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Hydroxyurea Is a Carbonic Anhydrase Inhibitor

Andrea Scozzafava and Claudiu T. Supuran*

Università degli Studi di Firenze, Dipartimento di Chimica, Laboratorio di Chimica Bioinorganica, Via della Lastruccia 3, Rm. 188, I-50019 Sesto Fiorentino, Firenze, Italy

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Abstract—The interaction of hydroxyurea with the cytosolic isozymes of carbonic anhydrase (CA), hCA I and hCA II has been investigated by means of kinetic and spectroscopic techniques. Hydroxyurea acts as a weak, non-competitive inhibitor of both isozymes, for the 4-nitrophenyl acetate esterase activity, with inhibition constants around 0.1 mM for both isozymes. The spectrum of the adduct of hydroxyurea with Co(II)-hCA II is similar to the spectra of tetrahedral adducts (such as those with sulfamide, acetazolamide or cyanamide), proving a direct interaction of the inhibitor molecule with the metal center of the enzyme, whose geometry remains tetrahedral. Based on the X-ray crystal structure of the adducts of hCA II with ureate and hydroxamate inhibitors, the hypothetical binding of hydroxyurea is proposed to be achieved in deprotonated state, with the nitrogen atom coordinated to Zn(II), and the OH group of the inhibitor making a hydrogen bond with Thr 199. This binding may be exploited for the design of both CA as well as matrix metalloproteinase (MMP) inhibitors, since hydroxyurea is the simplest compound incorporating a hydroxamate functionality in its molecule. Indeed, such inhibitors of the sulfonylated amino acid hydroxamate type have been generated, with potencies in the low nanomolar range for both type of enzymes, CAs and MMPs.
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Introduction

Although carbonic anhydrases (CAs, EC 4.2.1.1) are rather simple enzymes, possessing one polypeptide chain of around 260 amino acid residues (for the most abundant such proteins, red cell, cytosolic isozymes CA I and CA II) and a catalytically critical Zn(II) ion, and even if they catalyse a very simple physiological reaction—the interconversion between CO_2 and bicarbonate—paradoxically, some basic aspects regarding their catalytic/inhibition mechanism are still unknown.[1–3] In addition to the interconversion between CO_2 and bicarbonate mentioned above, CAs catalyze other unphysiological reactions. Due to the chemical simplicity of CO_2, it is difficult to design substrate analogues, as in the case of other enzymes. Among the different molecules which are isoelectronic (and/or isosteric) with CO_2, N_2O was shown by Khalifah not to act as inhibitor,[4] cyanate is a strong suicide inhibitor being slowly hydrolysed to carbamate (according to reaction 2) which remains bound within the active site,[5] whereas cyanamide possesses the most intricate behaviour (reaction 3 of Scheme 1).[6,7] It has initially been shown that cyanamide binds to the metal ion within the CA active site,[6] adding to the coordination sphere, not substituting the metal-bound solvent molecule [the Zn(II) or Co(II) ion of the cobalt-enzyme presumably possess a trigonal bipyramidal geometry in such adducts].[6] Cyanamide thereafter undergoes a nucleophilic attack from a hydroxide ion interacting with the catalytical metal ion, forming urea which remains bound to the metal, as observed in the X-ray crystal structure of hCA II soaked in cyanamide solutions for several hours.[6] The urea molecule was shown to be directly coordinated to the active site Zn(II) ion through a nitrogen atom, whereas a network of six hydrogen bonds involving active site residues Thr 199, Thr 200 as well as three water molecules (Wat 99, Wat 122 and Wat 123) further stabilize this adduct.[6] Only recently the three-dimensional structure of the intermediate in the hydration reaction of cyanamide to urea catalyzed by hCAII has been determined by cryo-

$$O{=}C{=}O + H_2O \Leftrightarrow HCO_3^- + H^+ \quad (1)$$

$$O{=}C{=}N^- + H_2O \Leftrightarrow H_2NCOO^- \quad (2)$$

$$HN{=}C{=}NH + H_2O \Leftrightarrow H_2NCONH_2 \quad (3)$$

$$RCONHR' + H_2O \Leftrightarrow RCOOH + H_2NR' \quad (4)$$

Scheme 1.

*Corresponding author. Tel.: +39-055-4573005; fax: +39-055-4573385; e-mail: claudiu.supuran@unifi.it

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crystallographic techniques.[7] A hCAII-cyanamide-water ternary complex (Fig. 1A), where cyanamide bound to the catalytic zinc ion, is approached by a water molecule/hydroxide ion in a five-coordinate adduct, was shown to be the intermediate of the conversion. This ternary adduct probably represents an intermediate state of the catalyzed reaction where the water molecule/hydroxide ion is about to perform a nucleophilic attack on the zinc-activated cyanamide substrate. The structural evidence is consistent with the kinetic data previously reported[6] about this recently described hydrolytic reaction catalyzed by hCAII, and indicates that a different mechanism with respect to that generally accepted for the physiologic carbon dioxide hydration reaction may be adopted by the enzyme, depending on the substrate chemical properties. This was the first report of a transition state intermediate in a reaction catalyzed by CAs, and may open interesting new directions for the design of inhibitors.[7] Thus, the two X-ray crystallographic studies mentioned above[6,7] allowed the thorough characterization of both state A (ternary adduct of CA with cyanamide and water nucleophilically activated by the metal center), as well as state D (hCA II–ureate adduct) shown in Fig. 1, but opened new questions regarding the interaction of these enzymes with small molecules possessing appropriate geometry and chemical affinity for Zn(II) in order to act as zinc binding functions. One such molecule is hydroxyurea, the simplest compound incorporating the hydroxamate functionality present in the molecules of many classes of potent matrix metalloproteinase (MMP) and CA inhibitors,[8–11] and this urea derivatives has never been investigated for its interaction with such zinc enzymes. It should be mentioned that CAs do not possess protease activity (reaction 4 is not catalyzed by CAs), as recently explained by our group.[8]

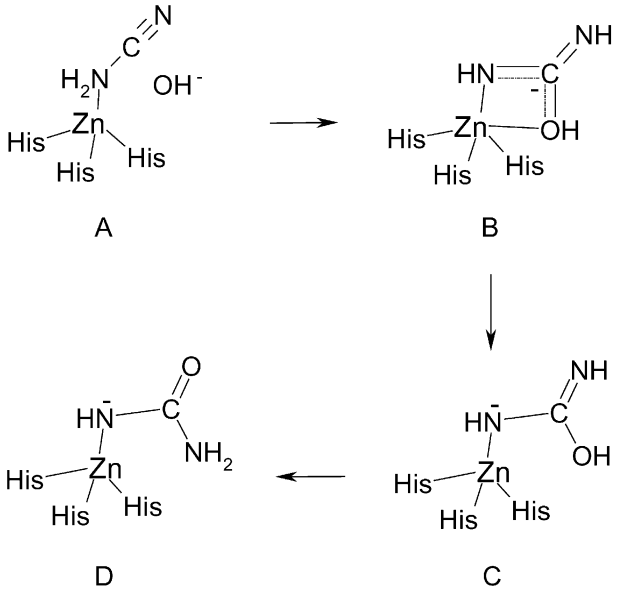
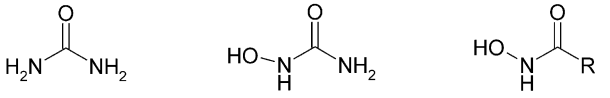


Figure 1. Mechanism of CA-catalyzed cyanamide hydration to urea (A–D). In A, the hydroxide ion acting as nucleophile in the hydration reaction is generated by a water molecule (Wat 51) acting as a distant, fifth ligand of the zinc ion. Ureate, strongly coordinated to the metal ion is generated in this way (D)—see text for discussion.[6,7]

In this paper, we investigated the interaction of the two cytosolic isozymes hCA I and hCA II with hydroxyurea by means of spectroscopic and kinetic techniques, showing that this compound may be considered as an interesting lead for the design of CA inhibitors with new zinc binding functions.



Results

Kinetic parameters for the interaction of hCA I and hCA II (for the 4-nitrophenyl acetate hydrolysis reaction) with hydroxyurea are shown in Table 1.

In Table 2, the electronic spectra of the adducts of cyanamide, urea, hydroxyurea and other classical inhibitors with Co(II)-hCA II are shown, as compared to the spectrum of the pure cobalt enzyme or its adducts with sulfonamide inhibitors, in rigorously the same conditions, concentrations and pH.

Inhibition constants against the esterase activity of hCA I and hCA II with urea and hydroxyurea are shown in Table 3.

Discussion

Similarly to urea,[6] hydroxyurea acts as a non-competitive inhibitor with the substrate 4-nitrophenyl acetate, both against hCA I as well as hCA II (Table 1). This result was expected since by means of X-ray crystallography it has been shown that urea (as ureate ion—Fig. 2) directly binds to the Zn(II) ion of the enzyme,[6] whereas substrates (CO_2 or esters) are thought to bind in hydrophobic pocket in the neighborhood of the zinc ion, lined by residues Val 121, Leu 141, Val 143, Leu 198, Val 207 and Trp 209 (in isozyme II).[1–3] As seen from data of Table 1, Michaelis–Menten constants are identical with or without inhibitor, which proves the non-competitive interaction of hydroxyurea with the CA-catalyzed 4-nitrophenyl acetate hydrolysis.

Table 1. Kinetic parameters for the interaction of hydroxyurea with isozymes hCA I and hCA II

System	K_m (mM)[a]	$10^6 \times V_{max}$ (M min^{-1})[a]	k_{enz} (min^{-1})[a]
hCA I[b]	15.4±0.83	55.9±4.7	6.57±0.50
hCA I[b] + hydroxyurea[c]	15.3±1.27	30.2±4.1	3.74±0.32
hCA II[d]	2.92±0.30	84.7±6.3	28.4±1.1
hCA II[d] + hydroxyurea[e]	2.94±0.24	51.9±4.5	18.5±1.0

[a]Mean±standard deviation (from three measurements).
[b]8.5 μM, pH 7.8 (Tris buffer, 10 mM).
[c][hydroxyurea] = 25 mM.
[d]3.0 μM, pH 7.8 (Tris buffer, 10 mM).
[e][hydroxyurea] = 25 mM.

Table 2. Electronic spectroscopic data of Co(II)-hCA II and its adducts with different inhibitors, among which also cyanamide, urea and hydroxyurea

Adduct	pH	Absorption maximum, nm (ε, M^{-1} cm^{-1})			
Co(II)-hCA II	7.20	520 (240)	540 (310)	616.5 (220)	640 (200)
+ cyanamide[a]	7.20	525 (45)	573 (50)	605 sh (30)	
+ urea[b]	7.20	520 (240)	540 (300)	616 (220)	642 (200)
+ hydroxyurea[c]	7.20	534 (280)	559 (335)	605 sh (315)	
+ acetazolamide[d] **1**	7.20	518 (390)	549 (220)	574 (530)	595 sh (500)
+ sulfamide[d] **2**	7.20	518 (210)	550 (270)	600 (215)	
+ thiocyanate[d]	7.20	465 (100)	529 sh (90)	571 (100)	689 (9)
+ nitrate[d]	7.20	470 (100)	515 (80)	555 (110)	709 (9)

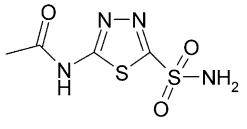
[a]Cyanamide = 240 mM, no incubation.
[b]Urea = 500 mM, incubation of enzyme and inhibitor solutions for 6 h at 4 °C.
[c]Hydroxyurea = 10 mM; no incubation.
[d]Enzyme concentrations were in the range 0.1–0.4 mM, at pH values specified in each case. Inhibitors concentrations were in the range of 0.001–2 mM.

The interaction of hydroxyurea with the metal center of CA has been confirmed by the spectroscopic data on the adduct of Co(II)-hCA II with this and other inhibitors (Table 2). It has thoroughly been investigated that sulfonamides, such acetazolamide **1** or sulfamide **2** bind to the Co(II) ion within the CA active site giving rise to a (pseudo)tetrahedral geometry of the metal ion.[19,21] Such adducts are characterized by intense spectra with molar absorbances above 300 M^{-1} cm^{-1}.[19,20] The four absorption maxima in the spectrum of the pure enzyme (at 520, 540, 616.5 and 640 nm, respectively) undergo notable changes when such an inhibitor is coordinated to the metal ion. Thus, especially the last two maxima are changed dramatically after complexation, colapsing into a unique, broad maximum centered at 574–575 nM, and a shoulder at 595–605 nm.[19–21] The tetrahedral geometry of such E–I adducts has been confirmed by X-ray crystallographic data for some of these complexes (of the native or metal-substituted enzymes).[21–23] Co(II) on the other hand is pentacoordinated in the adducts with thiocyanate,[24] nitrate,[25] acetate or benzoate.[19,20] Such spectra are characterized by molar absorbances under 150 M^{-1} cm^{-1},[19,20] and a different pattern of the four absorption maxima: thus, a maximum appears under 490 nm (generally at 465–480 nm), whereas the two strong maxima in the spectrum of pure Co(II)-hCA II at 540 and 616.5 nm, appear as a weak band, with the absorption maximum at 555–575 nm. Additionally, another maximum at 689–709 nm is seen in the electronic spectra of pentacoordinated Co(II) of such E–I adducts. The pentacoordination of the metal ion in some of these complexes has been then confirmed by the report of the X-ray structure for some of these adducts: hCA II–thiocyanate by Liljas group,[24] and hCA II–nitrate by Mangani's group.[25] As seen from the data of Table 2, the spectrum of the hydroxyurea–Co(II)hCA II is typical for tetrahedral adducts, with three intense maxima at 534, 559 and 605 (shoulder) nm, respectvely, being quite similar with the spectrum of the Co(II)hCA II–sulfamide adduct, previously investigated by us.[26] The Co(II)hCA II–hydroxyurea spectrum is very different from the spectrum of the adduct with cyanamide, previously reported by this group.[6] On the other hand, the adduct of Co(II)–hCA II with urea is practically identical to that of the uncomplexed enzyme, even after incubation of enzyme and inhibitor solutions for 24 h.[6,7] Obviously urea and hydroxyurea, although very similar structurally, show a completely different behavior in their interaction with this enzyme.

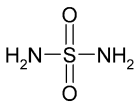
Table 3. Inhibition parameters of the esterase activity of hCA I and hCA II with urea, hydroxyurea and sulfamide (10 mM Tris buffer, pH 7.80, 25 °C)

Inhibitor	K_I (mM)[a]	
	hCA I	hCA II
Urea[b]	445 ± 15	460 ± 20
Hydroxyurea[c]	0.10 ± 0.003	0.12 ± 0.004
Sulfamide[c] **2**	0.035 ± 0.005	0.082 ± 0.008

[a]Mean ± standard deviation from three determinations.
[b]Preincubation of the enzyme and inhibitor solutions for 24 h at 4 °C.
[c]Preincubation of the enzyme and inhibitor solutions for 15 min at room temperature.



1



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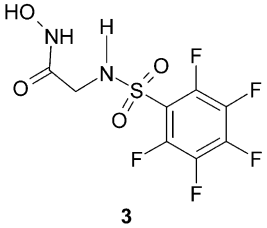
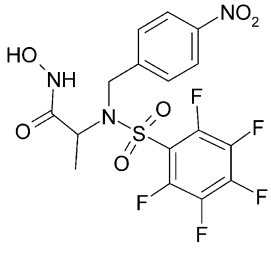


3

K_I (MMP-1) = 145 nM
K_I (hCA II) = 8 nM



4

K_I (MMP-1) = 4 nM
K_I (hCA II) = 5850 nM

Hydroxyurea acts as a weak inhibitor of the esterase activity of hCA I and hCA II, with K_I of around 0.10 mM against both these isozymes (Table 3). Its potency is thus similar to that of sulfamide **2**, which is around 1.50–2.85 times a stronger inhibitor as compared to hydroxyurea (Table 3). On the other hand, both these compounds are much more potent inhibitors as compared to urea, which—as seen from data of Table 3—even when incubated for prolonged periods (24 h) with

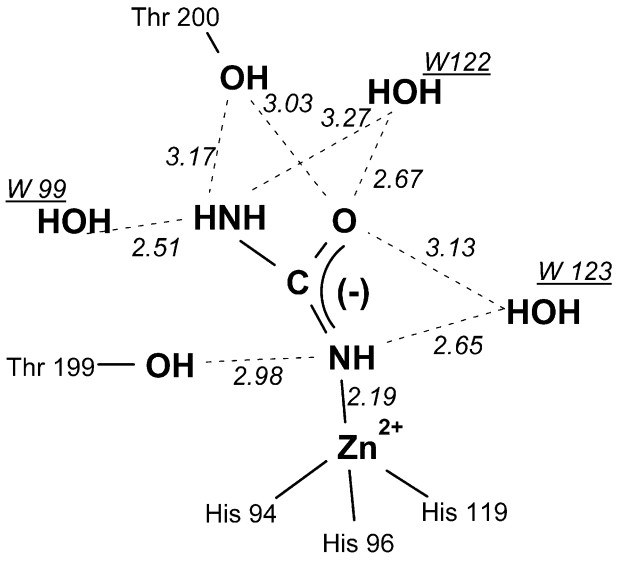
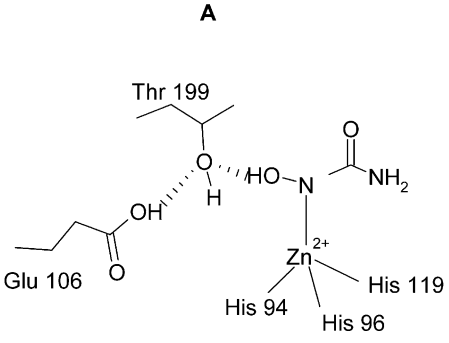


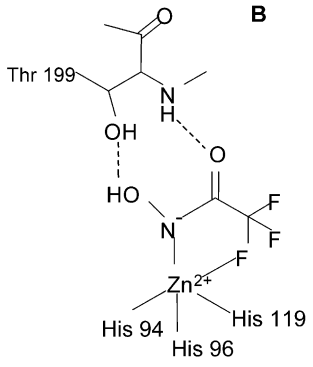
Figure 2. Schematic binding of ureate to hCA II, as determined by X-ray crystallography.[6] The deprotonated ureate nitrogen is coordinated to the catalytical Zn(II) ion, whereas a network of eight hydrogen bonds involving three water molecules (W 99, W 122 and W 123) and residues Thr 199 and Thr 200, fix the inhibitor within the active site (figures represent distances in Å). This makes ureate, potentially the strongest CA inhibitor ever described, whereas its conjugate acid, urea is a particularly weak inhibitor.

the enzyme, exerts a very weak inhibitory activity. This is clearly due to the fact that in aqueous medium, at pH around 7–8, it is practically impossible to generate the ureate anion, which on the other hand has a very high affinity for the enzyme active site,[6] as shown in Fig. 2. Practically this anion—generated by the suicide inhibitory action of cyanamide on CA—is one of the strongest CA inhibitors ever evidenced, being fixed within the active site by a network of eight hydrogen bonds involving three water molecules and the active site residues Thr 199 and Thr 200, in addition to the coordinative bond to the metal center (Fig. 2).[6] In fact, even nanomolar sulfonamide inhibitors, such as acetazolamide **1**, are unable to displace ureate from its adduct with CA,[6,7] and this is a good proof that ureate may be a sub-nanomolar CA inhibitor.

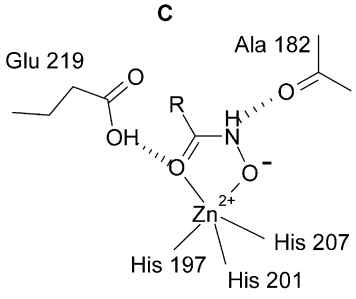
At this point, we may hypothesize a binding of hydroxyurea to CA as shown in Figure 3A, based on the above kinetic/spectroscopic findings, and also considering the interaction of hydroxamates (such as the simple trifluoromethylhydroxamate with CA II,[27] or more complicated hydroxamates of the succinyl or amino acid sulfonyl type, with the related enzymes, the MMPs)[8,28] (Fig. 3B and C). Indeed, hydroxamates interact very differently with CAs and MMPs. In the case of the last



CA II- hydroxyurea adduct



CA II- trifluoromethylhydroxamate adduct



MMP-8 - hydroxamate adduct

Figure 3. Hypothetic binding of hydroxyurea within the hCA II active site (A), based on the hCA II–urea structure,[6] as compared to the binding of trifluoromethyl hydroxamate to hCA II (B),[27] and that of a hydroxamate inhibitor within the MMP-8 active site (C), both determined by means of X-ray crystallographic techniques.[8,28]

enzymes—as shown by X-ray crystallography—the oxygen-deprotonated hydroxamate moiety acts as a bidentate ligand to the catalytic Zn(II) ion of the protease, the 'carbonyl' oxygen participates in a hydrogen bond with the carboxylic group of Glu 219, whereas the NH functionality of the hydroxamate makes another hydrogen bond with the backbone CO of Ala 182 (MMP-8 numbering).[28] Very different is the coordination of hydroxamates to CA II, as shown by the crystallographic work of Christianson's group.[27] In this case, the nitrogen-deprotonated hydroxamate is coordinated to Zn(II), whereas the OH functionality interacts with Thr 199 OH moiety. The carbonyl oxygen of the hydroxamate also participates to a hydrogen bond with the Thr 199 amide NH, whereas a fluorine atom of the hydroxamate is in the coordination sphere of zinc. This may explain the micromolar affinity of this hydroxamate for hCA II.[27] At this point we may also consider the pK_a of these different zinc binding functions: sulfonamides typically show pK_a values of the SO_2NH_2 proton in the range of 7–9, and they clearly bind in the SO_2NH^- form to Zn(II) in the CA active site.[1–3,22] The simple hydroxamates shown to bind to hCA II by Christianson's group[28] also possess a pK_a between 8 and 9,[29] whereas hydroxyurea has a pK_a of 6.2.[30] It is clear that all these compounds may easily be deprotonated at the physiologic pH, and as anions, interact with the Zn(II) ion of the hCA II active site. Indeed, hydroxyurea, probably binds in a similar manner to CA II, with its deprotonated nitrogen atom interacting with the zinc ion, and the OH functionality making a hydrogen bond with the OH group of Thr 199. Since hydroxyurea is about a 100 times weaker inhibitor as compared to trifluoromethylhydroxamate, we presume that the other hydrogen bond is not formed, and also that the NH_2 moiety of the inhibitor is not in the coordination sphere of Zn(II) (the same situation is valid for the adduct of hCA II with sulfamide, when the deprotonated NH group is coordinated to Zn(II), whereas the second NH_2 group is not).[21]

Thus, hydroxyurea may be considered a lead molecule for the design of both CA as well as MMP inhibitors. For the CAs, such inhibitors have recently been reported by this group.[11] For example, sulfonylated amino acid hydroxamates of the type RSO_2NX-AA-CONHOH (X = H; benzyl; substituted benzyl; AA = amino acid moiety, such as those of Gly, Ala, Val, Leu) act as low nanomolar MMP inhibitors when X = benzyl or substituted benzyls, and are much more ineffective when X = H.[8,10] Sulfonylated amino acid hydroxamate act on the other hand as good CA inhibitors (with inhibition constants in the range of 5–40 nM, against the human isozymes hCA I and hCA II, and 10–50 nM against bovine CA IV),[11] when the X moiety in the above formula is H, and potency is drastically reduced for the benzyl/substitutedbenzyl corresponding derivatives. This is rather well illustrated for hydroxamates **3** and **4**, one of which is a good CA inhibitor and a rather ineffective MMP inhibitor (compound **3**), whereas the other one (**4**) possess just the reversed behavior, being a low nanomolar MMP-1 inhibitor and a micromolar CA II inhibitor. Compound **3** may be formally considered a hydroxyurea derived CA inhibitor in which the NH_2 moiety of the lead has been converted to a longer moiety able to interact with the zinc ion, probably by means of the SO_2NH group, which is known to possess good zinc-binding properties.[1–3] Obviously, **3** may interact with the CA II active site in many other ways, but the difference in activity between the two compounds is dramatic: **3** is a 8×10^5 times more potent CA II inhibitor as compare to hydroxyurea. The design of dual enzyme inhibitors (for example MMP and TACE inhibitors) seems to be of great interest for the treatment of some diseases, such as cancer,[31] and since some CA isozymes are predominantly found in cancerous tissues (and lack from their normal counterparts)[32,33] the design of dual CA/MMP inhibitors may constitute another future approach for the management of this disease.

In conclusion, we explain here why hydroxyurea binds to the active site of CA, investigating this interaction by means of kinetic and spectroscopic techniques. Although this compound is a weak inhibitor (with inhibition constants around 0.1 mM against the cytosolic isozymes hCA I and hCA II), its binding to the CA active site may be exploited for obtaining good inhibitors directed both towards these enzymes as well as against the MMPs, zinc enzymes that possess a metal binding site quite similar to that of the CAs.

Experimental

Buffers (Tris, Hepes, phosphate), 4-nitrophenylacetate, cyanamide, urea, hydroxyurea and pyridine-2,6-dicarboxylic acid were from Sigma; acetonitrile from Acros, and were used without further purification. All buffers used in the kinetic measurements were brought to a ionic strength $\mu = 0.1$, by addition of K_2SO_4. Cobalt(II)-hCA II was prepared as described in the literature by removing zinc from the native enzyme in the presence of pyridine-2,6-dicarboxylic acid, followed by dialysis against metal-free Tris–H_2SO_4 buffer, and addition of the stoichiometric amount of Co(II) salt.[12]

Human CA I (hCA I) and CA II (hCA II) cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II as described in the literature (the two plasmids were a gift from Prof. Sven Lindskog, Umea University, Sweden).[13] Cell growth conditions were those described by Lindskog's group and enzymes were purified by affinity chromatography according to the published method.[14,15] Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of 49 mM^{-1} cm^{-1} for hCA I and 54 mM^{-1} cm^{-1} for hCA II, respectively, based on $M_r = 28.85$ kDa for CA I, and 29.30 kDa for CA II, respectively.[16,17]

Initial rates of 4-nitrophenyl acetate hydrolysis were monitored spectrophotometrically, at 400 nm, using an SX.18MV-R Applied Photophysics stopped flow instrument.[18] Solutions of substrate were prepared in anhydrous acetonitrile; the substrate concentrations

varied between 2×10^{-2} and 1×10^{-6} M, working at 25 °C. A molar absorption coefficient ε of 18,400 M^{-1} cm^{-1} was used for the 4-nitrophenolate formed by hydrolysis, in the conditions of the experiments (pH 7.80), as reported in the literature.[18] Non-enzymic hydrolysis rates were always subtracted from the observed rates. Triplicate experiments were done for each activator concentration, and the values reported throughout the paper are the mean of such results. The enzymatic rates (without inhibitor) were obtained as the mean from at least five experiments.

Acknowledgements

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