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RESEARCH ARTICLE

Kinetics of the exchange reaction catalyzed by 2-amino-3-ketobutyrate CoA ligase

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Abstract **2-Amino-3-ketobutyrate CoA ligase (KBL) of *Escherichia coli* is a member of the α-oxoamine synthase family; it catalyzes the condensation reaction between glycine and acetyl CoA to yield 2-amino-3-ketobutyrate. We have previously shown that KBL catalyzes the exchange of *pro-R* hydrogen of glycine with protons in the medium; however, the kinetics of this reaction has never been determined. In this study, we calculated the kinetic parameters of this exchange reaction by using different concentrations of [2*RS*- $^3H_2$: 2-^{14}C] glycine. The rate of the exchange reaction was determined by measuring the $^3H/^{14}C$ ratio in recovered [2*S*- ^3H: 2-^{14}C]glycine. The Lineweaver-Burk plot showed that K_m and k_{cat} of this reaction were 3.8×10^{-3} M and 0.22 S^{-1}, respectively. On the other hand, K_m and k_{cat} values of the overall KBL-mediated catalysis were correspondingly 1.23×10^{-2} M and 1.19 S^{-1}. Thus, the rate of the exchange reaction was almost five times lower than that of overall KBL catalysis.**

Keywords enzyme, 2-amino-3-ketobutyrate CoA ligase, kinetics, exchange reaction

Introduction

The pyridoxal phosphate (PLP)-dependent enzyme 2-amino-3-ketobutyrate CoA ligase (KBL) catalyzes the condensation reaction between glycine and acetyl CoA to yield 2-amino-3-ketobutyrate (Mukherjee and Dekker, 1987; Marcus and Dekker, 1993; Schmidt et al., 2001). This enzyme has been characterized in different prokaryotes and eukaryotes, such as *Escherichia coli* and humans, and its role in threonine degradation/synthesis has been well established both *in vivo* and *in vitro* (Mukherjee and Dekker, 1987; Marcus and Dekker, 1993; Edgar and Polak, 2000).

KBL is one of the four members of the α-oxoamine synthase family. Other members of this family include 5-aminolevulinate synthase (ALAS) (Gibson et al., 1958; Riddle et at., 1989), 8-amino-7-oxononanoate synthase (AONS) (Alexeev et al., 1998; Kerbarh et al., 2006), and serine palmitoyltransferase (SPT) (Hanada et al.,1998; Gable et al., 2000; Hanada et al., 2000). These enzymes play an important role in biosynthesis of sphingolipids, tetrapyrroles, and biotin. All four enzymes catalyze a Claisen-type condensation reaction between different amino acids and acyl-CoA thioesters followed by a decarboxylation reaction (Alexeev et al., 1998; Edgar and Polak, 2000) (supplement Scheme S1). However, KBL only catalyzes the condensation reaction and not the decarboxylation of 2-amino-3-ketobutyrate (Scheme 1), which is in contrast to the reactions catalyzed by the other three members of the α-oxoamine synthase family(Marcus and Dekker, 1993; Bashir et al., 2006). It has been shown that 2-amino-3-ketobutyrate is a very unstable intermediate that is either converted into threonine by L-threonine dehydrogenase (TDH) or decarboxylates (non-enzymatically) into aminoacetone (Marcus and Dekker, 1993) (supplement Scheme S1).

Previously, our laboratory has shown that KBL from *E. coli* catalyzes exchange of *pro-R* of glycine with protons of the medium (Bashir et al., 2006; Jamil et al., 2010), but the rate of this exchange reaction has never been determined (Scheme 1). Therefore, we sought to study kinetic parameters of this exchange reaction, which is one of the steps of the overall biochemical process catalyzed by KBL and TDH. Information about these parameters will contribute to a better understanding of the threonine degradation pathway.

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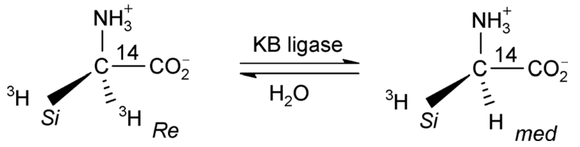

Scheme 1 Exchange reaction catalyzed by KBL. In this reaction, KBL catalyzes the exchange of *pro-R of* [2*RS*-$^3H_2$: 2-^{14}C]glycine with protons of the medium.

Methods

Purification of KBL and TDH

Expression and purification of KBL were carried out according to methods outlined by Mukherjee and Dekker (1987) and Jamil et al. (2010). Recombinant *KBL*-pET and *TDH*-pET vectors were transformed and expressed in *E. coli* separately. Expression of both genes was induced by 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Translated proteins were soluble in water. The proteins were purified by using anion, hydrophobic, and gel filtration chromatography. The purified samples were analyzed by polyacrylamide gel electrophoresis (PAGE) followed by staining with Coomassie Brilliant Blue.

Molecular mass analysis of purified KBL

Molecular mass of purified KBL was obtained using a 6224 TOF LC/MS system (Agilent Technologies, USA) equipped with a dual electro-spray ionization source. Positive ions were produced by using a dual ESI-voltage of 3.5 kV at 325°C, gas flow 5 L/min, and nebulizer pressure of 30 psig. Data were collected at a rate of 1.03 spectra/s. The flow injection analysis of the apoenzyme was performed by injecting 20 μL of the KBL solution (5 μg of protein/μL in 10 mM Tris-HCl, pH 8) at a flow-injection rate of 0.2 mL/min using a mixture of 0.1% formic acid (70%) and acetonitrile solution in 0.1% formic acid (30%). The multiply charged spectrum was extracted from TIC using Agilent Mass Hunter qualitative analysis software and processed for deconvolution.

Rate of exchange of *pro-R* hydrogen of [2RS-$^3H_2$: 2-^{14}C] glycine catalyzed by KBL

One milliliter of the reaction mixture that contained KBL (0.6 U) and [2*RS*-$^3H_2$: 2-^{14}C]glycine (different concentrations in different reactions) was incubated at 37°C in 50 mM Tris-HCl (pH 8.0). Aliquots (200 μL) were taken from the reaction mixture at 0, 15, 30, 60 and 120 min after the start of the reaction. The samples were placed in boiling water for 5 min to quench the reaction and converted to benzyloxycarbonyl glycine as described in Jamil et al. (2010). A portion of benzyloxycarbonyl glycine crystals was dissolved in the scintillation fluid for radioactivity measurements and changes in the ^{3}H/^{14}C ratio were used to calculate the rate of the exchange reaction.

The rate of the coupled reaction of KBL and TDH

The overall catalytic activity of the recombinant KBL was assayed as described by Marcus and Dekker (1993). The reaction mixture contained 50 mM Tris-HCl (pH 8.0), 200 mM glycine, 1 mM acetyl CoA, 0.5 mM NADH, KBL (0.6 U), and TDH (1 U) in a final volume of 1 mL. This mixture was incubated at 37°C and changes in NADH absorbance were monitored at 340 nm (= 6.2 mM$^{-1}\cdot$cm^{-1}) for 10 min. One unit was defined as the quantity of enzyme, which catalyzed the formation of 1 μmol NAD$^+$ per min at 37°C. For kinetic measurements, different glycine concentrations were used in separate reactions to calculate K_m and V_{max} values.

Results and discussion

Induction with 0.2 mM IPTG induced KBL expression in *E. coli* but the enzyme was inactive. After sonication, the cell extract was incubated with 0.1 mM PLP in 50 mM Tris HCl buffer (pH 8.0). After activation, the enzyme was purified by precipitation with 45% ammonium sulfate followed by anion and hydrophobic column chromatography. The purified enzyme produced a single 43 kDa band on the 12% SDS PAGE gel (Fig. 1). Analysis of purified KBL by MALDI-TOF indicated that its molecular mass was 43117.15 ± 1 Da (Fig. 2). This value perfectly matched previous KBL amino acid sequence data reported by Mukherjee and Dekker (1987).

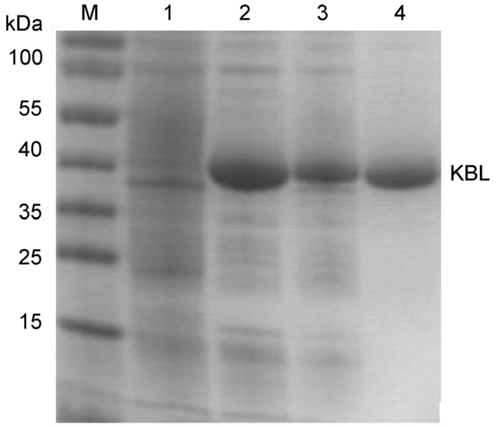

Figure 1 12% SDS-PAGE gel of purified KBL stained with Coomassie Brilliant Blue. Lane M: Protein marker; Lane 1: Non-induced *E. coli* cells having pET-21a/*KBL*, Lane 2: crude extract (induced with IPTG), Lane 3: 45%–65% ammonium sulfate precipitates after dialysis against 50 mM Tris-HCl, pH 8.0, Lane 4: after anion exchange column.

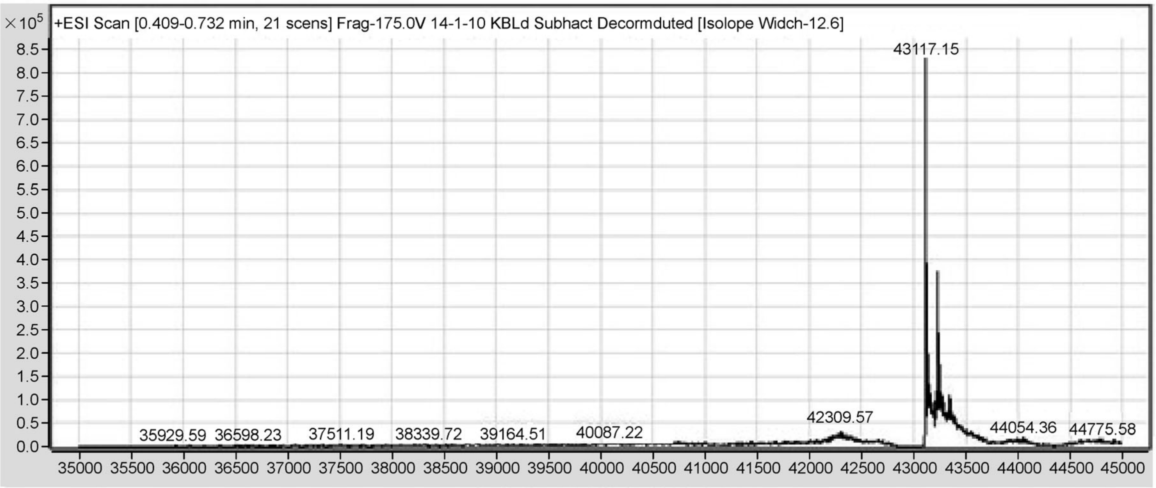

Figure 2 ESI mass spectrum of the KB ligase (apoenzyme).

The rate of the exchange reaction

The incubation of [2*RS*-$^3H_2$: 2-^{14}C]glycine with KBL resulted in a gradual decrease of the $^3H/^{14}C$ ratio of recovered glycine. This decrease may be attributed to the exchange reaction catalyzed by KBL, in which *pro-R* tritium is substituted by protons of the medium (Scheme 1). The reaction was carried out at different concentrations of [2*RS*-$^3H_2$: 2-^{14}C]glycine. We observed that the rate of the exchange reaction with 8 mM [2*RS*-$^3H_2$: 2-^{14}C]glycine was almost 20 times higher than that in presence of 2 mM glycine (Fig. 3). The method that was used for rate determination by an example in which 2 mM [2*RS*-$^3H_2$: 2-^{14}C]glycine (24137 ^{14}C counts per min) was incubated with KBL, and an aliquot was removed after 10 min of incubation and converted to benzyoxyglycine for radioactivity loss measurement.

The $^3H/^{14}C$ ratio at start of reaction = 100%
After 15 min of incubation with KBL,
the $^3H/^{14}C$ ratio inrecovered benzyloxycarbonylglycine = 70%
The change in the $^3H/^{14}C$ ratio = 30%
The rate of the exchange reaction =

$$\frac{\text{Decrease of } {}^3\text{H}/{}^{14}\text{C}(\%) \times \text{Conc. of the glycine used}}{\text{Half of the } {}^3\text{H}/{}^{14}\text{C ratio } (50\%) \times \text{Time of reaction}}$$

Therefore, the rate of the exchange reaction =
$(30 \times 2) / (50 \times 15) = 0.08$ µmol/min

Similarly, the rates of the exchange reaction under different concentrations of [2*RS*-$^3H_2$: 2-^{14}C]glycine were obtained (Table 1) and the Lineweaver-Burk Plot showed that K_m (1/*x*-intercept: 1/0.26) and k_{cat} (1/*y*-intercept: 1/4.5) comprised 3.8 mM and 0.22 s^{-1}, respectively (Fig. 4).

Table 1 The time course of the exchange (H_{Re}) of [2*RS*-$^3H_2$: 2-^{14}C] glycine with KBL. The % age decrease of the $^3H/^{14}C$ ratio is used to find the rate of exchange reaction

Time (min)	Concentrations of the [2*RS*-$^3H_2$: 2-^{14}C]glycine				
	2 mM	3.5 mM	5.7 mM	6.5 mM	8 mM
	Relative $^3H/^{14}C$ (% age)				
0	100	100	100	100	100
15	70	76	82	84	85
30	62	68	76	78	81
60	57	65	71	75	77
120	55	62	69	72	73

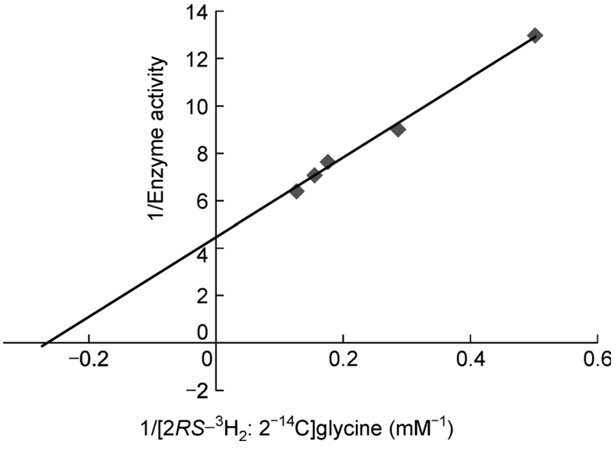

Figure 3 Rate of exchange of H_{Re} of [2*RS*-$^3H_2$: 2-^{14}C]glycine. Rates of the exchange reaction obtained with different concentrations of glycine.

The rate of the coupled reaction of KBL and TDH

The overall catalysis rate of KBL was determined by coupling it with TDH. In the coupled reaction, KBL catalyzed condensation of glycine and acetyl CoA to 2-amino-3-ketobutyrate that was reduced to threonine by TDH and NADH. By keeping concentrations of all substrates constant, it was observed that the rate of the coupled reaction

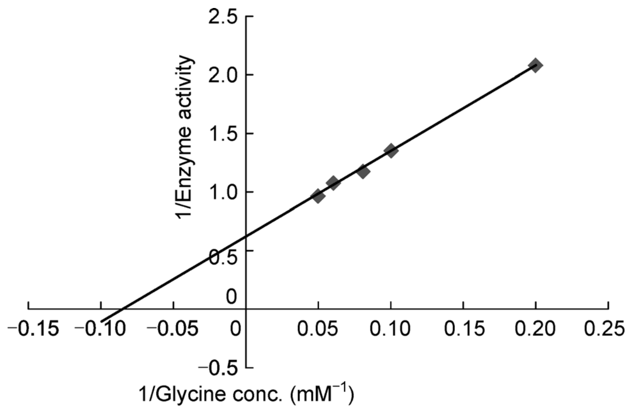

Figure 4 Lineweaver-Burk plot for the exchange reaction. Calculated K_m and k_{cat} values were 3.8 mM and 0.22 s^{-1}, respectively.

accelerated with the increase in the concentration of glycine. The rate of the coupled reaction was almost 2.3 times higher in 25 mM glycine than that the rate obtained in presence of 5 mM glycine. By using coupled reaction rates calculated at different concentrations of glycine, the values of K_m (1/0.08) and k_{cat} (1/.84) were found to be 12.3 mM and 1.19 s^{-1}, respectively.

This showed that the rate of exchange of glycine *pro-R* hydrogen comprised 18.5% of the overall KBL catalysis rate (Table 2). Such a slow rate could be attributed to the absence of acetyl CoA. In the case of ALAS, the rate of an identical process appeared to be very slow although it had not been determined quantitatively. For AONS, the rate of exchange was 10 times lower than the overall biosynthesis rate (Ploux and Marquet, 1996). In comparison with these enzymes, the exchange of C-2 hydrogen of glycine catalyzed by KBL was apparently faster.

Table 2 Kinetic parameters of the coupled and exchange reaction

	K_m (M)	k_{cat} (S^{-1})	k_{cat} / K_m (M^{-1}·s^{-1})
Coupled reaction	1.23×10^{-2}	1.19	9.6×10
Exchange reaction	3.8×10^{-3}	0.22	5.8×10

Abbreviations

KBL: 2-amino-3-ketobutyrate CoA ligase; ALAS: aminolevulinate synthase; AONS: 8-amino-7-oxononanoate synthase; SPT: serine palmitoyltransferase; TDH: L-threonine dehydrogenase, PAGE: polyacrylamide gel electrophoresis.

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Compliance with ethics guidelines

This study does not contain any experiment on human and animals by the author.

References

Alexeev D, Alexeeva M, Baxter R L, Campopiano D J, Webster S P, Sawyer L (1998). The crystal structure of 8-amino-7-oxononanoate synthase: a bacterial PLP-dependent, acyl-CoA-condensing enzyme. J Mol Biol, 284(2): 401–419

Bashir Q, Rashid N, Akhtar M (2006). Mechanism and substrate stereochemistry of 2-amino-3-oxobutyrate CoA ligase: implications for 5-aminolevulinate synthase and related enzymes. Chem Commun (Camb), (48): 5065–5067

Edgar A J, Polak J M (2000). Molecular cloning of the human and murine 2-amino-3-ketobutyrate coenzyme A ligase cDNAs. Eur J Biochem, 267(6): 1805–1812

Gable K, Slife H, Bacikova D, Monaghan E, Dunn T M (2000). Tsc3p is an 80-amino acid protein associated with serine palmitoyltransferase and required for optimal enzyme activity. J Biol Chem, 275(11): 7597–7603

Gibson K D, Laver W G, Neuberger A (1958). Initial stages in the biosynthesis of porphyrins. 2. The formation of delta-aminolaevulic acid from glycine and succinyl-coenzyme A by particles from chicken erythrocytes. Biochem J, 70(1): 71–81

Hanada K, Hara T, Fukasawa M, Yamaji A, Umeda M, Nishijima M (1998). Mammalian cell mutants resistant to a sphingomyelin-directed cytolysin. Genetic and biochemical evidence for complex formation of the LCB1 protein with the LCB2 protein for serine palmitoyltransferase. J Biol Chem, 273(50): 33787–33794

Hanada K, Hara T, Nishijima M (2000). Purification of the serine palmitoyltransferase complex responsible for sphingoid base synthesis by using affinity peptide chromatography techniques. J Biol Chem, 275(12): 8409–8415

Jamil F, Gardner Q A, Bashir Q, Rashid N, Akhtar M (2010). Mechanistic and stereochemical studies of glycine oxidase from *Bacillus subtilis* strain R5. Biochemistry, 49(34): 7377–7383

Kerbarh O, Campopiano D J, Baxter R L (2006). Mechanism of oxoamine synthases: identification of the intermediate Claisen product in the 8-amino-7-oxononanoate synthase reaction. Chem. Comm, 60–62

Marcus J P, Dekker E E (1993). Threonine formation via the coupled activity of 2-amino-3-ketobutyrate coenzyme A lyase and threonine dehydrogenase. J Bacteriol, 175(20): 6505–6511

Mukherjee J J, Dekker E E (1987). Purification, properties, and N-terminal amino acid sequence of homogeneous *Escherichia coli* 2-amino-3-ketobutyrate CoA ligase, a pyridoxal phosphate-dependent enzyme. J Biol Chem, 262(30): 14441–14447

Ploux O, Marquet A (1996). Mechanistic studies on the 8-amino-7-oxopelargonate synthase, a pyridoxal-5′-phosphate-dependent enzyme involved in biotin biosynthesis. Eur J Biochem, 236(1): 301–308

Riddle R D, Yamamoto M, Engel J D (1989). Expression of δaminolevulinate synthase in avian cells: separate genes encode erythroid-specific and nonspecific isozymes. Proc Natl Acad Sci USA, 86(3): 792–796

Schmidt A, Sivaraman J, Li Y, Larocque R, Barbosa J A, Smith C, Matte A, Schrag J D, Cygler M (2001). Three-dimensional structure of 2-amino-3-ketobutyrate CoA ligase from *Escherichia coli* complexed with a PLP-substrate intermediate: inferred reaction mechanism. Biochemistry, 40(17): 5151–5160