

SHORT COMMUNICATION

Carbonic anhydrase inhibitors: purification and inhibition studies of pigeon (*Columba livia* var. *domestica*) red blood cell carbonic anhydrase with sulfonamides

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Abstract

A carbonic anhydrase (CA, EC 4.2.1.1) from red blood cells of pigeons (*Columba livia* var. *domestica*), cICA, was purified to homogeneity. Its kinetic parameters for the CO₂ hydration reaction were measured. With a k_{cat}/K_m of $1.1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$, and a k_{cat} of $1.3 \times 10^6 \text{ s}^{-1}$, cICA has a high activity, similar to that of the human isoform hCA II. A group of 25 aromatic/heterocyclic sulfonamides incorporating the sulfanilamide, homosulfanilamide, benzene-1,3-disulfonamide, and acetazolamide scaffolds showed variable inhibitory activity against the pigeon enzyme, with K_i s in the range of 1.9–3460 nM. Red blood cells of pigeons, like those of ostriches, contain just one CA isoform, unlike the blood of mammals, which normally contain two isoforms, one of low (CA I-like) and one of very high activity (CA II-like). However, from the sulfonamide inhibition viewpoint, the pigeon enzyme was more similar to hCA II than to the ostrich enzyme.

Keywords: Carbonic anhydrase, sulfonamide, pigeon, inhibitor, enzyme inhibition

Introduction

The metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) is involved in pH buffering of extra and intracellular spaces by catalyzing the interconversion of CO₂ to HCO₃⁻ with generation of protons¹⁻⁴. The equilibration between these chemical species is constantly maintained by the catalytic activity of these enzymes, across the whole phylogenetic tree, in prokaryotes and eukaryotes⁵⁻⁹. CAs are in fact encoded by five distinct, evolutionarily unrelated gene families: the α -, β -, γ -, δ - and ζ -CAs^{1,5-9}. The α -CA gene family is present in many organisms, starting with bacteria and plants, protozoa, or invertebrates and ending with higher vertebrates, including humans¹⁻⁴. The ubiquity of these enzymes in all these organisms is clearly due to their involvement in basic physiological processes, in which the three chemical species mentioned above (CO₂, HCO₃⁻, and the H⁺ ions) are involved¹⁻⁴.

It is thus not surprising that in mammals 16 different α -CA isozymes have been described, with very different catalytic activity, subcellular localization, tissue distribution, physiological/pathological roles, and susceptibility to inhibitors^{1-4,8,9}. Among them, are five cytosolic catalytically-active forms (CA I-III, CA VII, and CA XIII), five membrane-bound isozymes (CA IV, CA IX, CA XII, CA XIV, and CA XV), two mitochondrial ones (CA VA and CA VB), a secreted CA isozyme, CA VI (in the saliva and milk), as well as three acatalytic proteins, CA VIII, X and XI, and CA-related proteins¹⁻⁴. CA XV is not present in primates but is found in other vertebrates, such as rodents, birds, and fish⁹. These different isoforms are involved in physiological processes connected with the respiration and transport of CO₂/HCO₃⁻ between metabolizing tissues and lungs, pH and CO₂ homeostasis, electrolyte secretion in a variety of

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tissues/organs, biosynthetic reactions (e.g., gluconeogenesis, lipogenesis, and ureagenesis), bone resorption, calcification, tumourigenicity, epileptogenesis, tumourigenesis, etc¹⁻⁹.

Vertebrate genomes encode only for α -CAs (which are zinc enzymes), but generally a rather high (and variable) number of isoforms is present in these organisms, and their precise number is not known for most orders^{1-4,8}. With the exception of humans and rodents (for which 15 and respectively 16 CAs have been described and characterized in detail, as outlined above)¹⁻⁴, few other vertebrates have been thoroughly investigated regarding the number of isoforms, their catalytic activity, and inhibition susceptibility with various classes of inhibitors^{1,10,11}. Indeed, some literature data are available for several fish species¹⁰ (such as the model animal zebra fish, *Danio rerio*¹¹, the rainbow trout *Oncorhynchus mykiss*¹², the sea bass *Dicentrarchus labrax*¹³ or the haemoglobinless Antarctic icefish *Chionodraco hamatus*¹⁴). Among birds, the red blood cell enzyme from the ratid *Struthio camelus* (ostrich)¹⁵ has been investigated in some detail, but as outlined above, these data for enzymes which are of non-human or non-rodent origin, are rather scarce. In the case of birds (*Aves*), there are several reports on CAs from chicken (*Gallus gallus*) and ostrich (*S. camelus*)¹⁶⁻²⁰ but no attempts were made to isolate, characterize, and study the inhibition of these enzymes were done, except for the ostrich enzyme which has been investigated in detail in this study¹⁵. It is interesting to note that Thomas¹⁷ reported previously in a preliminary work about some CA activity of pigeon erythrocyte.

In a previous work, we have reported on the kinetic parameters for the CO₂ hydrase activity of the red blood cell CA from *S. camelus* (scCA) and its inhibition with a series of aromatic and heterocyclic sulfonamides, one of the main class of CA inhibitors (CAIs)¹⁵. Here, we extend this type of investigation to another enzyme of the avian origin, and report on the purification, characterization, and inhibition susceptibility to sulfonamides of a CA isolated from red blood cells of the pigeon, *Columba livia* var. *domestica*.

Materials and methods

Sulfonamide inhibitors

Acetazolamide (AZA), ethoxzolamide (EZA), and sulfanilamide (SA) were from Sigma-Aldrich (Milan, Italy). Compounds **1-5** used in the assay were previously reported by one of our groups²¹⁻²⁷.

Preparation of the pigeon blood haemolysate

Blood samples from *C. livia* erythrocytes were anticoagulated with acid-citrate-dextrose and centrifuged at 1848g for 20 min at 4°C. The supernatant was removed. The packed red cells were washed with NaCl (0.9%) three times and the erythrocytes were haemolysed with three times the volume of cold water for 1 h. The ghost and

intact cells were removed by centrifugation at 18,924g for 25 min at 4°C, and the pH of the haemolysate was adjusted to 8.5 with solid Tris buffer. The haemolysate was applied to an affinity column containing sepharose-4B-L-tyrosine-sulfonamide and equilibrated with 25 mM Tris-HCl/0.1 M Na₂SO₄ (pH 8.5). The affinity gel was washed with a solution of 25 mM Tris-HCl/22 mM Na₂SO₄ (pH 8.5). The CA was eluted with a solution of 0.1 M CH₃COONa/0.5 M NaClO₄ (pH 5.6). The enzyme was dialyzed extensively for 3 days against 20 mM Tris buffer (pH 7.5) until all the acetate and perchlorate were removed¹⁵.

Determination of protein content

After scanning at 280 nm, the tubes with the significant absorbance were pooled and a quantitative protein determination was done by the Coomassie brilliant blue G-250 method²⁸.

CA assay

The change in absorbance of a pH indicator was measured by an SX.18MV-R (Applied Photophysics, Leatherhead, Surrey, UK) stopped-flow instrument in order to determine the initial velocities of the CO₂ hydration reaction which is catalysed by different CA isozymes, including the newly purified clCA²⁹. Phenol red (at a concentration of 0.2 mM) was used as an indicator, working at the absorbance maximum of 557 nm, with 10 mM HEPES(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; pH 7.5) as buffer, 0.1 M Na₂SO₄ (for maintaining constant the ionic strength), following the CA-catalyzed CO₂ hydration reaction for a period of 10–100 s. Saturated CO₂ solutions in water at 20°C were used as substrate. The CO₂ concentrations ranged from 1.7–17 mM for the determination of the catalytic 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 inhibitors were prepared at a concentration of 1–3 mM (in DMSO-water 1:1, v/v) and dilutions up to 0.01 nM were done with the assay buffer mentioned above. The kinetic k_{cat} and $k_{\text{cat}}/K_{\text{m}}$ were obtained by non-least square methods using PRISM3 (La Jolla, CA) whereas the inhibition constants were obtained by non-linear least-squares methods using PRISM 3, from Lineweaver-Burk plots, as reported earlier¹⁵, and represent the mean from at least three different determinations.

Results and discussion

Among the higher vertebrate CAs, the human and mouse isozymes have generally been thoroughly characterized both with regard to their kinetic properties for the CO₂ hydration reaction, as well as for their interaction with sulfonamide inhibitors¹⁻⁹. However,

few isozymes of other origin have been investigated in detail. This is particularly true for birds (*Aves*), for which different reports mention the role of CAs in the eggshell formation for chicken (*Gallus gallus*) and ostrich (*S. camelus*), but few attempts have been undertaken to isolate, characterize, and investigate the inhibition of these enzymes were done^{15–20}. Here, we report on the purification of a CA from pigeon red blood cells as well as on the first inhibition study of this enzyme with a series of aromatic and heterocyclic sulfonamides. This study is of interest in order to understand potential differences between diverse CAs from higher vertebrates such as mammals and birds, which diverged evolutionarily more than 350 million years ago.

As the sulfonamides represent a major class of CAIs, we also investigated the interaction of cICA with three sulfonamide drugs known to possess relevant CA inhibitory properties^{1–3}, AZA, EZA, and SA, as well as with a library of sulfonamides of types **1–5**, reported on earlier by one of our groups^{21–27}.

Blood from domestic pigeons has been used for the isolation and purification of the enzyme, as described in detail in the Materials and Methods section. Only one CA isoform, with a molecular weight of around 30 kDa (the same as those of the human (h) CA isoforms hCA I and II^{1–3}) was isolated and purified from pigeon blood by sulfonamide affinity chromatography (Figure 1). The catalytic activity of this preparation was measured by using a stopped-flow spectrophotometric method²⁹, monitoring the physiologic reaction catalyzed by these enzymes, i.e., CO₂ hydration to HCO₃[–] and protons. The data in Table 1 show the kinetic parameters (k_{cat} and K_{m}) for the physiologic reaction catalyzed by various vertebrate CA isoforms, such as the primate ones hCA I–hCA III, and the bird enzymes from ostrich (scCA)¹⁵ and pigeon, denominated here cICA (see Experiment for details).

Data of Table 1 show the pigeon enzyme cICA to possess a very high catalytic activity as CO₂ hydrase, with a $k_{\text{cat}}/K_{\text{m}}$ of $1.1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$, similar to that of the most active human isoform hCA II (the physiologically dominant one)^{1–4}, which is also one of the most effective catalysts known in nature (with a $k_{\text{cat}}/K_{\text{m}}$ of $1.5 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$)^{1,10}. The pigeon enzyme has a first order rate constant of $1.3 \times 10^6 \text{ s}^{-1}$, slightly lower than hCA II (k_{cat} of $1.4 \times 10^6 \text{ s}^{-1}$), whereas the Michaelis–Menten constants (K_{m}) of the two enzymes are also rather similar, of 11.8 mM for the pigeon enzyme and 9.3 mM for hCA II. It should be observed that cICA is catalytically much more active than other vertebrate enzymes investigated earlier, such as the human ones hCA I, III, and IV or the ratid one scCA, which showed turnover numbers in the range of 2.5×10^5 – $5.1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (Table 1). The pigeon enzyme, similar to all other CAs explored to date with the exception of CA III³⁰, was also significantly inhibited by the clinically used sulfonamide CAI AZA (1,3,4-thiadiazole-2-sulfonamide) with an inhibition constant of 71 nM (Table 1).

Sulfonamides constitute one the most important class of CAIs, with many representatives used as human or

veterinary drugs, as diuretics, antiglaucoma, antiobesity or antiepileptic agents^{1–3}. It appeared thus of interest to investigate inhibition of the pigeon enzyme isolated here, cICA, with some of these compounds, many of which are widely used drugs^{1–3}. We have included in this study, three clinically used derivatives, AZA, EZA, and SA, as well as a group of 22 other derivatives^{21–27}, of types **1–5**, some of which are aromatic (**1–3**) and heterocyclic (**4** and **5**) sulfonamides, obtained by the tail approach^{31,32}, considering SA and AZA as lead molecules. These compounds have been investigated earlier as inhibitors of the human isoforms hCA I and II^{21–27} and more recently for their interaction with the ostrich enzyme scCA¹⁵. In Table 2, the inhibition data of three vertebrate CAs with these 25 sulfonamides are shown: hCA II and ostrich enzyme scCA inhibition data are shown for comparison reasons, together with the new data of the pigeon enzyme isolated here for the first time.

Several interesting structure-activity relationship features were observed for the inhibition of cICA with the set of compounds investigated here: (i) SA and two other derivatives, the thioureas **1n** and **1p**, showed weak inhibitory action against cICA, with inhibition constants of 621–3460 nM. It is interesting to note that



Figure 1. SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) for cICA purified from red blood cells (lane 4) compared to ladder (lane 1), hCA I (lane 2) and hCA II (lane 3). The hCAs were from Sigma-Aldrich.

Table 1. Kinetic parameters for CO₂ hydration reaction catalysed by the cytosolic α -CA isozymes of mammalian and avian, origin and their susceptibility to acetazolamide (AZA) inhibition.

Isozyme	Activity level	k_{cat} (s ⁻¹)	$k_{\text{cat}}/K_{\text{m}}$ (M ⁻¹ .s ⁻¹)	K_{i} (AZA) (nM)
hCA I*	Moderate	2.10 ⁵	5.10 ⁷	250
hCA II*	Very high	1.4.10 ⁶	1.5.10 ⁸	12
hCA III**	Low	1.0.10 ⁴	3.10 ⁵	300,000
scCA***	High	1.2.10 ⁶	1.8.10 ⁷	303
cICA	High	1.3.10 ⁶	1.1.10 ⁸	71

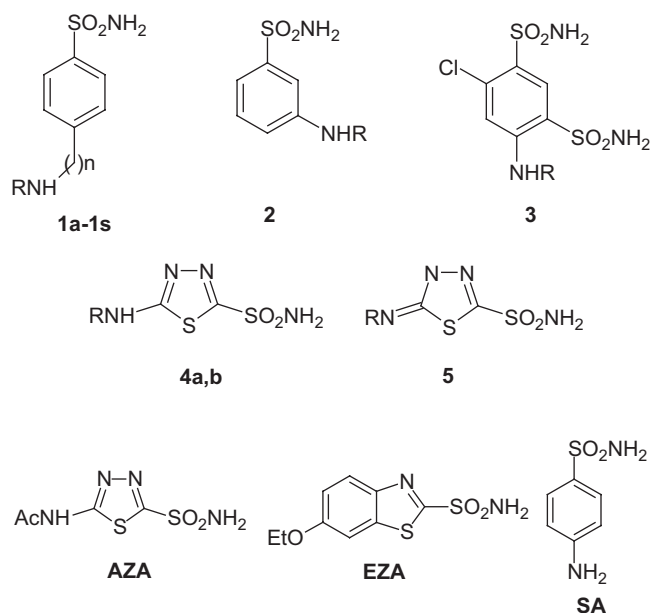
*From refs.^{1–3}.

**From ref.³⁰.

***From ref.¹⁵.

CA, carbonic anhydrase; cl, pigeon enzyme; h, human; m, murine; sc, ostrich isozymes.

Table 2. hCA II, scCA, and cICA inhibition data with sulfonamides **1**–**5**, acetazolamide (AZA), ethoxzolamide (EZA) and sulfanilamide (SA). Data of hCA II and scCA are from refs.^{15,21–27}.



No	n	R	K _i (nM)		
			hCA II ^a	scCA ^b	cICA ^{c*}
1a	0	CH ₃ CO	246	4550	467 ± 12
1b	0	CF ₃ CO	133	4700	325 ± 14
1c	0	EtCO	232	3600	382 ± 21
1d	0	<i>n</i> -PrCO	227	397	355 ± 16
1e	0	<i>i</i> -PrCO	258	573	369 ± 20
1f	0	<i>n</i> -BuCO	214	260	360 ± 17
1g	0	<i>t</i> -BuCO	230	540	147 ± 8
1h	0	PhCO	37	3245	95 ± 6
1i	0	MeSO ₂	64	66	137 ± 12
1j	0	PhSO ₂	49	2430	41 ± 3
1k	0	4-AcNHC ₆ H ₄ SO ₂	82	2600	36 ± 3
1m	1	PhSO ₂	40	318	31 ± 2
1n	1	PhNH-C(=S)	12	2970	679 ± 48
1p	2	PhNH-C(=S)	53	2980	621 ± 31
1q	2	PhNH-C(=O)	75	39	238 ± 12
1r	2	4-H ₂ NO ₂ SC ₆ H ₄ NH-C(=S)	4	3030	16 ± 1
1s	2	4-H ₂ NO ₂ SC ₆ H ₄ CO	5	25	15 ± 0.8
2	—	PhNH-C(=O)	150	3650	156 ± 10
3	—	PhNH-C(=O)	100	69	79 ± 7
4a	—	4-BrC ₆ H ₄ SO ₂	2	62	1.9 ± 0.2
4b	—	4-O ₂ NC ₆ H ₄ SO ₂	1	72	3.5 ± 0.3
5	—	Furan-2-yl-CO	6	768	3.6 ± 0.2
AZA	—	—	12	303	71 ± 6
EZA	—	—	8	3.9	12 ± 1
SA	—	—	300	570	3460 ± 68

*Mean ± standard error (from three assays).

^aHuman, recombinant enzyme, data from ref.^{21–27}.

^b*Struthio camelus* red blood cell enzyme, data from ref.¹⁵.

^c*Columba livia* red blood cells enzyme, this study.

the two thioureas incorporate the same scaffold but have one or respectively two CH₂ moieties between the benzenesulfonamide and phenylthiourea fragments of the molecule. These two compounds also act as very effective, low nanomolar hCA II inhibitors (K_is of 12–53 nM), whereas they are weak inhibitors for the ostrich enzyme (K_is of 2970–2980 nM). Thus, the two avian enzymes are more similar with each other with regard to their interaction with these two inhibitors. However, SA acts as a much more potent scCA inhibitor (K_i of 570 nM) compared to its inhibition of the pigeon enzyme; (ii) A rather large group of derivatives, i.e., **1a–1g**, **1i**, **1q** and **2**, showed medium inhibitory action against the pigeon enzyme, with K_is in the range of 137–467 nM. They include, the acylated SAs **1a–1g** incorporating aliphatic moieties, the disulfonamide **1i**, as well as the ureas **1q** and **2**. Thus, the introduction of acyl or alkylsulfonyl moieties in the SA scaffold, dramatically enhances the cICA inhibitory activity with these compounds, compared to the lead. Furthermore, an increase in the alkyl chain in compounds **1a–1g** from one to four carbon atoms, leads to an increase of the inhibitory activity against the pigeon enzyme. It should be noted that most of these compounds are rather weak ostrich CAIs, whereas their affinity for the human enzyme hCA II is better, in the same range as for the pigeon one (Table 2). (iii) Potent cICA inhibition, with K_is in the range of 1.9–95 nM, has been observed with the following compounds investigated here: **1h**, **1j–1l**, **1m**, **1r**, **1s**, **3**, **4**, **5**, AZA, and EZA. They include the benzoylated SA **1h**, as well as the structurally related sulfonylated SAs **1i–1m**, the *bis*-sulfonamides **1r** and **1s**, the urea incorporating the benzene-1,3-disulfonamide scaffold **3**, the bromo-nitro-substituted benzolamides **4a** and **4b**, the furanyl analogue of methazolamide **5** as well as AZA and EZA. Several inhibitors with K_is < 5 nM for the pigeon enzyme have been identified, such as **4a**, **4b**, and **5**. It should be mentioned that most of these compounds are much weaker inhibitors for the ostrich enzyme, whereas their affinity for hCA II is most of the time in the low nanomolar range.

In conclusion, we report here that the red blood cells of the pigeon contain just one CA isoform, unlike the blood of mammals, which usually contain two such isoforms, one of low activity (CA I-like) and one of high or very high activity (CA II-like). Pigeons, similar to ostriches, have thus a high activity, CA II-like isoform in the blood. cICA purified here showed kinetic parameters for the CO₂ hydration reaction typical of a highly effective catalyst, with a k_{cat}/K_m of $1.1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$, and a k_{cat} of $1.3 \times 10^6 \text{ s}^{-1}$. A group of 25 aromatic/heterocyclic sulfonamides incorporating the SA, homosulfanilamide, benzene-1,3-disulfonamide, and AZA scaffolds, showed variable inhibitory activity against the pigeon enzyme, with K_is in the range of 1.9–3460 nM. The least effective inhibitor was SA, whereas the highly effective ones incorporated

benzamide- and AZA-like scaffolds. The pigeon enzyme showed inhibitory properties more similar to the human than the ostrich enzyme for this class of sulfonamides.

Declaration of interest

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