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THE DECARBOXYLATION OF α-KETO-γ-METHIOLBUTYRATE IN RAT LIVER MITOCHONDRIA

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SUMMARY: The decarboxylation of α-keto-γ-methiolbutyrate, the α-keto acid of methionine has been studied in rat liver. Most (70%) of the decarboxylation activity is in the mitochondrial fraction. The Km for the decarboxylation of α-keto-γ-methiolbutyrate in intact mitochondria is 0.6 mM. Pyruvate, α-keto-butyrate and α-ketoisocaproate significantly inhibit this activity. The inhibition due to α-ketoisocaproate is 3.3 fold that of pyruvate and 1.1 fold that of α-ketobutyrate at a concentration of 0.4 mM.

INTRODUCTION

L-methionine is catabolized via activation to S-adenosylmethionine with subsequent conversion of its carbon to homocysteine and cystathionine, and its sulfur to cysteine through the transsulfuration pathway (1,2). Recently, the metabolism of methionine has been reinvestigated. In vitro experiments with rat liver homogenates and slices have raised the question of whether methionine may also be oxidized via pathways other than the transsulfuration pathway (3-5). Attempts to discern another pathway for methionine metabolism led to investigating transamination as a viable route of oxidation (4,5). The ability of methionine to act as a substrate for transamination with formation of its α-keto acid, α-keto-γ-methiolbutyrate, has now been shown for a number of aminotransferases (6-9). Most recently the decarboxylation product of α-keto-γ-methiolbutyrate, 3-methylthiopropionate, has been isolated as an intermediate in the metabolism of methionine in rat and monkey liver homogenates (10).

Abbreviations: KMBA - α-keto-γ-methiolbutyrate

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In the present study experiments are described which investigate α-keto-γ-methiolbutyrate (KMBA) metabolism in a mitochondrial preparation. The metabolism of the α-keto acid of methionine is discussed in relation to the metabolism of pyruvate, α-ketobutyrate and the branched-chain α-keto acids.

MATERIALS AND METHODS

Male Holtzman rats (100-250g) were decapitated and their livers removed, chilled and 20% homogenates prepared by homogenizing 4 grams of liver with 16 ml of a 0.025 M mannitol-0.075 M sucrose-0.05 M EDTA medium with a Potter-Elvehjem glass tube and motor-driven teflon pestle. Mitochondria were isolated from the homogenate by the method of Sedgwick and Hubscher (11).

The decarboxylation of [1-^{14}C] KMBA was measured by collecting $^{14}CO_2$ from incubations of rat liver mitochondria because preliminary experiments with cell fractions revealed that the mitochondrial fraction contained approximately 70% of the homogenate KMBA decarboxylation activity. [1-^{14}C] α-keto-γ-methiolbutyrate was prepared by incubating [1-^{14}C] L-methionine (Amersham/Searle) with 1 unit of L-amino acid oxidase (Worthington) and 8000 units of catalase (Sigma) in 1 ml 0.2 M Tris buffer, pH 7.2, for two hours at 37°C. The keto acid was isolated on a Dowex 1 X8-400 column (12 X 0.8 cm), acetate form, and eluted with 0.0-1.0 N HCl gradient as described previously (4). The peak corresponding to KMBA was monitored with a Packard model 3043 Flow Cell Adaptor, collected, lyophilized until about a ml remained, neutralized with KOH to approximately pH 5.5 and stored under nitrogen at -70°C. Purity was determined by spotting aliquots onto TLC silica gel plates (#13179 Eastman Kodak) and developing the chromatogram with 95% ethanol at 4°C in the dark. KMBA accounted for 85-95% of the radioactivity with the majority of the remaining counts cochromatographing with α-keto-γ-methiolbutyrate sulfoxide. All nonradioactive α-keto acids were obtained from Sigma Chem. Co., St. Louis, Mo.

Incubations were done in 6 X 1.2 cm test tubes with side arms connected to scintillation vials containing 1 ml ethanolamine: methylcellosolve (1:2) to trap the evolved $^{14}CO_2$. The assay was started by adding mitochondria. The tubes were then sealed with serum caps and incubated at 37°C in a shaking water bath. The reaction was stopped by injecting 100 ul of a 30% trichloroacetic acid solution through the serum cap. The tubes were kept in the shaking bath for an additional 1.5 - 2 hours to insure complete trapping of the $^{14}CO_2$ released. From 95-100% of a $Na^{14}CO_3$ standard was recovered with this procedure. Boiled tissue extracts incubated with the assay mixture served as blanks. The incubation mixture contained, except where noted, the following in a final volume of 400 ul: 62.5 mM KPO_4 buffer (pH 7.9), 0.20 mM NAD, 3.75 mM $MgCl_2$, 1.67 mM EDTA and [1-^{14}C] KMBA substrate at the indicated concentration.

Trapped $^{14}CO_2$ was counted by liquid scintillation spectrometry using Tritosol (12) or the scintillation fluid described by Jeffay and Alvarez (13). The counting efficiency of each sample was determined using the automatic external standardization method. Protein was assayed by the method of Lowry *et al.*, using crystalline bovine serum albumin as the standard (14).

RESULTS

The release of $^{14}CO_2$ from [1-^{14}C] KMBA by mitochondria was linear with time of incubation up to 40 min (Fig 1). By 40 min 12.6% of the substrate

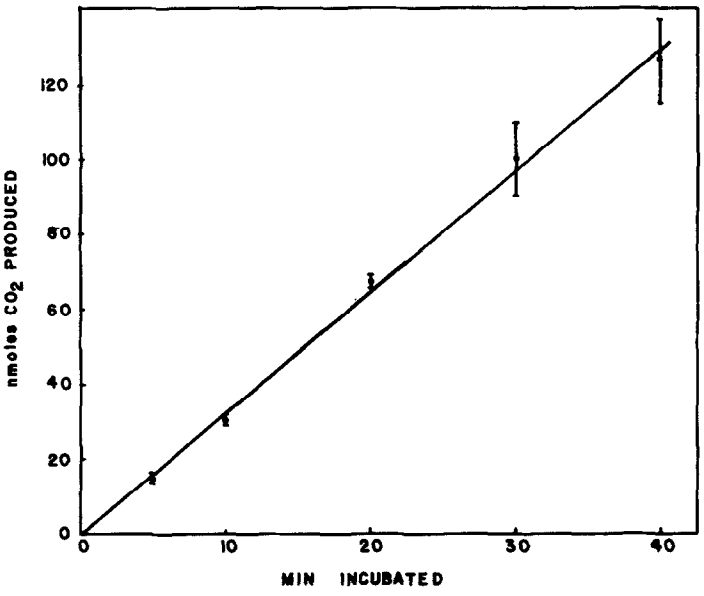

Fig 1 Production of $^{14}CO_2$ from [1-^{14}C] KMBA (2.5 mM) incubated with mitochondria: time linearity. Mitochondria (0.89 mg protein) were incubated as described in Methods except that 0.5 mM CoA was present. The points represent the mean of three incubations ± SD. The linear regression line is $y = 0.0032\ \chi - 2.25 \times 10^{-5}$; $r = 0.99$. The decarboxylation activity was 3.43 nmoles/mg protein/min.

was decarboxylated. Production of $^{14}CO_2$ was also linear with the amount of mitochondrial protein added to the incubation. The reaction mixture contained NAD and in some instances coenzyme A. The rationale for their inclusion is based on a similar assay described for the branched-chain α-keto acid dehydrogenase (15).

Figure 2 depicts the Lineweaver-Burk plot of $^{14}CO_2$ production versus KMBA concentration. The Km for the mitochondrial decarboxylation of KMBA was calculated to be 0.6 mM under the conditions of the assay and thus a KMBA concentration of 2.5 mM was used in subsequent experiments. Experiments were performed to determine whether other α-keto acids could affect $^{14}CO_2$ production from KMBA incubated with mitochondria (Fig 3). At moderate concentrations the four α-keto acids inhibited CO_2 production by mitochondria incubated with 2.5 mM KMBA. Pyruvate and phenylpyruvate inhibited less strongly than did α-ketobutyrate and α-ketoisocaproate. Although the inhibition of KMBA

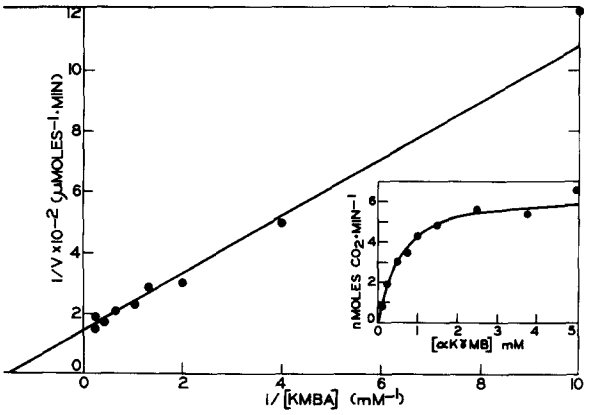

Fig 2 Lineweaver-Burk plot of the production of $^{14}CO_2$ to the KMBA concentration shown in inset. Mitochondria (1.95 mg protein) were incubated as described in Methods except that 0.37 mM CoA was present. The values are the average of duplicate observations after 10 min of incubation. The decarboxylation activity at a KMBA concentration of 5 mM was 3.83 nmoles/mg protein/min.

decarboxylation is gradually increased as pyruvate concentration is increased, it is interesting that the addition of 2.5 mM pyruvate does not substantially further depress CO_2 production from KMBA over a 1 mM pyruvate addition. At a concentration of 0.4 mM, α-ketobutyrate and α-ketoisocaproate strongly inhibited KMBA decarboxylation to 30% and 24% of control activity, respectively. Increasing the concentration of these two α-keto acids above 0.4 mM did not result in a further significant reduction in KMBA decarboxylation. The more severe inhibition of KMBA decarboxylation observed with α-ketobutyrate and α-ketoisocaproate may indicate that they are either more potent competitive inhibitors than pyruvate or that pyruvate directly or through its metabolism inhibits by a different mechanism.

Fig 4 illustrates the effect of pyruvate concentration at different levels of KMBA. The pattern does not intersect precisely on the y-axis. However, it does suggest that at high pyruvate concentrations (5 mM) the pyruvate inhibition is via a competitive mechanism. As the pyruvate inhibition is competitive, the failure of pyruvate to significantly further suppress KMBA decarboxylation at concentrations over 5 mM indicates that the pyruvate

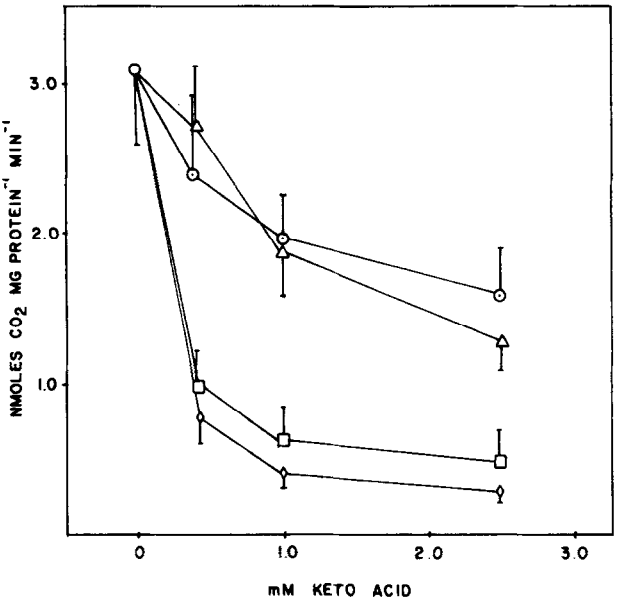

Fig 3 Effect of α-keto acids on $^{14}CO_2$ production from 2.5 mM [1-^{14}C] KMBA incubated with mitochondria (1.3-1.7 mg protein). Mitochondria were incubated as in Methods for 4 min with pyruvate (●), phenylpyruvate (Δ), α-ketobutyrate (□), or α-ketoisocaproate (◊) added at 0.4, 1.0, or 2.5 mM concentrations. The values represent the mean ± SEM of experiments from 3 rats. The control activity of these 3 preparations was 3.10 ± 0.54 nmoles/mg protein/min.

inhibition of KMBA decarboxylation is approaching saturation at 5 mM pyruvate and that the residual KMBA decarboxylation activity is due to another enzymatic activity.

The nature of the KMBA decarboxylation activity which cannot be suppressed with pyruvate is investigated in Fig 5 where the effect of pyruvate and α-ketoisocaproate separately and together are depicted. Pyruvate at 5 mM depressed $^{14}CO_2$ production to 53% of control activity while 10 mM pyruvate only depressed KMBA decarboxylation to 47% of control. At 1 mM, α-ketoisocaproate depressed $^{14}CO_2$ production from KMBA to 22% of control activity while 2.5 mM α-ketoisocaproate depressed decarboxylation to 19% of control. When 2.5 mM pyruvate and 2.5 mM α-ketoisocaproate were added simultaneously, inhibition of KMBA decarboxylation was almost 100%. These data suggest that two processes are involved in the decarboxylation of KMBA by mitochondria--one re-

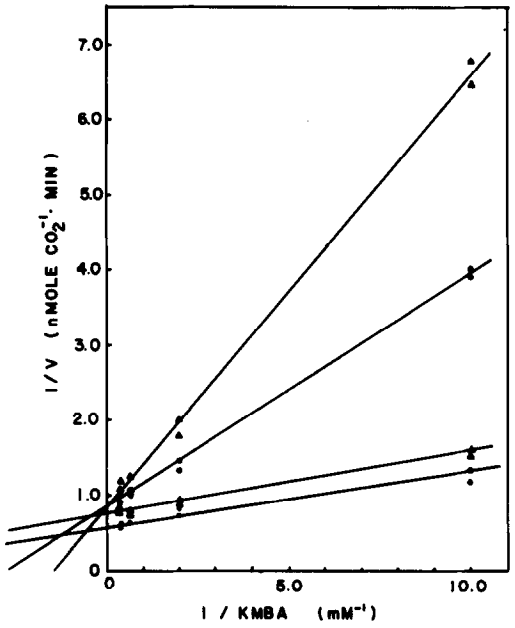

Fig 4 Lineweaver-Burk representation of the inhibition of $^{14}CO_2$ production from [1-^{14}C] KMBA by pyruvate. Mitochondria (0.67 mg protein) were incubated for 10 min as in Methods except that 0.5 mM CoA was present. The pyruvate additions were 0 mM (•), 1 mM (Δ), 5 mM (o), or 10 mM (▲). The decarboxylation activity at 2.5 mM KMBA without added pyruvate was 2.37 nmoles/mg protein/min.

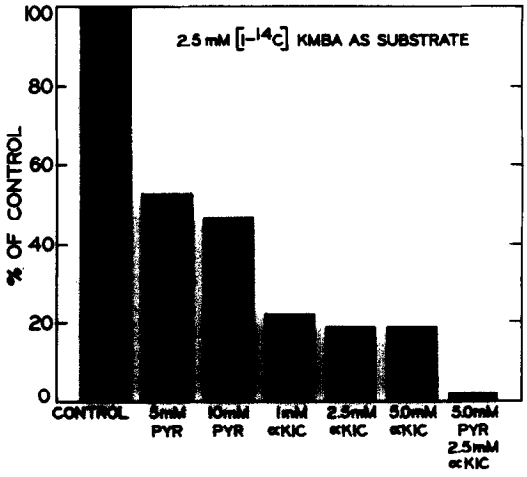

Fig 5 The effect of addition of pyruvate or α-ketoisocaproate or both simultaneously on the mitochondrial decarboxylation of KMBA. Mitochondria (0.3 mg protein) were incubated for 10 min as in Methods. The control activity was 5.5 nmoles/mg protein/min.

sponding to pyruvate addition while the other responds to α-ketoisocaproate addition, as KMBA decarboxylation could only be completely suppressed when both α-keto acids were added.

DISCUSSION

An assay for the decarboxylation of [1-^{14}C] KMBA to $^{14}CO_2$ is described. The Km for the mitochondrial decarboxylation of KMBA was 0.6 mM. Pyruvate, phenylpyruvate, α-ketobutyrate and α-ketoisocaproate inhibited $^{14}CO_2$ production from [1-^{14}C] KMBA. The data indicates that α-ketoisocaproate is capable of inhibiting the residual KMBA decarboxylation activity that is observed after pyruvate inhibition is saturated at 5 mM. Therefore, it appears that KMBA decarboxylation in intact mitochondria may be carried out by two separate systems. The inhibition experiments described here may be interpreted in different ways. Firstly, it might be that the two saturable processes depicted in Fig 5 are due to separate transport systems located in the mitochondrial inner membrane. Secondly, the data may be interpreted as representing more than one independently saturable dehydrogenase activity. If the latter interpretation is correct then it appears that KMBA may be decarboxylated by two dehydrogenase systems. Preliminary experiments with mitochondria incubated under conditions that produce membrane swelling, thus eliminating inner mitochondrial transport effects, indicate that 2 enzymatic activities are responsible for the differential pattern observed in Fig 5.

The decarboxylation of the α-keto acid of methionine by intact rat liver mitochondria which can be inhibited by both α-ketoisocaproate and pyruvate is a new observation. Its physiological significance is not fully understood but the metabolism of methionine via a transaminative route which involves oxidative decarboxylation of KMBA may be important in metabolic conditions in which the metabolism of methionine is altered. For example, in the disease hypermethioninemia and certain types of homocystinuria (16,17), high levels of methionine are seen and, methionine and homocysteine via methionine may be catabolized via this pathway.

The reaction mechanism for the decarboxylation of KMBA and the location of the enzyme within the mitochondrion has not yet been investigated. Studying cofactor requirements may help in determining whether the mechanism is similar to that of the branched-chain α-keto acid and pyruvate dehydrogenases (15,18,19). If the enzyme is located on the matrix side of the inner mitochondrial membrane, addition of nucleotide cofactors should not stimulate the decarboxylation of KMBA as their concentrations are fairly well maintained in intact mitochondria and added coenzyme A or NAD cannot cross the inner mitochondrial membrane.

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