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The synthesis of pABA: Coupling between the glutamine amidotransferase and aminodeoxychorismate synthase domains of the bifunctional aminodeoxychorismate synthase from *Arabidopsis thaliana*

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ABSTRACT

Aminodeoxychorismate (ADC) synthase in plants is a bifunctional enzyme containing glutamine amidotransferase (GAT) and ADC synthase (ADCS) domains. The GAT domain releases NH_3 from glutamine and the ADCS domain uses NH_3 to aminate chorismate. This enzyme is involved in folate (vitamin B9) biosynthesis. We produced a stable recombinant GAT–ADCS from *Arabidopsis*. Its kinetic properties were characterized, and activities and coupling of the two domains assessed. Both domains could operate independently, but not at their optimal capacities. When coupled, the activity of one domain modified the catalytic properties of the other. The GAT activity increased in the presence of chorismate, an activation process that probably involved conformational changes. The ADCS catalytic efficiency was 10^4 fold higher with glutamine than with NH_4Cl, indicating that NH_3 released from glutamine and used for ADC synthesis did not equilibrate with the external medium. We observed that the GAT activity was always higher than that of ADCS, the excess of NH_3 being released in the external medium. In addition, we observed that ADC accumulation retro-inhibited ADCS activity. Altogether, these results indicate that channeling of NH_3 between the two domains and/or amination of chorismate are the limiting step of the whole process, and that ADC cannot accumulate.

Introduction

Folates are a family of cofactors that are essential for all cellular one-carbon transfer reactions. They are involved in several metabolic pathways, all of prime importance, such as the synthesis of purines, thymidylate, pantothenate, methionine and the interconversion of serine and glycine [1,2]. Folate cofactors are made of three distinct parts: a pterin ring, a *p*-aminobenzoate (pABA)[2] moiety, and a glutamate residue, to which is usually attached a γ-linked polyglutamyl tail of up to about six residues [3]. These cofactors belong to the group of B vitamins (vitamin B9) and their biosynthesis is a matter of concern for at least two reasons. First, folate deficiency in humans is a worldwide health problem and because plant foods are a major source of folate, it is important to understand how these cofactors are synthesized to develop strategies aimed to enhance folate content in plants [4–6]. Second, the biosynthesis of folate derivatives is a target of choice for antibiotics, anti-parasites and herbicides. Up to now, the antifolates that have been developed and used in therapy target only two enzymes of the pathway, namely dihydropteroate synthase (targeted by pABA analogs, such as sulfonamide compounds), and dihydrofolate reductase (targeted by pteridin analogs, such as methotrexate and trimethoprim). However, the increasing resistance seen in both bacterial and eukaryotic pathogens, including the apicomplexans *Plasmodium falciparum* and *Toxoplasma gondii* [7], requires the identification of new targets.

Among the potential targets, the enzymes involved in the pABA branch of the folate pathway are of great interest. Indeed, the only known metabolic fate of pABA is its commitment in folate synthesis and it was recently shown that the production of pABA in plants, together with the production of pterins, is rate limiting for the whole folate pathway [6,8]. pABA is synthesized from chorismate, a metabolite also involved in *p*-hydroxybenzoate and aromatic amino acid synthesis [9–11]. As shown in Fig. 1A, the first step is the amination of chorismate to form 4-amino-4-deoxychorismate (ADC). In a second step, ADC is aromatized with loss of pyruvate to form pABA.

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[2] *Abbreviations used*: ADC, 4-amino-4-deoxychorismate; ADCS, ADC synthase; ADCL, ADC lyase; GAT, glutamine amidotransferase; AtADCS, *Arabidopsis thaliana* ADC synthase; EcADCL, *Escherichia coli* ADCL; pABA, *p*-Aminobenzoate; PLP, pyridoxal phosphate.

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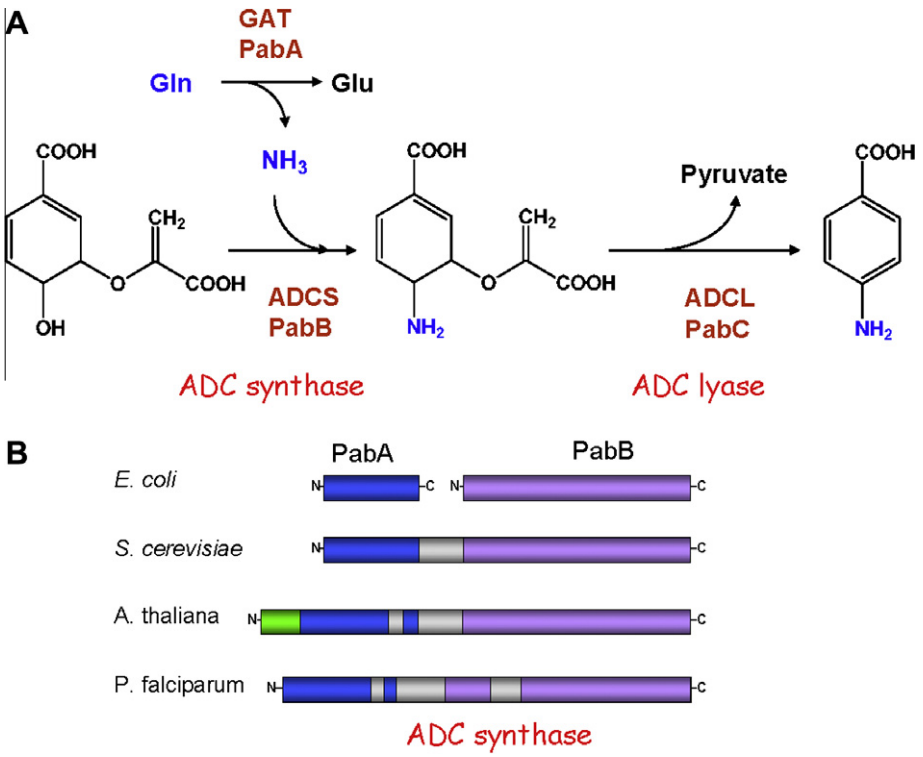

Fig. 1. The two steps synthesis of pABA from chorismate. (A) The first step is catalyzed by PabA and PabB in *E. coli* and by ADCS in eukaryotic organisms. This first step involved two reactions: a GAT activity to provide the NH_3 moiety, and an ADCS activity for the replacement of the hydroxyl group at the C4 position of chorismate by NH_3. (B) Representation of the ADCS from various organisms, illustrating the bi-functionality found in eukaryotes. Note that the plant enzyme has an additional N-terminal extension corresponding to a plastidial transit peptide (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In bacteria such as *Escherichia coli* or *Bacillus subtilis*, this biosynthesis requires three separate proteins: PabA (the GAT), PabB (the ADCS), and PabC (the ADCL). Thus, the synthesis of ADC in bacteria requires the cooperation of two proteins, PabA and PabB forming a heterodimeric complex [12]. While PabA alone has no GAT activity [12], PabB alone can convert chorismate to ADC in the presence of ammonia [13]. The mechanism used for ADC synthesis is common to three other chorismate-utilizing enzymes, salicylate synthase, isochorismate synthase and anthranilate synthase [14–17]. It involves the formation of an intermediate generated by addition of a nucleophile to C2 of chorismate and elimination of the C4 hydroxyl group [18,19]. In *E. coli* PabB, the nucleophile is the K274 residue, whereas in *B. subtilis* it is free NH_3 provided by PabA, forming in this last situation an aminodeoxyisochorismate intermediate, as for anthranilate synthase [20]. In this last case, two NH_3 (and thus two glutamines) are therefore required for the formation of ADC, one for the nucleophilic attack of the chorismate ring, and one for its amination. Based on this mechanism, strategies have been developed to irreversibly inhibit PabB activity by chorismate analogs [21] such as 2-fluorochorismate [22]. Although the mechanism of ADC synthesis is now rather well understood, the way the two activities, GAT and ADCS, are coordinated is less clear. By comparison with the carbamoyl phosphate synthetase, an other class I aminotransferase enzyme, it is possible that NH_3 is channeled between the two domains [23,24], a hypothesis reinforced by the high K_m value (representative of a low affinity) recorded for NH_3 [25]. Also, there is to date no evidence for any regulation of pABA synthesis. Indeed, neither PabA or PabB activities have been shown to be feedback controlled by the end products of their reactions, or by products of folate metabolism [26].

In eukaryotes, the situation appears different. Indeed, in plants and lower eukaryotes such as yeast and apicomplexa, the synthesis of ADC is catalyzed by a single protein (Fig. 1B) containing the two domains, GAT in the N-terminal part and ADCS in the C-terminal part [27,28]. Only the plant enzyme activity has been partially characterized [29]. It is a monomeric protein located in plastids, together with the ADCL, making this organelle the unique site of pABA synthesis in plants [27,30]. Preliminary studies with the plant recombinant protein confirmed the bifunctional nature predicted by genomic analyses [29]. However, the low expression of the enzyme in *E. coli* and its instability prevented detailed examination of the activities and coupling of the two domains. In particular, the catalytic properties of the ADCS domain were not examined. To further understand the kinetic mechanisms operating in this class of enzymes, we produced at high yield a stable recombinant plant GAT–ADCS, and we characterized in details some of its properties, estimating the activities, limiting steps and coupling of the two domains through various methods of measurement. Also, we investigated for the first time the impact of ADCL upon the GAT and ADCS activities.

Materials and methods

Expression of the recombinant AtADCS in E. coli

Arabidopsis cDNA encoding AtADCS starting at S44 was amplified by PCR from a cDNA library synthesized from total RNA isolated from Arabidopsis leaves (*Arabidopsis thaliana*, ecotype Columbia), using the following pair of primers: 5′-TAGGCTAGCT GTCGTAGTAAAACTACGAGGAA (forward) and 5′-CCCTCGAGC TATTGTCTCCTCTGATCACTACAAAACTCC (reverse). The PCR product was ligated into the expression vector pET28b (Novagen) between the *NheI* and *XhoI* restriction sites. Using this cloning strategy, one