Detection of Covalent Intermediates Formed in the Reaction of 4-Amino-4-deoxychorismate Synthase

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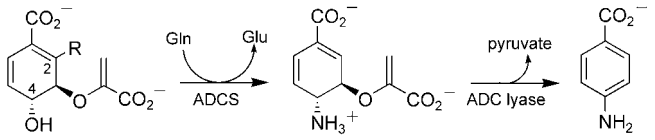
The enzyme 4-amino-4-deoxychorismate synthase (ADCS) catalyses the first committed step on the folate biosynthesis pathway in microorganisms and plants. ADCS converts chorismate (**1**), the product of the shikimate pathway, to 4-amino-4-deoxychorismate (ADC, **2**; Scheme 1).[1–3] ADC lyase then catalyses



Scheme 1. *Reactions catalyzed by ADCS and ADC lyase.*

the elimination of pyruvate from ADC to produce the folate precursor *p*-aminobenzoate (**3**).[4] A mechanism for ADCS involving an enzyme-bound covalent intermediate **4** was recently proposed (Figure 1, panel 1).[5] In this communication we provide the first direct experimental evidence for this covalent intermediate.

Escherichia coli ADCS is a heterodimer consisting of a glutamine amidotransferase (GATase) subunit, PabA (21 kDa), and a chorismate aminating subunit, PabB (51 kDa).[6] PabA produces ammonia for the amination reaction catalyzed by PabB. It is assumed that the ammonia then passes through a channel to the PabB active site, by analogy with anthranilate synthase.[7–9] PabA only exhibits significant GATase activity when bound to PabB in a 1:1 complex.[10] PabA is a class I or G-type GATase, with a catalytic triad of cysteine, histidine and glutamate at the active site. This triad consists of Cys79, His-168 and Glu-170 for *E. coli* PabA.[11] A general mechanism for the class I GATases has been proposed that proceeds via a glutamyl thioester enzyme intermediate **5** (Figure 1, panel 3).[12] The presence of the glutamyl–enzyme intermediate in the ADCS reaction has been confirmed by using [^{14}C]-glutamine.[10]

The PabB subunit of ADCS catalyses the amination of chorismate at C4 with retention of stereochemistry (Scheme 1).[1–3] PabB is able to catalyze the ADCS reaction in vitro without PabA when provided with a supply of ammonia. However, there is an associated increase in k_{cat}, and decrease in K_M for chorismate on formation of the PabA–PabB complex.[13]

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Until recently there was little detailed information on the mechanism of PabB. Retention of stereochemistry at C4 precludes direct S_N2 displacement of the C4 hydroxyl by ammonia. Several mechanisms have been proposed including a double S_N2 displacement involving an active-site nucleophile.[14] The publication of the *E. coli* PabB crystal structure revealed a candidate for this active-site nucleophile.[15] Modelling of chorismate into the PabB active site showed that the ω-amino group of Lys274 would be positioned close to C2 of the substrate, where it could play a direct role in the mechanism.[5]

Site-directed mutagenesis of PabB confirmed that Lys274 was involved in catalysis.[5] A key observation was that the K274A mutant formed a mixture of ADC and the isomeric 2-amino-2-deoxyisochorismate. It was assumed that ammonia reacts at C2 in place of Lys274. This study led to a detailed mechanistic proposal for ADCS that involved two S_N2" displacements with Lys274 acting as the active-site nucleophile (Figure 1, panel 1). The involvement of Lys274 was further supported by studies with the irreversible inhibitor 2-fluorochorismate (**6**), which was shown to covalently modify Lys274.[16]

Although both these studies are consistent with the mechanism shown in Figure 1, neither provided direct experimental evidence for the putative covalent intermediate **4**. We have obtained that evidence by using electrospray mass spectrometry (ESMS) to detect enzyme-bound intermediates on both PabA and PabB during the ADCS-catalyzed reaction. We reasoned that intermediate **4** would form on PabB before nascent ammonia was generated on PabA, and that if ammonia were excluded from the system, it might be possible to detect **4**.

Solutions of *E. coli* PabA and PabB were mixed in a 1:1 ratio to form a 50 μM sample of ADCS (see Supporting Information for details). This sample was injected directly into an electrospray mass spectrometer. The molecular masses observed for PabB and PabA were 50953 ± 2 Da and 20771 ± 1 Da, respectively (Figure 1, panel 2a), in agreement with the calculated masses of 50954 Da and 20772 Da. A small amount of PabA (10–15%) in the ADCS sample had undergone a 32 Da modification to give a mass of 20803 ± 1 Da. This is almost certainly due to oxidation of the active site cysteine (see below). The rate of formation of this adduct was accelerated by the incubation of PabA with PabB.

On addition of chorismate (100 μM) to the ADCS solution (50 μM), the mass of approximately 70% of the PabB subunits increased to 51161 ± 2 Da, which corresponds to a covalent modification of 208 Da (Figure 1, panel 2b). This agrees exactly with the expected mass increase for the formation of the covalent intermediate **4**. There was no change in mass of the PabA subunit. To determine whether the covalent intermediate detected on PabB is competent in the ADCS reaction, the second substrate, glutamine, was added to the chorismate-modified ADCS. Upon addition of glutamine (10 mM), the 208 Da covalent adduct on PabB disappeared immediately, and a covalent modification of 130 Da appeared in approximately 55% of the unoxidized PabA (Figure 1, panel 2c). This change in mass corresponds to the mass of the glutamyl thioester intermediate (**5**) expected in the GATase reaction (Figure 1, panel 3). The proportion of PabA that forms the glutamyl–enzyme inter-

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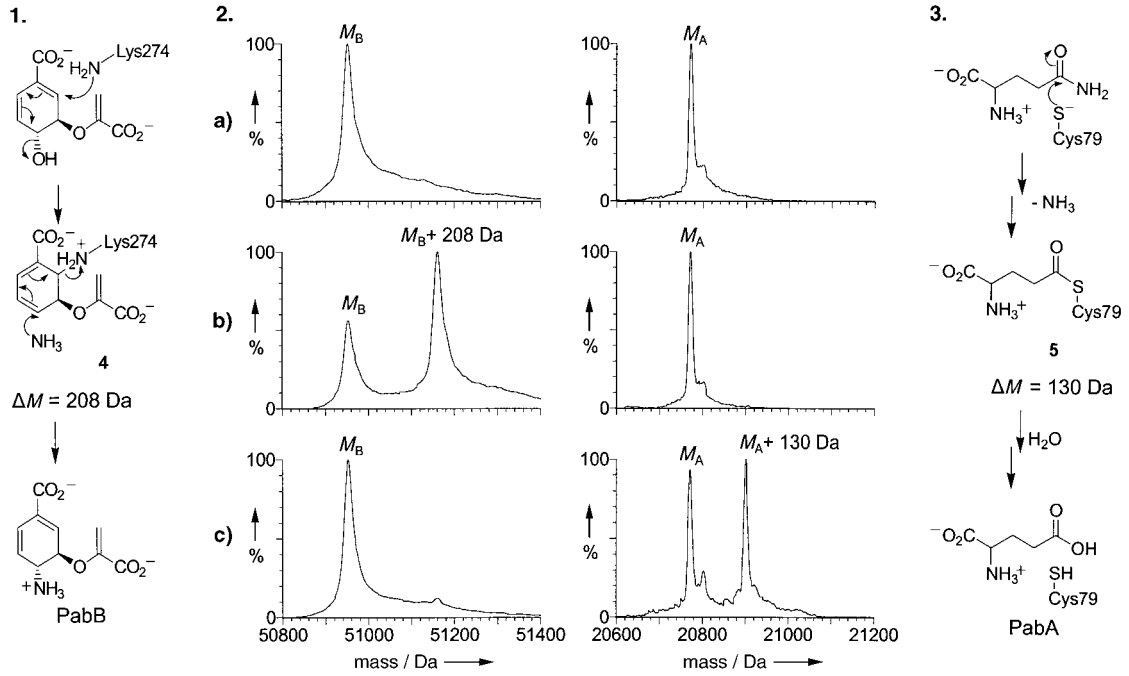

Figure 1. *Panel 1: Proposed mechanism for the reaction catalyzed by the PabB subunit of ADCS.[5] Panel 2: Charge-deconvoluted electrospray-mass spectra of covalent intermediates formed in the ADCS reaction on PabB (left-hand column) and PabA (right-hand column) a) 50 μM ADCS, b) 50 μM ADCS treated with 100 μM chorismate, c) 50 μM ADCS treated with 100 μM chorismate followed by 10 mM glutamine. Samples were incubated for 5 min following the addition of substrates and were prepared for ESMS by a 20-fold dilution in methanol (10%, v/v):formic acid (1%, v/v). The mass of unmodified PabB was 50953 ± 2 Da (M_B) and that of PabA was 20771 ± 1 Da (M_A). Panel 3: Proposed mechanism for the reaction catalyzed by the PabA subunit of ADCS.[12]*

mediate is in accord with the 56% for steady-state turnover observed previously by using [^{14}C]glutamine.[10] The oxidized PabA (mass 20803 Da) did not undergo further modification, consistent with oxidation of the active-site cysteine.

The formation of covalent intermediate **4** on PabB was further investigated by using the catalytically active K274A mutant of PabB. The experiments detailed above were repeated with a 1:1 mixture of K274A PabB and wild-type PabA. On addition of chorismate to this preparation, no change in the mass of the K274A PabB was observed (see Supporting Information). When this sample was further treated with glutamine, the expected 130 Da modification of PabA occurred. This result provides strong evidence that the 208 Da covalent enzyme intermediate observed on PabB forms on Lys274.

In order to ensure that the disappearance of the modification on PabB, on addition of glutamine, is due to the production of ammonia by PabA, further control experiments were performed by using the glutamine analogue 6-diazo-5-oxo-L-norleucine (DON). DON rapidly and irreversibly inhibits PabA.[10] Tandem mass-spectrometry sequencing of peptides from a digest of DON-inactivated PabA confirmed that DON covalently modifies Cys79 of PabA by 125 Da (see Supporting Information). DON (10 mM) was added to chorismate-treated ADCS in which PabB had been modified by 208 Da. Over the time course of the experiment, the mass of approximately 70% of the PabA increased by 125 Da. The 208 Da modification of PabB was not altered. The results of this experiment suggest that the disappearance of the covalent intermediate on PabB when glutamine is added is due to the production of ammonia by PabA and the turnover of the intermediate to the product ADC.

This communication details the first direct observation of the covalent intermediate formed between chorismate and PabB during the ADCS reaction. The ability to detect covalent intermediates on both the PabB and PabA subunits of ADCS establishes a platform for further detailed studies into the mechanism of action of the enzyme.

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