Identification of a novel β-replacement reaction in the biosynthesis of 2,3-diaminobutyric acid in peptidylnucleoside mureidomycin A

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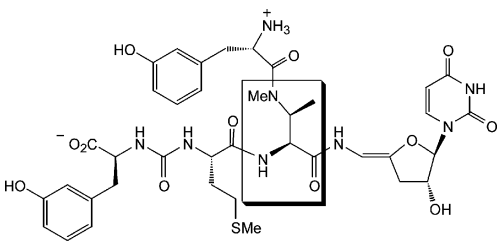
2,3-Diaminobutyric acid (DABA) is an unusual di-amino acid component of mureidomycin A, a member of the peptidylnucleoside antibiotic family produced by *Streptomyces flavidovirens* SANK 60486. Radiochemical assays using cell-free extract from *S. flavidovirens* revealed that ^{14}C-L-Thr is converted into radiolabelled DABA by an ammonia-dependent β-replacement activity, and not *via* oxidation to 3-keto-2-aminobutyric acid. The substrate specificity of partially purified enzyme was assayed using a spectrophotometric assay, and β-replacement activity was inhibited by known inhibitors of PLP-dependent enzymes. These data imply that DABA is biosynthesised from L-Thr by a PLP-dependent β-replacement enzyme, using ammonia as a nucleophile. These results are consistent with literature proposals for the biosynthesis of 2,3-diaminopropanoic acid from the viomycin biosynthetic gene cluster.

Introduction

Three families of peptidyl nucleoside antibiotics, the mureidomycins, pacidamycins, and napsamycins, are potent inhibitors of phosphoMurNAc-pentapeptide translocase (MraY), an essential enzyme in bacterial cell wall peptidoglycan biosynthesis.[1,2] They each contain a 3′-deoxyuridine sugar attached *via* an enamide linkage to an *N*-methyl-2,3-diaminobutyric acid (DABA) residue, which is acylated on both nitrogen substituents by a peptide chain containing unusual features.



Mureidomycin A (**1**)

The mureidomycins A–D were isolated from *Streptomyces flavidoviridens* SANK 60486, and were found to be active against strains of *Pseudomonas* (MIC 0.1–3.13 μg mL^{-1}), protecting mice against *Pseudomonas aeruginosa* infection.[3–5] Mureidomycin A (MrdA, **1**) selectively and specifically inhibits bacterial MraY, showing no inhibition of bacterial teichoic acid or mammalian glycoprotein biosynthesis.[6,7] Pacidamycins 1–7 were isolated from *Streptomyces coeruleorubidus* strain AB 1183F-64,[8–10] and pacidomycins D, 4N and 5T were later isolated from *Streptomyces coeruleorubidus* strain NRRL 18730.[11] The configuration of each of the amino acids in pacidamycin-D was determined by semi-synthetic work to be L (*S*) for all the natural amino acids, and *S*,*S* for the 2-amino-3-*N*-methylaminobutyric unit.[12,13]

Kinetic studies have shown that mureidomycin A (**1**) is a slow-binding inhibitor (K_i 35 nM, K_i* 2 nM) of solubilised *E. coli* MraY.[14] Inhibition is competitive *versus* both the UDPMurNAc-pentapeptide soluble substrate and the polyprenyl phosphate lipid substrate.[15] Structure–function studies on both mureidomycin A[16] and pacidamycin D[13] have revealed that the enamide functional group is not primarily responsible for MraY inhibition. Analogues of MrdA containing the amino-terminal peptide chain were found to inhibit MraY, leading to the proposal that the mechanism of inhibition of translocase MraY by MrdA involves binding of the amino terminus to the Mg^{2+} binding site, and maintenance of an active conformation *via* a *cis*-amide linkage.[17,18]

Having previously carried out structure–function studies on mureidomycin A,[14–18] we wished to investigate the biosynthesis of this unusual natural product. There have been no reported studies on the biosynthesis of the mureidomycins, pacidamycins, or napsamycins. The unusual diamino acid 2,3-diaminobutyric acid (DABA) is found in a family of cyclic non-ribosomal peptide natural products that includes the friulimicins,[19] the amphomycins,[20] tsushimycin,[21] and aspartocin,[22] and is also found in feldamycin, a tripeptide natural product.[23] *meta*-Tyrosine is not found in other non-ribosomal peptide natural products, but the urea-Xaa-CO_2H motif is found in muraymycin, another peptidylnucleoside inhibitor of MraY,[24] and in chymostatin, a *Streptomyces* peptide aldehyde which acts as an inhibitor of serine proteases.[25] This paper describes studies to elucidate the biosynthesis of the 2,3-diaminobutyric acid (DABA) component of mureidomycin A.

Results

Bio-assay for mureidomycin A production

Mureidomycin A is known to be selective against species of *Pseudomonas*, with highest activity against *Pseudomonas aeruginosa*. We have found that soil bacterium *Pseudomonas putida* ATCC 4359 is sensitive to mureidomycin A, providing a convenient bio-assay for mureidomycin A production. Supernatant from broths of *Streptomyces flavidovirens* was transferred to diluted cultures of

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Pseudomonas putida in a microtitre plate reader, and cell growth monitored at 595 nm. Production of mureidomycin A commenced after 4 days growth, reaching a maximum level of production after 6 days growth (see Fig. 1).

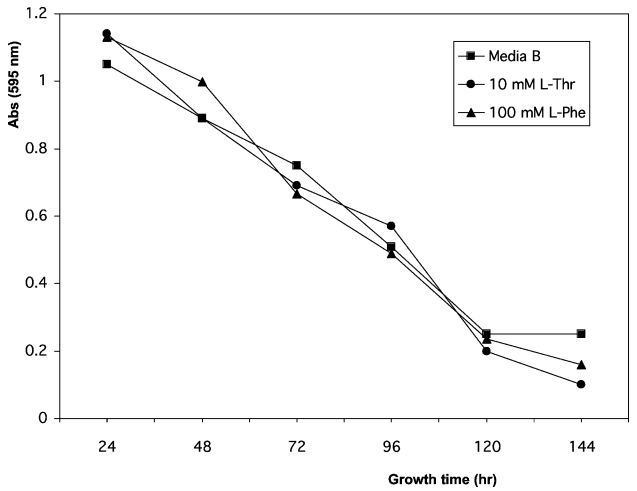


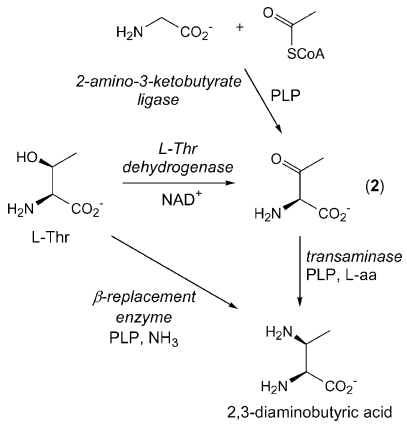
Fig. 1 Bio-assay data for antibiotic production from *Streptomyces flavidovirens* over 1–6 days, with and without growth additives.

The effect of various possible biosynthetic precursors on mureidomycin A production was investigated using this bio-assay. Addition of 10 mM L-threonine or 100 mM L-phenylalanine to the growth media was found reproducibly to enhance antibiotic production at day 5 and 6 (see Fig. 1), but no enhancement was observed in the presence of L-methionine, *meta*-tyrosine, uracil, or sodium acetate, at 0.1% w/v concentration. No enhancement of antibiotic production was observed using D-threonine or D-phenylalanine. Several attempts to isolate mureidomycin A in sufficient quantities to carry out stable isotope incorporation experiments were unsuccessful. Nevertheless, the observation that antibiotic production was enhanced by addition of L-threonine prompted an investigation into the possible conversion of L-Thr into 2,3-diaminobutyric acid.

Investigation of hypotheses for biosynthesis of 2,3-diaminobutyric acid from L-threonine

The biosynthesis of the unusual amino acid 2,3-diaminobutyric acid (DABA) is not known. The observation that antibiotic production is enhanced by L-threonine, a 4-carbon amino acid containing a β-hydroxyl group, suggested that DABA might be biosynthesised from L-Thr, prior to assembly of the peptide chain. There are three plausible hypotheses for the biosynthesis of DABA, illustrated in Scheme 1.

The first pathway involves the enzyme 2-amino-3-ketobutyric acid (AKB) ligase, a pyridoxal phosphate-dependent enzyme that is known to be responsible for L-Thr catabolism. AKB ligase catalyses the reversible condensation of glycine with acetyl CoA to form 2-amino-3-ketobutyrate (**2**).[26] Transamination (or reductive amination) of 2-amino-3-ketobutyrate would then form DABA. A second pathway also proceeds *via* 2-amino-3-ketobutyrate, but *via* oxidation of L-threonine, catalysed by L-threonine dehydrogenase, a known catabolic enzyme.[27] The third pathway involves the direct conversion of L-threonine to DABA by a β-replacement reaction.



Scheme 1 Three pathways for biosynthesis of 2,3-diaminobutyric acid.

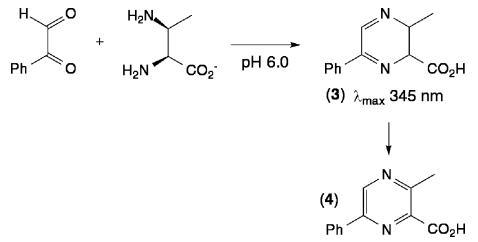
Several β-replacement reactions are known in primary and secondary metabolism,[28] notably by β-cystathionine synthase,[29] but usually involving sulfur nucleophiles.

Cell-free extract from *Streptomyces flavidovirens* SANK 60486 was prepared, and used to investigate the proposed pathways. AKB ligase activity was assayed using a coupled assay, in which release of free coenzyme A was detected using DTNB.[26] AKB ligase activity was detected, but activity showed little change over days 1–6 of antibiotic production, indicating no apparent correlation with MrdA production. Incubation of cell-free extract with ^{14}C-glycine and acetyl CoA, with or without added L-glutamic acid or L-alanine (in order to support transamination of AKB to DABA), gave no new spot corresponding to DABA by radiochemical thin layer chromatography.

Assays for L-threonine dehydrogenase, required for the putative oxidation of L-Thr, failed to detect any activity in cell-free extracts of *Streptomyces flavidovirens* SANK 60486. Furthermore, incubation of cell-free extract with ^{14}C-L-threonine and acetyl CoA, with or without added L-glutamic acid or L-alanine, gave no new spot for DABA by radiochemical cellulose thin layer chromatography. It was observed, however, that incubation of cell-free extract in 50 mM ammonium bicarbonate buffer with ^{14}C-L-threonine (R_f 0.15) gave rise to a new ^{14}C-labelled spot (R_f 0.05) after autoradiography, consistent with the formation of a more polar diamino acid.

In order to characterise the reaction product formed, derivatisation of the suspected diamine product was carried out using phenylglyoxal(2-keto-phenylacetaldehyde), known to react with diamines.[30] Treatment of the reaction product with phenylglyoxal at pH 6.0 gave rise to a new spot by cellulose TLC at R_f 0.23. Analysis of the derivatisation product by UV–vis spectroscopy showed a new species with λ_{max} 345 nm, corresponding to 2,3-dihydropyrazine derivative **3** (see Scheme 2). The same λ_{max} was observed upon reaction of ethylene-1,2-diamine with phenylglyoxal under the same conditions, and agrees with literature λ_{max} values of 345–358 nm for similar 2,3-dihydropyrazine derivatives.[31] Analysis of the derivatised product by negative ion LC-MS gave a major peak corresponding to the oxidised pyrazine (**4**, m/z 213 $[M - H]^-$, 169 $[M - H - CO_2]^-$).

The formation of a UV-active derivatisation product allowed the monitoring of the β-replacement reaction using a stopped enzyme assay, in which the DABA product was derivatised by



Scheme 2 Derivatisation of DABA by phenylglyoxal.

phenylglyoxal, and the absorbance at 340 nm measured. The measured A_{340} in this assay increased linearly *versus* reaction time, and was dependent upon protein concentration in the cell-free extract.

Characterisation of L-Thr β-replacement enzyme activity

The UV–vis assay described above was used to analyse samples of *S. flavidovirens* cell-free extract at different times during the growth. Highest activity was observed at day 2 and day 3, as shown in Fig. 2, prior to the production of mureidomycin A, indicating that DABA is produced prior to assembly of the antibiotic. Using cell-free extract harvested at day 2, the β-replacement enzyme activity was partially purified by Q sepharose anion exchange chromatography. Active fractions were collected at 0.5–0.65 M KCl, and were pooled and concentrated. The partially purified enzyme, of specific activity 0.80 absorbance units min^{-1} (mg protein)$^{-1}$, was then used for kinetic assays.

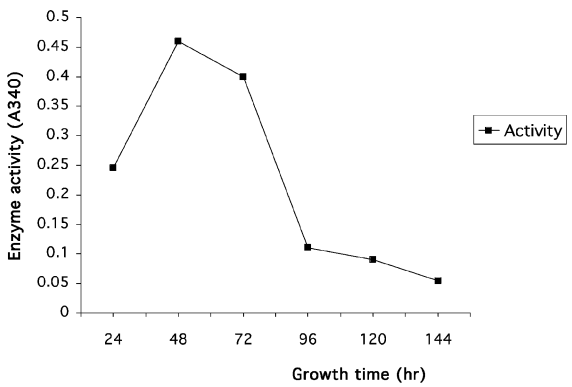


Fig. 2 Time course of β-replacement enzyme activity *vs.* growth time.

The substrate specificity of the enzyme was examined, using a range of β-substituted amino acid substrates. As shown in Table 1, the highest activity was observed using L-threonine, but high levels of activity were also observed using L-cysteine and DL-*allo*-threonine. Activity was observed with amino acid substrates containing β-hydroxyl groups (L-Thr, L-Ser) or a β-*O*-acyl group (*O*-acetyl-L-Ser), or substrates with a thiol (L-Cys) or chlorine substituent (β-chloroalanine) at the β-position.

Measurement of rate *versus* substrate concentration for L-Thr showed Michaelis–Menten kinetic behaviour (see Fig. 3A), and a K_m value of 12 μM was measured. The dependence upon ammonium ion concentration was investigated (see Fig. 3B), and maximum activity was observed at 50 mM ammonium bicarbonate.

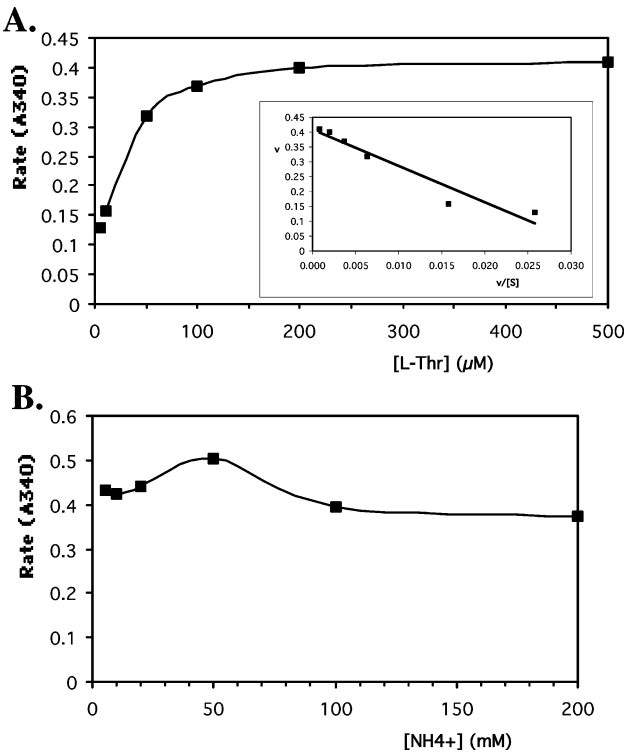


Fig. 3 Steady-state kinetic data for DABA synthase. A. Michaelis—Menten kinetic data for L-Thr (inset: Eadie–Hofstee plot of *v vs. v*/[*S*]). B. Dependence of activity upon NH_4^+ concentration.

Several compounds were tested as inhibitors or activators of the β-replacement enzyme, as shown in Table 2. Addition of pyridoxal 5′-phosphate was found to increase enzyme activity by 29%, consistent with a role for PLP as a coenzyme in the β-replacement reaction. Carbonyl reagent 2,4-dinitrophenylhydrazine reduced activity by 25%, and treatment with L-Thr and sodium

Table 1 Substrate specificity data for DABA synthase. Activity measured using stopped spectrophotometric activity, as described in the Experimental, relative to L-threonine, all at 10 mM concentration

Substrate	Relative activity
L-Threonine	100
D-Threonine	81.6
allo-DL-Threonine	66.3
L-Serine	56.5
O-Acetyl-L-serine	53.2
L-Cysteine	75.0
β-Chloro-D-alanine	48.4

Table 2 Inhibition data for DABA synthase. Activity measured using stopped spectrophotometric activity, as described in the Experimental, corrected for control lacking enzyme. Additives each at 10 mM concentration

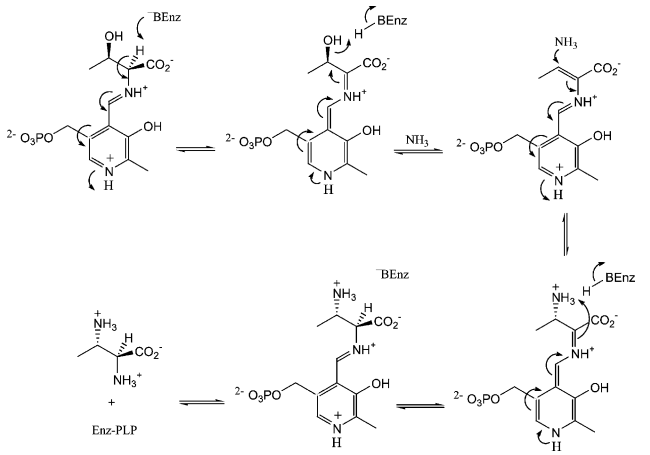
Addition	Activity/AU	Relative
Enz + Thr + NH_3	0.52	100
Enz + Thr	0.06	12
+ Pyridoxal 5′-phosphate	0.67	129
+ 2,4-Dinitrophenylhydrazine	0.39	75
+ 4-Hydrazinobenzenesulfonic acid	0.43	82
+ Sodium borohydride	0.06	12
+ β-Chloro-D-alanine	0.16	31
+ DL-Vinylglycine	0.04	8

borohydride reduced activity by 88%, consistent with the involvement of a PLP-threonine imine adduct in catalysis. Two possible suicide substrates for PLP-dependent enzymes, β-chloro-D-alanine[32] and vinylglycine,[33] were also found to inhibit the enzyme strongly, by 69% and 92% respectively, at 10 mM concentration. β-Chloro-D-alanine also has activity as a substrate (see Table 1), so it appears to act as a suicide substrate for DABA synthase.

Discussion

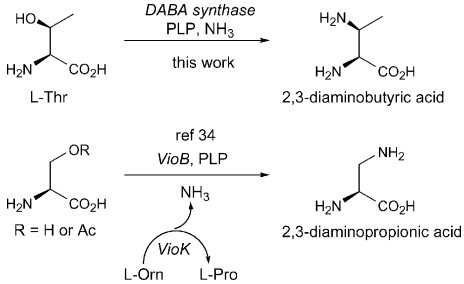
Despite the identification and study of several nucleoside natural product antibiotics that target the MraY reaction in bacterial cell wall peptidoglycan biosynthesis,[1,2] there is currently no information regarding the biosynthesis of these natural products. Although in our hands the levels of production of mureidomycin A were very low, we have observed that addition of L-threonine to the growth media of *S. flavidovirens* SANK 60486 increases the production of antibiotic, suggesting that L-Thr might be converted biosynthetically into the unusual diamino acid DABA. Three hypotheses for conversion of L-Thr into DABA were investigated using radiochemical tracer experiments. No evidence was found for the conversion of L-Thr or glycine into 2-amino-3-ketobutyrate, which could be converted to DABA *via* transamination. However, evidence has been obtained for conversion of ^{14}C-L-Thr and ammonia to ^{14}C-DABA, *via* a β-replacement reaction.

A stopped UV–vis assay for this reaction has been developed, using phenylglyoxal as a derivatising agent. The reaction is time-dependent, protein-dependent, and shows Michaelis–Menten kinetic behaviour for L-Thr. The reaction is promoted by addition of pyridoxal 5′-phosphate, and is strongly inhibited by carbonyl reagents and suicide inhibitors for PLP-dependent enzymes, consistent with the involvement of PLP in this reaction. The role of PLP in this reaction would be to facilitate deprotonation of the amino acid α-proton by formation of a PLP-amino acid imine linkage, and hence allow an addition–elimination mechanism, shown in Scheme 3. To our knowledge, this is the first demonstration of a PLP-dependent β-replacement enzyme that uses a nitrogen nucleophile.[28]



Scheme 3 Proposed mechanism for PLP-dependent DABA synthase reaction.

Thomas *et al.* have reported the presence in the biosynthetic gene cluster for viomycin, a non-ribosomal peptide natural product containing 2,3-diaminopropanoic acid, of a VioB gene product, which bears sequence similarity to PLP-dependent enzymes cysteine synthase and serine dehydratase.[34] They propose that VioB catalyses a β-replacement reaction upon either L-serine or a derivative thereof (*e.g. O*-acetyl L-serine), using ammonia as a nucleophile, to form diaminopropanoic acid.[34] Also present in the gene cluster is a VioK gene product which bears sequence similarity to ornithine deaminase, which might generate ammonia for the β-replacement reaction,[34] as shown in Scheme 4.



Scheme 4 Biosynthesis of 2,3-diamino acids *via* β-replacement reactions.

The proposal of Thomas *et al.* for 2,3-diaminopropanoic acid biosynthesis agrees with our biochemical data for DABA biosynthesis, suggesting that PLP-dependent β-replacement reactions could be responsible for the biosynthesis of both 2,3-diamino acids in non-ribosomal peptide natural products. Recent studies have shown that combinatorial libraries based upon fragments of the mureidomycin scaffold yield biologically active compounds,[35] therefore an understanding of the biosynthesis of these nucleoside natural products could in future lead to the engineered biosynthesis of new biologically active nucleoside analogues.

Experimental

Materials

Streptomyces flavidovirens SANK 60486 was a gift of Dr M. Inukai (Sankyo Ltd.).

Growth of *Streptomyces flavidovirens* SANK 60486

S. flavidovirens was maintained on nutrient agar (Difco) or oatmeal agar (2% oatmeal, 0.5% yeast extract, 1.5% Bacto agar). For antibiotic production, 50 ml cultures of *S. flavidovirens* were grown in the following media: 0.1% glucose, 0.5% yeast extract, 0.5% soya bean meal, 0.1% $CaCO_3$, 2.4% soluble starch, 0.3% beef extract, 0.5% tryptone, and 1% antifoam (Sigma). Cultures were grown at 30 °C at 220 rpm shaker speed, for 5–6 days.

Bioassay for antibiotic production

Cultures (5 ml) of *Pseudomonas putida* ATCC 4359 were grown overnight at 30 °C in Luria broth (LB), then diluted 100-fold into sterile LB media. 1 ml samples of *S. flavidovirens* culture were centrifuged (5500 rpm, 10 min), and the supernatant (50 μl) was added to 50 μl of diluted *P. putida* in a well of a 96-well microtitre plate, with four duplicate samples, and with control samples containing water in place of *S. flavidovirens* supernatant. The microtitre plate was grown at 30 °C for 24 hr, and absorbance was read at 595 nm.

Preparation of cell-free extract and enzyme purification

Cultures (50–500 ml) were harvested by centrifugation (5500 rpm, 10 min). the cell pellet was re-suspended in 100 mM potassium phosphate buffer (pH 7.5, 20–50 ml) containing 50 mM NaCl. Lysozyme (1 mg) was added, and the cells were left at 4 °C for 30 min, then broken by sonication (4 × 1 min). Centrifugation at 15 000 rpm for 30 min yielded the cell-free extract, used for radiochemical enzyme assays.

DABA synthase was partially purified by application of extract (20 ml) to a Q sepharose anion exchange column, and elution with a 200 ml gradient of 50 mM potassium phosphate buffer (pH 7.5) containing 0–1.0 M KCl. Fractions (5 ml) were collected and assayed for DABA synthase activity (see below). Active fractions (at 0.5–0.65 M KCl) were pooled, and concentrated to 2.0 ml using an Amicon ultrafiltration device. Partially purified enzyme was used for enzyme kinetic assays.

Radiochemical enzyme assays

Assays for ^{14}C-glycine incorporation (50 µl total volume) contained cell-free extract (25 µl), 50 mM potassium phosphate buffer (pH 7.5), 20 mM $MgCl_2$, 200 µM acetyl CoA, and ^{14}C-glycine (50 000 cpm, specific activity 50 µCi µmol^{-1}). Assays for coupled transaminase activity also contained 1 mM L-glutamic acid or L-alanine. Assays for ^{14}C-L-threonine incorporation (50 µl) contained cell-free extract (25 µl), 50 mM potassium phosphate buffer (pH 7.5), 20 mM $MgCl_2$, ^{14}C-L-threonine (50 000 cpm, specific activity 50 µCi µmol^{-1}), and either 1.0 mM NAD^+ or 50 mM ammonium bicarbonate. Assays were incubated for 30 min at 20 °C, then applied to a cellulose thin layer chromatography plate (20 × 20 cm), dried, then eluted by solvent containing 60% *n*-butanol, 25% ethanol, 15% acetic acid. The eluted plate was exposed to film for 24 hr, and the film was developed.

Coupled enzyme assays

AKB ligase assays (1.0 ml) containing cell-free extract (100 µl), 50 mM potassium phosphate buffer (pH 7.5), 20 mM $MgCl_2$, 200 µM acetyl CoA, and 15 mM glycine were incubated at 37 °C for 5–30 min. Aliquots (100 µl) were removed, and added to 50 mM potassium phosphate buffer (pH 7.5), 20 mM $MgCl_2$, and 100 µM DTNB [5,5′-dithio-bis(2-nitrobenzoic acid)], and the absorbance was measured at 412 nm.

Threonine dehydrogenase assays (1.0 ml) contained cell-free extract (300 µl), 50 mM potassium phosphate buffer (pH 7.5), 15 mM L-threonine, and 5 mM NAD^+. The reaction was monitored directly at 340 nm.

DABA synthase assays (1.5 ml) containing cell-free extract (300 µl), 50 mM potassium phosphate buffer (pH 7.5), 10 mM L-threonine, and 10 mM ammonium acetate were incubated at 37 °C for 10–20 min. A 300 µl aliquot of phenylglyoxal (50 mM solution in 1 : 1 H_2O–acetic acid, pH 6.0) was then added, and incubated for a further 10 min, and the absorbance at 340 nm was measured.

Kinetic assays of DABA synthase

DABA synthase assays were carried out in a 96-well microtitre plate. Assays (200 µl) containing partially purified enzyme (50 µl), 50 mM potassium phosphate buffer (pH 7.5), L-threonine (10 mM), and 10 mM ammonium acetate were incubated at 37 °C for 10 min. A 50 µl aliquot of phenylglyoxal (50 mM solution in 1 : 1 H_2O–acetic acid, pH 6.0) was then added, and incubated for a further 10 min, and the absorbance at 340 nm was measured. Alternative substrates L-cysteine, L-serine, *O*-acetyl L-serine, D-threonine, *allo*-DL-threonine, and β-chloro-D-alanine were each tested at 10 mM concentration. K_m determination for L-threonine was carried out at 5–5000 µM concentrations. K_m and k_{cat} parameters were determined by Eadie–Hofstee plots. Rate measurements were also carried out at varying concentrations (5–500 mM) of ammonium acetate. Inhibition studies were carried out in the presence of 10 mM concentrations of the following compounds: 2,4-dinitrophenylhydrazine, 4-hydrazinobenzenesulfonic acid, sodium borohydride, β-chloro-D-alanine, and DL-vinylglycine.

Acknowledgements

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