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Discovery of a new family of carbonic anhydrases in the malaria pathogen *Plasmodium falciparum*—The η-carbonic anhydrases

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ABSTRACT

The genome of the protozoan parasite *Plasmodium falciparum*, the causative agent of the most lethal type of human malaria, contains a single gene annotated as encoding a carbonic anhydrase (CAs, EC 4.2.1.1) thought to belong to the α-class, *Pf*CA. Here we demonstrate the kinetic properties of *Pf*CA for the CO_2 hydration reaction, as well as an inhibition study of this enzyme with inorganic and complex anions and other molecules known to interact with zinc proteins, including sulfamide, sulfamic acid, and phenylboronic/arsonic acids, detecting several low micromolar inhibitors. A closer examination of the sequence of this and the CAs from other *Plasmodium* spp., as well as a phylogenetic analysis, revealed that these protozoa encode for a yet undisclosed, new genetic family of CAs termed the η-CA class. The main features of the η-CAs are described in this report.

Carbonic anhydrases (CAs, EC 4.2.1.1) are metalloenzymes present in all life kingdoms, with five genetically distinct families described to date in various organisms.[1–3] Most of them are zinc-containing enzymes, but Fe(II) may be present at the active site of the γ-CAs (described so far in Bacteria, Archaea and plants), whereas Cd(II) or Zn(II) ions seem to be equally effective for promoting catalysis in the ζ-CAs (diatoms encode for this class of CAs).[4–6] The metal ion is coordinated by three His residues (in the α-, γ- and δ-class enzymes) or by one His, and two Cys residues (in the β- and ζ-CAs), with the fourth ligand being a water molecule/hydroxide ion.[4–13] The main difference between the α-, γ- and δ-class enzyme families, where three His ligands coordinate to zinc, is the spacing between the His residues in the protein sequence. For example, in all α-CAs investigated so far the His ligands are at positions *x*, *x* + 2 and *x* + 25 (for example in the human isoform I, hCA I, these are His94, His96 and His119).[1–3] For the γ-CAs the positions of His residues coordinating the metal ion are always *x*, *x* + 36 and *x* + 41, respectively, whereas for the δ-CAs, the zinc ligands are positioned at residues *x*, *x* + 3 and *x* + 112, respectively.[1,4,5]

CAs belonging to various classes have been cloned, purified and characterized from many pathogenic organisms such as bacteria, fungi and worms,[14–18] in order to investigate whether inhibition of such enzymes is crucial for their survival or pathogenesis. Indeed, for CAs from most organisms it has been demonstrated that inhibitors belonging to the sulfonamide class (the most investigated CA inhibitors (CAIs))[1–4] interfere with the growth, possessing interesting anti-infective properties.[19–22]

Few protozoan parasites have been investigated for the presence and druggability of CAs up until now. The causative agent of human malaria, *Plasmodium falciparum*, was one of the first to be investigated.[23–25] A truncated form of the *P. falciparum* CA gene was cloned, expressed and purified in 2004 by Krungkrai's group,[23a] who showed that it is an active enzyme, possessing a good esterase activity with 4-nitrophenylacetate as a substrate, and also was inhibited by known sulfonamide-based CA inhibitors such as acetazolamide. The same authors concluded that the enzyme belongs to the α-class of CAs.[23a] Subsequent studies from Krungkrai's and our laboratories showed that different *Plasmodium* spp. encode CAs, all considered to belong to the α-class, and that primary sulfonamides inhibited in vitro and in vivo the growth of Plasmodium parasite.[23b–25] The *P. falciparum* CA, the only Plasmodium CA investigated to date, has been denominated *Pf*CA.[23] Some benzenesulfonamide derivatives showed effective

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Figure 1. 'Forced' alignment of the amino acid sequences of the truncated *P. falciparum* CA (*Pf*CA), which contains three additional C-terminal amino acids (GNK) not present in the native sequence, with the human α-CA isoforms I and II (hCA I and hCA II). In order to align the Zn(II) ion ligands (in red) of the three enzymes, six 'missing' residues have to be inserted in the protozoan enzyme sequence, and five in the mammalian enzymes sequences. It may be seen however that the other residues crucial for the catalytic mechanism of the α-CAs (i.e., the proton shuttle residue His64 shown in blue) and the gatekeeper residues (Glu106 and Thr199, in orange) are not conserved in the protozoan enzyme (except for Glu106). The hCA I numbering system has been used.[29–34] Amino acid sequence cryptonym is indicated in Table 3.

in vitro inhibition of the esterase activity of *Pf*CA, and also inhibited the in vitro growth of the parasite.[23b–25] Furthermore, some of these sulfonamides were effective as antimalarial agents in mice infected with *Plasmodium berghei*, an animal model of human malaria infection, with an efficacy similar to that of the clinically used drug chloroquine.[24] CAIs were considered to possess antimalarial activity because their inhibition of the first step of pyrimidine nucleotide biosynthesis in the protozoan parasite, that is, the CA-mediated carbamoylphosphate biosynthetic pathway.[24] However, this has not been experimentally confirmed and *Pf*CA has never been investigated for its catalytic activity with CO_2 as substrate up until now.

More recently, an α-CA has also been cloned and characterized in another unicellular protozoan, *Trypanosoma cruzi*, the causative agent of Chagas disease.[26] This enzyme, denominated *Tc*CA, had a high catalytic activity for the CO_2 hydration reaction, although it is devoid of the His64 proton shuttle, an amino acid residue involved in the catalytic cycle of α-CAs.[1–3,26] The thiols, another class of CAIs, were the most potent in vitro inhibitors of *Tc*CA (K_Is of 21.1–79.0 nM) and some of them also inhibited the epimastigotes growth of two *T. cruzi* strains in vivo.[26] Thus, protozoan CA inhibition may be a valid strategy to control infection with protozoans causing diseases such as malaria and Chagas disease.[24,26]

Anions constitute another important class of CAIs.[27–30] As there are no anion inhibition studies of *Pf*CA, here we undertook such an investigation, including in our study the common metal-complexing anions, the halides and pseudohalides, as well as some complex anions and small molecules known to interact with these enzymes, such as sulfamide, sulfamic acid, phenylboronic acid, diethyldithiocarbamate, etc.[31] Up until this study, it should be noted that the catalytic activity of *Pf*CA had only been investigated for the esterase reaction catalyzed by the enzyme, with 4-nitrophenylacetate as a substrate.[24] Here we present the first kinetic study of the CO_2 hydrase properties of this enzyme, together with the anion inhibition data mentioned above. As discussed above, based on amino acid sequence comparisons, *Plasmodium* CA enzymes have until now been classified as members of the α-CA family. A closer look at the amino acid sequence and phylogeny of CAs present in *P. falciparum* and other *Plasmodium* spp., led us to the conclusion that *Pf*CA was erroneously assigned as being an α-class enzyme. Here we propose that *Plasmodia* encode for CAs belonging to a new genetic family that we call the η-CA class.

The *Pf*CA full length enzyme contains 600 amino acid residues (PlasmoDB: PF3D7_1140000), in contrast to hCA I and II which have 260 and 259 residues, respectively.[25] An alignment of the amino acid sequences of a truncated[23a] *Pf*CA sequence, with the two human α-CAs, hCA I and II, is shown in Figure 1, in order to identify some features of the protozoan enzyme previously assigned to the α-class.[23b] The truncated *Pf*CA sequence can be aligned to that of hCA I and II, but only if gaps are added to the amino acid sequence regions in which the zinc coordinating histidines are located: six gaps must be placed in the *Pf*CA sequence (after residue 96—the hCA I numbering system is used throughout the paper), and five gaps in the hCA I/II sequences (after Leu118). However, we consider this to be a 'forced' alignment that has erroneously led to the assignment of *Pf*CA as belonging to the α-class. Indeed, the sequence alignment of Figure 1 demonstrates that other features of α-CAs are not present in the *Pf*CA sequence, such as the proton shuttling residue in position 64 (His in most, but not all α-CAs, but Gln in *Pf*CA), or the Thr199 residue. The dyad Glu106—Thr199 is conserved in all α-CAs investigated so far, being involved in the orientation of the substrate for the nucleophilic attack by the zinc hydroxide species of the enzyme.[1–4] It may be observed that the side chain of Lys present in position 199 in the *Pf*CA sequence in the forced alignment would be too bulky for assuring the correct hydrogen bonding network with Glu106, when CO_2 is bound in the hydrophobic pocket of α-CAs.[2] All these features of *Pf*CA which are not typical of an α-class enzyme, prompted us to compare the sequences of other *Plasmodium* spp. CAs. An alignment of CA sequences available in the Plasmodium genome database, as well as *Plasmodium reichenowi* and *Plasmodium vinckei* sequences, is shown in Figure 2.[32] These alignment data indicate that the CAs present in these protozoa are not α-CAs, and that they belong to a yet undescribed, new CA genetic family, for which we propose the name η-CA. The main features of this new enzyme class are as follows:

(i) the predicted metal ion coordinating residues are His94, His96 and His118 (again the hCA I numbering system is used for allowing us to better describe the differences between