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Carbonic anhydrase inhibitors. Interaction of isozymes I, II, IV, V, and IX with phosphates, carbamoyl phosphate, and the phosphonate antiviral drug foscarnet

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Abstract—A detailed inhibition study of five carbonic anhydrase (CA, EC 4.2.1.1) isozymes with inorganic phosphates, carbamoyl phosphate, the antiviral phosphonate foscarnet as well as formate is reported. The cytosolic isozyme hCA I was weakly inhibited by neutral phosphate, strongly inhibited by carbamoyl phosphate (K_I of 9.4 μM), and activated by hydrogen- and dihydrogenphosphate, foscarnet and formate (best activator foscarnet, K_A = 12 μM). The cytosolic isozyme hCA II was weakly inhibited by all the investigated anions, with carbamoyl phosphate showing a K_I of 0.31 mM. The membrane-associated isozyme hCA IV was the most sensitive to inhibition by phosphates/phosphonates, showing a K_I of 84 nM for PO_4^{3-}, of 9.8 μM for HPO_4^{2-}, and of 9.9 μM for carbamoyl phosphate. Foscarnet was the best inhibitor of this isozyme (K_I of 0.82 mM) highly abundant in the kidneys, which may explain some of the renal side effects of the drug. The mitochondrial isozyme hCA V was weakly inhibited by all phosphates/phosphonates, except carbamoyl phosphate, which showed a K_I of 8.5 μM. Thus, CA V cannot be the isozyme involved in the carbamoyl phosphate synthetase I biosynthetic reaction, as hypothesized earlier. Furthermore, the relative resistance of CA V to inhibition by inorganic phosphates suggests an evolutionary adaptation of this mitochondrial isozyme to the presence of high concentrations of such anions in these energy-converting organelles, where high amounts of ATP are produced by ATP synthetase, from ADP and inorganic phosphates. The transmembrane, tumor-associated isozyme hCA IX was on the other hand slightly inhibited by all these anions.

1. Introduction

Anions represent the second class of inhibitors of the metallo-enzyme carbonic anhydrase (CA, EC 4.2.1.1), in addition to the sulfonamides/sulfamates/sulfamides with clinical applications as antiglaucoma, antiepileptic, antiobesity, or antitumor drugs.[1–6] Indeed, both metal-complexing anions such as cyanide, cyanate, thiocyanate, halides, azide, hydrogensulfide, etc., as well as anions with a lower tendency to bind metal ions, such as perchlorate, tetrafluoroborate, or sulfate among others,[6–11] were shown to act as inhibitors of the many CA isozymes isolated so far in diverse organisms all over the phylogenetic tree (14 such isozymes are presently known in humans).[1–3,5]

Anions bind to the metal ion within the active site of CA, as shown by means of X-ray crystallography[10] or electronic spectroscopy using the Co(II)-substituted CAs.[12,13] Two types of behavior have been observed so far in α-CAs, including the diverse human isozymes (hCAs) investigated: (i) substitution of the nonprotein fourth zinc ligand (a hydroxide ion or water molecule) by the inhibitor, as anionic species, with formation of a tetrahedral adduct (Fig. 1, Eq. 1, and A, exemplified by the hydrogensulfide adduct[10a]), and (ii) addition of

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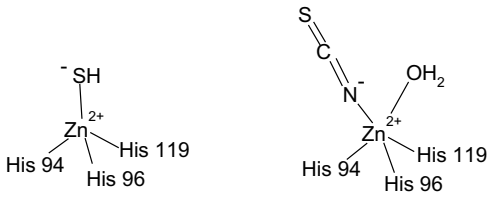
$$\text{E-Zn}^{2+}\text{-OH}_2 + \text{I} \Leftrightarrow \text{E-Zn}^{2+}\text{-I} + \text{H}_2\text{O} \qquad \textbf{(substitution)} \qquad (1)$$

Tetrahedral adduct

$$\text{E-Zn}^{2+}\text{-OH}_2 + \text{I} \Leftrightarrow \text{E-Zn}^{2+}\text{-OH}_2\text{(I)} \qquad \textbf{(addition)} \qquad (2)$$

Trigonal-bipyramidal adduct



Tetrahedral adduct **Trigonal-bipyramidal adduct**

A **B**

Figure 1. CA inhibition mechanism by anions: both tetrahedral (Eq. 1 and hydrogensulfide adduct, A) and trigonal–bipyramidal (Eq. 2 and thiocyanate adduct) species of Zn(II) may be formed.

the inhibitor to the metal coordination sphere, with formation of trigonal–bipyramidal adducts, in which the metal ion within the enzyme active site is coordinated in addition to the three histidine residues (His 94, 96, and 119) by a water molecule and the anion inhibitor (the best studied example of trigonal–bipyramidal coordination is the thiocyanate adduct reported by Liljas and co-workers,[10b] Fig. 1, Eq. 2, and B).

Inhibition of CAs by anions is also important from the physiological point of view, considering the high concentrations of some anions (bicarbonate, chloride, phosphate, etc.) in different tissues, as well as the association of diverse CA isozymes with proteins involved in the transport of anions, such as the anion exchangers (AE) or the sodium bicarbonate co-transporter proteins NBC1 and NBC3, with the formation of metabolons, recently investigated in detail by Casey's group.[14–17] For example, a physical interaction has been identified between hCA II, and the erythrocyte membrane Cl^-/HCO_3^- anion exchanger, AE1, mediated by an acidic motif in the AE1 carboxy terminus.[14–17] It has been proven that the presence of hCA II attached to AE1 accelerates AE1 HCO_3^- transport activity, as AE1 moves bicarbonate either into or out of the cell.[14–17] Functional and physical interactions were also shown to occur between CA II or CA IV and Na^+/HCO_3^- co-transporter isoforms NBC1 and NBC3 by the same group.[14–17] Another metabolon was also recently reported by Sly et al. for the mitochondrial isozyme CA V,[18] which interacts with some gluconeogenic enzymes such as malate dehydrogenase and pyruvate carboxylase.

Among the physiologically important anions which have not been investigated in detail for their interaction with different CA isozymes, are the phosphates, both the inorganic ones as well as the organic phosphates. Except for the investigation of Rowlett et al.[19] regarding CA III interaction with dianions, including hydrogenphosphate, and the work of Carter et al.[20] on the interaction of carbamoyl phosphate with isozymes I, II, and III, no detailed data on this topic are available. Here we report a detailed such study, incorporating a large number of isozymes, such as the cytosolic hCA I and II, the membrane-bound one hCA IV, the mitochondrial isozyme hCA V, as well as the tumor-associated, transmembrane hCA IX, together with the three inorganic species of phosphate, carbamoyl phosphate, the antiviral phosphonate foscarnet,[21] as well as formate (foscarnet, phosphonoformic acid trisodium salt, also contains a carboxylate moiety, and this was the reason why formate has been included in our study). The rather bulky nucleoside-mono-, di-, or tri-phosphates (such as ATP, ADP, GTP, etc.) have not been included in this work since preliminary molecular modeling (data not shown) suggests that they are too sterically impaired for being able to bind within the CA active site.

2. Chemistry

Buffers and metal salts (sodium hydrogenphosphate, dihydrogenphosphate, formate, trisodium phosphate, carbamoyl phosphate disodium salt) were of highest purity available, from Sigma–Aldrich (Milano, Italy) and were used without further purification. Foscarnet (trisodium salt) was from Pharmacia Co.

3. CA inhibition data

Inhibition data against five CA isozymes, that is, hCA I, hCA II (cytosolic forms), hCA IV (membrane-associated), hCA V (mitochondrial), and hCA IX (transmembrane),[22] with the above mentioned anions are shown in Table 1. Inhibition data for bicarbonate (one of the CA substrates) and carbonate are also provided for comparison, as they were recently reported by this group.[7,8]

Table 1. Inhibition constants of inorganic phosphates, carbamoyl phosphate, foscarnet, formate, and bicarbonate/carbonate against isozymes hCA I, II, IV, V, and IX, for the CO_2 hydration reaction, at 20 °C[22]

Inhibitor*	K_I# (mM)				
	hCA I[a]	hCA II[a]	hCA IV[a]	hCA V[b]	hCA IX[c]
PO_4^{3-}	0.78	0.53	8.4×10^{-5}	6.87	0.56
HPO_4^{2-}	A	13.2	9.8×10^{-3}	4.49	3.67
$H_2PO_4^-$	A	3.06	7.57	3.30	21.1
$H_2NCOOPO_3^{2-}$	9.4×10^{-3}	0.31	9.9×10^{-3}	8.5×10^{-3}	3.06
$^-OOC–PO_3^{2-}$**	A	14.2	0.82	41.7	2.21
$HCOO^-$	A	24.0	1.25	9.97	1.22
HCO_3^-	12	85	6.6	82	13
CO_3^{2-}	15	73	5.7	95	NT

A = activator: K_A ($H_2PO_4^-$ = 1 mM; K_A (foscarnet) = 12 μM, K_A (formate) = 5 mM (K_A = activation constant). Monohydrogenphosphate is a very weak activator and K_A could not be determined (a maximal activation of 120% has been achieved at 20 mM of HPO_4^{2-}). NT = not tested.

* As sodium salts.

** Foscarnet (trisodium salt).

Errors were in the range of 3–5% of the reported values, from three different assays.

[a] Human cloned isozymes.

[b] Recombinant, full-length form of hCA V.

[c] Catalytic domain of the human, recombinant isozyme.

Data of Table 1 allow us to draw the following conclusions regarding CA interaction with the phosphates/phosphonate investigated here: (i) isozyme hCA I is inhibited by neutral phosphate (PO_4^{3-}) and carbamoyl phosphate, and it is activated by hydrogen- and dihydrogenphosphate, foscarnet, and formate. Thus, PO_4^{3-} is a more effective hCA I inhibitor as compared to bicarbonate or carbonate (K_I of 0.78 mM), whereas carbamoyl phosphate is indeed a very potent hCA I inhibitor, with an inhibition constant of 9.4 μM, of the same order of magnitude as many sulfonamide CA inhibitors.[1–5] Thus, this last data confirms the previous work of Carter et al.[20] who also investigated the interaction of carbamoyl phosphate with hCA I, II, and III. What appears rather surprising at a first glance is the activatory properties of some of the investigated anions on hCA I, such as hydrogen- and dihydrogenphosphate, foscarnet and formate. CA activators have been described from many classes of compounds, such as amines, amino acids, peptides, azoles, and anions,[23,24] and the X-ray crystal structure of two adducts of activators with isozyme hCA II have been reported by this group.[25,26] Thus, it is presently generally accepted that activators bind at a distinct site of that of the inhibitors within the enzyme active cavity[25,26] and participate to the rate-limiting step of the CA catalytic cycle, a proton transfer reaction between the active site and the reaction medium, shuttling protons by means of groups possessing an appropriate pK_a. Indeed, Rowlett et al.[19] showed that hydrogenphosphate acts as a weak activator (K_A of around 1 mM) of isozyme CA III, and it seems that this anion, as well as the other ones mentioned above (dihydrogenphosphate, foscarnet, and formate) show the same behavior against isozyme hCA I. Still, this is the first example in which it is observed that structurally related anions such as the phosphates/phosphonate/carboxylate investigated here may act either as inhibitors or as activators of the same isozyme (e.g., all these anions act only as inhibitors of the other isozymes investigated here, see later in the text). Hydrogenphosphate acts as a very weak CA I activator (a maximal activation of around 120% is achieved at about 20 mM concentration of activator—Table 1), dihydrogenphosphate and formate are weak activators (activation constants in the range of 1–5 mM), whereas foscarnet is a potent activator, with an activation constant of 12 μM, in the same range as that of histamine, the best studied CA activator.[25] The most remarkable of all these data are the potent inhibitory properties of carbamoyl phosphate against hCA I, and the good activatory properties of foscarnet, but it is rather difficult to hypothesize whether this may have physiological consequences, due to the fact that the precise physiological function of this quite abundant isozyme is very much unknown;[5] (ii) against hCA II, one of the physiologically most important isozymes, all the anions investigated here show inhibitory properties (Table 1). Thus, the best hCA II inhibitors are PO_4^{3-} and carbamoyl phosphate (inhibition constants in the range of 0.31–0.53 mM), followed by dihydrogenphosphate (K_I of 3.06 mM), whereas hydrogenphosphate, foscarnet, and formate are weaker anionic inhibitors (K_Is in the range of 13.2–24.0 mM), being much more effective than carbonate and bicarbonate, which show inhibition constants in the range of 73–85 mM; (iii) the most susceptible isozyme to inhibition by phosphates seems to be hCA IV (Table 1). Thus, PO_4^{3-} acts as a very potent hCA IV inhibitor, with an inhibition constant of 84 nM, being one of the best anion inhibitor ever reported for any CA isozyme.[6] HPO_4^{2-} and carbamoyl phosphate are also very effective hCA IV inhibitors, with inhibition constants in the range of 9.8–9.9 μM, whereas foscarnet and formate are weak inhibitors (K_Is in the range of 0.82–1.25 mM). Weak CA inhibitors are also bicarbonate, carbonate, and dihydrogenphosphate, with inhibition constants in the range of 5.7–7.57 mM. It is rather difficult to explain this very different behavior of isozyme hCA IV toward the phosphates, and work is in progress in this laboratory for determining the X-ray crystal structure of adducts of these anions with hCA IV, in order to rationalize this behavior. It is also noteworthy to mention that the isozyme most sensitive to inhibition by foscarnet is just

hCA IV, which is very abundant in the kidneys, where it plays an important physiological function in the bicarbonate reabsorption and secretion of ammonium ions into urine among others.[5] Since foscarnet shows an appreciable renal toxicity,[21] it may be hypothesized that hCA IV inhibition by this antiviral drug may also play a role in these side effects of foscarnet. It is not possible to understand at this point whether foscarnet interacts with the zinc ion of CA by means of its phosphonate or by means of its carboxylate moiety (both foscarnet and formate show rather similar CA IV inhibitory properties). Thus, an X-ray crystal structure of this drug with hCA IV is highly desirable; (iv) the mitochondrial isozyme hCA V is also inhibited by all the investigated anions (Table 1). The best inhibitor is by far carbamoyl phosphate, which with an inhibition constant of 8.5 μM, is at least three orders of magnitude a better inhibitor as compared to other phosphates/phosphonate investigated here. Indeed, the inorganic phosphates as well as formate are weak hCA V inhibitors (K_Is in the range of 3.30–9.97 mM), whereas foscarnet has the lowest affinity for this isozyme, as compared to all other CAs investigated here (K_I of 41.7 mM), being anyhow a better inhibitor as compared to bicarbonate/carbonate (K_Is in the range of 82–95 mM). These data allow us to refute the hypothesis of Chegwidden et al.[27] according to which CA V is involved in the reaction catalyzed by carbamoyl phosphate synthetase I (CPS I). It is stated[27] that this mitochondrial enzyme (CPS I) catalyzes the formation of carbamoyl phosphate from ammonium ions, bicarbonate, and ATP, and that the bicarbonate needed for this carboxylation (not CO_2, which is not the real substrate of CPS I) is generated inside the mitochondria through the activity of CA V (the only CA isozymes present within mitochondria). From our data, it is obvious that CA V is highly inhibited by very low concentrations of carbamoyl phosphate (hCA V is the isozyme with the highest affinity for this compound, together with hCA I and IV), so that it is probable that this isozyme cannot play the role hypothesized by the cited authors.[27] From data of Table 1, it may be observed that the only nontumor associated CA isozyme that is resistant to carbamoyl phosphate inhibition is CA II (K_I of 0.31 mM), and that probably the bicarbonate needed for this mitochondrial biosynthetic step catalyzed by CPS I is generated in and transported from the cytosol to the mitochondria by means of mechanisms which remain to be identified, in which CA V or one of its metabolons[18] may be involved. On the other hand, the relative resistance of CA V to inhibition by inorganic phosphates (as compared to hCA IV, e.g.) may be due to an evolutionary adaptation of this isozyme to the presence of high concentrations of such anions in these energy-converting organelles, where high amounts of ATP are produced by ATP synthetase, from ADP and inorganic phosphates;[28] (v) the tumor-associated, transmembrane isozyme hCA IX is also inhibited by all the anions investigated here. Thus, PO_4^{3-}, HPO_4^{2-}, carbamoyl phosphate, foscarnet, and formate are weak CA IX inhibitors (K_Is in the range of 0.56–3.67 mM), whereas dihydrogenphosphate and bicarbonate are even weaker (K_Is in the range of 13–21.1 mM).

4. Conclusion

We report here the first detailed inhibition study of five CA isozymes with inorganic phosphates, carbamoyl phosphate, the antiviral phosphonate foscarnet as well as formate. The cytosolic isozyme hCA I was weakly inhibited by neutral phosphate, strongly inhibited by carbamoyl phosphate (K_I of 9.4 μM), and activated by hydrogen- and dihydrogenphosphate, foscarnet, and formate (the best activator was foscarnet, with an activation constant $K_A = 12$ μM). The cytosolic isozyme hCA II was weakly inhibited by all the investigated anions, with carbamoyl phosphate the best inhibitor, showing a K_I of 0.31 mM. The membrane-associated isozyme hCA IV was the most sensitive to inhibition by phosphates/phosphonates, showing a K_I of 84 nM for PO_4^{3-}, of 9.8 μM for HPO_4^{2-}, and of 9.9 μM for carbamoyl phosphate. Foscarnet was the best inhibitor of this isozyme (K_I of 0.82 mM), which is highly abundant in the kidneys, and which may explain some of the renal side effects of this antiviral drug. The mitochondrial isozyme hCA V was weakly inhibited by all phosphates/phosphonates, except carbamoyl phosphate, which showed a K_I of 8.5 μM. Thus, CA V cannot be the isozyme involved in the carbamoyl phosphate synthetase I biosynthetic reaction, as hypothesized some years ago. Furthermore, the relative resistance of CA V to inorganic phosphate inhibition suggests an evolutionary adaptation of this mitochondrial isozyme to the presence of high concentrations of such anions in these energy-converting organelles, where high amounts of ATP are produced by ATP synthetase, from ADP and inorganic phosphates. The transmembrane, tumor-associated isozyme hCA IX was only slightly inhibited by all these anions.

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