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Investigations of the esterase, phosphatase, and sulfatase activities of the cytosolic mammalian carbonic anhydrase isoforms I, II, and XIII with 4-nitrophenyl esters as substrates

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Abstract—The esterase, phosphatase, and sulfatase activities of carbonic anhydrase (CA, EC 4.2.1.1) isozymes, CA I, II, and XIII with 4-nitrophenyl esters as substrates was investigated. These enzymes show esterase activity with 4-nitrophenyl acetate as substrate, with second order rate constants in the range of 753–7706 M^{-1} s^{-1}, being less effective as phosphatases (k_{cat}/K_M in the range of 14.89–1374.40 M^{-1} s^{-1}) and totally ineffective sulfatases. The esterase/phosphatase activities were inhibited by sulfonamide CA inhibitors, proving that the zinc-hydroxide mechanism responsible for the CO_2 hydrase activities of CAs is also responsible for their esterase/phosphatase activity. CA XIII was the most effective esterase and phosphatase. CA XIII might catalyze other physiological reactions than CO_2 hydration, based on its relevant phosphatase activity.

Carbonic anhydrases (CAs, EC 4.2.1.1) are among the most efficient catalysts known in Nature.[1] Some of the 16 isozymes presently characterized so far in mammals,[1–3] catalyze CO_2 hydration to bicarbonate and a proton (the physiological reaction in which CAs participate, reaction 1 in Scheme 1) with turnover numbers close to the limits of the diffusion controlled processes, that is, of around 10^8 M^{-1} s^{-1} (for example the human isozyme hCA II, see Table 1).[1–3] On the other hand, hCA II, one of the most effective and best studied such enzymes, is not only a very effective catalyst for the physiological reaction (Table 1), but also shows some catalytic versatility, participating in other hydrolytic processes which presumably involve non-physiological substrates. Some of these reactions include the hydration of cyanate to carbamic acid (reaction 2, Scheme

$O=C=O + H_2O \Leftrightarrow HCO_3^- + H^+$	(1)
$O=C=NH + H_2O \Leftrightarrow H_2NCOOH$	(2)
$HN=C=NH + H_2O \Leftrightarrow H_2NCONH_2$	(3)
$RCHO + H_2O \Leftrightarrow RCH(OH)_2$	(4)
$RCOOAr + H_2O \Leftrightarrow RCOOH + ArOH$	(5)
$RSO_3Ar + H_2O \Leftrightarrow RSO_3H + ArOH$	(6)
$ArF + H_2O \Leftrightarrow HF + ArOH$ (Ar = 2,4-dinitrophenyl)	(7)
$PhCH_2OCOCl + H_2O \Leftrightarrow PhCH_2OH + CO_2 + HCl$	(8)
$RSO_2Cl + H_2O \Leftrightarrow RSO_3H + HCl$ (R = Me; Ph)	(9)
$ArOPO_3H_2 + H_2O \Leftrightarrow ArOH + H_3PO_4$	(10)

Scheme 1. Reactions (1–10) catalyzed by α-CAs.

Keywords: Carbonic anhydrase; Cytosolic isozyme; Esterase; Phosphatase; Sulfatase; 4-Nitrophenyl esters.
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Table 1. Kinetics parameters for the CO_2 hydration reaction catalyzed by the cytosolic mammalian CA isozymes I, II, III, and XIII at 25 °C and pH 7.4, and their inhibition with acetazolamide

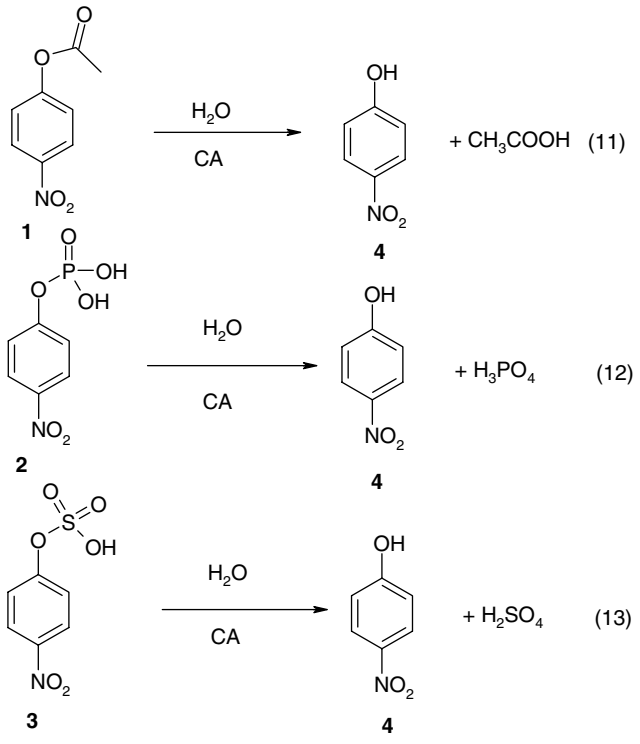
Isozyme*	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})	K_I (acetazolamide) (nM)	Reference
hCA I	2.0×10^5	4.0	5.0×10^7	250	1
hCA II	1.4×10^6	9.3	1.5×10^8	12	1
hCA III	1.3×10^4	52.0	2.5×10^5	2×10^5	12
hCA XIII	1.5×10^5	13.8	1.1×10^7	16	Unpublished results
mCA XIII	8.3×10^4	19.3	4.3×10^6	17	13

* h = human, m = mouse isoform.

1),[4] or of cyanamide to urea (reaction 3, Scheme 1),[5] the aldehyde hydration to *gem*-diols (reaction 4);[6] the hydrolysis of some carboxylic,[7,8] or sulfonic acid esters (reactions 5 and 6),[8] as well as other less investigated hydrolytic processes in which aryl halides, chloroformates or sulfonyl chlorides[1–3,9] act as substrates, as described by Eq. 7–9 of Scheme 1.

A rather controversial issue regards the possible phosphatase activity of CA III, an isozyme with very low CO_2 hydration activity (Table 1), which has originally been reported to be a phosphatase (with 4-nitrophenyl phosphate as substrate, reaction 10 in Scheme 1)[10] but subsequently this has been retracted,[11] being considered that the phosphatase activity is due to another protein impurity present in the CA III preparations used in the earlier investigations.[10] It has also been originally considered that the phosphatase activity of CA III is not due to the zinc-hydroxide functionality present in the active site of this and all other α-CAs from mammals[10b] (which acts as a strong nucleophile—equivalent to the hydroxide ion in solution—against many electrophiles, but at pH values in the physiological range, since the Zn(II) ion in the hydrophobic environment of the enzyme active site strongly acidifies the coordinated water molecule, which acquires a pK_a of around 7, as compared to the normal pK_a of bulk water of 14).[1] Indeed, Pullan and Noltmann[10b] considered the phosphatase activity of CA III to be due to a secondary catalytic site (different of the zinc-hydroxide one) of the enzyme, containing one or two Arg residues (presumably Arg67 and/or Arg91), based on their inactivation experiments with the arginine-specific reagent phenylglyoxal. However, no other more recent investigations on the phosphatase activity of CA III (and related cytosolic isozymes, such as CA I and II) are available in the literature at this moment, and the possible phosphatase activity of various CA isozymes remained a controversial and poorly investigated issue.

Some other unresolved questions regarding this family of highly investigated[1–5] proteins concern the rather high number of isozymes present in mammals (16 in non-primates and 15 in primates),[2–14] their very different catalytic activity for the physiologic reaction (Table 1), and the fact whether some of them might possess physiologic relevance for other reactions than CO_2 hydration to bicarbonate. Indeed, considering some of the cytosolic isoforms presented in Table 1, it can be seen that in addition to the perfect catalyst which is CA II (k_{cat}/K_M of 1.5×10^8 M^{-1} s^{-1}), the isozyme CA III (highly abundant in the muscles)[12] has a very low catalytic activity for the CO_2 hydration reaction (around 0.16% of that of CA II), whereas CA I and XIII show intermediate activities between those of the very effective (CA II) and very ineffective catalysts (CA III). Considering that only CA II (and some of its active site mutants) was investigated in some detail for their esterase activity with 4-nitrophenyl esters of aliphatic C_1–C_6 carboxylic acids as substrates,[15,16] we decided to investigate comparatively the physiologically relevant cytosolic isozymes hCA I, hCA II and mCA XIII (h = human, m = mouse isozyme) for their esterase, phosphatase, and sulfatase activities (reactions 11–13, Scheme 2) with 4-nitrophenyl esters **1–3** as substrates. This study may bring some insights whether other reactions than CO_2 hydration may have physiological relevance for organs/tissues where CAs are present, and to better understand catalysis of the non-physiological reactions in which CAs might be involved, a field poorly investigated at this moment. For example, it has been reported[17] that



Scheme 2. Hydrolytic reactions (11–13) investigated in the present work under catalytic conditions employing cytosolic CA isozymes I, II and XIII.

the total erythrocyte CA esterase activity as well as isozyme I concentrations are reduced by 50% in patients with diabetes mellitus type II as compared to healthy individuals. There are also various reports regarding the different temporal expression of various CA isozymes (e.g., CA II, IX, XII, and XIII among others) in different tumors, with some of them decreasing (e.g., CA II and XIII) and others increasing (e.g., CA IX and XII) during progression of tumors from low to high grade.[18]

We report here a kinetic study on the hydrolysis of 4-nitrophenyl acetate **1**, phosphate **2**, and sulfate **3** in the presence of three cytosolic, physiologically relevant CA isozymes, hCA I, hCA II, and mCA XIII (h = human, m = murine isoforms). In solution, these esters are hydrolyzed by the nucleophilic attack of water (or hydroxide ions) to the central atom (carbonyl CO for acetate **1**, phosphorus for phosphate **2** and sulfur for sulfate **3**) with formation of a transition state from which the 4-nitrophenoxide is released. Considering the fact that CAs contain the equivalent of a strong base (hydroxide ions, HO^- coordinated to the zinc ion) at neutral pH, due to the powerful activation of water by the zinc ion from the active site cavity and the hydrophobic environment of the protein, in principle, hydrolytic reactions 11–13 of Scheme 2 should have the same mechanism as the hydrolysis catalyzed by bases in solution. Thus, our working hypothesis was that the hydrolytic processes described by Eqs. 11–13 of Scheme 2, involve the active site $Zn^{2+}(OH)^-$ functionality of the enzyme, that is, the same one responsible of the CO_2 hydration activity of CAs.

Indeed, data of Table 2 show that all three investigated CA isozymes show esterase activity with 4-nitrophenyl acetate **1** as substrate, with second order rate constants (k_{cat}/K_M) in the range of 753–7706 M^{-1} s^{-1}.[19,20] The weakest esterase activity was observed for hCA I, followed by hCA II (which was 3.46 times more active as an esterase as compared to hCA I), whereas mCA XIII was the best esterase, with an activity 10.23 times higher than that of hCA I and 2.95 times higher than that of hCA II. Only for CA II such kinetic data are available in the literature,[15,16] for wild type and mutant enzymes of human and bovine origin, which are in good agreement with our data (k_{cat}/K_M in the range of 2050–2100 M^{-1} s^{-1}, were reported in different conditions of pH and ionic strength by Host et al.[15] and Gould and Tawfik,[16] respectively, which compare well with our hCA II data of 2607 M^{-1} s^{-1}. The observed difference may be due to the much higher buffer concentrations used in the experiments reported in the cited studies[15,16] (50 mM Tris) and different pH values (8.5) as compared to our experiments performed in 10 mM Hepes and 10 mM Tris, at the physiologic pH of 7.4, at which hCA II has a maximal enzymatic activity).[1] Comparing data of Tables 1 (hydrase activity with CO_2 as substrate) and 2 (esterase activity with **1** as substrate), it is clear that the various CA isoforms have enzymatic activities which do not parallel with each other. Indeed, hCA II is the best catalyst for CO_2 hydration, followed by hCA I, with mCA XIII being a much less effective one. On the contrary, mCA XIII is the best esterase, followed by hCA II, with hCA I having the worst activity of the esterase type. The fact that this esterase activity is due to the zinc-hydroxide functionality of the enzyme is proved also by the fact that it is inhibited by the sulfonamide inhibitor acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), with IC_{50} values in the range of 28–1210 nM. In fact, sulfonamides bind in deprotonated form to the Zn(II) ion of the CA active site, replacing the hydroxide ion/water molecule coordinated to the metal ion, with the enzyme losing its catalytic activity due to the lack of the nucleophilic species from the active site.[1–5,21–24]

Data of Table 3 show that these CA isoforms also possess a weak but significant phosphatase activity with ester **2** as substrate, with second order rate constants (k_{cat}/K_M) in the range of 14.89–1374.40 M^{-1} s^{-1}.[20] This time the weakest phosphatase activity was shown by the excellent catalyst for CO_2 hydration hCA II, with hCA I possessing an intermediate activity (4.40 times higher than that of hCA II), whereas mCA XIII was a much more active phosphatase with 4-nitrophenyl phosphate **2** as substrate. Indeed, mCA XIII was 92.3 times a better phosphatase as compared to hCA II, and a 20.9 times better one as compared to hCA I (Table 3). Again, as a proof that this enzyme activity is due to the zinc-hydroxide functionality of the enzyme, we performed inhibition experiments with the classical, clinically used inhibitor acetazolamide (Table 3). This sulfonamide is an effective inhibitor of the phosphatase activity of hCA I, II, and mCA XIII, with IC_{50} values in the range of 63–1050 nM.

To our greatest surprise, all three investigated CA isoforms showed no sulfatase activity at all with ester **3** as substrate, although the isostructural phosphate **2** and acetate **1** were readily hydrolyzed by these enzymes. Experiments were performed in the pH range of 5.4–8.5

Table 2. Kinetic parameters for the hydrolysis of 4-nitrophenyl acetate 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*	k_{cat}/K_M (M^{-1} s^{-1})	K_M (mM)	IC_{50} (nM)
hCA I	753 ± 31	3.025 ± 0.014	1210 ± 76
hCA II	2607 ± 85	30.53 ± 2.10	28 ± 1.1
mCA XIII	7706 ± 324	1.132 ± 0.015	490 ± 33

The data are provided as means ± standard deviation (from at least 3 different assays).[19]

* h = human, m = murine isoform.

Table 3. Kinetic parameters for the hydrolysis of 4-nitrophenyl phosphate 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*	k_{cat}/K_M (M^{-1} s^{-1})	K_M (mM)	IC_{50} (nM)
hCA I	65.55 ± 5.2	0.935 ± 0.10	330 ± 14
hCA II	14.89 ± 0.54	2.195 ± 0.20	63 ± 5
mCA XIII	1374.40 ± 62	0.232 ± 0.02	1050 ± 76

The data are provided as means ± standard deviation (from at least 3 different assays).[20]

* h = human, m = murine isoform.

with **3** as substrate, but the sulfatase activity could not be detected.

It is interesting to note that esters **2** and **3** (as sodium salt, used in these experiments) are conjugate bases of strong acids. Thus, probably both **2** and **3** are in anionic form at all pH ranges in which experiments have been performed. It is thus difficult to rationalize the different behavior of the phosphate **2** and sulfate **3** as possible substrates of CAs. A possible electrostatic repulsion between the oxygen sulfate atom(s) of **3** and the zinc hydroxide species of the enzyme cannot be taken into account, since phosphate **2** is probably also bound in anionic form to the CA active site and acts as a CA substrate. Obviously, acetate **1** is bound as neutral species to the CA active site, allowing the strong nucleophilic ($Zn^{2+}(OH)^-$) attack, without any electrostatic repulsions, being thus able to effectively hydrolyze it according to Eq. 11 of Scheme 2.

In conclusion, we investigated in detail the esterase, phosphatase, and sulfatase activities of three cytosolic CA isozymes, hCA I, II, and mCA XIII with 4-nitrophenyl esters as substrates. These enzymes showed 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 (k_{cat}/K_M in the range of 14.89–1374.40 $M^{-1}\,s^{-1}$) and totally ineffective as sulfatases. These esterase/phosphatase activities were inhibited by sulfonamide CA inhibitors, proving that the zinc-hydroxide mechanism responsible for the CO_2 hydrase activities of these enzymes is also responsible of their esterase/phosphatase activity. CA XIII was the most effective esterase and phosphatase, although it showed reduced hydrase activity as compared to CA I and II. It is probable that CA XIII might catalyze other physiological reactions than CO_2 hydration, based on its relevant phosphatase activity reported here.

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19. Pocker, Y.; Stone, J. T. *Biochemistry*, **1968**, *7*, 2936. Ester **1** hydrolysis was monitored by measuring the absorbance at 405 nm of the chromogenic group 4-nitrophenol **4** which is released according to Eq. 11, Scheme 2 (extinction coefficient of 10,510 M^{-1}).[16] Reactions were performed at 25 °C in a quartz cuvette with 1 cm lightpath, 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, that is, concentrations of ester **1** of 0.3–5.0 mM) by fitting the data to the Michaelis–Menten model (Eq. 14):

$$Vo = (k_{cat}[E]_0[S]_0)/([S]_0 + K_M) \qquad (14)$$

Otherwise, k_{cat}/K_M values were derived from a linear fit (Eq. 15) by using PRISM[16]:

$$Vo = [E]_0[S]_0(k_{cat}/K_M) \quad (15)$$

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 and 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 vs. molarity of inhibitor.[15,16]

20. The phosphatase activity with **2** as substrate has been assayed by a variant of the method used by Pullan and Noltmann,[10b] measuring the absorbance at 405 nm of the chromogenic group 4-nitrophenol **4**, which is released according to Eq. 12. The assay has been performed at 25 °C (not at 30 °C)[10b] in the same buffer used for the esterase activity measurements described above,[19] working at substrate concentrations of 0.08–5 mM. Kinetic parameters were determined as described above,[19] and the rates of spontaneous hydrolysis (without enzyme) were subtracted from the measured enzymatic rates. Inhibition with acetazolamide has again been used as a control, being performed as described above, by titration of the enzymes with acetazolamide solutions in the concentration range between 10 nM and 100 μM. IC_{50} values were calculated as described above.[19] The sulfatase activity with ester **3** as substrate, described by Eq. 13 of Scheme 2, has been investigated in an analogous manner, and working at various pH values (in the range of 5.4–8.5, data not shown) but was totally absent with all three investigated enzymes, in all experimental conditions employed.

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