of k_{cat} for all three investigated isoforms, that is, hCA I, hCA II, and hCA VI. Thus, for hCA I, this parameter for the pure enzyme is of 2.0×10^5 s^{-1},[10] whereas in the presence of activators **3** and **4** at a concentration of 10 μM, it becomes of $2.3–19.8 \times 10^5$ s^{-1}. For hCA II, the enhancement of k_{cat} from the value of the pure enzyme (1.4×10^6 s^{-1}) is in the range of $5.2–5.7 \times 10^6$ s^{-1}, whereas for hCA VI, this enhancement is of $4.9–10.3 \times 10^5$ s^{-1}, from the initial value of 3.4×10^5 s^{-1} for the pure enzyme (Table 2).

Table 2. Kinetic parameters for the activation of hCA isozymes I, II, and VI with L- and D-Phe, at 20 °C and pH 7.5, for the CO_2 hydration reaction

Isozyme	k_{cat}[a] (s^{-1})	$(k_{cat})_{L\text{-Phe}}$[b] (s^{-1})	$(k_{cat})_{D\text{-Phe}}$[b] (s^{-1})	K_A[c] (μM)	
				L-Phe	D-Phe
hCA I[d]	2.0×10^5	19.8×10^5	2.3×10^5	0.07	86
hCA II[d]	1.4×10^6	5.7×10^6	5.2×10^6	0.013	0.035
hCA VI[e]	3.4×10^5	10.3×10^5	4.9×10^5	1.23	16

[a] Observed catalytic rate without activator. K_M values in the presence and the absence of activators were the same for the various CA isozymes (data not shown).
[b] Observed catalytic rate in the presence of 10 μM activator.
[c] The activation constant (K_A) for each isozyme was obtained as described in the Section 5, and represents the mean from at least three determinations by a stopped-flow, CO_2 hydrase method.[19] Standard errors were in the range of 5–10% of the reported values.
[d] Human recombinant isozymes.
[e] Human recombinant full length isozymes.

hCA VI activation constants for a series of structurally related amino acids and amines of types **1-17** are shown in Table 3. The activation constants for the ubiquitous isozymes hCA I and hCA II are also provided for comparison. These compounds were shown earlier to act as activators of the cytosolic isozymes hCA I and II.[7,30–33] All of them possess protonatable moieties of the primary amino or heterocyclic amine type (or both of them), being thus able in principle to participate in proton transfer processes leading to the generation of the nucleophilic species of the enzyme, with hydroxide coordinated to the active site zinc ion. It should be noted that the amines included in our study possess aminoethyl or aminomethyl moieties, in addition to aromatic/heterocyclic groups, the last of which usually incorporate nitrogen atoms that can be protonated at pH values in the physiological range. Similarly with the inhibition constant K_I (for the enzyme inhibitors),[1–4] the activation constant K_A measures the affinity of the activator for the enzyme. The lower this parameter is, most strong is the activator against the corresponding isoform.[7,30–33]

Table 3. Activation constants of hCA I, hCA II, and hCA VI with amino acids and amines **1–17**

No.	Compound	K_A (μM)[a]		
		hCA I[b]	hCA II[b]	hCA VI[c]
1	L-His	0.03	10.9	32
2	D-His	0.09	43	13
3	L-Phe	0.07	0.013	1.23
4	D-Phe	86	0.035	16
5	L-DOPA	3.1	11.4	18
6	D-DOPA	4.9	7.8	4.58
7	L-Trp	44	27	15
8	D-Trp	41	12	39
9	L-Tyr	0.02	0.011	9.31
10	4-H_2N-L-Phe	0.24	0.15	5.32
11	Histamine	2.1	125	6.50
12	Dopamine	13.5	9.2	21
13	Serotonin	45	50	19
14	2-Pyridyl-methylamine	26	34	14
15	2-(2-Aminoethyl)pyridine	13	15	18
16	1-(2-Aminoethyl)-piperazine	7.4	2.3	9.54
17	4-(2-Aminoethyl)-morpholine	0.14	0.19	42

[a] Mean from three determinations by a stopped-flow, CO_2 hydrase method.[19] Standard errors were in the range of 5–10% of the reported values.
[b] Human recombinant isozymes, stopped flow CO_2 hydrase assay method.[19]
[c] Full length, human recombinant enzyme, stopped flow CO_2 hydrase assay method.[19]

Data of Table 3 show that amines and amino acids investigated here, of type **1–17**, act as CAAs against all three investigated isozymes, but with quite different activity profile for the newly characterized isozyme hCA VI. Thus, the following SAR can be observed from data reported in Table 3: (i) several compounds investigated here, such as L-His **1**, D-Trp **8**, dopamine **12**, and the morpholine derivative **17**, behaved as quite weak CA VI activators, with K_As in the range of 21–42 μM. It should be noted that all these derivatives generally act as much better activators of isozymes CA I and II (except D-Trp against CA I, for which it behaves again as a weak CAA), sometimes with affinities in the nanomolar range (e.g., L-His against hCA I); (ii) medium potency CA activatory effects against hCA VI were observed for D-His **2**, D-Phe **4**, L-DOPA **18**, L-Trp **15**, serotonin **19**, and the pyridine derivatives **14** and **15**, which showed K_As in the range of 13–19 μM. Again these derivatives showed an activatory efficacy quite different against isozymes I and II. Thus, for example, D-His **2** acts as a very potent hCA I activator (K_A of 0.09 μM), a quite inefficient CA II activator (K_A of 43 μM), and a medium potency CA VI activator (K_A of 13 μM) (Table 3); (iii) a group of compounds, such as L-Phe **3**, D-DOPA **6**, L-Tyr **9**, 4-amino-L-phenylalanine **10**, histamine **11**, and the piperazine **16**, showed efficient hCA VI activating properties, with K_As in the range of 1.23–9.54 μM. The best CA VI activator detected up to now was L-Phe, which with an activation constant of 1.23 μM is roughly 13 times a better activator as compared to its enantiomer, D-Phe, acting as a medium potency CA VI activator. The same type of differences of activation power was observed for other pairs of enantiomers investigated here as CA VI activators (derivatives **1–8**). In fact, it has been documented by means of X-ray crystallography that such enantiomers (such as for example L- and D-His,[28,31a] or L- and D-Phe[31b]) bind in a different manner within the enzyme active site, interacting with diverse amino acid residues and participating in the proton transfer processes by means of different pathways. It may be also noted that

the stereochemistry of the amino acid pair of compounds was not the crucial element in the CA VI activating efficacy, since in some cases the L-enantiomer was a better CA VI activator (L-Phe and L-Trp over the corresponding D-enantiomers), whereas in other cases the D-enantiomer was a better activator as compared to the corresponding L-one (D-His and D-DOPA, over the corresponding L-enantiomers, respectively). There was also not a notable difference of activity between amino acids and structurally related amines (compare L-His, D-His, and histamine, or L-DOPA, D-DOPA, and dopamine, respectively), as both types of derivatives showed good CA VI activating properties. Obviously, as this is the first CA VI activation study reported, the number of investigated compounds is rather limited for allowing a detailed SAR discussion. It must also be noted that only micromolar CA VI activators were detected for the moment, although for CA I and II, many of the investigated amines/amino acids act as nanomolar activators.

But what is the significance of our results? As mentioned in the introduction, the saliva secreted isozyme hCA VI is the least understood isozyme of this large family of metalloproteins. However, several important physiological studies showed the involvement of this enzyme in olfaction, taste, and pH regulation in the oral cavity.[10–15] Our results show CA VI to possess an enzymatic activity for the CO_2 hydration reaction much higher than previously reported. In fact CA VI has around 33% of the activity of hCA II, a very efficient catalyst for this crucial physiologic reaction. Furthermore, CA VI has the same catalytic power as CA I, another very abundant and ubiquitous CA isoform.[1–4] CA VI has a good affinity for many amines or amino acid activators investigated here, some of which are present in many body tissues in rather high concentrations.[1–7] Thus, further studies are warranted for starting to understand the role of CA VI activation processes from the physiologic or pathologic viewpoints.

4. Conclusions

The first hCA VI activation study is presented here. The full length enzyme has a high catalytic activity with the following kinetic parameters at 20 °C and pH 7.5, for the CO_2 hydration reaction: $k_{cat} = 9.5 \times 10^5\ s^{-1}$, and $k_{cat}/K_M = 9.8 \times 10^7\ M^{-1}\ s^{-1}$, showing a significant catalytic activity for the physiological reaction, of the same order of magnitude as the ubiquitous isoform CA I or the transmembrane, tumor-associated isozyme CA IX. A series of amino acids and amines were shown to act as CA VI activators, with variable efficacies. L-His, L-Trp, and dopamine showed weak CA VI activating effects (K_As in the range of 21–42 μM), whereas D-His, D-Phe, L-DOPA, L-Trp, serotonin, and some pyridyl-alkylamines were better activators, with K_As in the range of 13–19 μM. The best CA VI activators were L-Phe, D-DOPA, L-Tyr, 4-amino-L-Phe, and histamine, with K_As in the range of 1.23–9.31 μM.

5. Experimental

5.1. Chemistry

Compounds **1**–**17** are commercially, highest purity available derivatives from Sigma–Aldrich (Milan, Italy) and were used without further purification.

5.2. Cloning and purification of hCA VI

The cDNA fragment encoding the open reading frame of hCA VI was amplified from polyA(+) RNA obtained from human salivary gland (Clontech, Palo Alto, CA, USA) by using a commercial RT-PCR kit (Takara, Kyoto, Japan) with adopter primers including *Eco*RI and *Sal*I recognition sequences (underlined in the following sequences, respectively): 5′-CGGAATTCCCATGAGGG CCCTGGTGCTTCT-3′ and 5′-GCGTC GACTCAGTT CAATGCTCTTCT-3′. The PCR was hot-started with incubation for 5 min at 94 °C and consisted of 35 cycles of 30 s at 94 °C, 30 s at 57 °C, and 90 s at 72 °C. The PCR products were cleaved with *Eco*RI and *Sal*I, purified, and cloned in-frame into the pGEX-4T2 vector (Amersham). The cDNA sequence of the hCA VI insert included in the vector was reconfirmed by DNA sequencing. The constructs were then transfected into *E. coli* strain BL21 for production of the GST-hCA VI fusion protein, similarly to the procedure already described for hCA VB, IX, and XII. Following induction of the protein expression by addition of 1 mM isopropyl-β-D-thiogalactopyranoside, the bacteria were harvested and sonicated in PBS. The cell homogenate was incubated at room temperature for 15 min and homogenized twice with a Polytron (Brinkmann) for 30 s each at 4 °C. Centrifugation at 30,000*g* for 30 min afforded the supernatant containing the soluble proteins. The obtained supernatant was then applied to a prepacked Glutathione Sepharose 4B column (Amersham). The column was extensively washed with buffer and then the GST-hCA VI fusion protein was eluted with a buffer consisting of 5 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0. Finally the GST part of the fusion protein was cleaved with thrombin. The advantage of this method is that hCA VI is purified easily and the procedure is quite simple. The obtained hCA VI was further purified by sulfonamide affinity chromatography,[18] the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate.[19]

5.3. CA activation assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each activator 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 activators **1**–**17** (10 mM) were prepared in distilled-deionized water and dilutions up to 0.1 nM were done thereafter with distilled-deionized water. Activator 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–A complex. The activation constant (K_A), defined similarly with the inhibition constant K_I,[3] may be obtained by considering the classical Michaelis–Menten equation (Eq. 1), which has been fitted by non-linear least squares by using PRISM 3

$$v = v_{max}/\{1 + K_M/[S](1 + [A]_f/K_A)\} \quad (1)$$

where $[A]_f$ is the free concentration of activator.

Working at substrate concentrations considerably lower than K_M ($[S] \ll K_M$), and considering that $[A]_f$ can be represented in the form of the total concentration of the enzyme ($[E]_t$) and activator ($[A]_t$), the obtained competitive steady-state equation for determining the activation constant is given by Eq. 2:[7]

$$v = v_0 \cdot K_A/\{K_A + ([A]_t - -0.5\{([A]_t + [E]_t + K_A) - ([A]_t + [E]_t + K_A)^2 - 4[A]_t \cdot [E]_t)^{1/2}\}\} \quad (2)$$

where v_0 represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator.[7]

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