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ACCELERATED PUBLICATION Completing the folate biosynthesis pathway in *Plasmodium falciparum*: *p*-aminobenzoate is produced by a highly divergent promiscuous aminodeoxychorismate lyase

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Enzymes that produce or recycle folates are the targets of widely used antimalarial drugs. Despite the interest in the folate metabolism of *Plasmodium falciparum*, the molecular identification of ADCL (aminodeoxychorismate lyase), which synthesizes the *p*-aminobenzoate moiety of folate, remained unresolved. In the present study, we demonstrate that the

plasmodial gene *PF14_0557* encodes a functional ADCL and report a characterization of the recombinant enzyme.

Key words: *p*-aminobenzoate, aminodeoxychorismate lyase, antifolate, catalytic promiscuity, malaria, pyridoxal phosphate.

INTRODUCTION

Tetrahydrofolate and related cofactors, collectively termed folates, are used by enzymes that mediate the transfer of onecarbon units and are strictly required for a number of basic metabolic processes, in particular the biosynthesis of purines and of thymidylate [1].

Humans are unable to synthesize folates *de novo* and must obtain these cofactors from the diet. In contrast, most pathogenic micro-organisms produce folates through specific pathways [1,2], which therefore appear to be suitable sites of action for selective antimicrobial therapy. Indeed, some enzymes involved in the biosynthesis and metabolism of folates are already targeted by established antimicrobial drugs, such as the antibacterials sulfonamides and trimethoprim and the antimalarials pyrimethamine and proguanil [3].

Analogous to bacteria, the malaria parasite *Plasmodium falciparum* assembles tetrahydrofolate from pterin, PABA (*p*-aminobenzoate) and glutamate [4] (Figure 1A). To obtain the final product, the pterin moiety is first synthesized from GTP and then condensed with PABA to form dihydropteroate, which is subsequently glutamylated and reduced. PABA is also produced endogenously by *P. falciparum* [5]. Recently, a study where a specific inhibitor of PABA synthesis was tested on *P. falciparum* found an impaired growth of treated parasites [6], implying that enzymes involved in this metabolic branch may be potential drug targets.

In *Escherichia* coli, PABA is derived from chorismate in two steps (Figure 1B). The first step is the synthesis of ADC (4amino-4-deoxychorismate) by ADC synthase, a heterodimeric complex formed by a glutamine amidohydrolase subunit and by a chorismate aminase subunit [2,7]. The second step is carried out by ADCL (aminodeoxychorismate lyase), an enzyme dependent on PLP (pyridoxal 5'-phosphate) that catalyses the β -elimination of pyruvate and the aromatization of the ADC ring to yield PABA [8] (Figure 1B).

In *P. falciparum*, ADC synthase consists of a single polypeptide, with a glutaminase domain and a chorismate aminase domain. The encoding gene was identified over a decade ago [9] as it shows substantial sequence similarity to ADC synthases from other organisms. In contrast, there is no clear orthologue of the bacterial or eukaryotic ADCLs in the plasmodial genome [4,10]. Therefore this represents a missing piece in our understanding of malaria parasite metabolism and may indicate either that plasmodia use a different enzyme (for instance, non-PLP-dependent) to catalyse the synthesis of PABA, or that they possess a PLP-dependent but highly divergent ADCL, which is not easily recognizable on the basis of sequence.

In the present study, we provide the bioinformatic and biochemical demonstration that *PF14_0557* is the ADCL-encoding gene in the *P. falciparum* genome, as well as a first characterization of the recombinant enzyme.

EXPERIMENTAL

Materials

Rabbit muscle LDH (lactate dehydrogenase) was from Fluka. Chorismic acid was from Sigma. All other reagents were from Fluka or Sigma–Aldrich.

A pET30a (Novagen) plasmid bearing the sequence of *E. coli* chorismate aminase (commonly known as PabB) and a pRSETa (Invitrogen) plasmid bearing the sequence of *E. coli* ADCL were provided by Professor Chris Abell and Dr Nigel Howard (Department of Chemistry, University of Cambridge, Cambridge, U.K.). The clones were used to transform *E. coli* BL21-CodonPlus cells (Stratagene) and the recombinant His₆-tagged proteins were expressed and purified as described in [11].

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Abbreviations used: ADC, 4-amino-4-deoxychorismate; ADCL, aminodeoxychorismate lyase; BCAT, branched-chain amino acid transaminase; DAAT, D-amino acid aminotransferase; LDH, lactate dehydrogenase; PABA, *p*-aminobenzoate; PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine 5'-phosphate. ¹ Present address: Institute of Clinical Chemistry and Pathobiochemistry, Technical University of Munich, 81675 Munich, Germany.

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Figure 1 Structure of monoglutamylated 5,6,7,8-tetrahydrofolate and the PABA biosynthesis pathway

(A) Structure of monoglutamylated 5,6,7,8-tetrahydrofolate, a typical folate, highlighting the three components from which is assembled. (B) The PABA biosynthesis pathway in *E. coli* and *P. falciparum. In vivo*, the ammonia molecule for the synthesis of ADC comes typically from the hydrolysis of L-glutamine, carried out by a specialized subunit or domain of ADC synthase.

Bioinformatic analyses

Similarity searches were performed using the BLAST software at the National Center for Biotechnology Information [12]. Protein searching and recognition was performed by consulting the B6 database [13]. Pairwise sequence alignments were performed with EMBOSS Needle at the EBI. Multiple sequence alignments were created with ClustalW2 [14] and displayed with BoxShade (http://www.ch.embnet.org/software/BOX_form.html).

Subcloning the PF14_0557 coding sequence

A synthetic ORF with the codon-optimized sequence of *PF14_0557* was provided by Professor Pradip Rathod (Department of Chemistry, University of Washington, Seattle, WA, U.S.A.). The sequence was amplified using the following primers: 5'-ATAATTAAT<u>CGGTCCG</u>ATGGCCATCCTCA-TCAAGGA-3' (forward; the start codon of the coding sequence is shown in bold) and 5'-TAAAATATA<u>CGGACCG</u>TAATTA-ACGGCCGGGTACCT-3' (reverse). Both primers carried 5'-tails such that the amplification products contained CpoI target sequences (underlined) near to both ends.

The amplified product was digested with CpoI and subsequently ligated into a pET28-CpoI plasmid. This vector is a derivative of pET28 (Novagen) and contains a single CpoI restriction site in the cloning region, downstream of a sequence encoding a His₆ tag [15]. As CpoI cleavage generates two non-identical 3' overhangs (GTC on one strand and GAC on the other), the CpoI-cleaved fragment was cloned in-frame and directionally into the plasmid.

The ligated plasmid was used to transform Tuner *E. coli* cells (EMD Biosciences) and selection was carried out on LB agar plates with 50 μ g/ml kanamycin. The plasmid from a positive clone was extracted and its insert was sequence verified to confirm that it contained the correct PF14_0557 coding sequence. This clone was used for all subsequent experiments.

Recombinant expression of PF14_0557

A subculture of transformed bacteria was used to inoculate 1 litre of LB broth, containing 50 μ g/ml kanamycin. Growth was conducted at 37 °C until the D_{600} reached ~0.8, at which point

IPTG was added to a final concentration of 1 mM. Induced cells were transferred to 20 °C and grown for 16 h, then harvested by centrifugation (10000 g for 10 min) and resuspended in 80 ml of lysis buffer (50 mM Tris/HCl, pH 8.5, 300 mM NaCl, 40 μ M PLP and 5 mM 2-mercaptoethanol) supplemented with 0.1 mg/ml hen's egg white lysozyme (Fluka). The bacterial suspension was stored on ice for 30 min before sonicating for 15 min. After sonication, the soluble lysate fraction was loaded on to a Talon cobalt-affinity resin (Clontech) equilibrated in lysis buffer, and the recombinant His₆-tagged protein was purified following the manufacturer's instructions. The protein fractions were analysed by gel electrophores is and those fractions with a purity >90%were pooled and dialysed against storage buffer [50 mM Tris/HCl, pH 8.5, 300 mM NaCl, 4 μ M PLP, 1 mM DTT and 5 % (v/v) glycerol], concentrated by ultrafiltration and stored at -80 °C. The final yield was \sim 40 mg of purified PF14_0557 per litre of bacterial culture.

ESI–MS analysis

The recombinant PF14_0557 protein $(1 \ \mu M)$ was placed in 500 μ l of reaction buffer (100 mM bicine, pH 8.5, 5 mM MgCl₂, 5 mM DTT and 20 μ M PLP) also containing 100 mM (NH₄)₂SO₄, 100 μ M chorismate and 5 μ M chorismate aminase (PabB from *E. coli*). In parallel, two control reactions were conducted, omitting PF14_0557 and chorismate aminase respectively.

The solutions were incubated at 30 °C for 1 h. Then the samples was ultrafiltered to remove proteins [Vivaspin 5000 Da MWCO (molecular mass cut-off), Sartorius] and 2 μ l of phosphoric acid were added to each ultrafiltrate to reach pH ~3.5. At this pH, PABA exists mostly in the uncharged form [16]. Each sample was subsequently extracted three times with 500 μ l of ethyl acetate. Control experiments using authentic PABA showed that such a treatment was sufficient to completely remove PABA from the aqueous phase. The organic phase (~1.5 ml total) was then dried using a speed vacuum.

Before MS, all samples were redissolved in 200 μ l of methanol (HPLC grade) and supplemented with a drop of formic acid [17]. The samples were directly perfused into an ESI–MS single quadrupole SQ detector (Waters) in positive scan using the following settings: capillary voltage 1.95 kV, cone voltage 30 V, source temperature 150 °C, desolvation temperature 300 °C.

ADCL activity assays

The ADCL activity was assayed in the presence of chorismate aminase (PabB from *E. coli*) which continuously fed the ADCL reaction by converting chorismate into 4-amino-4-deoxychorismate.

Reaction kinetics were monitored spectrophotometrically (Cary 400, Varian) by two alternative methods [18]: a continuous absorption assay at 278 nm that monitors directly the formation of PABA, and a coupled assay with LDH where formation of pyruvate is measured.

Assays at 278 nm were carried out at 30 °C in reaction buffer (100 mM bicine, pH 8.5, 5 mM MgCl₂, 5 mM DTT and 20 μ M PLP) also containing 100 mM (NH₄)₂SO₄ and 5 μ M PabB. After adding ADCL (but not chorismate) to this mixture, spectra were collected for 15 min to make sure that absorption in the 278 nm region was stable. Finally, the reaction was started by adding chorismate (100 μ M final concentration) and spectra were collected at 2 min intervals for another 20–35 min.

Activity measurements exploiting the LDH-coupled assay were usually carried out at 30 °C in reaction buffer containing 100 mM (NH₄)₂SO₄, 5 μ M PabB, 0.2 mM NADH and 5 units/ml LDH.

This mixture was supplemented with ADCL (10–500 nM) and equilibrated for a few minutes at 30 °C. Then chorismate (typically 200 μ M final concentration) was added to start the reaction, and the kinetics of NADH disappearance were followed at 340 nm for 5–15 min. Least-squares fittings of the data were performed using Sigma Plot (SPSS).

Half-transamination assays

To assess the occurrence of half-transamination reactions between PF14_0557 and branched-chain amino acids or D-amino acids, solutions containing the enzyme ($\sim 20 \,\mu$ M) in 100 mM bicine (pH 8.5) were supplemented with the compound under examination (typically, 5 mM final concentration). Reactions were carried out at 30 °C and changes in the PLP absorption spectrum were monitored using a Cary 400 spectrophotometer. To measure the kinetics of half-transamination reactions, spectra were collected at regular intervals for up to 30 min after mixing the enzyme with the amino acid.

BCAT (branched-chain amino acid transaminase) activity assay

The kinetic experiments to assess BCAT activity were performed with a coupled assay using glutamate dehydrogenase. The reaction mixture contained 100 mM bicine, pH 8.5, 5 mM DTT, 5 mM L-glutamate, 15 mM NH₄Cl, 0.25 mM NADH and 5 units/ml glutamate dehydrogenase, in addition to PF14_0557 (5 μ M) and 5 mM 4-methyl 2-oxovalerate (the oxoacid of leucine). Reactions were conducted at 30 °C, and the rate of 2-oxoglutarate formation was assessed by monitoring spectrophotometrically the coupled disappearance of NADH at 340 nm.

DAAT (D-amino acid aminotransferase) activity assay

D-aspartate:2-oxoglutarate aminotransferase activity was measured via a coupled assay with malate dehydrogenase. The assay mixture contained 100 mM bicine, pH 8.5, at 30 °C, 0.1 or 1 mM 2-oxoglutarate, 0.25 mM NADH and 25 units/ml malate dehydrogenase, in addition to PF14_0557 (2 μ M) and D-aspartate at several different concentrations. The rate of oxaloacetate formation was measured by monitoring spectrophotometrically the coupled disappearance of NADH at 340 nm.

For the D-alanine:2-oxoglutarate aminotransferase activity, the kinetic experiments were performed by a coupled assay that exploits LDH. The reaction mixture contained 100 mM bicine, pH 8.5, 5 mM 2-oxoglutarate, 0.25 mM NADH and 5 units/ml LDH, in addition to PF14_0557 (5 μ M) and D-alanine at several different concentrations.

The D-glutamate:2-oxaloacetate or D-glutamate:pyruvate aminotransferase reactions were assessed through a coupled assay with glutamate dehydrogenase. The buffer used was 100 mM bicine, pH 8.5, at 30 °C. The reaction mixture also contained 1 or 10 mM oxoacid, 0.25 mM NADH, 15 mM NH₄Cl and 5 units/ml glutamate dehydrogenase, in addition to PF14_0557 (2 μ M) and D-glutamate at several different concentrations.

RESULTS

Bioinformatic identification of PF14_0557 as the candidate plasmodial ADCL

The ADCL proteins validated so far [8,19–21] show little sequence conservation. Indeed ADCL sequences from proteobacteria such as *E. coli* [8] are scarcely alignable with those from actinomycetes [20] or from fungi such as *Saccharomyces cerevisiae* [19], suggesting that these enzymes have undergone divergent evolutionary dynamics. Despite this, all validated ADCLs belong to the same structural subgroup of PLP-dependent enzymes, designated 'fold-type IV' [22]. This subgroup is known to comprise just two enzyme types in addition to ADCL, namely DAAT and BCAT [13].

According to the B6 database [13], the genome of *P. falciparum* encodes only 12 PLP-dependent enzymes in total (Table 1). For ten of these gene products, function could be assigned with good confidence based on homology. The last two gene products, however, show only weak similarity to functionally validated enzymes, making the bioinformatic association to specific activities unreliable. The two genes in question are *PFD0285c*, whose predicted product is a very large protein with a decarboxylase domain, and *PF14_0557*, which encodes a hypothetical protein that belongs to fold-type IV and is most similar to validated DAATs.

The deduced PF14_0557 protein shares no more than 22% identity with the ADCLs from either *E. coli* or *S. cerevisiae*. Furthermore, although two of the three amino acid residues proposed to be functionally essential in all fold-type IV enzymes (Lys¹⁴⁰ and Glu¹⁷³, numbered according to the *E. coli* ADCL sequence [23]) are conserved in the plasmodial sequence, the third one (Arg⁴⁵) is not conserved and is replaced by an asparagine residue (Supplementary Figure S1 at http://www.biochemj. org/bj/455/bj4550149add.htm).

Nevertheless, PF14_0557 is apparently the only fold-type IV protein encoded in the *P. falciparum* genome and it also retains a threonine residue (Thr²⁸) that is important in the catalytic mechanism of *E. coli* ADCL [23]. For these reasons, PF14_0557 seemed the only reasonable candidate to be the parasite ADCL, assuming that, along evolution, plasmodia had preserved this enzyme as PLP-dependent.

Inspection of the transcriptomic data available at PlasmoDB (http://plasmodb.org/) showed that $PF14_0557$ is expressed at significant levels in various phases of the *P. falciparum* life cycle, particularly in blood stages such as the late trophozoite and early schizont stages, when DNA synthesis is intense and antifolates are mostly active [24]. The actual production of the protein in the trophozoite stage is confirmed by proteomics [25].

The recombinant PF14_0557 protein has ADCL activity

To establish whether the *PF14_0557* gene product is a functional ADCL, we overexpressed a His₆-tagged version of the protein in *E. coli* cells, and purified it by immobilized metal-affinity chromatography. The recombinant protein, obtained in good yield and in soluble form, exhibited a characteristic PLP absorption spectrum.

Since the substrate of ADCL (4-amino-4-deoxychorismate) is unstable and not commercially available, we assayed the ability of PF14_0557 to produce PABA in the presence of chorismate aminase (PabB from *E. coli*) which can use ammonia to convert chorismate into ADC. We incubated the recombinant PF14_0557 with chorismate, PabB and free ammonia for 1 h, extracted the reaction products and subjected then to ESI–MS analysis.

The mass spectrum of the enzymatic reaction mixture comprised a very intense peak with an m/z ratio of 138.23, identical within error to that expected for PABA (M + 1 species). Such a peak was undetectable in the mass spectra of control reactions where either chorismate aminase or PF14_0557 had been omitted (Supplementary Figure S2 at http://www.biochemj.org/bj/455/ bj4550149add.htm). These findings unambiguously proved that PF14_0557 possesses ADCL activity. The virtual absence of PABA from reaction samples that did not contain the plasmodial

Table 1 Inventory of the plasmodial genes that encode PLP-dependent enzymes

The complete set of PLP-dependent proteins encoded in the genome of *Plasmodium falciparum* 3D7 [37] was taken from the B6 database (http://bioinformatics.unipr.it/cgi-bin/bioinformatics/ B6db/home.pl) and obtained as described in [13]. The B6 database groups PLP-dependent enzymes into 'families', defined as monophyletic groups of sequences endowed with the same enzymatic function, and usually the family name comprises the four-digit EC number of the corresponding activity [13]. In this Table, the 'family' column reports the name of the family whose hidden Markov model scored best when compared with the plasmodial query.

Putative activity	Family	Protein accession number	E-value	Gene
Glycine hydroxymethyltransferase*	2.1.2.1	XP 001350750	8.4×10 ⁻²⁴⁹	PFL1720w
Glycine C-acetyltransferase	2.3.1.29	XP_001348328	2.5×10 ⁻⁴³	PF14 0155
5-Aminolaevulinic acid synthase	2.3.1.37	XP_001350846	3×10 ⁻¹⁵⁸	PFL2210w
Aspartate aminotransferase	2.6.1.1 c	XP_001349556	1.5×10 ⁻⁷²	PFB0200c
Ornithine-oxoacid aminotransferase+	2.6.1.13	XP_966078	9.9×10 ⁻²⁵⁵	PFF0435w
Cysteine desulfurase	2.8.1.7 a	XP_001349169	7.5×10 ⁻²¹⁸	MAL7P1.150
Ornithine decarboxylase:	4.1.1.17 1	XP_001347606	4.7×10^{-80}	PF10_0322
Selenocysteine lyase	4.4.1.16 a	XP_001349069	1.1×10 ⁻⁵⁴	PF07_0068
O-phospho-L-seryl-tRNASec:L-selenocysteinyl-tRNA synthase	Sec synthase	XP_001350460	2.3×10 ⁻²⁵²	PFL0255c
Unclassified activity	Uncharacterized family Prosc§	XP_001352068	5×10 ⁻¹⁶⁶	PFI0965w
Unassigned	n/a	XP_001351370	1.9×10 ⁻¹⁷	PFD0285c
Unassigned	n/a	XP_001348731	2.3×10^{-05}	PF14_0557
Unassigned	n/a	XP_001348731	2.3×10^{-05}	PF14_0557

*Validated experimentally [38].

[†]Validated experimentally [39].

*Validated. Part of a bifunctional enzyme that possesses both ornithine decarboxylase and S-adenosyl-L-methionine decarboxylase activities [40,41].

§'Prosc' (PROline Synthase Co-transcribed) takes its name from a *Pseudomonas aeruginosa* gene that is transcribed together with a known proline biosynthetic gene. Although the yeast homologue was reported to show some amino acid racemase activity [42], the actual function of this ubiquitous group of PLP-dependent enzymes remains unknown.

enzyme further implied that any spontaneous conversion of ADC into PABA was negligible under our reaction conditions.

We also showed that the ADCL activity of PF14_0557 could be monitored by two distinct spectrophotometric methods: a continuous absorption assay at 278 nm that follows the formation of PABA, and a coupled assay with LDH where formation of pyruvate is measured [18]. Although the rates determined with the two methods were comparable, the coupled assay was more reliable and less subject to interferences, so it was used in preference for the subsequent kinetic studies.

Comparing the activities of PF14_0557 and of the E. coli ADCL

The impossibility of performing the ADCL reaction in the presence of well-defined concentrations of substrate precluded the determination of the standard parameters describing kinetic efficiency (k_{cat} or k_{cat}/K_m) for PF14_0557. To obtain, at least, limits for such parameters, we first needed to establish conditions under which the overall process of PABA formation was limited by the lyase step rather than by the preceding ADC synthesis. We hence carried out a set of reactions using a relatively high concentration of chorismate aminase (i.e. the *E. coli* PabB; 5 μ M) and much lower concentrations of PF14_0557 (nanomolar range).

Figure 2 shows how the observed reaction rate changed as a function of either the concentration of PF14_0557 or of the *E. coli* ADCL, which was used as a control and reference. The plot can be split into three parts, evidently reflecting different kinetic regimes. At lyase concentrations >100 nM (right-hand part of the plot) the observed reaction rate was independent of the concentration of either lyase. This implies that the overall reaction was completely limited by the synthase step, a hypothesis supported by the observation that the rate in this region of the plot is very close to that calculated on the basis of the known catalytic parameters of PabB [18].

In the central part of the plot (10–100 nM range), the observed rate declined somewhat with decreasing lyase concentration, suggesting that under these conditions the lyase step was becoming partially rate limiting.



Figure 2 The reaction rate of either PF14_0557 or the *E. coli* ADC lyase in the presence of 5 μ M PabB and 200 μ M chorismate (pH 8.5, 30 °C)

In the left-hand part of the plot the observed rate of reaction increases linearly with the concentration of the lyases, with a slope of $\sim 5 \text{ s}^{-1}$ (plasmodial enzyme) or $\sim 15 \text{ s}^{-1}$ (*E. coli* ADCL). In the right-hand part of the plot, the observed reaction rate is constant ($\sim 215 \text{ nM} \cdot \text{s}^{-1}$) and close to the expected reaction rate of PabB (at pH 8.5, 25 °C, the enzyme was reported to show $k_{cal} = 0.032 \text{ s}^{-1}$ and $K_m^{\text{chrismale}} = 58 \ \mu\text{M}$ [18]).

Finally, in the left-hand part of the plot, the observed rate of product formation depended linearly on the concentration of PF14_0557 (or of its *E. coli* orthologue), as expected if the lyase step was completely rate-limiting under these conditions. The slopes in this part of the plot were ~5 s⁻¹ for PF14_0557 and ~15 s⁻¹ for the *E. coli* ADCL. These slopes represent lower limits for k_{cat} by the two lyases. Furthermore, since in the reaction mixtures the concentration of ADC was necessarily <200 μ M (the initial concentration of chorismate), conservative lower limits for k_{cat}/K_m can be estimated at 2.5×10⁴ M⁻¹ · s⁻¹ for PF14_0557 and at 7.5×10⁴ M⁻¹ · s⁻¹ for the *E. coli* ADCL. Overall, these data imply that PF14_0557 is an efficient enzyme, with an intrinsic ADCL activity comparable with that of its bacterial counterpart.

PF14_0557 shows a secondary DAAT activity

Given the similarity of PF14_0557 to DAATs and BCATs mentioned above, and given the tendency of PLP-dependent



Figure 3 Transaminase activity of PF14_0557 towards p-aspartate

(A) Progressive spectral changes observed upon reacting recombinant PF14_0557 (17 μ M) with 5 mM p-aspartate (100 mM bicine buffer, pH 8.5, 30 °C). Inset: time course of the half-transamination at two different wavelengths. The curves are best fits of the data points to exponential functions, yielding in both cases $k_{obs} = 0.012 \text{ s}^{-1}$. (B) Michaelis–Menten plot for the p-aspartate:2-oxoglutarate transamination catalysed by PF14_0557 (100 mM bicine buffer, pH 8.5, 30 °C). The titrations collected at 0.1 mM 2-oxoglutarate (\bigcirc) or 1 mM 2-oxoglutarate (\bigcirc) were nearly undistinguishable, implying that the second part of the transamination process (transfer of the amino group from PMP to 2-oxoglutarate) was not rate-limiting under these experimental conditions. The calculated catalytic parameters were $V_{max} = 155 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ (corresponding to $k_{cal} \approx 0.1 \text{ s}^{-1}$) and $K_m^{\text{p-aspartate}} = 18 \text{ mM}$.

enzymes to show catalytic promiscuity [26], we next aimed at testing whether PF14_0557 retained any activity as an aminotransferase.

In PLP-dependent enzymes, the absorption properties of PLP can be exploited to monitor substrate binding and the formation of catalytic intermediates (e.g. [27]). For example, when an aminotransferase reacts with its amino-group donor substrate (in the absence of amino-group acceptors) it undergoes a half-transamination, whereby the PLP form of the cofactor ($\lambda_{max} \approx 412$ nm) is converted into the pyridoxamine form [PMP (pyridoxamine 5'-phosphate); $\lambda_{max} \approx 330$ nm]. Accordingly, we performed a first test of the PF14_0557 propensity to transaminate branched-chain amino acids or D-amino acids by incubating the enzyme in the presence of these compounds and monitoring the ensuing spectral changes.

L-leucine, L-isoleucine and L-valine caused essentially no changes in the enzyme spectrum over a period of 20 min (Supplementary Table S1 at http://www.biochemj.org/bj/455/bj4550149add.htm), implying that PF14_0557 is unable to transaminate these branched-chain amino acids. The enzyme also failed completely to carry out a transamination reaction between L-glutamate and 4-methyl 2-oxovalerate (the oxoacid of leucine), a reaction that is typical of *bona fide* BCAATs [15].

In contrast with these results, some D-amino acids (in particular D-alanine, D-aspartate and D-glutamate) did elicit the formation of substantial quantities of PMP, indicative of a half-transamination



Figure 4 Effect of p-glutamate pre-incubation on the ADCL activities of recombinant PF14_0557 and of its *E. coli* counterpart

Activity was measured at 25 °C through the LDH-coupled assay, as described in the Experimental section, using 0.1 μ M lyase, 5 μ M PabB and 200 μ M chorismate. Before starting the reaction, the lyases were incubated for 5 min in just the reaction buffer (controls; open bars) or in the reaction buffer supplemented with 500 μ M D-glutamate (+D-Glu, grey bars). The results represent the means \pm S.D. of three replicates. The loss of activity by PF14_0557 is consistent with the D-glutamate-induced conversion of PLP into PMP, which is not an active form of the cofactor in the ADCL reaction. Conversely, the lack of effect of D-glutamate on the *E. coli* ADCL is consistent with the reported inability of this enzyme to react with D-glutamate [28].

process (Supplementary Table S1). Although accumulation of a 330 nm band took minutes to occur (Figure 3A and results not shown), the process was at least one order of magnitude faster than the reported half-transamination between the *E. coli* ADCL and D-alanine [28].

We confirmed the actual ability of PF14_0557 to carry out a complete transamination between D-alanine and 2oxoglutarate (using an LDH-coupled assay, which detects the formation of pyruvate as product of the transaminase reaction) or between D-aspartate and 2-oxoglutarate (using a malate dehydrogenase-coupled assay). For the latter reaction, a k_{cat} of 0.1 s^{-1} and a k_{cat}/K_m of $6 \text{ M}^{-1} \cdot \text{s}^{-1}$ were determined (Figure 3B). For the opposite process (D-glutamate:2-oxaloacetate transamination), we measured a slightly lower k_{cat} , but estimated a 20–100-fold higher k_{cat}/K_m , owing to much tighter binding of D-glutamate ($K_m < 0.1 \text{ mM}$; results not shown). Not unexpectedly, D-glutamate, at submillimolar concentrations, also caused a timedependent loss of the ADCL activity of PF14_0557, although it had no effect on the activity of the *E. coli* enzyme (Figure 4 and results not shown).

DISCUSSION

The identity and even the existence of a plasmodial enzyme catalysing the ADCL reaction have long remained uncertain [4,6,10,29]. In the present study we have positively identified PF14_0557 as the *P. falciparum* ADCL and shown that, despite its weak similarity to known ADCLs, the recombinant plasmodial enzyme converts ADC into PABA with efficiency comparable with that of its well-characterized *E. coli* counterpart. While the present paper was in preparation, the attribution of an ADCL function to PF14_0557 was also tentatively proposed in two different reviews [30,31].

A catalytically promiscuous divergent enzyme

We also explored the catalytic promiscuity of PF14_0557, demonstrating that it transaminates some D-amino acids, in particular D-glutamate and D-aspartate. The efficiency of this secondary activity is low, but not negligible, and might have some physiological significance. For example, it could contribute somehow to the degradation of exogenous D-amino acids, which are toxic to *Plasmodium* [32].

Even though the lyase and transaminase reactions appear drastically different, in terms of both substrates and outcome, the ability of ADCLs to show some DAAT activity is not unprecedented and may be inherent to these enzymes. As noted, the E. coli ADCL can carry out a very slow half-transamination with D-alanine [28]. An even more striking situation is found in Arabidopsis thaliana, where the same enzyme has been classified as ADCL by one group [33] and as DAAT by another group [34]. This promiscuity presumably depends on the similarities in both structure and reaction mechanism between ADCLs and DAATs. Similar to DAATs, ADCLs bind their substrate by forming a Schiff base between PLP and the substrate amino group, then proceed to deprotonate the amino carbon. Since ADC is subsequently aromatized (with the elimination of pyruvate), it cannot undergo a half-transamination reaction [23], but D-amino acids are not subject to this constraint, making transamination feasible.

This catalytic promiscuity suggests a couple of considerations on the evolution of ADCLs, on their divergence and on their intricate phylogenetic relationships with other fold-type IV enzymes. First, since catalytic promiscuity often begets the emergence of novel functions [35], it seems reasonable to assume that DAATs originated from ancestral ADCLs, possibly in multiple instances over the course of evolution. If this is the case, some extant ADCLs may well share more sequence similarity to DAATs than to their own orthologues in distantly related lineages.

We also speculate that in some organisms that lack a proper DAAT, but benefit nevertheless from low levels of that activity, the DAAT role might be surrogated by promiscuous ADCL enzymes. This would presumably enhance and favour the divergence between ADCLs. It has been argued enzyme orthologues that, in some lineages, possess a 'moonlighting', but important, function would be subject to lineage-specific selective pressures and to peculiar evolutionary trajectories [36].

Implications for drug targeting

Antifolates are already a mainstay of antimalarial chemotherapy, even though no new drugs of this type have been introduced since the middle of the last century [4,29]. Today, as P. falciparum strains resistant to the current antimalarials are becoming more and more diffused, there is an urge to identify new drugs and drug targets. With this perspective, inhibitors of PABA production may be a valuable addition to the antimalarial repertoire [6]. Although a shutdown of the PABA biosynthesis branch might by itself have only a limited effect on the vitality of P. falciparum (which can also import PABA from the host), such a shutdown could potentiate the efficacy of other antifolate drugs. The specific impact of PF14_0557 inhibition may be also exalted if really the additional activities of the enzyme are important for P. falciparum fitness. We have found that PF14_0557 can react with and be inhibited by D-cycloserine, an off-the-shelf antibiotic (G. Magnani and A. Peracchi, unpublished work). This process is relatively inefficient and unspecific (D-cycloserine is known to react with a number of PLP-dependent enzymes), but may represent a starting point for the development of more effective inhibitors.

Our findings also make obvious the identification of ADCLs in not only other *Plasmodium* species, but also different disease-causing protists such as *Toxoplasma gondii* (gene: *TGME49_081500*) and *Giardia lamblia* (gene: *GL50803_29078*), unveiling potential drug targets for the treatment of these parasitic diseases.

AUTHOR CONTRIBUTION

Giovanni Magnani carried out the majority of the experimental work and assisted the analysis of the data. Michela Lomazzi performed the ESI–MS experiments and analysis. Alessio Peracchi planned the research, conducted some kinetic experiments, analysed the data and wrote the paper. All authors participated in editing the paper in its final form prior to submission.

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SUPPLEMENTARY ONLINE DATA Completing the folate biosynthesis pathway in *Plasmodium falciparum*: *p*-aminobenzoate is produced by a highly divergent, promiscuous aminodeoxychorismate lyase

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PF14_0557	1MAILIKDNVPSLVELSVQDFIKLGIHEVYTMMKTVMSPEYIFNF
ADCL_S.cerevisiae	1 MSLMDNWKTDMESYDEGGLVANPNFEVLATFRYDPGFARQS SKKEI EAPDPRLELRDEDIRQQIINED
ADCL S.spFR-008	1MKIWLDGALRDAGTAQVSVLDHGLTVGDCVEEWKTVDCRALALTR
ADCL B. subtilis	1MIYVNGRYMEEKDAVLSPFDHGFLYGICVEFFRLYECCPFLLDW
ADCL P.aeruginosa	1MLDWVDGRPAAELSVRDRGLAYGDCLEALAVRACTPRLLER
ADCL E.coli	1MFLINGHKQESLAVSDRATOFGDCCTMARVIDCKVSLLSA
PF14_0557	45 SLHMNNTHKYCTNYLKVQENTNREHISKILKYLTVEDIYKNSSKN
ADCL S.cerevisiae	71 YSSYLRVREVNSGGDLLENIQHP AWKHD-CKTIVCORVEDMLQVIYERFFLLDEQYORIRIALSYFKID
ADCL S. SpFR-008	47HLDRIARSARGLGLPEPILDEVRRACAAVLAA-EPVPL
ADCL B. subtilis	46HIERLERALKDLOIEYTVSKHSILEMLDKLLKLNDIKDGNAR
ADCL P.aeruginosa	43HLARLEEGCRRLAIPIDTAALROELLAFCAA-LGDGV
ADCL E. coli	42 HIOSUODACORLMISC FWPOLEOEMKTLAAEOONGV
PF14_0557	91MIT-LTWDMNST-VDTLE
ADCL S.cerevisiae	140 FSTSLNDLLSLLVENLINCKEGNSEYHEKIOKMINEROCYKMRVLVSKTGDIRIEAIPMPMEPILKLTTD
ADCL S. SpFR-008	84G3LRITYTGGLSPLGS-DRGDDTPTLVVALGEAHPRPETTAVVTVD
ADCL B subtilis	88VELNI SAGI SDKGFVAOTYDKPTVLCFVNOLKPESLPLOKEGKVLS
ADCL P aeruginosa	79SPRPAYPERHWOOGVRLF
ADCL E coli	79TAVVISRGSGGRGYSTINSGPATRILSVTAVPAHYDRIRNEGITIA
ADCD_B.COII	
	4
PF14 0557	135 KYYSILCYIKCLPKHODHVOIDMMCGERKTPNIKYADVFOVRDKFLKLKNENS
ADCL S.cerevisiae	210 YDSVSTYFIKTMLNGFLIDSTINWDVVVSSEPLNASAFTSF/TTSRDHYARARVRMOTAINNLRGSEPTS
ADCL S. SpFR-008	129WTRNERGALSGL%TTSYAENVVALARAREOGA
ADCL B. subtilis	134IRRNTPEGSFRLASHHYLNNMYAKREIGNDPR
ADCL P. aeruginosa	125ACRTRIAEOPLIAGLISHLNRLEOVLARAEWSDAGH
ADCL E. coli	125ISPVRIGRNPHLAGIKHLNRLEOVLIRSHLEOTNA
PF14 0557	188HEVULFNESNOHTEGLSCHEFCFLNNTLYT-AKDELVLKGTIREOIINLCERENIKLKKDF
ADCL S. cerevisiae	280 SVSOCRIFSNKSGL MPGSITNVAVIOKDPNGSKKYVTPRLATGCLCGTMRHYLLRLGL
ADCL S. SpFR-008	161TPALFANTYGALCPGTGSNVFVVLDGEIHTPPVSSGCLAGITRALTAEWTGARETELPMDV
ADCL B. subtilis	166V2GUFLTEDGAWA2GIISNVFWRKGRCIYTPSLDTGILDGYTRFIIENAKDIGLELKTGRYELEA
ADCL P aeruginosa	160 ABC MI.DUHER. WEGVESN LIVI.DCTI.VAPDI.RECGVAGVMEAELLERAEGIGVPLATEDVSMAE
ADCL E coli	
ADCI_E.COII	
PF14 0557	248 IDIGTHKFEFSFICSTTRNILDIRKTILFTENRKEPYEMNUNHPITENKKEKVETYI
ADCL S cerevisiae	340 IEEE IDIGSLTVGNEVILENGVMGCIK-GTVKTKY-
ADCL S SDFR-008	222 LER DE TELTSSIRDVOGVHRVDORTLAADGDVTAKATRIF-AERSAADTDP
ADCL B subtilie	232 LINE DE AUMENCUTET DE RETEREN VCCCCE ARGAINET FANNE LEF
ADCL P seruginos	226 Interpretence of the treatment of the transfer of the treatment of the
ADCL F coli	220 MAT THE TE MONTOWST MUTADON OF COMPLEX VEVI ADD OF DOWN
ADCL_E.COII	220 SLY BENVICENDER WE VEROBUSE SATILIEILAPICERPN

Figure S1 Multiple sequence alignment of the PF14_0557 protein with functionally validated ADCLs from *Saccharomyces cerevisiae* (GenBank® NP_014016), *Streptomyces sp. FR-008* (GenBank® ACL50980), *Bacillus subtilis* (GenBank® NP_387957), *Pseudomonas aeruginosa* (GenBank® NP_251654) and *Escherichia coli* (GenBank® P28305)

Positions that are conserved in all functionally validated ADCLs are shaded, and the active-site lysine residue that forms a Schiff base with PLP is indicated by an arrow. A red shade highlights those conserved residues that are mutated in PF14_0557.

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Figure S2 ESI-MS identification of PABA produced from chorismate by the combined activities of chorismate aminase (PabB) and PF14_0557

Reactions were conducted in the presence of 100 μ M chorismate, as described in the Experimental section of the main text. (A) The reaction mixture contained 1 μ M PF14_0557 and no PabB. (B) The reaction mixture contained 5 μ M PabB and no PF14_0557. (C) The reaction mixture contained both 5 μ M PabB and 1 μ M PF14_0557. The grey circle indicates a newly formed peak with an apparent *m*/z ratio of 138.2. For comparison, (D) shows the ESI–MS spectrum of a solution containing authentic PABA (100 μ M) incubated for 1 h in reaction buffer, in the absence of chorismate and enzymes.

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Table S1 Half-transamination assay between PF14_0557 and a selected set of $\mbox{L-}$ and $\mbox{D-}amino$ acids

Potential ligands (5 mM) were mixed with the enzyme in 100 mM bicine buffer, pH 8.5, at 30 °C. Spectral changes were measured every 2 min for at least 20 min after mixing, looking for the disappearance of the main band of PLP (\sim 412 nm) and concomitant rise in absorbance at \sim 330 nm, indicative of PMP formation. The accumulation of the new spectroscopic species is indicated semi-quantitatively by the number of + signs ranging from – (negligible) to + + + (complete and fast).

Amino acid	New spectroscopic species
Glycine	-
L-Valine	-
L-Leucine	-
L-Isoleucine	-
L-Methionine	-
L-Alanine	-
L-Aspartate	-
L-Glutamate	-
D-Alanine	+ +
D-Serine	-
D-Threonine	-
D-Aspartate	+ + +
D-Asparagine	+
D-Glutamate	+ + +
D-Cysteine	-
D-Isoleucine	-
D-Methionine	-
D-Tyrosine	-
D-Iryptophan	-
D-HISTIGINE	-
D-Arginine	-

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