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pH-Dependent Decarboxylation of 2-Amino-3-ketobutyrate, the Unstable Intermediate in the Threonine Dehydrogenase-Initiated Pathway for Threonine Utilization

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2-Amino-3-ketobutyrate can be readily formed enzymatically by the action of L-threonine dehydrogenase. A convenient assay for determining the half-life of this β-keto acid is afforded by its rapid and quantitative conversion to glycine (+ acetyl CoA), as catalyzed by 2-amino-3-ketobutyrate CoA lyase. Using this system, we have found the half-life of 2-amino-3-ketobutyrate varies with pH from 8.6 minutes at pH 5.9 to 140 minutes at pH 11.1 yielding a theoretical titration curve that predicts a pK_a value of 8.15 for the α-amino group of this intermediate. These data are considered relevant to discussions pertaining to a threonine dehydrogenase/2-amino-3-ketobutyrate CoA lyase enzyme complex in the threonine utilization pathway and to mechanistic aspects of the 5-aminolevulinate synthase-catalyzed reaction where 2-amino-3-ketoadipate is involved.

Threonine dehydrogenase (TDH) (EC 1.1.1.103) and 2-amino-3-ketobutyrate (AKB) CoA lyase (EC 2.3.1.29) initiate the primary route for threonine utilization in both prokaryotes and eukaryotes (1-3). This pathway accounts for approximately 90% of threonine catabolism in the liver of normally fed rats (4). The reactions catalyzed by TDH and AKB CoA lyase are shown in Scheme I. AKB, the intermediate in this sequence of reactions, can spontaneously decarboxylate to form aminoacetone (5). Several studies in which it was shown that aminoacetone is formed from added threonine in humans (6), intact rats or rat liver mitochondria (7, 8), and other biological systems (1, 9) infer that this aminoketone comes from the decarboxylation of the AKB involved as the intermediate in the TDH/AKB CoA lyase enzyme couple. It has been reported that humans excrete approximately 0.4 mg of aminoacetone daily (10); the physiological significance of this observation is unknown. Another unresolved question is whether the aminoacetone so formed participates in Vitamin B_{12} biosynthesis. Aminoacetone can be reduced to D-1-amino-2-propanol by a stereospecific oxidoreductase (11, 12) and, in

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Abbreviations used: TDH, threonine dehydrogenase; AKB, 2-amino-3-ketobutyrate; AKA, 2-amino-3-ketoadipate; SDS, sodium dodecyl sulfate.

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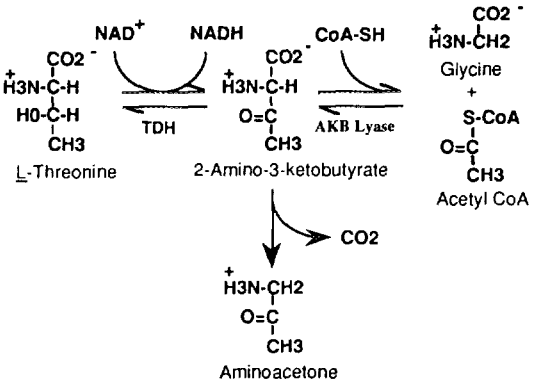


Scheme I

radiotracer studies, the incorporation of ^{14}C from threonine into the D-1-amino-2-propanol moiety of Vitamin B_{12} has been shown (13-15).

The efficiency of the TDH/AKB CoA lyase coupled enzyme system, as measured by its ability to yield glycine and preclude the formation of aminoacetone, has been examined in earlier studies (8,16). An important factor that was not addressed in such work, however, was the stability of AKB in solution. In strictly chemical studies, AKB was reported before to have a half-life of less than one minute at pH 7.0 (5). Since our lab has purified TDH and AKB CoA lyase to homogeneity and in sizable quantities from extracts of *E. coli* (3, 17), we were able to generate large pools of AKB enzymatically with TDH and subsequently to quantitate the pool size by measuring the glycine formed by the AKB CoA lyase-catalyzed reaction. Such experiments, reported here, allowed us to determine the half-life of AKB in solution over the pH range of 5.9 to 11.1, to predict a pK_a value for the α-amino group of AKB, and to assess the relevancy of the stability of this intermediate to AKB channeling in the TDH/AKB CoA lyase enzyme couple.

MATERIALS AND METHODS

TDH and AKB CoA lyase were purified to homogeneity from extracts of *E. coli* SBD-76 cells as previously described (17-19). The purity of enzyme preparations used in these studies was routinely established by SDS-polyacrylamide gel electrophoresis. L-Threonine, NAD^+, and CoA were products of Sigma Chemical Company (St. Louis, MO).

The following procedures were all carried out at 25° C. The overall approach consisted of using TDH to generate a pool of AKB which was then efficiently converted to glycine + acetyl CoA with excess CoA and AKB CoA lyase. The glycine so formed was subsequently quantitated with the use of a Beckman Model 120C automated amino acid analyzer (Beckman W1 resin). To that end, TDH (~40 mg) was added to a reaction mixture (5 ml) containing 2 mM threonine, 5 mM NAD^+, 90 mM alanine (as an internal standard in amino acid analysis), and 200 mM potassium phosphate buffer (pH 9.0). After ~320 nmoles of AKB had been generated (as inferred from the increase in O.D. to ~2 at 340 nm), TDH was removed from the reaction mixture by filtering it through a Centricon-30 microconcentrator (Amicon). The pH of individual portions of the reaction mixture containing AKB was then adjusted to the desired value in the range of 6 to 11 by careful addition of 1 N HCl or NaOH. Aliquots (200 µl) were removed at timed intervals and added to a solution containing 50 µl of phosphate buffer (1 M, pH 8.0) and sufficient acid or base to bring the pH to 8.0. Immediately thereafter, 200 µl of a mixture containing 0.1 mg of AKB CoA lyase (specific activity ≈ 60 µmol glycine formed

$min^{-1}mg^{-1}$) and 1 mmol of CoA were added to rapidly and quantitatively convert the AKB to glycine. After this solution was incubated for 5 min, the reaction was stopped by adding 150 µl of a 25% (w/v) solution of trichloroacetic acid. The glycine present was then determined quantitatively by automated amino acid analysis; its level was taken as a measure of the amount of AKB in the solution. To obtain the decarboxylation rate constant and thereby the half-life of AKB, the amounts of AKB found *versus* the time of incubation were fit to the following equation:

$$AKB_t = (AKB_0)\, e^{-kt}$$

where t = the incubation time (min), k = the decarboxylation rate constant (min^{-1}), and AKB_t and AKB_0 are the amounts of AKB at times t and 0 minutes, respectively.

RESULTS AND DISCUSSION

Determination of the Half-life of AKB at pH 7.0. Using the methods described above, we determined the half-life of AKB at pH 7.0 and were surprised to find it to be ~10 min. This value is at least ten times greater than what had previously been reported (*i.e.* < 1 min) by Laver *et al.* (5). Careful review of the experimental conditions used by these investigators suggests their determination is likely too low. In contrast to our procedures, Laver *et al.* used palladized charcoal to catalytically convert the AKB benzyl ester into free AKB in solution. They attempted to determine the rates of hydrogenation of the ester and decarboxylation of the free acid by measuring the changes in gas volume as hydrogen was absorbed and carbon dioxide was evolved. A problem with this method is that palladium might not only catalyze hydrogenation of the ester but also AKB decarboxylation. Indeed, certain polyvalent metal ions are known to catalyze the decarboxylation of β-keto acids (20, 21). Additionally, the amount of carbon dioxide recovered from the dehydrogenation and subsequent decarboxylation of their starting material only approached 60% of the theoretical value; a large correction factor was therefore needed to account for CO_2 presumably still dissolved in the reaction mixture. Furthermore, the catalytic hydrogenation reaction rate was slow ($k = 0.14\ min^{-1}$) in comparison with that determined for decarboxylation ($k > 0.69\ min^{-1}$) which also makes the decarboxylation measurements subject to error.

Decarboxylation of AKB as a Function of pH. In light of this somewhat surprising result, we examined the stability of AKB in solution over the pH range of 6 to 11. The results obtained show the half-life of AKB varies from a low of 8.6 min at pH 5.9 to a high of 140 min at pH 11.1 (see Table I). Such data are also not in agreement with the earlier report (5) which indicated that the half-life of AKB decreased at higher pH values.

The pH dependence of the AKB decarboxylation rate constant indicates that the state of protonation of the amino group plays an important role in determining the decomposition rate of this α-amino-β-keto acid. A plot of the decarboxylation rate constants *versus* pH yields a theoretical titration curve which predicts a pK_a value of 8.15 for the α-amino group of AKB (see Figure 1). The α-amino group of threonine, in contrast, has a $pK_a = 9.1$. The reason for this difference in pK_a values is due to the electron withdrawing potential of the hydroxyl versus the keto group located on the β-carbon. The more electronegative keto group of AKB inductively competes for the electrons on the α-carbon atom. This, in turn, affects the adjacent amino group making it more acidic.

Table I. Effect of pH on the Half-life of 2-Amino-3-ketobutyrate

pH	Half-life	Error margin %
5.9	8.57	3.4
7.0	9.85	2.6
8.1	14.15	2.4
8.8	36.64	0.4
9.4	78.13	1.4
9.9	111.1	2.1
11.1	140.5	5.6

Half-life values were determined as described under Materials and Methods. Error margin is based on non-linear least squares analysis from a single half-life determination.

A general mechanism for decarboxylation of β-keto acids, proposed by Westheimer and Jones in 1941 (22), helps to explain the effect of pH on this process; Scheme II shows this mechanism as applied to AKB. On protonation of the carboxylic moiety of AKB (structure I), an intermediate resembling a 6-membered ring (II) is formed which favors transfer of the proton from the carboxyl group to the β-keto oxygen atom. This makes the keto group positively charged (III) which, in turn, facilitates the electronic shift shown. The end result is decarboxylation of the β-keto acid forming 1-aminopropene-2-ol (IV), the enol form of aminoacetone (V). From an alternate plot of the data (using the pKa value of 8.15) shown in Figure 1, $k_{decarboxylation}$ values of 0.078 min^{-1} ($T_{1/2} = 8.83 \pm 0.37$ min) and 0.0050 min^{-1} ($T_{1/2} = 139 \pm 6$ min), respectively, are obtained for the protonated (*i.e.* NH_3^+ form) and

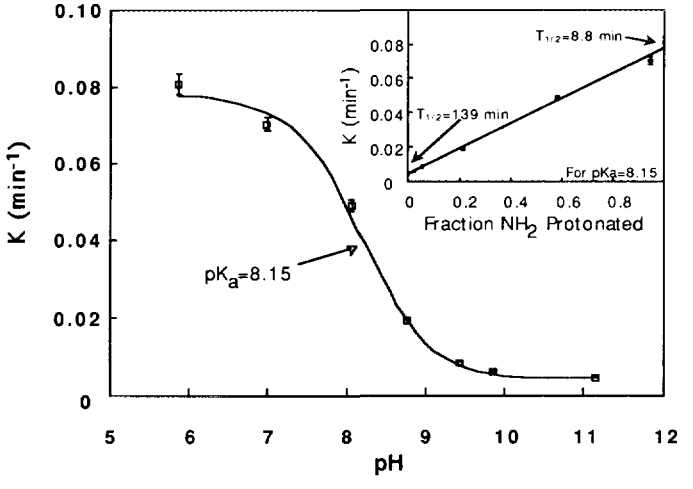
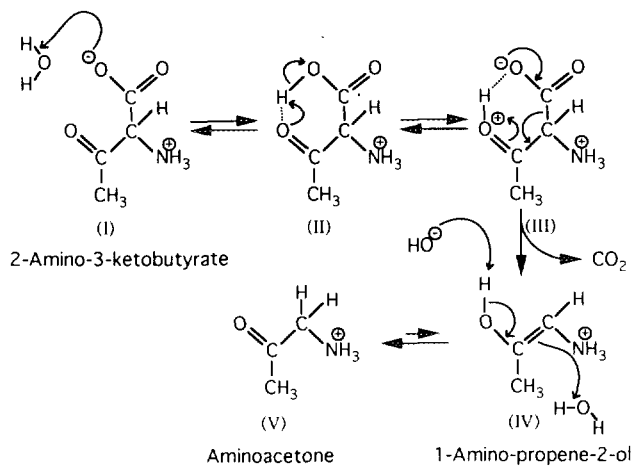


Figure 1. pH Dependence of the Decarboxylation Rate Constant for 2-Amino-3-ketobutyrate. The half-life (hence the rate constant) of AKB was measured at various pH values. The rate constant thus obtained was plotted *vs.* pH and the data fit to a theoretical titration curve. The fraction of the α-amino group that is protonated is related to the rate constant as shown in the inset. Errors bars represent non-linear least squares error estimations for single half-life determinations where the data were fit to the first order decay equation given in MATERIALS AND METHODS.



Scheme II

unprotonated amino group of AKB (see inset, Figure 1). As is evident in Scheme II, since protonation of this group withdraws electrons from the α-carbon, the electronic shift from the carboxyl group is enhanced (*i.e.* increased decarboxylation); the opposite is true when the amino group is unprotonated. Therefore, the greater the degree of protonation of the α-amino group, the less stable AKB is.

Biological Implications of These Results. Since AKB is the intermediate in the TDH and AKB CoA lyase-catalyzed reactions, the necessity of invoking a TDH/lyase enzyme complex is to a large degree dependent upon the stability of AKB. If this intermediate is very unstable, it would decarboxylate as it is released from TDH and not be available as a substrate for the lyase. It has been postulated that TDH and AKB CoA lyase exist as an enzyme complex in order to carry out substrate channeling of the unstable AKB intermediate (1, 16, 23-25). A half-life of 10 min at pH 7.0, however, would appear to allow ample time for AKB, once it is released from TDH, to diffuse to the lyase and be converted to glycine plus acetyl CoA. While a half-life of a metabolic intermediate that is relatively long with respect to enzyme turnover and diffusion rates inside the cell does not eliminate the possibility of an enzyme complex being involved, it does lessen the apparent necessity for such a complex to exist in order to allow for efficient catalytic coupling.

Substrate channeling is a process whereby the product of one enzyme reaction is passed directly as a substrate to the next enzyme in a given pathway. As such, the product/substrate does not equilibrate with the surrounding medium inside the cell. If in this instance AKB were being channeled directly from TDH to AKB CoA lyase, virtually no aminoacetone should be formed. Since AKB has a decarboxylation rate constant that is orders of magnitude smaller than enzyme turnover rates (< 0.1 min^{-1} vs. >1000 min^{-1}), it most likely would be converted into glycine plus acetyl CoA before it had a chance to undergo decarboxylation. Hence, although the possibility of TDH and AKB CoA lyase existing in the cell as a complex cannot be ruled out, the fact that aminoacetone is formed in both intact and disrupted biological systems (1, 6, 8, 9, 16,

23) warns against invoking substrate channeling simply to explain the efficiency of the coupled reaction in the direction of glycine formation.

It has been reported that with TDH purified from pork liver mitochondria, AKB is not released as the product; rather, CO_2 and aminoacetone are (23). If such is the case with the enzyme from this source, substrate channeling of AKB would seem to be involved. We, however, have found the opposite to be true with TDH from *E. coli*; a large pool of AKB could never have been generated if CO_2 and aminoacetone were the only products of the *E. coli* dehydrogenase-catalyzed reaction. All indications are that virtually no decarboxylation of AKB (or if any, a very trivial amount) occurs while it occupies the active site of the *E. coli* enzyme.

In summary, substrate channeling of AKB in the TDH/lyase-catalyzed coupled reactions need not be invoked to explain how AKB is formed as an intermediate and subsequently converted to glycine plus acetyl CoA (rather than undergoing decarboxylation). This net conversion can be explained as well by the relatively stable character of AKB in solution and its efficient utilization as a substrate in the lyase-catalyzed reaction. In other studies, we have found the lyase uses AKB with a V_{max} that is at least 20-50 times greater ($k_{cat} \approx 5000$ min^{-1}) than that for glycine utilization ($k_{cat} \approx 100$ min^{-1}) (26). Knowing, therefore, that the decarboxylation rate of AKB is relatively slow compared to enzyme turnover rates, that AKB is released as the true product of the TDH-catalyzed reaction in some biological systems, that it is utilized very efficiently as a substrate by AKB CoA lyase, and that aminoacetone is often a side product of this coupled reaction sequence in intact systems cautions against invoking direct substrate channeling between these two enzymes.

Relevance to 5-Aminolevulinate Synthase. Laver and coworkers also reported the half-life of 2-amino-3-ketoadipate, the product of the synthase-catalyzed reaction, to be "virtually instantaneous" ($T_{1/2} \approx 0$ at pH 7.4 whereas they reported a value of 0.85 min at pH 5.0) (5). As a β-keto acid, AKA also spontaneously decarboxylates yielding 5-aminolevulinate. Since its structure is very similar to AKB, having an α-amino group adjacent to a β-carbonyl, we would expect AKA to have a half-life and a pH-dependent stability profile comparable to that of AKB. An attempt to isolate AKA and to establish its properties would be enhanced by the findings reported here.

Knowing, therefore, the values for the half-life of AKB will not only facilitate further study of the TDH/AKB CoA lyase coupled enzyme system but also of analogous enzymes, such as 5-aminolevulinate synthase, where the decarboxylation of an α-amino-β-keto acid is involved.

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