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Carbonic anhydrase inhibitors: The inhibition profiles of the human mitochondrial isoforms VA and VB with anions are very different

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Abstract—The first anion inhibition study of the mitochondrial human carbonic anhydrase (hCA, EC 4.2.1.1) isoform hCA VB is reported. Fluoride, chloride, bromide, iodide, cyanate, thiocyanate, cyanide, azide, bicarbonate, carbonate, nitrate, nitrite, hydrogen sulfide, bisulfite, sulfate, sulfamide, sulfamic acid, phenylboronic acid and phenylarsonic acid were compared as inhibitors of the two mitochondrial isozymes hCA VA and hCA VB. These enzymes are involved in biosynthetic reactions leading to fatty acid and Krebs cycle intermediates biosynthesis in addition to acting as catalysts for the interconversion of carbon dioxide and bicarbonate. The anion inhibition profiles of the two isoforms are dramatically different. The best hCA VB inhibitors were cyanate, thiocyanate, cyanide and hydrogensulfide (K_Is of 80–76 μM) whereas the least effective ones were the halides (K_Is of 11–72 mM), with the best inhibitor being fluoride and the least effective ones bromide and iodide. Whereas hCA VA is not sensitive to bicarbonate inhibition (K_I of 82 mM) similarly to the cytosolic isoform hCA II, hCA VB is well inhibited by this anion, with a K_I of 0.71 mM. Overall, hCA VB is more sensitive to anion inhibitors as compared to hCA VA. Such data support prior suggestions that the two mitochondrial isozymes play different physiological functions.

1. Introduction

The two mitochondrial isoforms of carbonic anhydrase (CA, EC 4.2.1.1), CA VA and VB, act as catalysts for the interconversion between CO_2 and bicarbonate, being also involved in several biosynthetic processes.[1–3] In mammals, carboxylating reactions involving pyruvate carboxylase and acetylcoenzyme A carboxylase, which in the end lead to fatty acid and Krebs cycle intermediates biosynthesis, use bicarbonate not CO_2 as substrate for the transfer of acetyl groups from the mitochondria to the cytosol, and as thus are assisted by the catalytic activity of the two mitochondrial isoforms mentioned above together with that of the ubiquitous, housekeeping cytosolic isozyme CA II.[2c,3–5] Indeed, CAs are excellent catalysts for the interconversion of the two species (CO_2 and bicarbonate) at neutral pH, being among the most effective enzymes known in nature.[1–3] Thus, by providing bicarbonate to such carboxylating enzymes, CAs participate in the biosynthesis of fatty acids and Krebs cycle intermediates (Fig. 1).

Recently, in some plants such as the Australian dicotyledon *Flaveria bidentis*, a similar role in biosynthetic processes has also been evidenced for some β-CAs,[6] which are totally distinct enzymes as compared to the α-CAs of which the mammalian mitochondrial isoforms CA VA and VB are representatives.[4] Thus, the three enzymes recently identified in this plant, denominated CA1-3, play the following very distinct roles: CA3 is responsible for catalyzing the first step in the C4 pathway in the mesophyll cell cytosol; CA2 provides bicarbonate for anapleurotic reactions involving non-photosynthetic forms of phosphoenolpyruvate carboxylase in the cytosol of cells in both photosynthetic and non-green tissues; whereas CA1 carries out non-photosynthetic functions

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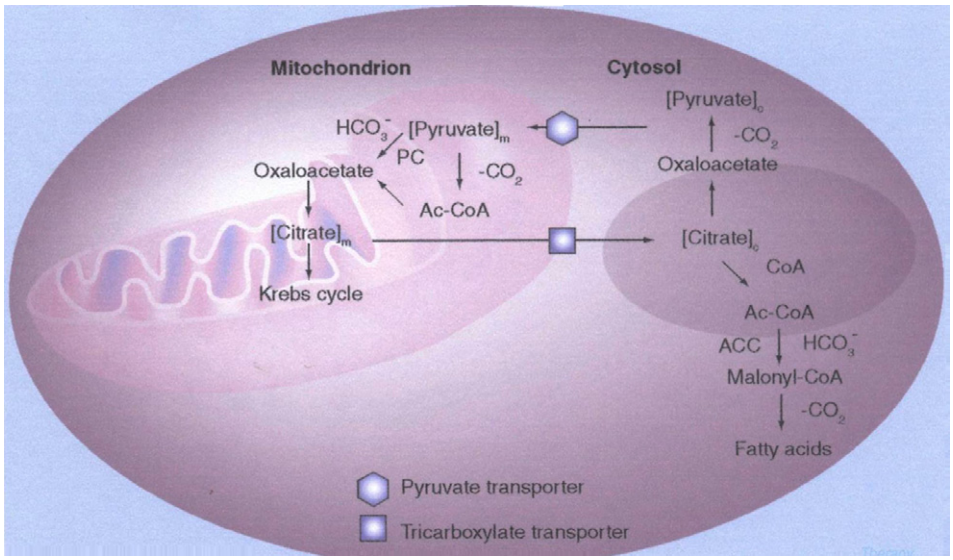

Figure 1. The transfer of acetyl groups from the mitochondrion to the cytosol (as citrate) for the provision of substrate for de novo lipogenesis. All steps involving bicarbonate also need the presence of CA isozymes, such as CA VA and CA VB, in the mitochondrion and CA II in the cytosol.[2c,3,5] PC, pyruvate carboxylase; Ac-CoA, acetyl-coenzyme A; CoA, coenzyme A; ACC, acetyl-coenzyme A carboxylase.

demonstrated also by C3 chloroplastic β-CAs, including lipid biosynthesis and antioxidant activity.[6] As both α- and β-CAs are highly abundant in bacteria, plants and fungi (and the α-class CAs also in mammals),[3–5] it is highly probable that the metabolic involvement of these enzymes may be exploited for designing novel types of pharmacological agents. In fact, the inhibition of lipid biosynthesis with sulfamates, sulfamides or sulfonamides targeting the human isoforms hCA VA/VB has recently been considered[2c,5e] as a possible therapeutic strategy for controlling obesity, a condition affecting an increasing number of persons worldwide.[7,8]

Isozyme CA VB was among the last mammalian α-CAs to be discovered, by Nishimori's group in 1999, who sequenced the genes encoding the protein, mapped it on chromosome X, expressed the enzymes and studied its tissue distribution.[9] CA VB has a much wider tissue distribution as compared to CA VA found only in hepatocytes, suggesting different physiological roles for the two mitochondrial CAs.[9] Indeed, Nishimori's and Sly's groups showed CA VB to be present in pancreas, kidney, salivary glands, spinal cord, heart and skeletal muscle, but not in the liver, where CA VA is present.[5b,9] Subsequently, our groups showed CA VB to be 3.3 times catalytically more efficient than CA VA for the physiological reaction, that is, CO_2 hydration to bicarbonate and a proton[10] and to be a target for inhibition by sulfonamide/sulfamate drugs[10] and activation by amines/amino acids,[11] similar to other α-CA isoforms.[1–3] However, no anion inhibition studies of this enzyme have been reported up until now. Metal complexing anions (such as cyanide, hydrogensulfide, azide, cyanate, etc.) as well as anions which bind less avidly cations in solution (such as perchlorate, sulfate, etc.) are known to act as inhibitors of many metalloenzymes, including all the investigated representatives of α-, β- and γ-CA classes.[12,13] The interactions between CAs and anions are rather complex: physical interactions have been identified between some mammalian isoforms, such as CA II, and the erythrocyte membrane Cl^-/HCO_3^- anion exchanger, AE1, mediated by an acidic motif in the AE1 C-terminus.[15] The presence of CA II complexed to AE1 in what is termed a metabolon accelerates the HCO_3^- transport activity of AE1 either into or out of the cells.[14] Thus, CA II, similar to CA XIII, is an isoform which is not easily inhibited by these anions (K_Is for Cl^- in the range of 156–200 mM, for bicarbonate in the range of 85–140 mM),[15] as shown earlier by our group,[15] in order to be able to function in such catalytic/transport processes mediated by the metabolon. Functional and physical interactions also occur between CA II and $Na^+HCO_3^-$ co-transporter isoforms NBC1 and NBC3.[14] More recently, it has also been demonstrated the involvement of some CA isoforms in thyroid hormone synthesis via regulation of the iodide transport across thyroidal cell membranes.[16] It is thus important to investigate the inhibition profile of all known CA isoforms with this simple class of inhibitors. Here we report the first anion inhibition study of human mitochondrial CA VB (hCA VB) with physiological and non-physiological anions, and compare the affinity for this class of inhibitors of the two mitochondrial isoforms hCA VA and hCA VB.

2. Results and discussion

The following anions, as metal salts, were used in our investigations: sodium or potassium fluoride, chloride, bromide, iodide, cyanate, thiocyanate, cyanide, azide, bicarbonate, carbonate, nitrate, nitrite, hydrogensulfide, bisulfite, and sulfate. They were of highest purity available and were used without further purification. Sulfamide, sulfamic acid, phenylboronic acid and phenylarsonic acid were also investigated as we showed

earlier that they behave similarly to the anionic inhibitors for the inhibition of several CA isoforms.[17–20] The two mitochondrial CA isozymes were recombinant ones prepared in-house as described earlier.[10,11,18–20]

The following should be noted regarding the anion inhibition data of isoform hCA VB shown in Table 1 (literature data[18–20] for the inhibition of the cytosolic hCA I and II and the other mitochondrial isozyme, hCA VA, are also shown for comparison, as they will be useful in the discussion): (i) potent hCA VB inhibitory activity was shown by the following anions: cyanate, thiocyanate, cyanide, hydrogensufide, sulfamide (as monodeprotonated species presumably)[21] and phenylarsonic acid (again in deprotonated form[20] at the pH at which the experiments were performed, i.e., 7.5). These derivatives possess inhibition constants in the range of 8–84 μM (Table 1). The highest affinity for hCA VB was shown by cyanide and hydrogensulfide (K_I of 8 μM), anions with a strong affinity for the Zn(II) ion also present in other metalloenzymes.[1,20] It may be observed that except for sulfamide and phenylarsonic acid, which are not excellent metal-complexing derivatives in solution, the other potent anion inhibitors of hCA VB are known for their ability to easily form coordination compounds with a wide range of metal ions, both when in solution or bound within the active sites of metalloenzymes[1,18–20]; (ii) medium-potency inhibitory properties against hCA VB were observed for a large number of anions, such as azide, bicarbonate, carbonate, nitrate, nitrite, hydrogensulfite, sulfate, sulfamic acid (probably sulfamate)[21a] and phenylboronic acid. These compounds showed inhibition constants in the range of 0.27–0.97 mM (Table 1). Again some of these medium-potency hCA VB inhibitors are known to act as good metal-complexing anions (e.g., azide, nitrite, carbonate), whereas others show less propensity for binding cations in solution (sulfate, sulfamic acid, phenylboronic acid)[18–20]; (iii) the weakest hCA VB inhibitors among these investigated anions were the four halides, from fluoride to iodide, which showed inhibition constants in the range of 11–72 mM (Table 1). It is interesting to note that in contrast to other investigated isoforms (e.g., hCA I, II and VA shown in Table 1), the halide hCA VB inhibitory activity dramatically decreases with the increase of the atomic weight of the halogen. This is difficult to explain, considering the fact that halides tend to possess better metal–ion complexing properties with the increase in their atomic weight, but the trend for hCA VB is quite clear: whereas fluoride shows weak but appreciable binding affinity for hCA VB (K_I of 11 mM), chloride is almost a four times less avid inhibitor (K_I of 43 mM) whereas bromide and iodide show the same weak activity, with K_Is of 71–72 mM (iv) the inhibition profile of hCA VB is very different from that of the related mitochondrial isoform, hCA VA (Table 1). Thus, halides were also weak hCA VA inhibitors, but as mentioned above, with the best inhibitor being iodide and the least effective one fluoride. The good metal complexants cyanate, cyanide and hydrogensulfide also act as very good hCA VA inhibitors (K_Is in the range of 15–28 μM), but thiocyanate is almost 10 times less effective (K_I of 0.74 mM) for hCA VA than hCA VB inhibitor. However, the greatest difference in inhibitory power is shown by bicarbonate, a physiologically relevant anion and also a CA substrate. Thus, hCA VB is

Table 1. Inhibition constants of anionic inhibitors against the cytosolic human isozymes hCA I, II, and the mitochondrial human isoforms hCA VA and B, for the CO_2 hydration reaction, at 20 °C and pH 7.5[17]

Inhibitor	K_I[a] (mM)			
	hCA I[b]	hCA II[b]	hCA VA[c]	hCA VB[d]
F^-	>300	>300	241	11
Cl^-	6	200	156	43
Br^-	4	63	50	72
I^-	0.3	26	25	71
CNO^-	0.0007	0.03	0.028	0.070
SCN^-	0.2	1.6	0.74	0.076
CN^-	0.0005	0.02	0.015	0.008
N_3^-	0.0012	1.5	0.30	0.27
HCO_3^-	12	85	82	0.71
CO_3^{2-}	15	73	0.72[e]	0.93
NO_3^-	7	35	16	0.72
NO_2^-	8.4	63	14	0.80
HS^-	0.0006	0.04	0.023	0.008
HSO_3^-	18	89	91[e]	0.72
SO_4^{2-}	63	>200	1.17[e]	0.83
H_2NSO_3H[f]	0.021	0.39	0.12	0.66
$H_2NSO_2NH_2$	0.31	1.13	0.84	0.084
$PhB(OH)_2$	58.6	23.1	1.04[e]	0.97
$PhAsO_3H_2$[e]	31.7	49.2	1.02[e]	0.082

[a] Errors were in the range of 3–5% of the reported values, from three different assays.
[b] Human recombinant isozyme, data from Ref. 18.
[c] Human recombinant full length isozyme, data from Ref. 18a.
[d] Human recombinant full length isozyme, this work.
[e] This work.
[f] As sodium salt.

115.5 times more susceptible to inhibition by this anion as compared to hCA VA (the inhibition of the two isoforms by chloride is not so dramatically different: hCA VB is only 3.6 times more avid for this anion as compared to hCA VA). The two isoforms on the other hand show the same inhibitory activity towards carbonate. Thus, the net difference in the inhibitory power of bicarbonate (and also chloride) of hCA VA and hCA VB points out to different physiological functions for the two mitochondrial isoforms: probably hCA VA participates in metabolon(s) involving the transport of bicarbonate/chloride in mitochondrial compartments (as it is not susceptible to inhibition by these anions, showing K_Is in the range of 82–156 mM), whereas hCA VB having a good sensitivity to bicarbonate (K_I of 0.71 mM) is not adapted to such a function (although its sensitivity to chloride is not too high). These data also pinpoint to the fact that hCA VB will be probably at least 50% inhibited at the bicarbonate concentrations required for mitochondrial pyruvate carboxylase (PC) activity, as the K_M for bicarbonate of sheep PC is around 1.2 mM.[21b] The same difference of activity between the two isozymes is also shown by the isoelectronic anion with bicarbonate, hydrogensulfite. Basically these two isostructural and isoelectronic anions show very similar hCA VA inhibitory activity (K_Is of 82–91 mM) and identical K_Is against hCA VB (of 0.71–0.72 mM). Overall, with few exceptions (for example cyanate), hCA VB is more avid to be inhibited by the anion inhibitors investigated here as compared to hCA VA; (v) the two cytosolic isozymes CA I and II also show inhibition profiles quite distinct of those of the mitochondrial enzymes CA VA and VB (Table 1). The most significant such differences are again related to the affinity of these enzymes for halides, bicarbonate and sulfate among others. Thus, both cytosolic isoforms are not at all inhibited by fluoride, whereas hCA VB shows good affinity for this inhibitor. Chloride, bromide and iodide are quite effective hCA I inhibitors but much weaker CA II, VA or VB inhibitors. Bicarbonate is a very good hCA VB inhibitor, appreciably inhibits hCA I, but has a quite low inhibitory activity against hCA II and hCA VA. Finally, sulfate does not inhibit hCA II; has very weak hCA I inhibitory properties, but acts as an efficient hCA VA/VB inhibitor (Table 1). These phenomena are clearly due to the different active site architecture of the two isoforms (Fig. 2).

Indeed, as shown in Figure 2, among the 36 active site residues that were previously[22] defined as forming the cavity of the enzyme, and represented by a combination of + (active-site hydrogen bond network), and Z (zinc-liganded histidine) signs, three residues of CA II, that participate in the hydrogen bond network, are replaced with different residues in the mitochondrial isozymes (shown in boxes). These residues (in positions 7, 64 and 67) are highly important both in the catalytic mechanism and for the binding of inhibitors.[1–3] Thus, the amino acid in position 7 is situated at the entrance of the active site cavity, the one in position 64 is a mobile one involved in the rate-determining step in the catalytic cycle of hCA I and II (being a His),[1–3] but it is a Tyr in the mitochondrial isoforms, an amino acid less prone to act as a good proton shuttle at physiological pH values. However, as the pH in the mitochondria is probably 8.5,[8] it is not impossible that a phenol OH may show such a function. But it should be mentioned that the proton transfer processes within the CA VA/B isoforms are less understood at this moment, as compared with the same processes in the mitochondrial isoforms CA I–III.[1–3] The amino acid residue in position 67 was also shown to be involved in the binding of many classes of inhibitors mainly in CA II.[23] In comparison with CA II, eight residues are substituted in CA VB and two additional substitutions are observed in CA VA (bold boxes in Fig. 2) which may thus explain the net differences in the inhibition profiles of the discussed isoforms even for this class of quite simple inhibitors. It is not improbable that the net differences of affinity for various anion inhibitors between the two mitochondrial isoforms are due to the different amino acids present in positions 62, 67, 131 and 204 (Fig. 2). In fact in isozyme hCA II, for which the highest number of adducts in complex with various inhibitors has been reported, it has been demonstrated that some of these residues are critically important for the binding of inhibitors.[1–3]

In conclusion, the first anion inhibition study of the mitochondrial human CA isoform hCA VB is reported here. Fluoride, chloride, bromide, iodide, cyanate, thiocyanate, cyanide, azide, bicarbonate, carbonate, nitrate, nitrite, hydrogen sulfide, bisulfite, sulfate, sulfamide, sulfamic acid, phenylboronic acid and phenylarsonic acid were compared as inhibitors of the two mitochondrial isozymes hCA VA and hCA VB. These enzymes are

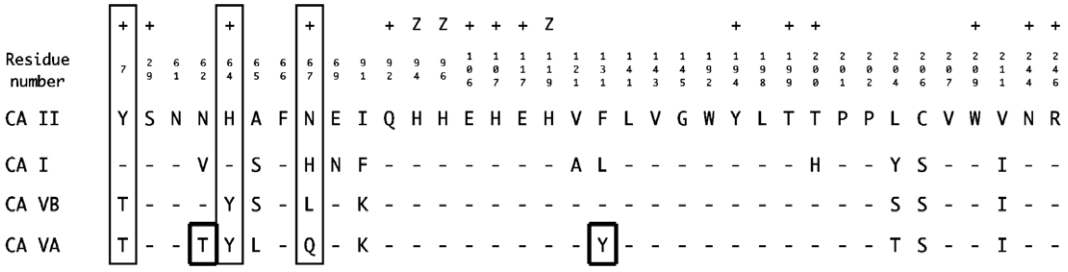

Figure 2. An alignment of active-site residues of CA VA and VB with CA II and CA I (all of human origin). Thirty-six active site residues that were previously[22] defined as forming the active site are aligned. Symbols: +, means active-site hydrogen bond network; Z, zinc-liganded histidine. Three residues of CA II, that participate to the hydrogen bond network, are replaced with different residues in the mitochondrial isozymes (shown in boxes). In comparison with CA II, 8 residues are substituted in CA VB and two additional substitutions are observed in CA VA (bold boxes). Residue numbers are based on the CA I sequence.[1–3]

involved in biosynthetic reactions leading to fatty acid and Krebs cycle intermediates biosynthesis in addition to acting as catalysts for the interconversion of carbon dioxide and bicarbonate. The anion inhibition profiles of the two isoforms are dramatically different. The best hCA VB inhibitors were cyanate, thiocyanate, cyanide and hydrogensulfide (K_Is of 8–76 μM) whereas the least effective ones were the halides (K_Is of 11–72 mM), with the best inhibitor being fluoride and the least effective ones bromide and iodide. Whereas hCA VA is not sensitive to bicarbonate inhibition (K_I of 82 mM) similar to the cytosolic isoform hCA II, hCA VB is well inhibited by this anion, with a K_I of 0.71 mM. Overall, hCA VB is more sensitive to anion inhibitors as compared to hCA VA. Such data confirm earlier suggestions that the two mitochondrial isozymes play distinct physiological functions.

3. Experimental

3.1. Chemistry

Buffers and metal salts (sodium or potassium fluoride, chloride, bromide, iodide, cyanate, thiocyanate, cyanide, azide, bicarbonate, carbonate, nitrate, nitrite, hydrogen sulfide, bisulfite, and sulfate) were of highest purity available from Sigma–Aldrich (Milan, Italy), and were used without further purification. Sulfamide, sulfamic acid, phenylboronic acid and phenylarsonic acid are also commercially available compounds from Sigma–Aldrich (Milan, Italy). The two mitochondrial CA isozymes, hCA VA and hCA VB (full length enzymes), were recombinant ones prepared in-house as described earlier.[10,11,18–20]

3.2. Enzyme inhibition assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity by the Khalifah method.[17] 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 or $NaClO_4$ (for maintaining constant the ionic strength), following 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 (50 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, from Lineweaver–Burk plots, as reported earlier,[18–21] and represent means from at least three different determinations.

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