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The mechanism of 7,8-diaminopelargonate synthase; the role of *S*-adenosylmethionine as the amino donor

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The product of the first transamination step in the reaction catalysed by diaminopelargonate (DAPA) synthase has been shown to be 4-(*S*-adenosyl)-2-oxobutanoate, which has been trapped as the corresponding alcohol.

The biosynthesis of biotin (vitamin H), the cofactor in most biological carboxylation and transcarboxylation reactions, has attracted considerable interest over the past decade. This is due not only to the novelty of the enzyme reactions involved, and implicitly their suitability as potential targets for selective antibiotics and herbicides, but also to the long term aim of producing the vitamin by fermentation.[1,2] Diaminopelargonate (DAPA) synthase is a pyridoxal 5′-phosphate (PLP) dependent enzyme, obligate in biotin biosynthesis, which catalyses the second committed step in the four step pathway, the stereospecific amination of (7*S*) 8-amino-7-oxononanoate **1** to give (7*S*,8*R*) 7,8-diaminononanoate (7,8-diaminopelargonate) **2**. This aminotransferase, first described by Eisenberg in 1970,[3] is unique in that it uses *S*-adenosylmethionine (SAM) as an amino donor. The usual roles that SAM performs in metabolic reactions are as a methyl donor in methyl transferases[4] and as a free radical initiator in Fe–S cluster enzymes such as biotin synthase.[5] The crystal structure of DAPA synthase has been determined[6] and its kinetics and its inhibition by the antibiotic amiclenomycin have been studied.[7,8] Despite the fact that it uses a unique amino donor, the reaction mechanism, shown in Scheme 1, is similar to that of other PLP-dependent aminotransferases.[9] However the identity of the SAM transamination product, produced in the first step, is based on *indirect* evidence from early studies which reported the isolation of 2-oxobutenoic acid, which could result from non-enzymatic elimination of 5′-methylthioadenosine from the oxo-acid **3**.[3] However this putative product has never been isolated and, since SAM itself is relatively unstable, it is conceivable that 2*S*-aminobutenoate, formed by elimination of 5′-methylthioadenosine, could be the actual amino donor. Here we describe studies which show that **3** is indeed the enzymatic product.

(±) 8-Amino-7-oxononanoate **1** was synthesised from 2-acetyl-1,3-dithiolane[10] by the route shown in Scheme 2 and the DAPA synthase gene was cloned from *E. coli* JM101 genomic DNA by PCR and inserted in a pET16 derived vector.

The protein was over-expressed in *E. coli* BL21 (DE3) cells. Purification by chromatography gave the essentially pure holo-enzyme which showed ping-pong kinetic behaviour.[7] The first half-reaction – the reaction of the PLP form of the enzyme with SAM to form the pyridoxamine phosphate (PMP) bound form and the putative transamination product **3** – was followed by UV-vis spectrophotometry which shows the rapid appearance of a transient peak at 487 nm (Fig. 1).

This peak can be ascribed to the intermediate quinonoid form (Enz.Q) formed from SAM and the cofactor. The first part of the half-reaction, transimination of the bound PLP to give the SAM external aldimine and deprotonation to

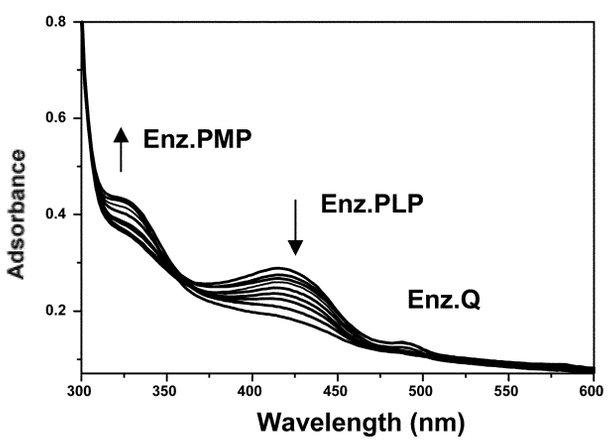
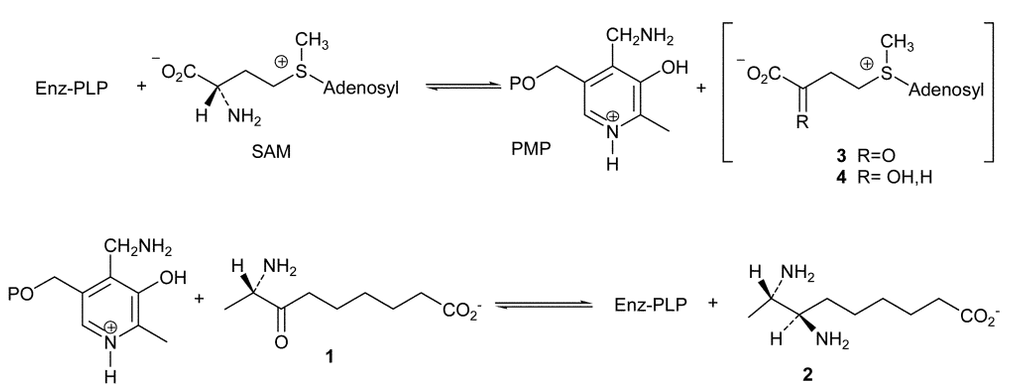


Fig. 1 Time course of the half-reaction of DAPA synthase (20 μM) with SAM (1 mM) at 25 °C in potassium phosphate buffer (20 mM, pH 7.5). Spectra were recorded on a HP8453 single beam diode-array spectrophotometer at 60 s intervals.



Scheme 1 The diaminopelargonate synthase reaction.

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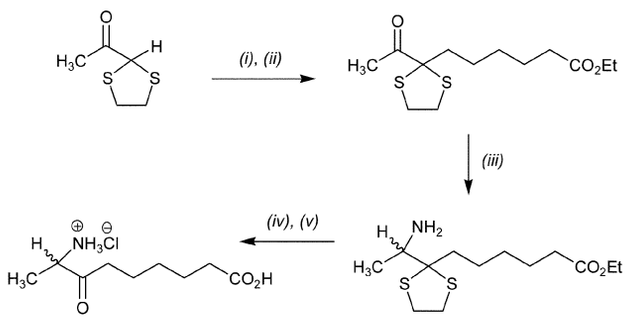

Scheme 2 *Reagents and conditions*: (*i*) LDA, THF, (*ii*) ethyl 6-iodohexanoate, HMPA, (*iii*) $TiCl_4$, Et_3N, $(Me_3Si)_2NH$, (*iv*) $NaCNBH_3$, DCM, (*v*) NBS, MeCN–H_2O (4–1), (*vi*) aq. HCl (6 M).

form the quinonoid is rapid but the subsequent hydrolysis to the bound PMP form is very slow and this appears to be the rate determining step in the overall catalytic reaction.

Under the conditions used in the enzyme experiments we found that SAM degrades slowly affording 5′-methylthioadenosine ($t_{1/2}$ ~ 20 h at 37 °C and pH 7.5).[11] Under steady state conditions[12] (both substrates at *ca* 5 × K_M) disappearance of SAM was accompanied by the appearance of an HPLC peak corresponding to a new product. On standing this compound decomposed to give 5′-methylthioadenosine much more rapidly than SAM suggesting that the oxo-acid **3** was indeed being formed and subsequently disproportionated. Since we were unable to find HPLC conditions[13] which gave satisfactory separation of this product from SAM, a series of incubations was carried out under non-optimum catalytic conditions with (±)-**1** (6 μM, 5 × K_M) in excess but where the SAM concentration (200–300 μM, 1.3–2.0 × K_M) was limited so that it was almost completely depleted from the incubation mixture within fifteen minutes, thus minimising the contamination of the product by SAM. HPLC-MS of the reaction mixtures showed that the unstable product had a M^+ peak at 400 amu. However this is very close to the molecular weight of the SAM cation itself (399 amu). So, to determine unambiguously whether the compound formed in the enzymatic reaction was indeed the putative oxo-acid, we elected to reduce the product *in situ* to afford the more stable hydroxy-acid **4**. Accordingly, authentic **4** was synthesised directly from SAM by partial diazotisation essentially as described by Zappia.[14,15]

The enzyme was incubated under the above conditions with the addition of [^{14}C-*methyl*]SAM (2 nmoles, 0.024 M Bq), the mixture was spin-filtered to remove the protein, and treated with $NaBH_4$ (5 mM, 5 min, on ice) to convert **3** to **4**.

Under conditions where virtually all of the SAM was turned over 88% of the radioactivity co-purified with **4** and 8% with 5′-methylthioadenosine. This provides compelling evidence that the first step in the reaction, the transfer of the amino group of SAM to PLP, generates the corresponding oxo-acid **3** as shown in Scheme 1.

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11 Commercially available SAM is a ~3 : 2 mixture of the *S* and *R* forms and can contain up to 10% 5′-methylthioadenosine. SAM used in these experiments was purified by HPLC and contained less than 1% 5′-methylthioadenosine. The unnatural *R* isomer is not a substrate (see ref. 7).

12 Steady state incubations were typically carried out at 37 °C in 20 mM potassium phosphate buffer (pH 7.5) containing 20 μM DAPA synthase (equilibrated with 100 μM PLP), 1 mM SAM and 6 μM (±)-**1**.

13 HPLC separations were typically carried out on a Phenomenex 5 μ C8 (150 × 4.6 mm) column eluted isocratically with 5% MeOH–H_2O for 5 minutes followed by a gradient to 60%. MeOH–H_2O, over 25 min. All eluents contained 0.1% TFA. For a flow rate of 0.5 cm^3 min^{-1} the retention times of SAM, **3**, **4** and 5′thioadenosine were 3.9, 4.0, 4.9 and 21.4 min respectively.

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15 *S*-Adenosyl-(2-hydroxy-4-methylthio)butanoic acid **4** was prepared as its TFA salt by treatment of the sulphate salt of SAM with excess $NaNO_2$ in citrate–phosphate buffer (1 M, pH 2.5) at 40 °C for 1 h. The product was purified by sequential ion exchange chromatography (AG15 × 8 resin, H^+ form) and HPLC. The ^{13}C NMR (125 MHz, ^2H_2O–H_2O) of **4** showed a methine resonance at 68.4 ppm. (*C*HOH) and no *C*HNH_2 signal (at 51.6 ppm in the spectrum of SAM). Fully concordant MS, NMR and UV data were obtained for the product. The stereochemistry of the 2-hydroxyl group was not determined.