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RESEARCH ARTICLE

Cloning, expression and purification of the complete domain of the η-carbonic anhydrase from *Plasmodium falciparum*

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Abstract

The antimalarial drugs are of fundamental importance in the control of malaria, especially for the lack of efficient treatments and acquired resistance to the existing drugs. For this reason, there is a continuous work in identifying novel, less toxic and effective chemotherapies as well as new therapeutic targets against the causative agents of malaria. In this context, a superfamily of metalloenzymes named carbonic anhydrases (CAs, EC 4.2.1.1) has aroused a great interest as druggable enzymes to limit the development of *Plasmodium falciparum* gametocytes. CAs catalyze a common reaction in all life domains, the carbon dioxide hydration to bicarbonate and protons ($CO_2 + H_2O \iff HCO_3^- + H^+$). *P. falciparum* synthesizes pyrimidines *de novo* starting from HCO_3^-, which is generated from CO_2 through the action of the η-CA identified in the genome of the protozoan. Here, we propose a procedure for the preparation of a wider portion of the protozoan η-CA, named PfCAdom (358 amino acid residues), with respect to the truncated form prepared by Krungkrai et al. (PfCA1, 235 amino acid residues). The results evidenced that the recombinant PfCAdom, produced as a His-tag fusion protein, was 2.7 times more active with respect the truncated form PfCA1.

Keywords

Carbonic anhydrase, η-class enzyme, hydratase activity, malaria, metalloenzymes, protozoa, protein expression, protonography, synthetic gene

History

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Introduction

Each year, there are hundreds of millions of people infected with disease-causing protozoa, particularly in tropical and subtropical regions of the world because humidity and high temperatures provide the necessary conditions for vectors and protozoans growth[1,2]. It has been estimated that approximately one million infected people die each year, due to protozoan infections, especially malaria that is caused by parasitic protozoans belonging to the genus *Plasmodium*[3–9]. Six different Plasmodium species infect humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and the zoonotic *Plasmodium knowlesi*[10,11]. *P. falciparum* is responsible for the most severe and life-threatening form of malaria. The antimalarial drugs are a mainstay in the control of malaria[12,13]. However, the lack of efficient treatments and acquired resistance to the existing drugs has stimulated efforts to identify new, less toxic, and more effective chemotherapies as well as novel therapeutic targets against the causative agents of malaria.

Druggable enzymes follow three major criteria that, in general, characterize many such proteins: (a) they interact with a drug-like molecule; (b) they are biomolecules essential for survival of the parasite/pathogen; and (c) they are sufficiently different from their closest counterparts in the human host, in order to be possible to inhibit them selectively[14]. In this context, a superfamily of metalloenzymes named carbonic anhydrases (CAs, EC 4.2.1.1) has aroused a great interest as druggable enzymes to limit the development of *P. falciparum* gametocytes. CAs catalyze a common reaction in all life domains, the carbon dioxide hydration to bicarbonate and protons ($CO_2 + H_2O \iff HCO_3^- + H^+$)[15–18]. Interestingly, several classes of CA inhibitors (CAIs) are known to date: (1) sulfonamides and their bioisosteres, such as sulfamates and sulfamides, which bind in a tetrahedral geometry to the metal ion of the CA active site in its deprotonated form. Thus, these compounds replace the metal-coordinated water molecule/hydroxide ion necessary for catalysis[8,9,19–25]; (2) anions, such as the inorganic metal-complexing ones or more complicated species, such as the carboxylates, are also known to bind to the CAs. Anions may bind either the tetrahedral geometry of the metal ion or as trigonal–bipyramidal adducts[5,19,26–31]; (3) dithiocarbamates (DTCs), which coordinate through one sulfur atom to the Zn(II) ion from the enzyme active site, and also interact with the conserved Thr199 amino acid residue[20–23]. In addition to the thorough investigations of various classes of CAIs, CAs started to be investigated in detail in pathogenic microorganisms, such as bacteria, fungi and protozoa[24,25,32–44], since it has been demonstrated that in many microorganisms, they are essential for the life cycle of the microorganism and that their

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inhibition leads to growth impairment or growth defects of the pathogen[16,17,45–52]. For example, *P. falciparum* during its exponential growth and replication in the erythrocytes needs purines and pyrimidines for DNA/RNA synthesis[40,45,53,54]. Unlike the purines, highly abundant in human erythrocytes, pyrimidines are present in only small concentrations. *P. falciparum* is able to perform *de novo* pyrimidine synthesis as it does not have active pathways for the salvage of pyrimidines from the host. *P. falciparum* synthesizes pyrimidines *de novo* from HCO_3^-, adenosine-5-triphosphate (ATP) and glutamine (Gln). HCO_3^-, generated from CO_2 through the action of a CA, is the substrate of the carbamoyl phosphate synthetase II (*Pf*CPSII), the first enzyme involved in the Plasmodia pyrimidine pathway[54]. Finally, we should mention that six different, genetically distinct CA families are known to date, the α-, β-, γ-, δ-, ζ- and η-CAs[17,55,56]. Thus, the last important aspect concerning the *P. falciparum* CA, classified as belonging to the η-CA class[17,40,45,53,57–60], is the observation that the metal ion coordination pattern is unique among all the genetic families encoding for such enzymes. As demonstrated by the homology modeling analysis, Zn(II) is coordinated by two His and one Gln residues, in addition to the water molecule/hydroxide ion acting as nucleophile in the catalytic cycle[53].

As aforementioned, it is readily apparent that *P. falciparum* CA not only meets the requirements of criteria *a* and *b* discussed above, but, as demonstrated by our groups, there are significant differences between η- and α-CAs, suggesting that η-CA fully meets criterion *c*, too. Thus, targeting *Plasmodium* CA for blocking the pyrimidine metabolic pathways might provide a promising route for novel drug development with high affinity and selectivity for the η-CAs over the human α-CAs[40,45,61,62].

The *P. falciparum* CA gene (accession number AAN35994.2, PlasmoDB: PF3D7_1140000) encodes a 600 amino acid polypeptide chain[63–67]. In 2004, Krungkrai et al.[63] cloned a truncated form of this gene encoding for a polypeptide chain formed by the amino acid residues from position 211 to 445 (235 amino acid residues) and it was named PfCA1. Here, we propose a procedure for the preparation of the recombinant PfCAdom starting from residue 181 to residue 538 and corresponding to 358 amino acid residues with respect to the truncated form prepared by Krungkrai et al.[63], which incorporated only 235 amino acid residues. Interesting, PfCAdom was catalytically more active than the PfCA1 truncated form for the physiologic hydration of CO_2 to form bicarbonate and protons.

Materials and methods

The identification of the gene encoding for *P. falciparum* η-CA (PfCAdom) was performed at the link http://www.ncbi.nlm.nih.gov/genome/selecting the genome of ''*P. falciparum*''. The η-CA gene of *P. falciparum* (accession number: AAN35994.2) was identified running the ''BLAST'' program and using as nucleotide query sequence a α-CA[68,69].

Cloning, expression and purification of PfCA

The GeneArt Company (Invitrogen, Milan, Italy), specialized in gene synthesis, designed the synthetic PfCAdom gene (PfCAdom-DNA) encoding for the PfCAdom (η-CA of 358 amino acid residues) containing four base-pair sequences (CACC) necessary fordirectional cloning at the 5′ end of the PfCAdom gene. The recovered PfCAdom gene and the linearized expression vector (pET-100/D-TOPO) were ligated by T4 DNA ligase to form the expression vector pET15-b/PfCAdom. Arctic Express DE3 competent cells (Agilent, Milan, Italy) were transformed with pET15-b/PfCAdom, grown at 20 °C and induced with 1 mM IPTG. After 30 min was added $ZnSO_4$ (0.5 mM) to the culture medium and the cells were grown for additional 6 h. Subsequently, cells were harvested and resuspended in the following buffer: 50 mM Tris/HCl, pH 8.0, 0.5 mM PMSF, and 1 mM benzamidine. Cells were then disrupted by sonication at 4 °C. After centrifugation at $12\,000 \times g$ for 45 min, the supernatant was incubated with His Select HF nickel affinity gel resin (Sigma, Milan, Italy) equilibrated in lysis buffer for 30 min. Following centrifugation at $2000\times g$, the resin was washed in wash buffer (50 mM Tris/HCl, pH 8.0, 500 mM KCl, 20 mM imidazole). The protein was eluted with the wash buffer containing 200 mM imidazole. Collected fractions were dialyzed against 50 mM Tris/HCl, pH 8.0. At this stage of purification, the protein was at least 85% pure and the obtained recovery was of 0.1 mg of the recombinant protein.

CA assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity[70]. Bromothymol blue (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10–20 mM TRIS (pH 8.3) as buffer, and 20 mM Na_2SO_4 for maintaining constant the ionic strength (this anion is not inhibitory and has a $K_I > 200$ mM against this enzyme), 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 and inhibition constants. For each measurement, 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 (1–10 mM) were prepared in distilled-deionized water and dilutions up to 0.01 μM 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 the Cheng-Prusoff equation whereas the kinetic parameters for the uninhibited enzymes from Lineweaver-Burk plots, as reported earlier, and represent the mean from at least three different determinations.

Protonography

SDS-PAGE was performed as described by Laemmli[71]. Briefly, wells of 12% SDS-gel were loaded with bCA, PfCA1 and PfCAdom mixed with Laemmli loading buffer without 2-mercaptoethanol and without boiling the samples, in order to prevent protein denaturation induced by heating. The gel was run at 180 V until the dye front ran off the gel[18]. Following the electrophoresis, the 12% SDS-gel was subject to protonography to detect the bCA, PfCA1 and PfCAdom hydratase activity on the gel as described by Capasso et al.[18].

Results and discussion

Purification of the recombinant PfCAdom

A wider portion of the CA domain (PfCAdom, 358 amino acid residues) identified in the genome of *P. falciparum* with respect to the truncated form (PFCA1, 235 amino acid residues with a theoretical molecular mass of 27.9 kDa) prepared by Krungkrai et al[62] was cloned, over-expressed and purified. In a homology model of PfCA, it was observed that residues of the full-length protein from 182 to 327 and from 397 to 535 could be modeled with known tridimensional CA structures as evidenced by the analysis carried out in our labs[53]. The truncated form (PfCA1), in fact, did not include the amino acid residues from 182 to 220 and from 446 to 538, containing only the amino acid residues from

```
        10         20         30         40         50
MKLLYLLYPI LLFYNVNVFI NYKKSRLMLE MIDKYNTHFV QTTKPYYEFN
        60         70         80         90        100
VTNLTNSKKK KKKKKRENHL IGSGENMQKK DEKNIKDFHI NDYEIDGKTI
       110        120        130        140        150
HNKENKDSFK MNKNKLNDNE ELFYMDNILS YKPNKKKLFT YSFSENEGNS
       160        170        180        190        200
EKEETLYNFK NMKNINSVQN NINKTFLYNK LKNVDYYEHG YNWDIGQCKT
       210 *      220        230        240        250
GKYQSPVDLP MKDLKERELK NISDVYLNLF DDDNYAWNNY NKPWMKGDFF
       260        270        280        290        300
YYYEYFIKKI VINRQNNIFQ IKAARDGIIP FGVLFTTEQP AMFYADQIHF
       310        320        330        340        350
HAPSEHTFQG SGNRREIEMQ IFHSTNYFYD IQDDKSKYKK KYGLHIYNNL
       360        370        380        390        400
KKNSKETSKK DSSRYHSYLM SFLMNSLSNE QLQNKYNKKK RIKKMKNQYE
       410        420        430        440      * 450
VISITFTSAE INASTINAFK KLPSEKFLRT IINVSSAVHV GSDPTLVELK
       460        470        480        490        500
DALNLDALMM MLNIEDMQFL SYQGSSTLPL CDENVSWKVA KQPLPVSTET
       510        520        530        540        550
ILNFYYLLKK HTPNYSGSDN DNYRSLQNVE DNTRHYRKFS LVQVFPIQVL
       560        570        580        590        600
ISSAISNIED KKVINIIKDI SPKNMSFTYY SKWDIYFILF IFYNIVLFLF
```

Figure 1. Complete translated amino acid sequences of *P. falciparum* η-CA. Legend: in red is reported the recombinant PfCA produced using a synthetic gene; * indicate the starting and ending points of the Krungkrai amino acid sequence (PfCA1, truncated form); in blue the amino acid residues of the catalytic site deduced by homology modeling[53].

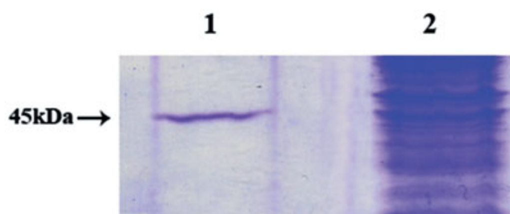

Figure 2. SDS-PAGE of the recombinant PfCAdom purified from *E. coli* cell extract. Lane 1, purified PfCAdom from His-tag affinity column; Lane 2, cell extract protein after induction with IPTG.

Table 1. Kinetic parameters for the CO_2 hydration reaction catalyzed by the human α-CAs (hCA I and hCA II) and protozoan η-CAs (PfCA1 and PfCAdom).

Isozyme	Class	Organism	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}.s^{-1}$)	K_I (acetazolamide) (nM)
hCA I	α	human	2.0×10^5	5.0×10^7	250
hCA II	α	human	1.4×10^6	1.5×10^8	12
PfCA1	η	protozoa	1.4×10^5	5.4×10^6	170
PfCAdom	η	protozoa	3.8×10^5	7.2×10^7	366

Errors in the range of ± 5% of the reported data from three different assays.
Acetazolamide (AAZ) inhibition data are also shown.

221 to 445 (see Figure 1). This prompted us to consider a wider portion of the plasmodium η-CA with a molecular mass of 42.3 kDa. In Figure 1, the amino acid sequences (accession number AAN35994.2, PlasmoDB: PF3D7_1140000) encoding for the complete *P. falciparum* CA is reported. Moreover, as described recently, the homology modeling analysis showed clearly that the metal ion coordination pattern of the η-CA involved two His and one Gln residues, in addition to the water molecule/hydroxide ion acting as nucleophile in the catalytic cycle[40,45,53,57,59,60]. The recombinant polypeptide chain PfCAdom starting from residue 181 to residue 538 (see Figure 1) was prepared designing a synthetic gene as described in ''Materials and methods'' and heterologously expressed as a His-fusion protein using the method reported earlier for several bacterial CAs[16,17]. The recombinant PfCAdom was isolated and purified to homogeneity from *Escherichia coli* (DE3) codon plus cells extract. Carbonic anhydrase activity was recovered in the soluble fraction of cell extract obtained after sonication and centrifugation. Using the affinity column (His-select HF Nickel affinity gel), PfCAdom was purified to apparent homogeneity, as indicated by SDS-PAGE (Figure 2, lane 3). Analysis by SDS-Page of PfCAdom showed a band of about 45 kDA (monomeric form) under reducing condition (Figure 2). The recovery of purified protein concentration was 0.1 mg of purified protein using the cloning, expression and purification aforementioned and starting from 2 L of bacterial culture.

Enzyme kinetics

Using the stopped-flow technique, the kinetic parameters were determined for the newly purified recombinant PfCAdom using CO_2 as a substrate. The activity of PfCAdom was compared to that of PfCA1 (truncated form) and with other α-CAs, such as the *Homo sapiens* isoforms hCA I and hCA II (Table 1). As shown in Table 1, the protozoan full-length domain (PfCAdom) had a k_{cat}/K_m ratio one order of magnitude higher respect to that of the truncated form, PfCA1 with a k_{cat} of 3.8×10^5 s^{-1}. This was something to be expected, since the truncated form lacked a Thr residue (Thr199 in hCA II corresponding to Thr 477 in PfCAdom), which is presumed to be crucial for catalysis and for orienting CO_2 in the proper mode for the nucleophilic attack from the zinc-coordinated hydroxide (Figure 3). Interesting to note that PfCA1 and PfCAdom obtained with two different cloning strategies are not well-affected by acetazolamide inhibition showing a K_I of 170 and 366 mM, respectively (Table 1).

Protonography

The hydratase activity of PfCAdom on the SDS–PAGE gel was investigated and compared with that obtained for PfCA1 (truncated form) and commercial bovine bCA (α-CA). The gels were run under denaturing and non-reducing conditions. The protonogram showed in Figure 4 was obtained loading on the on the SDS–PAGE samples of PfCAdom, PfCA1 and bCA at 10 μg/well. As described in the experimental section, the protonography is based on monitoring the pH variation in the gel due to the CA-catalyzed conversion of CO_2 to bicarbonate and protons. The protonogram was thereafter stained with bromothymol blue, which is a widely used pH indicator. This dye appears blue in its deprotonated form, while its color changes to yellow in the protonated form. Thus, the production of H^+ ions during the CO_2 hydration reaction, due to the CA hydratase activity, lowers the pH of the solution until the color transition point of the dye is reached, that is, at pH 6.8. The developed protonogram showed a yellow band at a molecular weight of 45 kDa corresponding to the η-hydrates activity of PfCAdom (Figure 4). The protonogram showed one band corresponding to the monomer of PfCAdom. η-CA fold resembles that of α-CA, which is active as a monomer.

Conclusions

These results confirmed that the PfCAdom fusion protein cloned as His-Tag fusion protein is an active recombinant protein. The yield and purification efficiency of PfCAdom fusion protein by His select Nickel affinity column allowed to recover about 0.1 mg of total protein in the cytoplasmic fraction of the *E. coli* cells used as host. The CO_2 hydratase activity detected on an SDS-PAGE by using the protonography, allows us to speculate that this technique

Figure 3. Alignment of the amino acid sequences of the PfCAdom (full length) and PfCA1 (truncated form) with the human α-CA isoforms I and II (hCA I and hCA II) and the bacterial α-CAs (NgonCA from *Nesseria ghonorrea* and TaCA from *Thermovibrio ammonificans*). It may be seen, however, that the catalytic triad crucial for the catalytic mechanism of the α-CAs are not conserved in PfCAdom. Amino acid residue Thr477 (in blue) is missing in PfCA1 (truncated form). Plasmodium CA numbering system was used.

```
PfCAdom     --------------------LK----NVDYYEHGYN-------W-----DIGQCKTGKYQS
PfCA1       -------------------------------------------------------------
hCAI        ---------------------------MASPDWGYDDKNGPEQWSKLYP----IANGNNQS
hCAII       ----------------------------MSHHWGYGKHNGPEHWHKDFP----IAKGERQS
NgonCA      MPRFPRTLPRLTAVLLLACTAFSAAAHGNHTHWGYTGHDSPESWGNLSEEFRLCSTGKNQS
TaCA        ---MKRVLVTLGAV----AALATGAVAGGGAHWGYSGSIGPEHWGDLSPEYLMCKIGKNQS

PfCAdom     PVDLPMKDLKE-RELKNISDVYLNLFDDDNYAWNNYNKPWMKGDFFYYYEYFIKKIVINR
PfCA1       -----MKDLKE-RELKNISDVYLNLFDDDNYAWNNYNKPWMKGDFFYYYEYFIKKIVINR
hCAI        PVDIKTSETKHDTSLKPISVSYNPA--------------------------TAKE-IINV
hCAII       PVDIDTHTAKYDPSLKPLSVSYDQA--------------------------TSLR-ILNN
NgonCA      pvnitet---vsgklpaikvnykps---------------------------mvd-venN
TaCA        pidinsada-vkaclapvsvyyvsd---------------------------aky-vvnN
                          *  :.  *                                  : *
                                               299 301
PfCAdom     QNNIFQIK--AARDGIIPFGVLFTTEQPAMFYADQIHFHAP------SEHTFQGSGNRRE
PfCA1       QNNIFQIK--AARDGIIPFGVLFTTEQPAMFYADQIHFHAP------SEHTFQGSGNRRE
hCAI        GH-SFHVNFEDNDNR----SVLKGGPFSDSYRLFQFHFHWGSTNEHGSEHTVDGVKYSAE
hCAII       GH-AFNVEFDDSQDK----AVLKGGPLDGTYRLIQFHFHWGSLDGQGSEHTVDKKKYAAE
NgonCA      GH-TIQVNYPEGGNT----LTVNGR----TYTLKQFHFHVP------SENQIKGRTFPMe
TaCA        GH-TIKVVMG-GRGY----VVVDGK----RFYLKQFHFHAP------SEHTVNGKHYPFe
             .  :.:              .:        :   *:***        **. ..      *
              320
PfCAdom     IEM-QIFHSTNYFYDIQDDKSKYKKKYGLHIYNNLKKNSKETSKKDSSRYHSYLMSFLMN
PfCA1       IEM-QIFHSTNYFYDIQDDKSKYKKKYGLHIYNNLKKNSKETSKKDSSRYHSYLMSFLMN
hCAI        LHVAHWNSAKY------------------------------------------------
hCAII       LHLVHWNT-KY------------------------------------------------
NgonCA      AHFVHLDEN--------------------------------------------------
TaCA        AHFVHLDKN--------------------------------------------------
             ..

PfCAdom     SLSNEQLQNKYNKKKRIKKMKNQYEVISITFTSAEINASTINAFKKLPSEKFLRTIINVS
PfCA1       SLSNEQLQNKYNKKKRIKKMKNQYEVISITFTSAEINASTINAFKKLPSEKFLRTIINVS
hCAI        -----------SSLAEAASKADGLAVIGVLMKVGEANPKLQKVLDALQAIKTK-------
hCAII       -----------GDFGKAVQQPDGLAVLGIFLKVGSAKPGLQKVVDVLDSIKTK-------
NgonCA      ---------------------KQPLVLAVLYEAGKTNGRLSSIWNVMPMTAGK-------
TaCA        ---------------------GNITVLGVFFKVGKENPELEKVWRVMPEEPGQ-------
                                     *:.:    .. :    .    :
                                                    477
PfCAdom     SAVHVGSDPTLVELKDALNLDALMMMLNIEDMQFLSYQGSSTLPLCDENVSWKVAKQPLP
PfCA1       SAVHVGSDPT--------------------------------------------------
hCAI        -----G------KRAPFTNFDP--STLLPSSLDFWTYPGSLTHPPLYESVTWIICKESIS
hCAII       -----G------KSADFTNFAA--RGLLPESLDYWTYPGSLTTPPLLECVTWIVLKEPIS
NgonCA      ------------V-KLNQPFDA--STLLPKRLKYYRFAGSLTTPPCTEGVSWLVLKTYDH
TaCA        ------------KRHLTARIDP--EKLLPENRDYYRYSGSLTTPPCSEGVRWIVFKEPVE


PfCAdom     VSTETILNFYYLLKKHTPNYSGSDNDNYRSLQNVEDNTRHYRK-
PfCA1       --------------------------------------------
hCAI        VSSEQLAQFRSLLSNVEGDNAVPMQHNNRPTQPLKGRTVRASF-
hCAII       VSSEQVLKFRKLNFNGEGEPEELMVDNWRPAQPLKNRQIKASFK
NgonCA      IDQAQAEKFTRAV----------GSENNRPVQPLNARVVIE---
TaCA        MSREQLEKFRKVM----------GFDNNRPVQPLNARKVMK---
```

Figure 4. SDS-PAGE protonography. The protonogram was obtained using bCA, PfCA1 and PfCAdom. The CA activity was detected by immersing the gel in CO_2-saturated ddH_2O. The yellow band corresponds to the CA position on the gel responsible for the drop of pH from 8.2 to the transition point of the dye in the control buffer. Legend: lane 1, bCA, commercial bovine CA; lane 2, PfCA1, truncated form from *P. falciparum*; lane 3, PfCAdom, full-length domain from *P. falciparum*.

allows the identification of CA activity belonging to different classes. Here, we also want stress the fact that Thr477 in PfCAdom is crucial for its catalysis as demonstrated by the fact that PfCAdom activity is higher than PfCA1.

Declaration of interest

The authors declare no conflict of interest. This work was supported in part by an FP7 European Union Project [Gums & Joints, Grant agreement Number HEALTH-F2–2010-261460].

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