Branched-chain 2-oxo acid dehydrogenase interferes with the measurement of the activity and activity state of hepatic pyruvate dehydrogenase

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Oxidative decarboxylation of pyruvate by branched-chain 2-oxo acid dehydrogenase can result in overestimation of the expressed and total activity of hepatic pyruvate dehydrogenase. Pyruvate is a poor substrate for branched-chain 2-oxo acid dehydrogenase relative to the branched-chain oxo acids; however, the comparable total activities of the two complexes in liver, the much greater activity state of branched-chain 2-oxo acid dehydrogenase compared with pyruvate dehydrogenase in most physiological states, and the use of high pyruvate concentrations, explain the interference that can occur in conventional radiochemical or indicator-enzyme linked assays of pyruvate dehydrogenase. Goat antibody that specifically inhibited branched-chain 2-oxo acid dehydrogenase was used in this study to provide a more specific assay for pyruvate dehydrogenase.

INTRODUCTION

Pyruvate has been well established [1,2] as a substrate for branched-chain 2-oxo acid dehydrogenase (BCODH; EC 1.2.4.4 + no EC number for dihydrolipoamide acyltransferase + EC 1.8.1.4). Kinetic parameters (apparent $K_m = 0.8$ mM, $V_{max} = 20\%$ of that for 3-methyl-2-oxobutanoate) indicate that physiological significance would depend on a high intramitochondrial pyruvate concentration. Investigators have used high pyruvate concentrations (up to 40 mM) to assay pyruvate dehydrogenase (PDH; EC 1.2.4.1 + EC 2.3.1.12 + EC 1.8.1.4), although the apparent K_m is very low (about 40 μ M) [3]. One would therefore expect a significant contribution by BCODH at high pyruvate concentrations to the overall rate of pyruvate oxidation (either flux or activity determinations) in those tissues where this enzyme activity is high relative to PDH.

The expressed activity of BCODH in liver is much larger than that of PDH as a consequence of two factors. First, total activity of the two enzymes is about the same (shown in the present study). Second, although BCODH is largely, if not entirely, in the active (dephosphorylated) state [4], PDH is largely inactive [5,6]. These factors result in significantly more expressed BCODH than PDH activity, and therefore in a significant overestimation of the latter at higher pyruvate concentrations. We hasten to point out that no direct evidence is presented that previous estimates of the activity state of PDH have been in serious error, nor that the observed changes in activity state have been qualitatively incorrect. However, there is the potential to be misled, especially when one is measuring subtle changes in activity or flux through PDH, by major changes in the activity state of BCODH.

MATERIALS AND METHODS

Sources of materials

Reagents were obtained as previously given [1,7] or from Sigma Chemical Co., St. Louis, MO, U.S.A.

2-Chloro-4-methylpentanoate was kindly given by Dr. Robert J. Strohschein and Dr. Ronald Simpson of Sandoz, East Hanover, NJ, U.S.A. Sodium 3-methyl-2-oxo[1- 14 C]butanoate and sodium [1- 14 C]pyruvate were from Amersham Corp., Arlington Heights, IL, U.S.A. Broad-specificity phosphoprotein phosphatase was isolated as previously given [8]. The purified phosphatase (16.8 mg/ml) was stored at -20°C in 50 mM-imidazole/HCl (pH 7.5)/0.5 mM-EDTA/0.5 mM-dithiothreitol/40% (v/v) glycerol. Though phosphatase instability has been reported [9], no loss of activity was detected 2 weeks after preparation.

Male Wistar rats with initial body weights of 170–185 g were purchased from Harlan Industries, Indianapolis, IN, U.S.A. Rats were given free access to water and fed *ad libitum* on chow diet (chow-fed animals; Purina Rodent Laboratory Chow 5001, minimum 23% protein), or low-protein (8%) diet described previously [10] for 48 h, or starved for 48 h. Re-fed animals were prepared exactly as described by Wieland *et al.* [6], i.e. chow-fed rats were starved for 24 h and then force-fed with 3 g of glucose 2 h before being killed. Goat antibody to rabbit liver BCODH lacking inhibitory activity against PDH was prepared as described by Paxton *et al.* [11].

Assay of hepatic BCODH and pyruvate decarboxylation activity

Rats were immobilized by cervical dislocation. Livers were removed and freeze-clamped (< 10 s) with Wollenberger clamps precooled in liquid N_2 . The tissue was pulverized to a fine powder in liquid N_2 with a mortar and pestle. The powder was suspended in ice-cold 50 mM-Hepes/KOH (pH 7.5 at 20°C) containing 3 mM-EDTA, 5 mM-dithiothreitol, 0.1 mM-2-chloro-4-methylpentanoate, 1 mM-tosyl-L-lysylchloromethane, trypsin inhibitor from egg white (0.1 mg/ml), 0.5 μ M-leupeptin, 0.5 μ M-pepstatin A, aprotinin (1 μ g/ml), 2% (v/v) rat serum and 2% (v/v) Triton X-100. The suspension was mixed, frozen by immersion in liquid N_2 , thawed, mixed

Abbreviations used: BCODH, branched-chain 2-oxo acid dehydrogenase; PDH, pyruvate dehydrogenase.

Table 1. Activity and activity state of pyruvate decarboxylation activity in rat liver homogenates before and after addition of goat anti-BCODH serum

Homogenates were assayed radiochemically as given in the Materials and methods section. Preincubations with antibody were for 30 min on ice with 23 μ g of IgG protein/mg wet wt. of tissue. Preincubations for phosphatase treatment were as given in the Materials and methods section, by using 0.1 M-KF or 25 μ g of phosphatase protein/mg wet wt. of tissue run in parallel at 30 °C for 20 min. Activity state is 100 $\times$ the rate without phosphatase treatment divided by the rate after phosphatase. Results are means $\pm$ S.E.M.; $n = 3$, except for the starved groups, where $n = 5$. * $P < 0.05$ versus corresponding re-fed group; † $P < 0.02$ versus corresponding starved group by unpaired Student's t test; ‡ $P < 0.05$ versus corresponding no-antibody group by paired Student's t test.

Nutritional state of rat	Antibody	Pyruvate decarboxylation activity (nmol/min per g wet wt.)		Activity state (% active)
		Without phosphatase	With phosphatase	
Re-fed	—	256 $\pm$ 15	1140 $\pm$ 90	23 $\pm$ 3
	+	151 $\pm$ 12†	1130 $\pm$ 110	14 $\pm$ 2‡
Starved	—	160 $\pm$ 32	1210 $\pm$ 90	12 $\pm$ 2*
	+	55 $\pm$ 5*†	1100 $\pm$ 100	5 $\pm$ 1*†
Low-protein diet	—	151 $\pm$ 26*	1060 $\pm$ 120	15 $\pm$ 4
	+	128 $\pm$ 25†	1050 $\pm$ 150	13 $\pm$ 3†

again, and homogenized on ice for a total of 45 s (three bursts of 15 s with intermittent cooling) at the slowest possible setting of a Polytron PT10 homogenizer. Samples of the extract were either assayed directly for BCODH and pyruvate decarboxylation activities, or preincubated with phosphatase to activate the complexes by dephosphorylation. Preincubation of control samples of the extracts without phosphatase was conducted in the presence of 0.1 M-KF to inhibit endogenous phosphatase activity. Samples of tissue extract (corresponding usually to about 10 mg wet wt. of tissue), preincubated with either KF or phosphatase, were assayed for BCODH and pyruvate decarboxylation activities in 30 mM-potassium phosphate (pH 7.5), containing 2 mM-MgCl₂, 0.4 mM-thiamin pyrophosphate, 0.4 mM-CoA, 1 mM-dithiothreitol, 1 mM-NAD⁺, 0.1% Triton X-100 and 1.25 units of pig heart dihydrolipoamide reductase (EC 1.8.1.4)/ml. Assay volume was 2 ml in 25 ml Erlenmeyer flasks sealed with rubber serum caps fitted with hanging centre wells. The flasks were preincubated for 10 min at 30 °C before initiation of the reaction by addition of either 1 mM-sodium 3-methyl-2-oxo[1-¹⁴C]butanoate for BCODH activity, or 5 mM-sodium [1-¹⁴C]pyruvate for pyruvate decarboxylation activity. The reaction was terminated 15 min later with the addition of 0.2 ml of β -phenethylamine/methanol (1:1, v/v) to the centre well and 1 ml of 5 M-H₂SO₄ to the reaction mixture. Incubations were continued for 1 h at 30 °C to collect CO₂ in the hanging centre well. The latter was removed and counted for radioactivity in 10 ml of xylene-based scintillation fluid (Fisher Scientific Co., Cincinnati, OH, U.S.A.) supplemented with 0.5 ml of methanol and 0.5 ml of β -phenethylamine. Linearity with respect to incubation time and amount of tissue extract used in the assay was established.

Expressed activities are taken as those after preincubation without phosphatase, and total activities are those obtained after incubation with phosphatase. The activity state (percentage of total activity) was taken as 100 $\times$ the activity without phosphatase divided by that after a preincubation with phosphatase.

Purified rabbit liver BCODH [1] and partially purified PDH (Sigma Chemical Co.) were assayed spectrophotometrically [12].

RESULTS AND DISCUSSION

Conditions were found for complete activation of BCODH and pyruvate decarboxylation activity in liver homogenates. Phosphatase (about 25 μ g of protein/mg of wet wt. tissue) was used to activate both enzymes. For pyruvate decarboxylation activity, 20 min preincubations resulted in complete activation. Substituting 5 mM free Mg²⁺ plus 0.1 mM free Ca²⁺ for the phosphatase resulted in the same degree of activation. BCODH activity in liver from low-protein-fed rats was completely activated by 30 min preincubations with the phosphatase, supplemented with 5 mM free Mg²⁺. Mg²⁺ was included as it was found to stimulate phosphatase activity. Preincubations conducted with KF but without phosphatase were associated with little change in the activity of either dehydrogenase.

BCODH activity (nmol/min per g wet wt.) was nearly the same among chow-fed, re-fed and starved animals for both the expressed activity (1210 $\pm$ 70, 13 observations) and the total activity (1330 $\pm$ 70, 13 observations), and the enzyme was almost completely active in these three nutritional states. In contrast, in low-protein-fed animals, expressed and total activities were significantly lower (190 $\pm$ 30 and 610 $\pm$ 30 nmol/min per g wet wt. respectively, for 3 observations) than the other nutritional states, as was the activity state (30 $\pm$ 7%).

Hepatic pyruvate decarboxylation activity was largely inactive in the re-fed and starved states, more so in the starved state, and the total activities were nearly the same (Table 1, no antibody). These activities are in good agreement with previous measurements of PDH with 20 mM-pyruvate [6]. Furthermore, hepatic pyruvate decarboxylation activity in the low-protein-fed rats was also largely inactive, whereas the total activity was nearly the same as the other nutritional states.

The antibody to BCODH prepared in this laboratory

Table 2. Effect of antibody to BCODH on the rate of pyruvate decarboxylation by PDH, BCODH and a mixture of these enzymes

Activity was measured spectrophotometrically as described in the Materials and methods section with 5 mM-pyruvate as substrate, 0.26 μg of PDH and 1.4 μg of BCODH. Antibody was added to a concentration of 20 μg of IgG protein/munit of activity towards the principal substrates (i.e. 1 mM-3-methyl-2-oxobutanoate, or, for PDH, 5 mM-pyruvate), based on the total amount of activity present. Enzymes were diluted in, and antibody incubations (30 min on ice before assay) were conducted in, 50 mM-Hepes/KOH/5 mM-dithiothreitol/50 μM -thiamin pyrophosphate/50 μM -EDTA/15% glycerol/0.1% Triton X-100, pH 7.5, at 20 °C. All determinations are in triplicate, expressed as means $\pm$ S.E.M.

Additions	Pyruvate decarboxylation activity (nmol/min)
PDH	2.1 $\pm$ 0.1
PDH + IgG	2.0 $\pm$ 0.1
BCODH	1.5 $\pm$ 0.1
BCODH + IgG	< 0.1
PDH + BCODH	3.5 $\pm$ 0.1
PDH + BCODH + IgG	2.2 $\pm$ 0.1

Fig. 1. Titration of BCODH activity in rat liver homogenates with antibody

Antibody incubations were for 30 min on ice before radiochemical assay of liver homogenates prepared from chow-fed (○) and low-protein-fed (●) rats, as described in the Materials and methods section. Enzyme present in homogenates from low-protein-fed rats was activated by treatment with the phosphatase as described in the Materials and methods section, before incubation with antibody.

has been demonstrated to inactivate BCODH almost completely at a concentration of 20 μg of IgG protein/munit of activity, but even higher concentrations did not inhibit bovine heart PDH activity [11]. To expand on this critical point, two experiments were conducted. First, substrate (pyruvate) saturation curves were determined

spectrophotometrically for bovine heart PDH, before and after incubation with 20 μg of IgG protein/munit of activity. The antibody had no discernible effect on activities measured through the range 20 μM –5 mM-pyruvate, indicating no effect on either K_m , or V_{max} , of the enzyme (results not shown). Second, to rule out the possibility of subunit interactions between these two similar multienzyme complexes in an experimental system containing both, as pertains in tissue homogenates, isolated enzymes were assayed spectrophotometrically, individually, or as a mixture, before and after antibody treatment, with pyruvate as the substrate for both enzymes (Table 2). Assayed individually, BCODH activity was decreased to below the level of sensitivity of the assay, whereas PDH activity was unaffected. Furthermore, although the mixture of enzymes had an activity accounted for by the individual activities present before addition of antibody, the activity remaining after antibody addition was equal to that of PDH only, since the antibody selectively inhibits BCODH. It seems likely that these findings apply in tissue homogenates.

The antibody was subsequently used to titrate BCODH activity, measured radiochemically, in liver homogenates from chow-fed and low-protein-fed rats. Complete inhibition of BCODH activity occurred with 23 μg of IgG protein/mg wet wt. of tissue (Fig. 1). Consequently, this concentration was used in the antibody treatment group of Table 1. BCODH activity was also measured after the same antibody treatment, both before and after activation by phosphatase. However, the activities were found to approach the sensitivity of the assay for BCODH, and are therefore not given in Table 2.

Pyruvate decarboxylation activity measured after antibody treatment (Table 1, plus antibody) should be a specific assay for PDH. The decrease in pyruvate decarboxylation activity with starvation, as measured by previous investigators, is not qualitatively different from the results seen in this study when assayed after antibody treatment. The expressed activities of PDH in the re-fed and starved animals, however, are considerably lower than in the absence of antibody, and this difference presumably reflects the activity of BCODH towards pyruvate. This difference (i.e. activity with or without antibody) is a substantial portion of the overall rate, because the expressed activity of BCODH in the re-fed and starved groups is much higher than that of PDH. On the other hand, the total activity of PDH in all three nutritional states is little affected by antibody, and this is understandable from the standpoint that, after dephosphorylation, both enzymes have about the same activity, so that the fractional contribution to the overall rate of pyruvate decarboxylation of BCODH is small. Such a small fractional change would be difficult to detect with the present method. No difference was measured in the total activity of hepatic PDH between the three nutritional states.

The expressed hepatic pyruvate decarboxylation activity of low-protein-fed animals was little affected by antibody, since the expressed BCODH activity in this state is lower, around that of PDH. The expressed activity of PDH in low-protein-fed animals was nearly the same as that in the re-fed animal, although it would have appeared similar to that of the starved animal in the presence of

interference by BCODH. This illustrates the point that large changes in the activity state of BCODH may be misinterpreted as small changes in that of PDH.

Hepatic PDH activity in the animal fed on low-protein high-carbohydrate diet was similar to that of the re-fed animal, possibly owing to the high carbohydrate content of the diet. To our knowledge, this is the first time this activity has been measured in the former animal.

At a pyruvate concentration of 5 mM, BCODH was observed to contribute to the overall rate of pyruvate oxidation in homogenates to the extent of 5–10% of its activity towards 3-methyl-2-oxobutanoate. Though the ability to extrapolate quantitatively from purified enzymes to tissue homogenates is admittedly limited, this value is predicted to reach a maximum of 20% at saturating concentrations of pyruvate. Quantitative extrapolation to flux determinations would be rough at best. With a K_m of 0.8 mM, the interference by BCODH may be insignificant at pyruvate concentrations much less than 1 mM.

The liver is probably the only routinely studied tissue where this interference may have been significant. The ratio of BCODH to PDH activity should be a reasonable indicator of the potential for interference. In the present study, the ratio for expressed activities is about 5, where interference is significant, whereas the ratio is about 1 after dephosphorylation, and the interference in total activity is not significant. Thus, if this ratio is much less than 1 in other tissues, interference is expected to be insignificant.

From data reported for expressed and total PDH activities [5,13,14], and expressed and total activities for BCODH [4,9,15,16], the ratio of PDH to BCODH was found to be less than 0.1 for expressed and total activities in adipose tissue, heart, brain and skeletal muscle. The ratio was higher in kidney, roughly 0.5 for expressed and total activities in this organ. Thus interference by BCODH in these tissues is expected to be insignificant, even at high pyruvate concentrations.

Intramitochondrial pyruvate concentrations have been reported as high as 1 mM [17,18], which suggests that BCODH in liver may oxidize pyruvate to a physiologically significant extent. However, whether this concentration is even achieved *in vivo* has yet to be determined, and therefore it is not clear whether the interference identified in the present study is of any physiological significance.

In summary, in the absence of a specific inhibitor of

either BCODH or PDH, the accurate determination of hepatic PDH is limited to lower pyruvate concentrations. In previous investigations using high concentrations of pyruvate, the rates of PDH were probably artifactually elevated by BCODH.

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