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Threonine Formation via the Coupled Activity of 2-Amino-3-Ketobutyrate Coenzyme A Lyase and Threonine Dehydrogenase

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The enzymes L-threonine dehydrogenase and 2-amino-3-ketobutyrate coenzyme A (CoA) lyase are known to catalyze the net conversion of L-threonine plus NAD^+ plus CoA to NADH plus glycine plus acetyl-CoA. When homogeneous preparations of these two enzymes from *Escherichia coli* were incubated together for 40 min at 25°C with glycine, acetyl-CoA, and NADH, a 36% decrease in the level of glycine (with concomitant NADH oxidation) was matched by formation of an equivalent amount of threonine, indicating that this coupled sequence of enzyme-catalyzed reactions is reversible in vitro. Several experimental factors that affect the efficiency of this conversion in vitro were examined. A constructed strain of *E. coli*, MD901 (*glyA thrB/C tdh*), was unable to grow unless both glycine and threonine were added to defined rich medium. Introduction of the plasmid pDR121 (*tdh*$^+$ *kbl*$^+$) into this strain enabled the cells to grow in the presence of either added glycine or threonine, indicating that interconversion of these two amino acids occurred. Threonine that was isolated from the total pool of cellular protein of MD901/pDR121 had the same specific radioactivity as the [^{14}C]glycine added to the medium, establishing that threonine was formed exclusively from glycine in this strain. Comparative growth rate studies with several strains of *E. coli* containing plasmid pDR121, together with the finding that k_{cat} values of pure *E. coli* 2-amino-3-ketobutyrate CoA lyase favor the cleavage of 2-amino-3-ketobutyrate over its formation by a factor of 50, indicate that the biosynthesis of threonine is less efficient than glycine formation via the coupled threonine dehydrogenase–2-amino-3-ketobutyrate lyase reactions.

Under normal conditions, threonine is synthesized by microbes from oxaloacetate via formation in sequence of the intermediates aspartate, aspartate-4-phosphate, aspartate semialdehyde, homoserine, and homoserine phosphate, which is finally converted to threonine in a reaction catalyzed by threonine synthase. Three major pathways for threonine degradation are known. Threonine aldolase activity is responsible for the conversion of threonine to acetaldehyde and glycine. The threonine dehydratase-catalyzed reaction leads to formation of α-ketobutyrate and eventually yields propionate or isoleucine. Alternatively, threonine dehydrogenase (TDH) (EC 1.1.1.103) catalyzes the NAD^+-dependent conversion of threonine to 2-amino-3-ketobutyrate (AKB), which then undergoes one of two reactions; it (plus coenzyme A [CoA]) may be a substrate for AKB CoA lyase (2-amino-3-oxobutanoate glycine-lyase; EC 2.3.1.29), yielding glycine and acetyl-CoA, or it can spontaneously decarboxylate, liberating CO_2 plus aminoacetone. Aminoacetone thus formed is thought to be incorporated into vitamin B_{12} after being reduced to D-1-amino-2-propanol (5).

The pathway initiated by TDH is recognized as the major route for threonine utilization in both prokaryotes (3, 12) and eukaryotes (7). The coupled reactions catalyzed by TDH and AKB CoA lyase make it possible for some strains of *Escherichia coli* and *Pseudomonas aeruginosa* to utilize threonine as the sole carbon and energy source (4, 6, 13); they also provide an efficient alternate route for glycine and serine biosyntheses (6, 20). This pathway accounts for 87% of the threonine catabolized in rat liver (2), and TDH activity is the only threonine catabolic reaction that is detected in chicken liver extracts (1). Expression of the TDH gene (*tdh*) in *E. coli* has been shown to be under control of the so-called leucine-responsive protein (or Lrp) (27), which induces the enzymes in this pathway seven- to ninefold (18, 19). While the importance of the TDH- and AKB CoA lyase-initiated pathway in threonine catabolism is being increasingly recognized, the threonine aldolase-catalyzed reaction seems to be much less significant than once thought because of its very low or even undetectable level of activity in a variety of systems (2, 10, 18, 28). The relative importance of threonine dehydratase to the overall degradation of threonine is also debatable, since the biosynthetic form of this enzyme is inhibited by small amounts of isoleucine (23, 24) and biodegradative threonine dehydratase is detectable only when cells are grown anaerobically or in the absence of glucose (9, 19).

Most of the attention given the TDH- and AKB CoA lyase-initiated pathway has focused on its role in providing acetyl-CoA and glycine to the cell, i.e., threonine breakdown. Although much work has been carried out to elucidate the regulation and properties of the enzymes involved in this pathway, to our knowledge very little if any work has been done to examine whether threonine can be formed by the action of these two enzymes, i.e., from glycine plus acetyl-CoA to threonine. In the studies presented here, we show that threonine formation can occur in this manner; some factors that influence the efficiency of this conversion are examined, and the significance of this mode of threonine formation is considered.

MATERIALS AND METHODS

Enzymes and chemicals. Homogeneous samples of AKB CoA lyase and L-TDH were prepared as described earlier (16). The purity of all samples was established by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (26). Acetyl-CoA synthetase from baker's yeast, DNase I and RNase I from bovine pancreas, and RNase T from *Aspergillus oryzae* were products of Sigma Chemical Co. Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* was procured from

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Boehringer Mannheim. α-Aminobutyric acid, 2-(*N*-cyclohexylaminoethane sulfonic acid (CHES), 3-(*N*-morpholino)propane sulfonic acid, *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and 2,3-dihydroxybenzoic acid were obtained from Sigma Chemical Co. [2-^{14}C]glycine and Ecolite+ scintillation cocktail were purchased from ICN Biomedicals, Inc. All other compounds were of the highest purity commercially available.

In vitro studies. (i) Quantitation of the conversion of glycine to threonine in vitro. Reaction mixtures containing AKB CoA lyase (0.4 mg), TDH (0.4 mg), [2-^{14}C]glycine (280 μM, 500 dpm/nmol), acetyl-CoA (10 mM), NADH (320 μM), and potassium phosphate buffer (200 mM, pH 8.0) were incubated for 40 min at 25°C. The reaction was stopped by adding 200 μl of 25% (wt/vol) trichloroacetic acid solution. Glycine and threonine concentrations in the supernatant fluid were determined by use of an automated Beckman 120C amino acid analyzer; levels of radioactivity present in elution peaks of interest were measured by liquid scintillation techniques. Changes in threonine concentrations during the course of the coupled enzyme reactions were measured spectrophotometrically by following the decrease in A_{340} (due to NADH oxidation). Aminoacetone, formed by the spontaneous decarboxylation of AKB, was quantitated by a colorimetric procedure with acetylacetone (8, 15).

(ii) Measurement of the efficiency of the coupled reactions in vitro. Unless otherwise specified, reaction mixtures were incubated at 25°C; for these studies, they typically contained AKB CoA lyase (0.1 mg), TDH (0.1 mg), glycine (300 mM), acetyl-CoA (200 μM), NADH (320 μM), and potassium phosphate buffer (200 mM, pH 8.0) in a total volume of 2 ml. The formation of threonine and aminoacetone was measured, and the efficiency of the coupled reaction is expressed as the ratio of threonine to aminoacetone (T/A). It should be noted that the assay for aminoacetone includes any AKB that is present because of its decarboxylation by trichloroacetic acid.

The effect of various levels of glycine and acetyl-CoA on the efficiency of the coupled reaction was ascertained under the standard conditions described above. Reactions were carried out until 260 nmol of NADH had been oxidized per ml of reaction mixture, and the average rates of threonine and aminoacetone formation, as well as the T/A ratio, were determined. In order to establish the effect of CoA inhibition on this coupled system, an acetyl-CoA-regenerating system was added; it consisted of acetyl-CoA synthetase (~0.3 U), potassium acetate (100 μM), ATP (50 mM), and $MgCl_2$ (40 mM).

The efficiency of the glycine-to-threonine conversion was also determined in the presence of added variable amounts of NAD^+. In addition, this parameter was examined by adding an NAD^+-to-NADH regenerating system; this system contained glucose-6-phosphate dehydrogenase (~0.4 U) and glucose-6-phosphate (4 mM) as well as the acetyl-CoA-regenerating system described above (in order to eliminate CoA buildup as a variable in the T/A ratio measurements).

(iii) Effect of AKB and CoA accumulation on the lyase-catalyzed reaction. The effect of AKB and CoA buildup on the rate of just the AKB CoA lyase-catalyzed condensation of acetyl-CoA with glycine was examined with standard reaction mixtures which contained 10, 30, or 100 μg of AKB CoA lyase. Aliquots of the mixtures were removed at timed intervals, the reaction was quenched by adding trichloroacetic acid, and the level of aminoacetone was measured to determine the rate of the reaction (8, 15, 16).

(iv) Determination of the K_i value for CoA towards AKB CoA lyase. The K_i for CoA towards AKB CoA lyase was obtained by measuring the apparent K_m for acetyl-CoA in the presence of appropriate CoA concentrations. Initial reaction velocities were measured by the fixed-point colorimetric assay for aminoacetone (8, 15). The reaction mixtures contained glycine (300 mM), different levels of CoA (0 to 300 μM), and various concentrations of acetyl-CoA (75 to 500 μM) for each level of CoA. AKB CoA lyase (10 μg) was added last. Aliquots of the reaction mixture were removed at timed intervals, and the level of aminoacetone was determined as before. Double-reciprocal plots of $1/v$ versus 1/[acetyl-CoA] yielded straight lines from which apparent K_m values for acetyl CoA were obtained; these values were then plotted against the various CoA concentrations.

(v) Determination of k_{cat} values for the AKB CoA lyase-catalyzed reaction with AKB as substrate. The TDH-catalyzed reaction was first used to form a large pool of AKB in solution; this was done at pH 9.5 since this unstable intermediate has a longer half-life at this pH (14). When a sufficient level of AKB had been generated, the reaction was stopped by removing TDH via rapid ultrafiltration, and the pH of the filtrate was adjusted to 7.5. The AKB formed was then quantified and used immediately as a substrate. Reaction mixtures contained CHES (200 mM, pH 9.5), potassium phosphate buffer (200 mM, pH 9.5), L-threonine (25 or 150 mM; added in order to ensure that residual threonine did not interfere with the lyase assay), NAD^+ (10 mM), alanine (50 μM; as an internal standard when measuring glycine formation), α-aminobutyric acid (1.0 mM; as an internal standard for quantifying the AKB pool), and CoA (10 mM). TDH (200 μg) was added last. A small portion of this solution was transferred to a thermostated microcuvette to monitor the reaction at 340 nm, while the remainder was incubated in a test tube at 25°C. When over 6 μmol of AKB had been formed, TDH was removed by ultrafiltration, the filtrates were pooled, and the solution pH was adjusted to 7.5 with 1 N HCl. Individual aliquots were then transferred to separate tubes and incubated at 25°C. At timed intervals thereafter (which allowed for the AKB concentration to be lowered as a result of its decarboxylation), aliquots were removed from the incubation mixtures and the level of AKB was determined (14). AKB CoA lyase (0.5 μg) was added 30 s later to the remaining incubation mixture, and after 1 min, the enzyme-catalyzed reaction was stopped by adding trichloroacetic acid. The amount of glycine formed (determined with an amino acid analyzer) was a measure of the rate of the lyase-catalyzed reaction. Appropriate corrections were made for dilutions and the spontaneous breakdown of AKB ($t_{1/2}$ = 11 min at pH 7.5) that occurred during this procedure. Plots of the rates of reaction versus the determined concentrations of AKB were fit to the Michaelis-Menton equation by nonlinear least-squares analyses to obtain a best-fit k_{cat} value for AKB as substrate for the lyase; K_m values are not reported since the presence of threonine was expected to interfere with this value.

The same method was used to obtain the K_m value for CoA except that various fixed concentrations of CoA were used; threonine was not expected to be a competitive inhibitor in these studies. Apparent V_{max} values for AKB consumption were determined for each concentration of CoA tested and were plotted against the CoA concentrations to provide the K_m value.

In vivo studies. The growth medium was prepared as specified by Neidhardt et al. (17) and modified by Wanner et al. (25). Sterilization was accomplished by passage through 0.2-μm filters. All the amino acids called for, except threonine, glycine, and valine, were included and, unless specified otherwise, 20× isoleucine (final concentration, 8 mM) was routinely added to all cultures in order to inhibit in vivo threonine dehydratase activity.

An agar medium for growth in petri dishes was made with

TABLE 1. Bacterial strains and plasmid used in these studies[a]

Strain or plasmid	Genotype	Phenotype	Phenotype with pDR121[b]	Source or reference
K-12 (W1485)	Prototroph	Prototroph	Prototroph	D. L. Oxender
SP915	***tdh::cat glyA***	**Gly$^-$** Can^r	**Gly$^+$**	20
SP1192	***tdh::cat glyA*** *ΔrecA srl*::Tn*10*	**Gly$^-$** Can^r Tet^r, UV^s	**Gly$^+$**	R. L. Somerville
NK5148	***thrB/C-34*::Tn*10***λ$^-$ IN(*rrnD-rrnE*)	**Thr$^-$** Tet^r	ND[c]	11
MD901	***tdh::cat glyA thrB/C-34*::Tn*10*** *recA$^+$*	**Thr$^-$ Gly$^-$** Tet^r Can^r	**Gly$^+$ or Thr$^+$**[d]	This work
pDR121	**tdh$^+$** ***kbl$^+$*** Amp^r		Plasmid allows strains to interconvert Gly and Thr	21

[a] Phenotypes and genotypes in boldface are those most relevant to this study.
[b] All plasmid-bearing strains were also Amp^r. Unless listed, other phenotypes remained the same.
[c] ND, not done.
[d] Either threonine or glycine must be supplied; when one is supplied, the other will be synthesized via the coupled-enzyme pathway introduced by the plasmid.

the same components and concentrations as described above except that 15 g of Bacto agar was added per liter of medium.

The genotypes and phenotypes of strains used in this study are given in Table 1. Strain MD901 was derived from SP915 by transduction with a phage P1-vir lysate of NK5148 which was procured from Nancy Kleckner through the *E. coli* Genetic Stock Center (Yale University, New Haven, Conn.) (11). Those colonies which exhibited resistance to tetracycline (50 μg/ml) were selected and tested to confirm the absence of endogenous threonine synthesis.

(i) Special growth requirements. In order to test the requirement for glycine and/or threonine in the plasmid complementation growth studies, either one or both of these amino acids were added to the medium at 20 times the normally recommended concentrations (threonine, 8 mM; glycine, 16 mM) along with isoleucine (8 mM). Colony growth on agar plates was monitored at 37°C.

(ii) Radioactivity incorporation studies with [^{14}C]glycine. [2-^{14}C]glycine (5 mM, 147 dpm/nmol) was added to growth medium lacking glycine and threonine. Cells were grown with shaking at 37°C to stationary phase, at which time they were disrupted by sonication and the crude homogenate was treated with a mixture of DNase I, RNase T, and RNase A. After this mixture was centrifuged, the soluble proteins were precipitated by adding trichloroacetic acid. A portion of the precipitated and washed protein was hydrolyzed in 6 N HCl at 150°C for 1 h, and the hydrolysate was analyzed on the amino acid analyzer; fractions collected in parallel allowed for quantitation by liquid scintillation counting and the normal ninhydrin reaction.

(iii) Measurement of growth rates of selected bacterial strains. The growth medium was inoculated with cell cultures that were grown overnight in the same medium. Rich media containing isoleucine with either 20× glycine (i.e., 20 times the normal amount specified [17, 25]), 20× threonine, or 20× glycine and threonine were used. The strains tested included MD901/pDR121, SP1192/pDR121, and K-12/pDR121. The flasks were shaken at 250 rpm and 37°C. Cell growth was monitored at 420 nm by removing aliquots of the cultures at timed intervals and diluting them with 0.2% formaldehyde solution. Initial optical density values of ~0.05 increased to as high as 1.0 during growth. Cell growth on agar plates was also monitored in order to visually confirm the results from growth rate studies.

RESULTS

Conversion of glycine (plus acetyl-CoA) to threonine in vitro. (i) Quantitation of the conversion of substrate to product. With the conditions and procedures described in Materials and Methods, ~36% conversion of glycine to threonine was observed within an incubation period of 40 min. This extent of conversion was established by determining that the initial level of 280 nmol of glycine at the start of the reaction was converted to 102 nmol of threonine (quantified by analysis on the amino acid analyzer). These results were corroborated by the amount of radioactivity recovered in the threonine peak as well as the amount inferred from the decrease in absorbance due to the oxidation of NADH. Of the total quantity of radioactivity added to the reaction mixture, 95% was recovered in either glycine or threonine; the amount of aminoacetone detected constituted <1% of the glycine added.

(ii) Factors affecting the efficiency of the in vitro conversion of glycine to threonine. As noted in Materials and Methods, the efficiency of this coupled-enzyme system was measured in terms of a T/A ratio (ratio of threonine formed to aminoacetone released). Since the amounts of enzymes and substrates in the system were the most obvious factors that would have an influence, they were examined first. As expected, higher levels of TDH increased the efficiency of the coupled lyase-TDH reaction; Table 2 shows that increased TDH levels enhanced the rate of threonine formation and reduced the amount of aminoacetone formed (because of a smaller AKB pool). AKB lyase levels increased the rates of both threonine and aminoacetone formation, but the overall efficiency of the reaction (T/A ratio) dropped since the rate of aminoacetone formation was raised to a greater degree.

The results presented in Fig. 1 show that the T/A ratio was progressively lower when higher concentrations of acetyl-CoA were added. Although the rates of threonine and aminoacetone formation both increase with more acetyl-CoA added, the rate of aminoacetone formation begins low and steadily increases, while the rate of threonine formation levels off. The

TABLE 2. Effect of various levels of TDH and AKB CoA lyase on the rates of threonine and aminoacetone syntheses and on the efficiency of the coupled reaction as indicated by the T/A ratio[a]

TDH (μg/ml)	AKB lyase (μg/ml)	Rate (nmol/min) of formation of:		T/A ratio
		Threonine	Aminoacetone	
16.8	26.5	9.1	22.2	0.41
336	26.5	17.9	7.6	2.36
16.8	1.77	2.2	3.9	0.56
336	1.77	5.3	2.2	2.41

[a] See Materials and Methods for a description of the conditions used in these experiments.

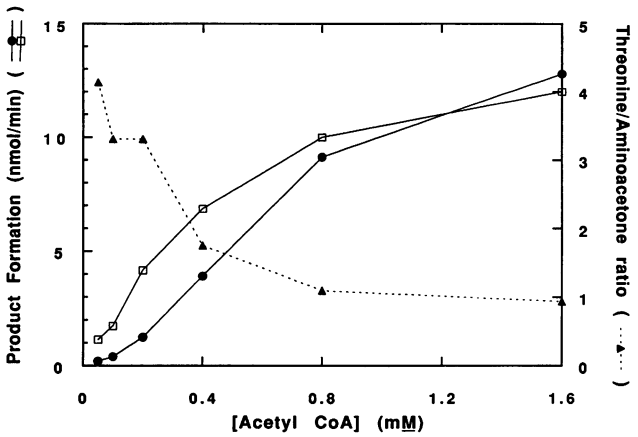

FIG. 1. Effect of various acetyl-CoA concentrations on the efficiency of the coupled TDH-AKB CoA lyase reaction in the direction of threonine formation. The concentrations indicated are those present in the reaction mixture. All other conditions are as given in Materials and Methods. Shown are the rates of threonine (□) and aminoacetone (●) formation as well as the T/A ratio as a measure of the efficiency of the reaction.

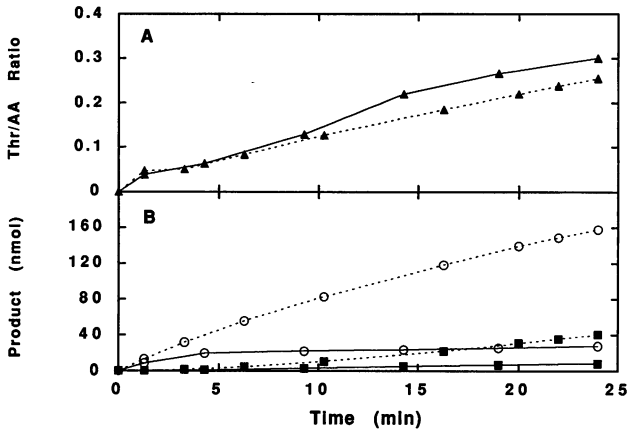

FIG. 2. Effect of CoA removal on the formation of threonine and aminoacetone and on the efficiency of the coupled reaction. Threonine and aminoacetone formation was measured with (– – – –) and without (——) removal of CoA by the acetyl-CoA-regenerating system described in Materials and Methods. (A) Change in the T/A ratio over the time course of the reaction. (B) Amounts of threonine (■) and aminoacetone (○) formed. When CoA is removed, the T/A ratio decreases as a result of the much larger AKB pool, evidenced by the increase in aminoacetone formation.

end result is that the T/A ratio drops substantially in the presence of increasing amounts of acetyl-CoA. The same trend was seen when increasing levels of glycine were added to the reaction mixture (data not shown).

Since other studies (described below) indicated that CoA is a good competitive inhibitor of the lyase-catalyzed condensation reaction between glycine and acetyl-CoA, the effect of including an acetyl-CoA-regenerating system was examined. The results obtained when CoA was removed with the acetyl CoA synthase system are presented in Fig. 2. Figure 2B shows a large difference in the amounts of threonine and aminoacetone formed over the time course of the reaction. Although the rates of aminoacetone formation (with and without the regenerating system) are similar when the reaction is first initiated, the rate falls rapidly when CoA is not removed (as compared with the system with acetyl-CoA synthase included). While the amounts of threonine and aminoacetone formed are both significantly higher when the acetyl-CoA-regenerating system is present, Fig. 2A shows that the T/A ratio is lowered over the time course of the reaction. The size of the AKB pool should be greater under these conditions; however, since the rate of the TDH reaction does not increase proportionally to this pool size, the efficiency of the reaction is lowered.

For the conversion of AKB to threonine by this enzyme couple, higher concentrations of NADH should facilitate greater use of AKB, thereby yielding a higher T/A ratio. When the NADH concentration was doubled from 100 to 200 μM in the initial reaction mixture (with other conditions standard), the T/A ratio increased 1.5-fold after 40 min of incubation. It was also expected that NAD^+, a product of the coupled reaction, would competitively inhibit the TDH-catalyzed reaction in the direction of threonine formation. Figure 3B shows that whereas threonine formation is lowered, aminoacetone levels are raised when NAD^+ is present. The consequence is a less efficient conversion of glycine to threonine as shown by the lower T/A ratio (Fig. 3A). In light of these results, the effect of including an NADH-regenerating system (i.e., glucose-6-phosphate dehydrogenase plus glucose-6-phosphate) in the standard reaction mixture was tested. Under these conditions, the AKB formed was utilized more effectively, resulting in a 3.2-fold increase in the T/A ratio after 60 min of reaction. This effect is due to increased formation of threonine while the amount of aminoacetone released remained relatively constant (data not shown).

(iii) Effect of AKB and CoA accumulation on the AKB CoA lyase-catalyzed reaction. When CoA was not removed from the coupled-enzyme system, it seemed reasonable that this product might inhibit the lyase reaction, thereby decreasing the rate of conversion of glycine plus acetyl-CoA to AKB (plus CoA). Figure 4 shows the effect of product accumulation on the rate of the lyase-catalyzed reaction (as measured by aminoacetone formation). As can be seen, the initial rates of

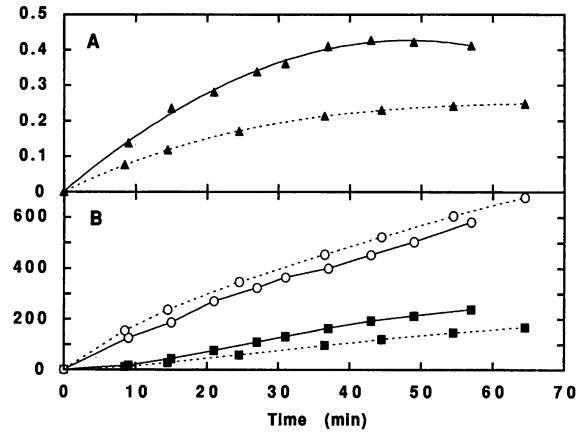

FIG. 3. Effect of various NAD^+ concentrations on the efficiency of the coupled AKB CoA lyase-TDH-catalyzed reactions. No NAD^+ (——) or 2 mM NAD^+ (– – – –) was added to standard reaction mixtures as described in Materials and Methods. (A) Efficiency (T/A ratio) of the coupled-enzyme system. (B) Amounts of threonine (■) and aminoacetone (○) formed. The T/A ratio falls in the presence of added NAD^+ because of the formation of less threonine and, consequently, more aminoacetone.

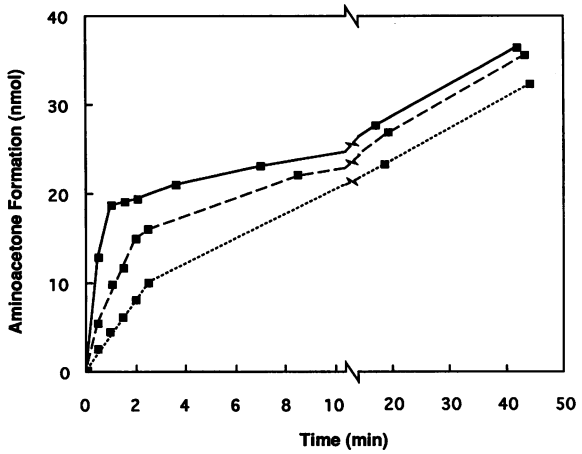

FIG. 4. Effect of AKB and CoA accumulation on the rate of the AKB CoA lyase-catalyzed reaction. Experimental details are described in Materials and Methods. Either 10 (------), 30 (– – – –), or 100 (——) μg of the lyase was added to reaction mixtures containing acetyl-CoA and glycine. Aliquots were subsequently assayed for aminoacetone at the times indicated.

reaction are highly dependent on the amount of enzyme included in the mixture. As the reaction continues, however, the rates become much less dependent on enzyme concentration and are almost identical. These results are consistent with the establishment of an equilibrium between substrates and products of the AKB CoA lyase-catalyzed reaction.

(iv) Competitive inhibition of the AKB CoA lyase-catalyzed reaction by CoA. The effect of CoA on AKB formation from glycine plus acetyl-CoA was measured as described in Materials and Methods. CoA was found to be a competitive inhibitor with respect to acetyl-CoA, exhibiting a K_i value of 30 μM. This value indicates the relatively tight binding of CoA by the lyase and provides an explanation for the effect it has on the T/A ratio in the coupled-enzyme system (Fig. 2).

(v) Determination of turnover numbers for AKB utilization and formation as catalyzed by *E. coli* AKB CoA lyase. Having established methods for preparing generous amounts of AKB in solution (14), for determining its stability over the physiologically and enzymatically relevant pH range (14), and for monitoring the rates of the AKB CoA lyase-catalyzed reaction in either direction by fixed-point assays, we measured the turnover number of this enzyme for both AKB formation and AKB utilization. At pH 7.5 and 25°C in 0.4 M potassium phosphate buffer, the k_{cat} for *E. coli* AKB CoA lyase with AKB as the substrate was ~5,000 min^{-1}. In contrast, when AKB was formed from glycine plus acetyl-CoA, the k_{cat} was found to be ~100 min^{-1}, clearly indicating that, under these experimental conditions, AKB utilization is favored over its formation.

In vivo conversion of glycine to threonine. Since it was evident that the lyase-TDH coupled-enzyme system can effectively catalyze the conversion of glycine to threonine in vitro, attempts were made to determine whether this same conversion could be observed in vivo. In the first experiments, *E. coli* SBD-76 cells (4) were grown in medium containing [2-^{14}C]glycine; subsequently, the threonine that had been incorporated into cellular protein was isolated and analyzed for the presence of radioactivity. Since no ^{14}C-labeled threonine was detected in these experiments, several strains of *E. coli* which lacked one or more enzymes that normally participate in threonine biosynthesis were constructed; the phenotypes and relevant growth requirements of the strains used in these studies are listed in Table 1.

(i) Plasmid complementation. Plasmid pDR121 (*tdh*$^+$ *kbl*$^+$) was introduced into strains SP1192, MD901, and K-12. As indicated in Table 1, the presence of this plasmid in strain SP1192 eliminates the requirement for glycine (Gly$^-$ becomes Gly$^+$), demonstrating that these cells can now synthesize this amino acid from threonine via the TDH-AKB CoA lyase pathway. Strain MD901, in contrast to SP1192, has an added requirement for threonine (because of its lack of a functional *thrB/C* gene). Introduction of plasmid pDR121 into this strain alleviates the requirement for either glycine or threonine but not both. These results show that the TDH-AKB CoA lyase enzyme couple is able to catalyze the interconversion of glycine and threonine in vivo.

(ii) Incorporation of ^{14}C from glycine into threonine. MD901/pDR121 cells were grown in defined rich medium (17) supplemented with [2-^{14}C]glycine. The cellular protein fraction was then isolated, washed, and hydrolyzed, and the hydrolysate was subjected to amino acid analysis. The specific radioactivities of the threonine and glycine so isolated were found to be 142 and 148 dpm/nmol, respectively, which compare very well with that of the glycine added to the medium (i.e., 147 dpm/nmol). With the appropriate bacterial strain, therefore, threonine formation from glycine can indeed occur in vivo.

(iii) Studies on the efficiency of the in vivo glycine-threonine interconversion. The relative growth rates of *E. coli* MD901, SP1192, and K-12 containing plasmid pDR121 were compared in various media supplemented with threonine, glycine, or both. The cell doubling times shown in Fig. 5 provide a qualitative measure of the rates of glycine-threonine interconversion. As can be seen, the doubling time for MD901/pDR121 is significantly longer when the strain is grown in the glycine-supplemented medium (threonine synthesis via the AKB lyase-TDH pathway is forced when threonine is not added) than when the strain is grown in a medium containing both threonine and glycine. Threonine synthesis is apparently a limiting factor in the growth of these cells. In addition, the doubling time of MD901/pDR121 in medium supplemented with threonine is almost the same as when both glycine and threonine are added; the growth of this strain, therefore, is not restricted by the availability of glycine synthesized via the TDH-lyase pathway. Since the normal mode for threonine biosynthesis from aspartate is functional in SP1192, its growth should not be affected adversely when threonine is not added. The only instance in which the growth rate of SP1192 might possibly be limited is when glycine synthesis is forced from threonine via the TDH-lyase pathway. Glycine biosynthesis by this pathway, however, must be very efficient since the cells supplemented with threonine alone grow at the same rate as when both threonine and glycine are added to the medium. Also, as expected, *E. coli* K-12 showed essentially the same growth rate on all three media tested. Taken together, these data indicate that the biosynthesis of threonine is less efficient than glycine formation via the TDH-AKB CoA lyase coupled-reaction pathway.

The forgoing studies were confirmed by observing the growth of strain MD901/pDR121 on agar plates containing equivalent supplementations. When threonine and glycine were both present in the medium (along with isoleucine), cell growth was normal. If glycine was excluded, the extent of growth was virtually the same, suggesting that glycine formation from threonine in these cells is not limiting. When, however, threonine was left out of the medium (i.e., threonine synthesis is forced), significantly less cell growth occurred after the 2-day incubation. No cell growth whatsoever was observed

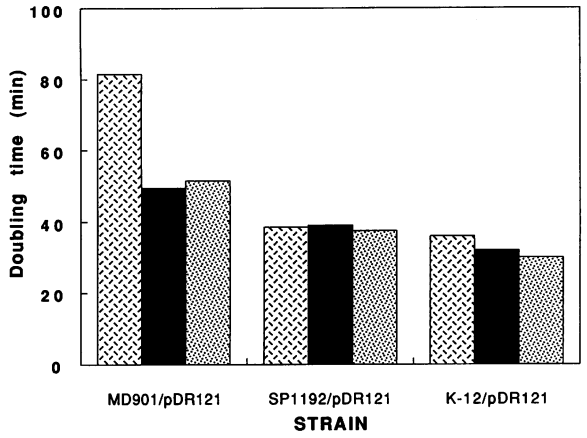

FIG. 5. Doubling times of *E. coli* strains cultured in medium supplemented with threonine and/or glycine. Strains containing the plasmid pDR121 were grown in rich defined medium containing glycine alone (▽▽), glycine plus threonine (■), or threonine alone (▒) as described in Materials and Methods. Growth rates for each strain should be compared with the case in which glycine and threonine were added. The doubling time of MD901 is significantly increased when only glycine is added (which forces this strain to obtain all of its threonine via the less efficient AKB lyase-TDH coupled pathway). The phenotypes of strains MD901 and SP1192 are given in Table 1.

if isoleucine was not present in medium lacking threonine (isoleucine was otherwise routinely added to all cultures).

DISCUSSION

L-Threonine dehydrogenase and AKB CoA lyase have been found in a wide variety of organisms. The reactions catalyzed by these two enzymes constitute an efficient pathway for the catabolism of L-threonine to glycine plus acetyl-CoA (termed the threonine utilization pathway). While one earlier report involving these two enzymes from mammalian sources indicated the possible conversion of glycine to threonine in vitro (22), to our knowledge, no studies have explored whether threonine formation from glycine can occur in vivo via a coupling of the AKB CoA lyase and TDH-catalyzed reactions. We therefore examined this possibility in vitro and in vivo (using *E. coli*). Attempts first to measure threonine biosynthesis from glycine plus acetyl-CoA in vitro showed that within an incubation period of 40 min, ~36% of the glycine added was indeed converted to L-threonine. This finding led us to consider what influence various factors might have on the system and subsequently whether the same net conversion could be observed in vivo.

In general, experimental factors affecting the efficiency of this conversion are characteristic of a coupled-enzyme system in which two reactions compete for the same product. In the case of TDH and AKB CoA lyase operating in the direction of threonine synthesis, AKB, the unstable intermediate involved, can have one of two fates; it can spontaneously decarboxylate to aminoacetone, or it may be utilized as a substrate in the TDH-catalyzed reaction to form threonine. Three factors affect the efficiency of the coupled reactions ending in threonine synthesis: (i) the rate of the lyase-catalyzed reaction, (ii) the stability of AKB, and (iii) the capacity of TDH to utilize AKB as a substrate. Thus, the efficiency of the net reaction is determined by how well TDH catalyzes the reduction of AKB before it spontaneously decarboxylates. Since the decarboxylation of AKB is a first-order decay process, the rate of aminoacetone formation will always be directly proportional to the size of the AKB pool; e.g., if the pool size is doubled, the rate of spontaneous breakdown should also double. The TDH-catalyzed reaction, on the other hand, involves a finite number of substrate-binding sites and will exhibit saturation kinetics; it therefore will not exhibit a proportionally higher rate when the pool of AKB is increased dramatically. Because of such considerations, the efficiency of the glycine-to-threonine conversion should be favored by a slow rate of AKB formation (the particular factor causing the slower rate of formation is of little consequence), a long half-life of the unstable AKB intermediate, and efficient capturing of the intermediate by TDH. These expectations are borne out by the data; for example, higher TDH or NADH concentrations or removal of NAD^+ inhibition of the TDH-catalyzed reaction each ends in a more efficient conversion of glycine to threonine. In contrast, when the lyase-catalyzed reaction proceeds at a faster rate, the efficiency of the coupled-enzyme system falls.

Several conclusions regarding the nature of this coupled sequence of reactions can be drawn from the data presented here. The first is that the lyase-catalyzed reaction appears to reach a state in which substrates and products are in equilibrium. This inference is supported by experiments in which the rate of condensation of glycine with acetyl-CoA proceeded very rapidly at first but then slowed considerably with the accumulation of products (Fig. 2). A second result that supports this conclusion is shown in Fig. 4; the initial rate of AKB formation is highly dependent on the concentration of enzyme for the first 1 to 2 min but not thereafter. Since the thermodynamic equilibrium for this reaction is reached shortly after the reaction is initiated (as soon as 1 min in the case of the highest level of lyase used), it appears that the equilibrium of the reaction lies in favor of glycine plus acetyl-CoA.

Other elements in the coupled-reaction system also support the view that the AKB CoA lyase-catalyzed reaction favors AKB cleavage rather than formation. First, the condensation reaction of glycine with acetyl-CoA has a comparatively low k_{cat} value (i.e., 100 min^{-1}), while the turnover number for AKB is on the order of 5,000 min^{-1}. Second, the K_m value for CoA (for the AKB → Gly reaction) with the lyase is somewhat lower than that for acetyl-CoA (for the Gly → AKB reaction) (30 versus 60 μM, respectively).

Since the formation of threonine from glycine in vivo can indeed be demonstrated, the pertinent question is whether this conversion is of biological significance. Threonine is an essential amino acid for mammals. Thus, there is no pathway capable of adequately meeting the need for threonine; the TDH and lyase enzymes, however, are present in higher eukaryotes (1, 2). The fact that mammalian organisms contain these two enzymes and yet require threonine in their diet indicates that very little or no synthesis of threonine occurs via this pathway. The findings presented here, concerning the efficiency of the interconversion of threonine and glycine via the action of TDH and AKB CoA lyase in *E. coli*, support the general view that these two enzymes normally assume a catabolic role in threonine metabolism.

When *E. coli* SP1192 (*glyA tdh*)/pDR121 (*tdh*$^+$ *kbl*$^+$) cells were grown overnight in a defined rich medium containing [2-^{14}C]glycine, no radioactivity was detected in the threonine isolated from the protein pool, indicating that threonine biosynthesis via this pathway does not operate to a significant degree when the aspartate-initiated pathway is synthesizing threonine. In fact, the specific radioactivity of the glycine (430 dpm/nmol) was actually diluted (to 250 dpm/nmol), apparently because of glycine formation from threonine via this pathway. When, however, normal threonine biosynthesis from aspartate

is blocked by introducing a transposon into the *thrB/C* gene, cells must utilize the lyase-TDH pathway to synthesize threonine. Under such conditions, cell doubling times are significantly longer. Such a result must be due to inefficient conversion of glycine to threonine in vivo. Conversely, when glycine is formed by the same pathway, no such increase in doubling time occurs. An added factor to be considered is that the *tdh* and *kbl* genes of the plasmid may form more TDH and AKB lyase than the chromosomal genes; thus, it is possible that strains without the plasmid would not be able to synthesize threonine from glycine in quantities sufficient to allow any detectable growth. Furthermore, in all cases in which threonine synthesis via the lyase-TDH pathway was necessary for growth, the medium needed to be supplemented with isoleucine. The ability of this amino acid to allow growth under these conditions is most likely due to its inhibition of the irreversible threonine dehydratase-catalyzed reaction. By inhibiting this reaction, the small amount of threonine that is formed by the AKB CoA lyase-TDH couplet is sufficiently spared so that it is available for protein synthesis or other necessary metabolic processes.

In conclusion, although the data presented show that AKB CoA lyase and TDH from *E. coli* can work together to catalyze the formation of threonine in vitro and in vivo, the primary role of this pathway is for threonine catabolism.

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