Supplementary Material (ESI) for Chemical Communications

Supplementary Information

Mechanism and substrate-stereochemistry of 2-amino-3-oxo(keto)butyrate CoA ligase: Implication for 5-aminolevulinate synthase and related enzymes.

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Materials. Radiochemicals were obtained from Amersham Biosciences and reagents for molecular biology from Fermentas International Inc. (830 Harrington Court, Burlington, ON L7N 3N4, Canada). Radioactivity was measured in Sigma-Fluor™ High Performance LSC Cocktail (St. Louis, Mo. USA) using Malisa scintillation counter (Raytest GmbH 7547 Straubenhardt 1, Germany) and correction for spill-over of ^{14}C counts into the ^{3}H channel was made manually, using standard samples of the two isotopes. Silica gel plates for thin layer chromatography (TLC) were from Fisher and were observed in ultraviolet light of 254nm. For enzyme purification AKTAexplorer system (Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom) together with the columns supplied by the same manufacturer were used.

Cloning and expression of the 2-amino-3-oxo(keto)butyrate ligase (kbl) and L- threonine dehydrogenase (tdh) genes. The total DNA isolated from *Escherichia coli* JM103 acted as the template and for the amplification of *kbl* , the primer pair F-1and R-1 and for *tdh* F-2 and R-2 were designed from the DNA sequence information available in the NCBI database (accession number X06690).

For amplification of 2-amino 3-ketobutyrate CoA ligase gene

Forward primer (F-1)

M R G E F Y Q Q

5' GCAGCATATGCGTGGAGAATTTTATCAGCAGT 3'

NdeI

Reverse primer (R-1)
5' GAGGATCCCTCTTCCGCTTTCAGTTTGG 3'
BamHI

For amplification of L-threonine dehydrogenase gene

Forward primer (F-2)
M K A L S K L K A
5' GCAGCATATGAAAGCGTTATCCAAACTGAAAGCG 3'
NdeI

Reverse primer (R-2)
5' GAGGATCCAAGAGATCAGCACAATTACGAACTC 3'
BamHI

The corresponding *kbl* and *tdh* genes were amplified by polymerase chain reaction (PCR) using the above primers. The PCR was performed at 72^0 C while denaturing and annealing was done at 94^0 C and 50^0 C in a thermal cycler. The products of 1244 base-pairs for *kbl* and of 1134 base-pairs for *tdh* were purified and ligated into pTZ57R/T. The recombinant plasmids were transformed into *E. coli* DH5α and clones containing the DNA inserts of desired sequences were restricted with *Nde*I and *Bam*HI and the products ligated into pET-21a also linearised with the same two enzymes. The resulting recombinant plasmids were transferred in *E. coli* BL21-CodonPlus(DE3). Single colonies from the plate were used to inoculate 5 ml of Luria-Bertani medium (tryptone 1%; yeast extract and NaCl 0.5% each) containing ampicillin at a final concentration of 100 μg/ml. The overnight grown cultures were then added to 500 ml of the above medium and grown at 37^0 C until an OD at 660 nm reached 0.4-0.6. The enzymes were then induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and after 4 hours cells were harvested, suspended in 25 ml of 50 mM Tris-HCl (pH 7.5) and sonicated for 5 min at 4^0 C and the supernatant then used for the purification of the ligase[1] and the dehydrogenase[2] essentially by the methods which have been used for the wild type enzymes, except that the ion exchange chromatography was performed on Resource Q, hydrophobic separation on Resource Iso and gel filteration on Superdex 200 HR10/30 columns of Amersham Biosciences. Both the enzymes gave single bands on 0.1 % sodium dodecyl sulphate-12% polyacrylamide gel electrophoresis. The specific activities of the ligase and dehyrogenase were 3.18 and 13.72 units per mg of protein, respectively.

Specific activity. The unit is defined as the activity that converts 1 μmole of the substrate into product in the standard assay system.

Preparation of [2-*R*-^{3}H: ^{14}C]glycine Serine transhydroxymethylase (SHM) was purified as described previously[3] and 0.3 units of the enzyme incubated at 37 ˚C in a final volume of 1ml containing 200 mM Tris-HCl buffer pH 7.5, 2 mM tetrahydrofolate and 5 mM [2-*RS*-$^{3}H_2$:^{14}C]glycine (^{3}H:^{14}C ratio of 19.89). Samples were removed at various time intervals, converted into benzyloxycarbonylglycine and subjected to the measurement of radioactivity. The loss of tritium plateaued after 60 minutes but the incubation was continued for another 180 minutes (Fig. 1). The bulk of the incubation mixture was stored at –20^{0} C and used for the stereochemical studies within two weeks.

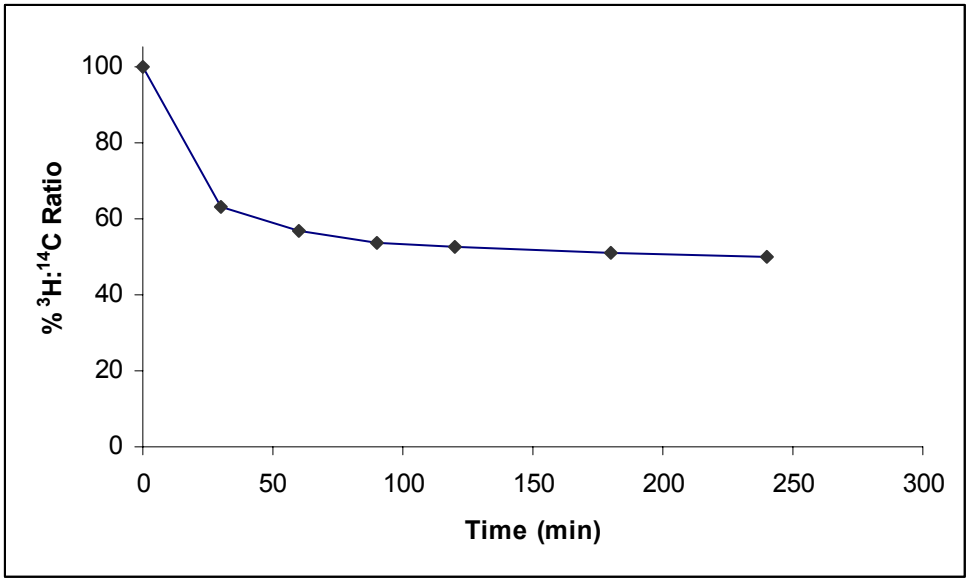

Fig.1 Stereospecific exchange of a hydrogen atom of glycine by SHM

The coupled enzyme system for the formation of L-threonine. In a final volume of 1 ml the incubation mixture contained 200 mM Tris-HCl buffer pH 7.5, 1 mM glycine, 1 mM acetyl CoA, 0.5 mM NADH, 0.5mg 2-amino 3-ketobutyrate CoA ligase and 0.5mg L-threonine dehydrogenase. Aliquots from the incubation mixture were removed at 0, 15 and 40 minute-post incubation, mixed with 1 mg each of glycine and L-threonine and added to a solution of $NaHCO_3$ (87.5 mg) in water (2 ml). To a vigorously stirred solution of the latter at 4^{o} C was added benzylchloroformate (0.1 ml). After 120 minutes the mixture was extracted with diethyl ether, the aqueous layer then acidified and extracted with ethyl acetate. The organic extract was dried over anhydrous Na_2SO_4 and after evaporation separated by TLC using chloroform:methanol:formic acid (20:1:0.5) as the solvent. The benzyloxycarbonyl derivatives of glycine and threonine, with R_f values of 0.5 and 0.3 respectively were removed for the determination of radioactivity.

The exchange of the *pro R* hydrogen atom of glycine. Samples of 1mM [2*RS*-$^{3}H_2$: ^{14}C] and [2-*R*-^{3}H]glycine were, in parallel, incubated in 1.0 ml of 200

mM Tris-HCl buffer pH 7.5 and KBL (0.5 mg). Aliquots were removed at various time intervals, mixed with carrier glycine (25 mg) and converted into benzyloxycabonylglycine using benzylchloroformate (0.075 ml) and NaOH (35 mg) in water (2 ml). After 30 minutes the reaction mixture was extracted with diethyl ether and the aqueous layer acidified to obtain a precipitate which was crystallized and a portion used for the determination of radioactivity. The data are shown below.

[2*RS*-$^{3}H_2$: ^{14}C]GLYCINE

Time (minutes)	^{3}H	^{14}C	$^{3}H/^{14}C$	% starting $^{3}H/^{14}C$
0	337045	16306	20.67	100
30	123853	9728	12.73	61.6
60	146303	13249	11.04	53.4
90	119701	11411	10.49	50.8
120	103262	10094	10.23	49.5
180	166045	16585	10.01	48.4
240	97752	9884	9.89	47.8

[2-*R*-^{3}H]GLYCINE

Time (minutes)	^{3}H	^{14}C	$^{3}H/^{14}C$	% starting $^{3}H/^{14}C$
0	267479	28132	9.51	100
30	388665	13403	2.88	30.5
60	31046	16684	1.86	19.6
90	24927	19062	1.31	13.8
120	24214	21060	1.15	12.1
180	14225	17168	0.83	8.7
240	11106	13541	0.82	8.6

1) J. G. Mukherjee and E. E. Dekker, *J. Biol. Chem.*, 1987, **262**, 14441-14447.
2) S. A. Boylan and E. E. Dekker, *J. Biol. Chem.*, 1981, **256**, 1809-1815.
3) M. Fujioka, *Biochim. Biophys. Acta*, 1969, **185**, 338-349.