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Determination of α-Ketoisocaproate Dioxygenase Activity in Mammalian Livers

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ABSTRACT

The α-ketoisocaproate dioxygenase (KICD) catalyzes the decarboxylation of α-ketoisocaproate (KIC), derived from transamination of leucine, to β-hydroxy-β-methylbutyrate (HMB) in the cytosol of the liver. The characterization of the purified KICD from rat and human livers indicated that this enzyme requires Fe^{2+} for its activity, but does not use CoA-SH and NAD^+ as cofactors that are required for the branched-chain α-keto acid dehydrogenase complex (BCKDC), which catalyzes the decarboxylation of KIC to isovalery-CoA in mitochondria. In the present study, a radiochemical assay was developed to measure the KICD activity in liver extracts. In this method, the decarboxylation of KIC by BCKDC was eliminated by addition of BCKDC-antiserum in the assay mixture. Evidence is provided here for establishment of improved assay conditions for KICD in liver extracts.

INTRODUCTION

It has been reported that the catabolic pathways of α-ketoisocaproate (KIC) were mediated by branched-chain α-keto acid dehydrogenase (BCKDC) and α-ketoisocaproate dioxygenase (KICD) in mitochondria and cytosol of livers, respectively (1-4). Relatively low activity of KICD in liver determines that a radiochemical assay, detecting the release of $^{14}CO_2$ from [1-^{14}C]KIC, should be used for measurement of KICD activity. However, the existence of BCKDC activity in tissue extracts would cause an overestimation of the KICD activity in the radiochemical assay, since BCKDC also decarboxylates KIC, producing CO_2. We hypothesized that the addition of the inhibitory antibody against BCKDC in the assay mixture could contribute to eliminate the BCKDC activity from the liver extracts.

It has been reported that relatively long reaction time is necessary for the determination of KICD activity in liver extracts, since the specific activity of KICD was fairly low (1). This finding raised us a question concerning whether the enzyme could be kept under optimal conditions during a long reaction time or not. We report here the development of an improved radiochemical assay method for the determination of KICD activity in liver extracts and further provide additional evidence for understanding the metabolic significance of KICD under various physiological conditions.

MATERIALS AND METHODS

[1-^{14}C]KIC was purchased from Amersham Japan (Tokyo). Antiserum against BCKDC was prepared as reported previously (5). All other reagents were of biochemical grade.

Male Sprague-Dawley rats, weighing from 350 to 400 g, were sacrificed by cervical dislocation, and livers were rapidly removed, freeze-clamped at liquid nitrogen temperature, and stored at -80°C until analysis.

Preparation of Tissue Extracts. Approximately 0.2 g of frozen liver was placed in 1.5 ml of ice-cold homogenizing buffer [0.25 M sucrose, 2.5 mM HEPES, 0.25 mM EDTA, 2% (v/v) bovine serum, 1 μM leupeptin, and 4 mg/l trypsin inhibitor, pH 7.5 at 25°C] and homogenized with a motor-driven Teflon pestle at maximum speed for 1 min, and then the homogenate was centrifuged at 10,000 × g for 30 min at 4°C. The supernatant obtained was used for the assay of KICD activity.

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KICD assay. The activity of KICD was measured radiochemically using [1-^{14}C]KIC as the substrate at 25°C. One unit of KICD catalyzed the production of 1 μmol of CO_2 /min. For the decarboxylation assay, a 2-ml Eppendorf tube containing 250 μl of 1.6 × assay buffer A (0.32 M Tris-maleate, pH 6.0), 25 μl of assay buffer B (32 mM $FeSO_4$, 8 mM ascorbic acid, 16 mM dithiothreitol, 1 μM leupeptin, and 4 mg/l trypsin inhibitor), and 100 μl of tissue extract was placed in a 20-ml glass (transparent) scintillation vial that contained 0.35 ml of 1.2 M KOH. After preincubation for 60 min at 25°C, the reaction was started by the addition of 25 μl of 16 mM [1-^{14}C]KIC pre-warmed at 25°C, and then the vial was immediately sealed with a rubber serum cap. After 60 min of incubation at 25°C with gentle shaking, 0.8 ml of a stop solution (20% trichloroacetic acid) was injected through the serum cap with a disposable syringe. A blank was prepared by adding [1-^{14}C]KIC and stop solution to the tube before the incubation for reaction.

RESULTS AND DISCUSSION

Since both KICD and BCKDC catalyze the decarboxylation of KIC and their activities are measured by quantifying the release of $^{14}CO_2$ from [1-^{14}C]KIC (1, 6), it was supposed that the generally used radiochemical assay for determination of the KICD activity would be subject to overestimation, owing to the interference of BCKDC activity. Although BCKDC is present in the mitochondrial fraction, the unavoidable damage to mitochondria during homogenization of tissues results in releasing of BCKDC into tissue extracts, especially preparing the extracts from frozen livers. This hypothesis was confirmed in the present study, in which a considerable activity of BCKDC was observed in a tissue extract prepared from freeze-clamped liver (Table 1). On the other hand, we found that the BCKDC activity was abolished by addition of 25 μl/ml BCKDC-antiserum in the assay mixture (Table 1). These results indicate that the addition of BCKDC-antiserum in the assay mixture is crucial for the accurate measurement of KICD activity in liver extracts.

As reported previously (1), KICD requires Fe^{2+} for its activity, but not CoA or NAD^+ as cofactors that are required for BCKDC. In the present study, we confirmed that the activity of KICD was completely inhibited by addition of the Fe^{2+} chelating reagent o-phenanthroline in the assay mixture, as shown in Table 2. This characteristic of the enzyme is contrast to that of the BCKDC, which needs Mg^{2+} for its activity. Moreover, we found that a color of the assay mixture turned reddish-brown when the pH was above 7.0, presumably due to oxidation of $FeSO_4$, resulting in a dramatic decrease in the activity of KICD as reported in (1). This is compatible with the optimal pH at 6.0 for the assay mixture of KICD (3).

Ammonium sulfate was used for the purification of KICD

Table 1: Effects of EDTA and BCKDC-antiserum on the activity of BCKDC in rat liver

Addition in the assay mixture	BCKDC activity	
	mU/g tissue	% of control
Control (optimal assay condition for BCKDC)	16	100%
+ 10 mM EDTA	3	20%
+ BCKDC-antiserum (25 μl/ml)	0	0

Note. Assay for the determination of BCKDC (5) was carried out in a 2-ml Eppendorf tube, containin the mixture of 200 μl of 2 × assay buffer, 100 μl of tissue extract and 100 μl of 4 mM [1-^{14}C]KIC, which was placed in a 20-ml glass (transparent) scintillation vial containing 0.35 ml of 1.2 M KOH, as described in MATERIALS AND METHODS. The reaction mixture was incubated for 20 min at 25°C.

Table 2: Effect of o-phenanthroline on the activity of KICD in rat liver

Addition/omission in the assay mixture	KICD activity	
	mU/g tissue	% of control
Control (optimal assay condition for KICD)	7.2	100%
+ 1 mM o-phenanthroline, -$FeSO_4$	0	0

Note. Assay for the determination of KICD activity was carried out in a 2-ml Eppendorf tube, containing the mixture of 250 μl of 1.6 × assay buffer A, 100 μl of tissue extract, 25 μl of assay buffer B and 25 μl of 16 mM [1-^{14}C]KIC, which was placed in a 20-ml glass (transparent) scintillation vial containing 0.35 ml of 1.2 M KOH. O-phenanthroline was added in the assay buffer at the final concentration of 1 mM and $FeSO_4$ was omitted from the assay buffer. The reaction mixture was incubated for 60 min at 25°C.

Table 3: Effect of ammonium sulfate on the activity of KICD in rat liver

Addition in the assay mixture	KICD activity	
	mU/g tissue	% of control
Control (optimal assay condition for KICD)	12	100%
+ 1.5 M ammonium sulfate	4	31%

Note. Assay for the determination of KICD activity was carried out in a 2-ml Eppendorf tube, containing the mixture of 250 μl of 1.6 × assay buffer A, 100 μl of tissue extract, 25 μl of assay buffer B and 25 μl of 16 mM [1-^{14}C]KIC, which was placed in a 20-ml glass (transparent) scintillation vial containing 0.35 ml of 1.2 M KOH. Ammonium sulfate was added to the assay buffer at the final concentration of 1.5 M. The reaction mixture was incubated for 60 min at 25°C.

(1, 7). The addition of 1.5 M ammonium sulfate to the assay mixture was assumed to be useful in stimulating the activity of KICD, as reported previously (7). However, we found that the addition of 1.5 M ammonium sulfate in assay mixture caused an obvious decrease in the activity of KICD to 31% of control (Table 3). It is worthy to note that Sabourin *et al.* (7) used partially purified enzyme for the assay of KICD activity. It was different from the present study, in which the tissue extracts were directly used for the assay of KICD activity.

Our previous study (3) suggested that a relatively long reaction time (1 hr for rat liver/2 hr for human liver) was necessary for the determination of KICD activity in tissue extracts. In order to keep the enzyme under the good conditions, we added protease inhibitors in homogenizing buffer and assay buffer B, as described in MATERIALS AND METHODS, because we found that the activity of KICD was somewhat decreased without protease inhibitors in the buffers (Table 4).

Finally, the optimal assay method for the determination of KICD activity in liver extracts was established and this assay was found to be linear with respect to both time (data not shown) and protein amount used in the assay (Fig. 1).

It is not clear whether KIC is a physiological substrate

Table 4: Effect of potease inhibitors on the activity of KICD in rat liver

Condition	KICD activity	
	mU/g tissue	% of control
Control (optimal assay condition for KICD)	14.5	100%
Homogenizing buffer without protease inhibitors	12.9	89%
Assay buffer B without protease inhibitors	14.0	97%
Homogenizing buffer and assay buffer B without protease inhibitors	12.0	83%

Note. Assay for the determination of KICD activity was carried out in a 2-ml Eppendorf tube, containing the mixture of 250 µl of 1.6 × assay buffer A, 100 µl of tissue extract, 25 µl of assay buffer B and 25 µl of 16 mM [1-^{14}C]KIC, which was placed in a 20-ml glass (transparent) scintillation vial containing 0.35 ml of 1.2 M KOH. Protease inhibitors were omitted from the buffers. The reaction mixture was incubated for 60 min at 25°C.

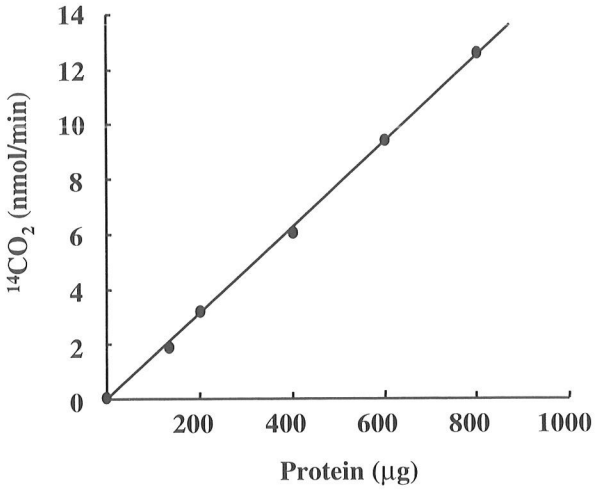

Figure 1: Proportionality of the decarboxylation of KIC to the amount of liver homogenate. The KICD activity was assayed under the optimal conditions as described in MATERIALS AND METHODS.

for KICD, because its K_m is very high (320 µM) compared to that for BCKDC (14.6 µM). It has been reported recently that the activities of KICD and 4-hydroxyphenylpyruvate (HPP) dioxygenase (HPPD), an important enzyme in the degradation of tyrosine (8), are due to a single enzyme (9). The K_m of HPP for HPPD is around 27 µM and the optimum pH for the HPPD activity is around 7.5 (10). Therefore, it is possible that the same enzyme may play the dual physiological functions, tyrosine and KIC degradation, under the different physiological conditions of liver. It should be noted that HMB is formed in vivo in man and rat (7, 11, 12) and that ~5% of leucine oxidation is reported to proceed via the catabolic pathway involving KICD under the normal conditions (13). Further studies are required to elucidate physiological functions of the KICD.

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