Mechanism and substrate stereochemistry of 2-amino-3-oxobutyrate CoA ligase: implications for 5-aminolevulinate synthase and related enzymes†

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The condensation process catalysed by 2-amino-3-oxobutyrate CoA ligase (KBL; also known as 2-amino-3-ketobutyrate ligase) involves the loss of the *pro-R* hydrogen atom of glycine and occurs with the inversion of stereochemistry; a similar scenario is envisaged for the condensation step of other α-oxoamine synthases.

Pyridoxal 5′-phosphate-dependent α-oxoamine synthases catalyse the conversion of Scheme 1 (reactions i to v) and presently known members of this class are: 5-aminolevulinate synthase (ALAS), 8-amino-7-oxononanoate synthase (AONS), serine palmitoyltransferase (SPT) and 2-amino-3-oxobutyrate CoA ligase (KBL). Our mechanistic and stereochemical studies carried out on ALAS in the 1970s[1,2] showed that the overall process catalysed by this enzyme (Scheme 1; R = H, Y = –CH_2–CH_2–COSCoA) involves two crucial bonding events; the first is a condensation reaction in which the *pro-R* hydrogen atom of pyridoxal phosphate-bound glycine **2** is removed to give a quinonoid intermediate that then attacks the carbonyl group of succinyl CoA to yield **4**. In the latter, all six carbon atoms of the two substrates are present as the pyridoxal phosphate-bound α-amino-β-oxoadipic acid moiety. The second reaction is a decarboxylaion process which is aided by the presence a carbonyl group at one of the β-positions and a vinylogous electron withdrawing pyridinium ring on the other, **4**, to give an ALA-pyridoxal phosphate complex **5** from which ALA is released by transimination reaction. When the ALA synthase reaction was carried out with stereospecifically tritiated glycines, one of the tritium atoms, in the *pro-R* position, was removed[1] and the other occupied the *pro-S* orientation[2] at C-5 of ALA **6** (R = H), which showed that the overall reaction had occurred with the inversion of stereochemistry, highlighting the fact that of the two bonding events one occurs by an inversion and the other by a retention of stereochemistry.

The mechanistic and stereochemical features defined for the ALA synthase reaction were subsequently shown to operate for two other enzymes, AONS[3] and SPT.[4] In these cases also the deprotonation–condensation–decarboxylation–reprotonation sequence of Scheme 1 (reactions i to v) is followed and the target carbon atom, the C-2 of amino acids, suffers one inversion and one retention of stereochemistry. Which of the two constituent reactions occurs with retention or inversion has remained elusive now for nearly three decades following these original studies. This facet, however, has been a focus of lively interest in the literature and predictions regarding the stereochemistry have been made, which are based either on precedents from enzyme catalysed condensation and decarboxylation reactions[3,5] or on X-ray based modelling studies.[6]

An enzyme originally known to catalyse the generic reaction characteristic of α-oxoamine synthases producing aminoacetone **9** from glycine and acetyl CoA has turned out to be 2-amino-3-oxobutyrate CoA ligase (KBL). This enzyme plays an important role in the interconversion of glycine and threonine in bacteria[7,8] as well as in animals.[9] The enzyme has sequence and structural similarities[10] to ALAS and ANOS but its physiological role is to catalyse only the condensation reaction (Scheme 1, reactions i, ii, iii and vi). Thus the cryptic enzyme-bound oxoacid intermediate of the type **4**, involved in an intermediary role in the reactions catalysed by other α-oxoamine synthases, in the case of the ligase, is hydrolysed and then used as a substrate for L-threonine dehydrogenase to give **8**. We have exploited this feature of the ligase to study the stereochemistry of the condensation process with the aid of variously tritiated glycine samples together with the use of recombinant *Escherichia coli* 2-amino-3-oxobutyrate CoA ligase and L-threonine dehydrogenase. For the cloning of the two enzymes their genes were amplified by polymerase chain reaction (PCR) using the total DNA of *E. coli* JM103 as the template and oligodeoxynucleotides corresponding to the border sequences of the coding regions of the genes as primers (see the Supplementary Material†). The amplified PCR products were cloned initially in pTZ57R/T and following DNA sequencing were transferred into the *nde*I and *bam*H1 sites of pET-21a for both the genes. The proteins from these plasmids were expressed in *E. coli* BL21-CodonPlus(DE3) as the host and purified to homogeniety.

A coupled enzyme system[7,8] was adapted in which 0.5 mg ml^{-1} of each enzyme was incubated with 1.0 mM [2-*RS*-$^3H_2$:^{14}C]glycine, 1.0 mM acetyl CoA and 0.5 mM NADH and the reaction was monitored at 340 nm to follow the oxidation of NADH. The loss of absorption plateaued at 15 min with the consumption of 210 nmol of NADH corresponding to the conversion of 19% of the original glycine into threonine. After the removal of an aliquot the incubation was continued for another 25 min. The samples of the reaction mixture were used for the conversion of the amino acids into the benzyloxycarbonyl derivatives that were separated by thin layer chromatography

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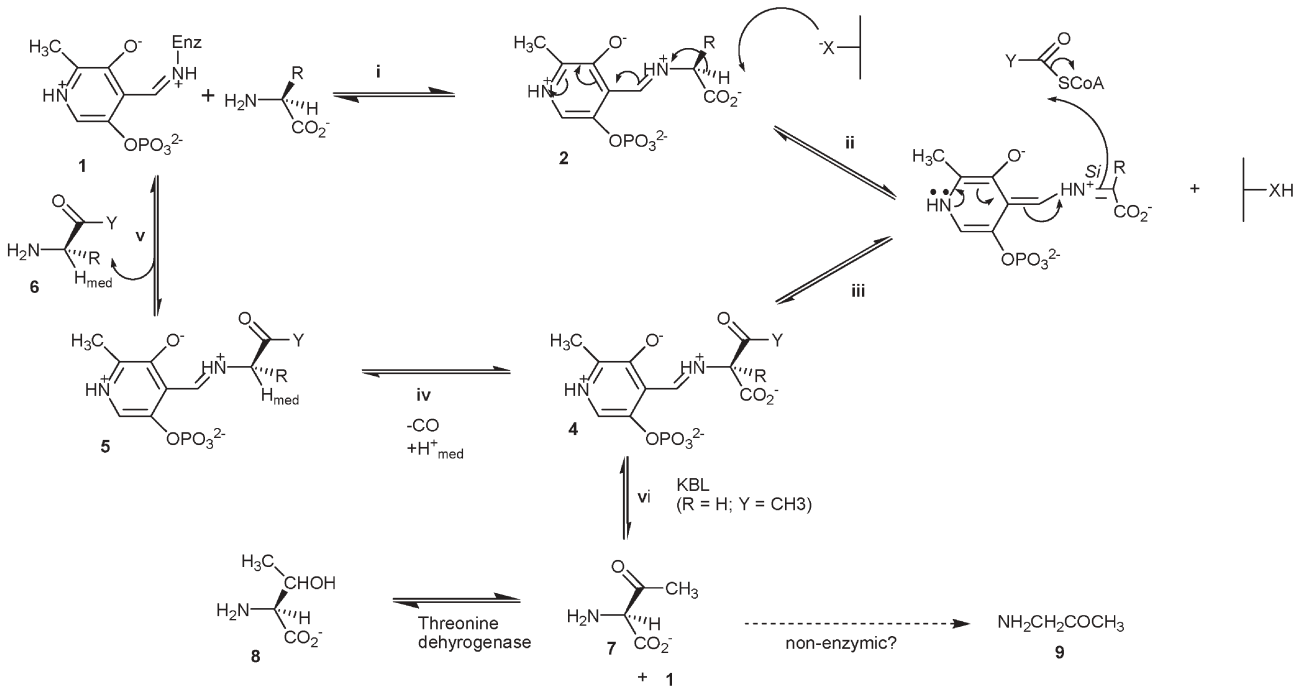

Scheme 1 Sequence of reaction for α-oxoamine synthases. For ALAS, R = H and Y = –CH2–CH2–COSCoA. H_{med} = hydrogen from the medium.

(TLC). It was found, from the 15 and 40 min post incubation experiments, that the ^{3}H:^{14}C ratio of threonine (7.13; Table 1) was about half (49%) that of the original glycine (^{3}H : ^{14}C ratio 14.6). Interestingly, glycine recovered from the 15 and 40 min post incubation experiments also showed progressive loss of ^{3}H which was 49% in the 40 min sample.

The experiments indicate that during the conversion of glycine into dehydrothreonine **7**—trapped as L-threonine **8**—a hydrogen atom of the former is stereospecifically exchanged with the protons of the medium and also that in the overall process, **1** to **7**, the cleavage of the C–H bond and its replacement with a C–C bond (**1** to **7**, R = H, Y = $–CH_3$) occurs in a stereospecific fashion.

In order to explore the absolute stereochemistry of the condensation reaction, advantage was taken of earlier work[11] in which it was shown that serine transhydroxymethylase (serine hydroxymethyltransferase), in the presence of tetrahydrofolate, catalyses the exchange of the *pro-S* hydrogen of glycine with the protons of the medium. [2-*RS*-$^{3}H_2$: 2-^{14}C]glycine was exchanged by the aforementioned protocol to give [2-*R*-$^{3}H_1$: 2-^{14}C]glycine which was converted into threonine using the coupled enzyme system. The last three entries in Table 1 show that threonine biosynthesised in this way had lost 98% of the *pro-R* tritium of the precursor and a similar loss was found for the recovered glycine.

Table 1 Stereospecific loss of a hydrogen atom of glycine in the formation of threonine by the coupled enzyme system

Compound	Time/min	^{3}H : ^{14}C ratio
[2-*RS*-$^{3}H_2$: 2-^{14}C]glycine	0	14.6
[2-*RS*-$^{3}H_2$: 2-^{14}C]glycine	40	7.45
Threonine	40	7.13
[2-*R*-$^{3}H_1$: 2-^{14}C]glycine	0	6.72
[2-*R*-$^{3}H_2$: 2-^{14}C]glycine	40	0.24
Threonine	40	0.19

The exchange of the C-2 hydrogen of glycine was then studied in a partial reaction in the absence of the second substrate, acetyl CoA as well as the dehydrogenase. Fig. 1 shows that while in the presence of the ligase the randomly tritiated glycine lost half of the isotope in the partial reaction, most of the ^{3}H was lost from the [2-*R*-$^{3}H_1$]glycine. The *pro-R* hydrogen exchanged in the partial reaction is thus the same that is lost in the overall condensation process. The knowledge of the substrate stereochemistry of the ligase reaction together with the known absolute stereochemistry of threonine, hence by implication that of dehydrothreonine, allows the deduction that the condensation process occurs (ii to iii) by the inversion of stereochemistry as shown by the comparison of structure **2** with **4**.

Cumulatively, the findings in this work show that in the ligase (KBL) catalysed process the pyridoxal phosphate-bound glycine rapidly exchanges its *pro-R* hydrogen—through the reversal of reactions i and ii—with the protons of the medium. This feature, to

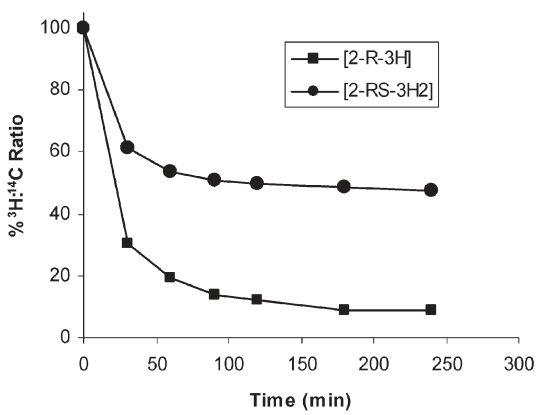

Fig. 1 Stereospecific exchange of a hydrogen atom of glycine by KBL.

varying degrees, is also observed in reactions catalysed by ALAS[12] and AONS.[3] It should, though, be born in mind that such exchange processes in addition to the operation of the aforementioned reactions, depend on the rate at which the conjugate acid (–X–H), formed following proton removal (reaction ii) exchanges with the hydrogen of water, a process which is slow in many enzyme catalysed reactions. This may be either due to the rate of the forward reaction being fast or that the conjugate acid is shielded from the medium during each catalytic turn-over.

In view of the sequence and structural similarity of the ligase[10] (KLB) to other α-oxoamine synthases it may be inferred that in the latter cases, the condensation step also occurs with inversion and the decarboxylation with retention, leading to an overall inversion at the C-atom involved in the bonding processes. Indeed, Baxter and colleagues have determined the X-ray structure[6] of the pyridoxal phosphate-bound form of AONS and using molecular replacement data have concluded that the CoA binding site in the enzyme may be located at the *si* face of the quinonoid intermediate, opposite to that from which a proton is removed. Such an architecture would favour inversion in the condensation step. In contrast to situation with α-oxoamine synthases the condensation reactions leading to the formation of threonine and serine by the pyridoxal phosphate-dependent serine transhydroxymethylase occur with the retention of stereochemistry.[11] Thus with the latter enzyme, deprotonation and delivery of an electrophile, from a bulky donor, methylenetetrahydrofolate, use the same face of the quinonoid intermediate.

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