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Paraoxon, 4-nitrophenyl phosphate and acetate are substrates of α- but not of β-, γ- and ζ-carbonic anhydrases

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ABSTRACT

Carbonic anhydrases (CAs, EC 4.2.1.1) belonging to α-, β-, γ- and ζ-classes and from various organisms, ranging from the bacteria, archaea to eukarya domains, were investigated for their esterase/phosphatase activity with 4-nitrophenyl acetate, 4-nitrophenyl phosphate and paraoxon as substrates. Only α-CAs showed esterase/phosphatase activity, whereas enzymes belonging to the β-, γ- and ζ-classes were completely devoid of such activity. Paraoxon, the metabolite of the organophosphorus insecticide parathione, was a much better substrate for several human/murine α-CA isoforms (CA I, II and XIII), with k_{cat}/K_M in the range of 2681.6–4474.9 M^{-1} s^{-1}, compared to 4-nitrophenyl phosphate (k_{cat}/K_M of 14.9–1374.4 M^{-1} s^{-1}).

Carbonic anhydrases (CAs, EC 4.2.1.1) catalyze a very simple but physiologically essential reaction in all life kingdoms, the hydration of carbon dioxide to bicarbonate and protons, with a high efficiency.[1,2] Although this reaction also occurs without a catalyst, it is too slow to account for physiologic requirements in many biological systems where this interconversion is needed (e.g., photosynthesis in plants, algae, diatoms and some bacteria;[3,4] various electrolyte-secreting tissues in mammals;[5–7] some tumors in which several CA isoforms are involved in tumor progression and metastasis,[8–10] biosynthetic processes such as gluconeogenesis, ureagenesis or lipogenesis,[1,5,6] etc.). Indeed, CO_2, bicarbonate and protons are essential molecules/ions in many important physiologic processes in all life kingdoms (*Bacteria*, *Archaea*, and *Eukarya*) throughout the phylogenetic tree, and high rates of their interconversion are necessary sometimes in conditions in which the spontaneous reaction cannot occur. For example, CA IX, a tumor-associated α-CA[8–10] has a very high turnover (of 7.5×10^7 M^{-1} s^{-1}) for CO_2 hydration to bicarbonate even at a pH of 6.5 (typical of hypoxic tumors in which this enzyme is over-expressed) although the spontaneous, non-catalytic CO_2 hydration at this pH value is very low.[10]

These enzymes are also excellent examples of convergent evolution, as five distinct genetic families (α-, β-, γ-, δ-, and ζ-CAs) were discovered so far.[1–4] Mammals possess 16 different α-CA isoforms, which are involved in many crucial physiological or pathological 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, bone resorption, calcification, tumorigenicity, etc.[1,2,5–7] Some of them are cytosolic (CA I, CA II, CA III, CA VII and CA XIII), others are membrane-bound (CA IV, CA IX, CA XII, CA XIV and CA XV), CA VA and CA VB are mitochondrial, and CA VI is secreted in saliva and milk. Three acatalytic isoforms are also known, denominated sometimes CA related proteins (CARP), that is, CA VIII, CA X and CA XI, which are cytosolic proteins too.[1,2,5–7]

In addition to CO_2 hydration to bicarbonate and protons, at least the α-CAs are known to possess other catalytic activities, such as among others esterase activity (with 4-nitrophenyl acetate, 4-NPA, and other activated esters).[11] The related ester 4-nitrophenyl phosphate is also a CA substrate, whereas 4-nitrophenyl sulfate is not at all hydrolyzed by these enzymes, as recently shown by our group.[11] Indeed, in a recent study we observed that the α-CA isozymes (of mammalian, human(h) or murine (m) origin), hCA I, hCA II and mCA XIII show a good esterase activity with 4-nitrophenyl acetate as substrate, with second order rate constants in the range of 753–7706 M^{-1} s^{-1}, being slightly less effective as phosphatases with 4-nitrophenyl phosphate (4-NPP) **1** as substrate (k_{cat}/K_M in the range of 14.89–1374.40 M^{-1} s^{-1}) and totally ineffective as sulfatases (with 4-nitrophenyl sulfate as substrate).[11] As shown in Scheme 1, hydrolysis of **1** mediated by CAs leads to the formation of 4-nitrophenol **2**.

It is presently not known whether enzymes belonging to other classes than the α-CAs possess this catalytic versatility mentioned

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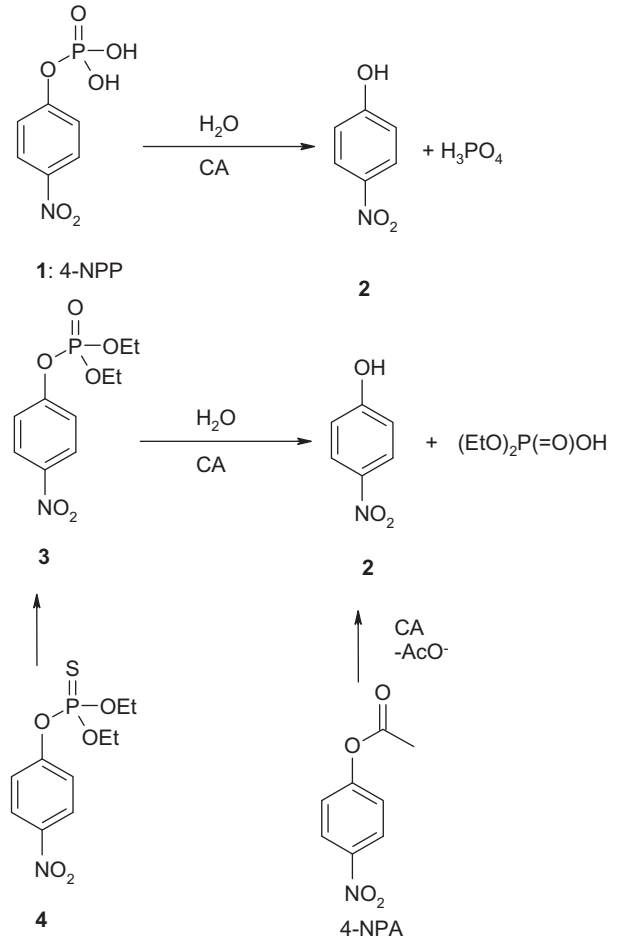

Scheme 1. Hydrolysis of the phosphate esters 4-nitrophenyl phosphate (4-NPP) **1** and paraoxon **3** to 4-nitrophenol **2**. The insecticide parathion **4** is metabolized to paraoxon **3**, which we demonstrate here to be a CA substrate. 4-Nitrophenylacetate (4-NPA) is also hydrolyzed by CAs with formation of **2** and acetate.

above, that is, in addition to the hydration of CO_2 to bicarbonate and protons, it is unknown whether the β-, γ-, δ-, and ζ-CAs may also act as esterases, phosphatases or eventually sulfatases. On the other hand, only 4-nitrophenyl phosphate **1** has been investigated to date as a possible substrate for the phosphatase activity of these enzymes,[11] although phosphate esters are highly relevant in a host of physiological processes (as recently reviewed by Blackburn,[12] some of the most important ones being: DNA stabilization; the RNA world, presumably the first forms of self-replication made possible by ribozymes; phospholipid; skeletal structures; organelle membrane recognition mediated by phosphoinositols; energy rich phosphates such as ATP, ADP; phosphorylation/dephosphorylation of proteins mediated by kinases/phosphatases; second messengers such as cGMP, cAMP, etc., just to mention the really essential ones).[12,13]

In this contribution we try to reply to some of the question raised above, that is, whether the non-α-CAs show catalytic versatility, possessing esterase and/or phosphatase activity. We also report here that paraoxon **3**, an organophosphorus compound acting as acetylcholinesterase inhibitor[14,15] (being the metabolite of parathion **4**, a widely used insecticide)[16,17] and structurally rather similar to 4-NPP **1**, is a much better α-CA substrate compared to the phosphate **1**.

In Table 1 the kinetic parameters (as k_{cat}/K_M) for several CAs belonging to various enzyme families (α-, β-, γ- and ζ-CA family) and found in organisms from the bacteria, archaea or eukarya domains, are shown, for the CO_2 hydration, 4-NPA hydrolysis and 4-NPP hydrolysis reactions.[18–27] The CO_2 hydrase activities of all these enzymes (human (h) and murine (m) CAs belonging to the α-class, under the form of several isoforms, such as hCA I, hCA II and mCA XIII;[11] an α-CA from the bacterium *Helicobacter pylori*, hpαCA,[21] the β-class CA from the same organism, hpβCA;[22] another bacterial enzyme, from *Brucella suis*, bsCA 1,[23] Cab, a β-CA from the archaeon *Methanobacterium thermoautotrophicum*; ScCA,[24] the β-CA from the yeast *Saccharomyces cerevisiae*;[25] Cam, the prototypical γ-CA from the archaeon *Methanosarcina thermophila*;[26] as well as the cadmium/zinc-containing CA1 repeat (Cd/Zn(II)-R1) from the marine diatom *Thalassiosira weissflogii*)[27] are quite high, with turnovers in the range of 8.3×10^4–1.6×10^8 M^{-1} s^{-1} (Table 1). We have included in our experiments enzymes belonging to very diverse organisms (bacteria, archaea, diatoms, yeasts and mammals) and four of the five classes (details on δ-CAs are very scarce in the literature and no enzyme from this family has been thoroughly characterized so far)[28] in order to investigate in detail the putative catalytic versatility of CAs for other reactions than CO_2 hydration. These enzymes show medium-low CO_2 hydrase activity (mCA XIII and Cab), moderate such activity (hCA I, the two enzymes from *H. pylori*, bsCA 1, ScCA and Cam) or very high CO_2 hydrase activity (hCA II, Cd(II)-R1 and Zn(II)-R1). Thus, the hydrase activity may be quite efficient irrespective whether the enzymes belong to the α-, β-, γ- and ζ-CA classes. However, as seen from data of Table 1, only the α-CAs show also esterase and phosphatase activity, whereas for the various enzymes belonging to the β-, γ- and ζ-CA family, such activities were not detected. The k_{cat}/K_M values for these reactions are on the other hand orders of magnitude lower compared to the CO_2 hydrase activity. Indeed, for 4-NPA hydrolysis the k_{cat}/K_M values ranged between 654 and 7706 M^{-1} s^{-1} (the best esterase was mCA XIII which possesses the lowest hydrase activity) whereas for the phosphate ester **1** hydrolysis, they were in the range of 38.9–1374.4 M^{-1} s^{-1} (again with isoform CA XIII the best phosphatase, Table 1). It should be mentioned that the phosphatase activity of all α-CAs with **1** as substrate showed a bell-shaped pH dependency with an optimal activity between pH 7.1 and 7.5. At pH values >8, this activity started to be highly diminished probably due to the fact that the zinc-hydroxide moiety of the enzyme has repulsive interactions with the ester **1** which is a dianion at that pH.

The explanation of this lack of esterase or phosphatase activity for the non-α-CAs is not straightforward, considering the fact that the metal ion coordination is rather similar at least between the α- and γ-CAs (three His residues and a water molecule/hydroxide ion) whereas for most of the β- and all ζ-CAs this is accomplished by two Cys, one His and the catalytically essential water molecule/hydroxide ion.[1–5] However, it should be mentioned that the active site dimensions are very much different between these enzymes, with the α-CAs possessing a wide and deep cavity of around 15×15 Å^2,[29] the β-CAs possess a channel of around 5 Å deep (much shallower compared to the α-CA active site),[30] whereas the γ-CA has the active site even smaller, a cleft at the interface between two adjacent subunits of the protein homotrimer.[3a–c] However, the ζ-CA shoes a bigger active site compared to the β- and γ-CAs, of around 7×7 Å^2.[4] Maybe, the access of substrate much bulkier than CO_2, such as the esters 4-NPA and 4-NPP, is very much hindered in the smaller active sites of the enzymes belonging to the β-, γ- and ζ-CA classes, whereas it may occur much easier in the wide and deep active site of the α-CAs. We have also investigated the sulfatase activity of the enzymes shown in Table 1, with 4-nitrophenyl sulfate as substrate, and all of them, similar to the mammalian ones investigated earlier,[11] are devoid of esterase activity (data not shown).

In order to see whether other organic phosphates may act as substrates of the α-CAs, we investigated whether paraoxon **3**, an ester structurally related to 4-NPP **1**, may be hydrolyzed enzymat-

Table 1
Kinetic parameters for various reactions catalyzed by CAs, at 25 °C[18–20]

Enzyme[a]	Class	CO_2 hydration	k_{cat}/K_M (M^{-1} s^{-1})		Ref.
			4-NPA hydrolysis	4-NPP hydrolysis	
hCA I	α	5.0×10^7	753	65.55	11
hCA II	α	1.5×10^8	2607	14.89	11
mCA XIII	α	8.3×10^4	7706	1374.4	11
hpαCA	α	1.5×10^7	654	38.9	This study, 21
hpβCA	β	4.8×10^7	0	0	This study, 22
bsCA 1	β	3.9×10^7	0	0	This study, 23
Cab	β	1.8×10^6	0	0	This study, 24
ScCA	β	9.8×10^7	0	0	This study, 25
Cam	γ	3.9×10^6	0	0	This study, 26
Cd(II)-R1	ζ	1.4×10^8	0	0	This study, 27
Zn(II)-R1	ζ	1.6×10^8	0	0	This study, 27

For the CO_2 hydration reaction the pH was of 7.4 for the α-, γ- and ζ-class enzymes and of 8.3 for the β-CAs (which show much lower activity at pH <8).[1–3] The CO_2 hydrase activities of all these enzymes were reported earlier,[11,22–27] whereas the esterase ones only for the mammalian CAs.[11] The esterase and phosphatase activities provided in the table were measured at a pH of 7.4 but many other pH values (between 5.5 and 9.0) have been investigated without measuring any catalytic enhancement in the presence of β-, γ- and ζ-CAs.

[a] h = human, m = murine isoforms; hpCA = *Helicobacter pylori* CA; bsCA = *Brucella suis* CA; Cab = *Methanobacterium thermoautotrophicum* enzyme; ScCA = *Saccharomyces cerevisiae* CA; Cam = *Methanosarcina thermophila enzyme*; Cd(II)-R1 = R1 repeat of cadmium-containing CA1 from *Thalassiosira weissflogii*; Zn(II)-R1 = R1 repeat of zinc-containing CA1 from *T. weissflogii*.

ically by α-CAs (hCA I, hCA II and mCA XIII, Table 2). It should be mentioned that there are enzymes, named paraoxonases (PON, EC 3.1.8.1), known to efficiently hydrolyze **3**.[31–33] At least three PON isoforms are known so far in humans (h), hPON 1–hPON 3. hPON1 is associated with high-density lipoprotein (HDL), and is a genetically polymorphic enzyme that has the ability to hydrolyze a wide range of organophosphates and carboxylic acid esters.[31] hPON 1 active site contains a Ca(II) ion critical for catalysis (and a second structural such ion) coordinated by five protein residues (the side chain oxygens of Asn224, Asn270, Asn168, Asp269 and Glu53) and by a water molecule which in deprotonated form (as hydroxide ion) acts as nucleophile for the hydrolysis of substrates.[31] Another potential ligand of the catalytic metal ion is an oxygen of the phosphate substrate which will undergo hydrolysis, but the rate determining step of the catalytic turnover is the deprotonation of the calcium-coordinated water molecule, assisted by a His–His dyad,[31] which very much resembling the CA catalytic cycle, in which a Zn(II) hydroxide species is the catalytically active nucleophile, which is generated by deprotonation of zinc-coordinated water assisted by a His residue from the enzyme active site.[1–3] Thus, two very different enzymes, with very diverse metal ions in the active site share a rather similar catalytic mechanism. This, and the fact that we showed earlier[11] that some α-CAs possess phosphatase activity with 4-NPP as substrate, prompted us to investigated paraoxon **3** as a possible substrate of CAs.

As seen from data of Table 2, paraoxon **3** is a good substrate for the three mammalian α-CAs investigated here, with k_{cat}/K_M values in the range of 2681.6–4474.9 M^{-1} s^{-1} (Table 2). Thus, paraoxon **3** is a much better substrate compared to 4-NPP **1**. Probably the presence of the two additional ethyl moieties in **3** allows a better interaction with the enzyme active site compared to the parent ester **1**. Indeed, it may be observed that the K_M values are rather different but of the same order of magnitude for the two esters and a particular CA isoform (except mCA XIII, for which there is a difference of almost one order of magnitude between the K_M of the two esters, with paraoxon showing a value of 2.20 mM and phosphate **1** of only 0.232 mM). The k_{cat}/K_M values are on the other hand very different when comparing the two substrates. Indeed, hCA I and hCA II showed a very weak phosphatase activity with 4-NPP **1** as substrate (turnovers of 14.89–65.55 M^{-1} s^{-1}), and as mentioned above, only mCA XIII had a relevant phosphatase activity with this ester. However, with **3** as substrate, all these enzymes behave as rather efficient phosphatases (Table 2), and it may be thus noted that they behave as 'paraoxonases'. It should be mentioned that CA XIII is the unique member of this enzyme family having a Val in position 200.[1,2] This amino acid residue (His in CA I and Thr in CA II) is essential for the binding of substrates and inhibitors to the CAa,[1,2] and its more hydrophobic nature in CA XIII may facilitate the binding of hydrophobic esters such as 4-NPP and paraoxon. Furthermore, this activity (with both phosphates as substrates) was inhibited by the clinically used sulfonamide acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), with IC_{50} values in the range of 59–1050 nM (Table 2). This is a proof that the zinc hydroxide mechanism responsible for the CO_2 hydrase activity of these enzymes is also responsible of the esterase and phosphatase activity of the α-CAs. The phosphatase activity of these α-CAs with paraoxon as substrate showed no pH dependency between pH 7.1 and 8.5, probably because at these pH values there is a substantial amount of the enzyme in the zinc hydroxide form, and paraoxon is in neutral form, unlike 4-NPP which may be a mono- or dianion, depending on the pH in the assay system.

In conclusion, we prove here that only CAs belonging to the α-class but not the β-, γ- and ζ-classes show esterase/phosphatase activity with 4-nitrophenyl acetate, 4-nitrophenyl phosphate and paraoxon as substrates. Paraoxon, the metabolite of the organophosphorus compound parathione, was a much better substrate for several human/murine α-CA isoforms (CA I, II and XIII), with k_{cat}/K_M in the range of 2681.6–4474.9 M^{-1} s^{-1}, compared to 4-nitrophenyl phosphate (k_{cat}/K_M of 14.9–1374.4 M^{-1} s^{-1}). α-CAs show thus a modest but significant paraoxonase activity.

Table 2
Kinetic parameters for the hydrolysis of 4-nitrophenyl phosphate (**1**) and paraoxon (**3**) in the presence of cytosolic CA isoforms I, II and XIII, at pH 7.4 and 25 °C, and inhibition data with acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide)

Isozyme[a]	Substrate	k_{cat}/K_M (M^{-1} s^{-1})	K_M (mM)	IC_{50} (nM)
hCA I	**1**	65.55 ± 5.2	0.935 ± 0.10	330 ± 14
hCA I	**3**	3893.0 ± 14.5	1.683 ± 0.12	338 ± 12
hCA II	**1**	14.89 ± 0.54	2.195 ± 0.20	63 ± 5
hCA II	**3**	4474.9 ± 58	1.645 ± 0.28	59 ± 4
mCA XIII	**1**	1374.4 ± 62	0.232 ± 0.02	1050 ± 76
mCA XIII	**3**	2681.6 ± 47	2.202 ± 0.14	1021 ± 63

Concentrations of the substrates **1** and **3** ranged between 0.08 and 5 mM. The data are provided as the mean ± standard deviation (from at least three different assays).[18,19]

[a] h = human, m = murine isoform.

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18. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561. An applied photophysics stopped-flow instrument has been used for assaying the CA catalysed 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 10–20 mM Hepes (pH 7.5, for α- and γ-CAs) or Tris (pH 8.3 for β-CAs) as buffers, and 20 mM Na_2SO_4 (for α-CAs) or 10–20 mM NaCl—for β-CAs (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. For each concentration of the substrate, 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 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, whereas the kinetic parameters for the uninhibited enzymes from Lineweaver–Burk plots, as reported earlier,[11] and represent the mean from at least three different determinations.
19. Pocker, Y.; Stone, J. T. *Biochemistry* **1968**, *7*, 2936. 4-Nitrophenyl acetate (4-NPA) hydrolysis was monitored by measuring the absorbance at 405 nm of the chromogenic compound 4-nitrophenol **2** which is released by the hydrolysis of the ester (extinction coefficient of 10510 M^{-1}).[11] Reactions were performed at 25 °C in a quartz cuvette with 1 cm light-path, using a Perkin Elmer Lambda Bio20 UV–vis spectrometer. Enzyme concentrations were between 0.10 and 5.0 μM and substrate concentration between 0.08 and 0.37 mM. The substrate was dissolved in freshly distilled acetonitrile and diluted with buffer (10 mM HEPES and 10 mM Tris, pH 7.4, maintaining the ionic strength constant by addition of 0.1 M sodium sulfate, in such a way that the final concentration of MeCN was of 5%). Kinetic parameters were determined (in cases in which the substrate solubility allowed this, i.e., concentrations of 4-NPA of 0.3–5.0 mM) by fitting the data to the Michaelis–Menten model (Eq. 1):

$$\mathrm{Vo} = (k_{\mathrm{cat}}[E]_0[S]_0)/([S]_0 + K_{\mathrm{M}}) \quad (1)$$

Otherwise, $k_{\mathrm{cat}}/K_{\mathrm{M}}$ values were derived from a linear fit (Eq. 2) by using PRISM:[11]

$$\mathrm{Vo} = [E]_0[S]_0(k_{\mathrm{cat}}/K_{\mathrm{M}}) \quad (2)$$

The rates of spontaneous hydrolysis (without enzyme) were subtracted from the enzymatic rates. Inhibition with acetazolamide has been used as a control, being performed as described above, by titration of the enzymes with acetazolamide solutions in concentration ranges between 10 nM to 100 μM. IC_{50} represents the molarity of inhibitor producing a 50% decrease of the enzyme activity and were determined from semilogarithmic plots of enzyme activity versus molarity of inhibitor.[11]
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