Preservation of the activity state of hepatic branched-chain 2-oxo acid dehydrogenase during the isolation of mitochondria

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A comparison was conducted of current methods for estimation of the activity states (proportion of enzyme in active, dephosphorylated, form) of hepatic branched-chain 2-oxo acid dehydrogenase. Practically all of the enzyme was active in freeze-clamped liver obtained from chow-fed and 48 h-starved rats, regardless of the presence of fluoride in the extraction and assay media to inhibit phosphatase activity. Likewise, the enzyme was almost completely active in mitochondria isolated by a conventional method from livers of chow-fed and starved rats. However, when fluoride and 4-methyl-2-oxopentanoate were included in the mitochondrial isolation medium, the activity state was decreased to 73% and 47% in mitochondria isolated from chow-fed and starved rats respectively. Furthermore, branched-chain 2-oxo acid dehydrogenase became partially inactivated upon incubation of isolated mitochondria on ice in fluoride- and/or 4-methyl-2-oxopentanoate-supplemented media. The rate of inactivation was greater in mitochondria prepared from starved than from chow-fed rats, which correlated with the lower activity state found in mitochondria of starved rats isolated in the fluoride- and 4-methyl-2-oxopentanoate-supplemented media. Thus the activity state of branched-chain 2-oxo acid dehydrogenase is underestimated in mitochondria isolated in media supplemented with fluoride plus 4-methyl-2-oxopentanoate.

INTRODUCTION

Branched-chain 2-oxo acid dehydrogenase complex (BCODH; EC 1.2.4.4+no EC number for dihydro-lipoamide acyltransferase+EC 1.8.1.4), an intramitochondrial enzyme, is subject to covalent modification, where phosphorylation inactivates and dephosphorylation activates the complex [1–3]. The degree of phosphorylation or activity state of the complex is obtained as the expressed activity assayed in tissue or mitochondrial extracts made with inhibitors of the kinase and phosphatase divided by the total activity obtained after activation by phosphatase [4–14]. The percentage of active form of BCODH has been determined in two general ways. The first involves rapidly freezing (i.e. freeze-clamping at liquid-N₂ temperature) the tissue to minimize alteration of the phosphorylation state and to limit proteolysis, followed by assay of the enzyme activity with and without phosphatase treatment [5–9]. The second method relies on activation of the complex *in situ* by intramitochondrial phosphatase. The latter requires isolation of intact mitochondria in two different media: one containing NaF to inhibit phosphatase, plus 4-methyl-2-oxopentanoate, 2-chloro-4-methylpentanoate or ADP to inhibit the kinase; and another medium without these agents, followed by incubation for various times, depending on the physiological conditions of the animal, to allow activation of the complex by the endogenous phosphatase [10–13]. The assumption is made that the kinase and phosphatase will be equally inhibited, since a change in the relative activity of either of these enzymes will result in a different steady state of BCODH phosphorylation/activity state. The activities obtained as units/g of mitochondrial protein have been

normalized to units/g wet wt. through the activity of citrate synthase in whole tissue and isolated mitochondria. Although both methods give, in general, similar total activities for certain tissues, the percentage active form of the enzyme is variable. With rats fed on chow diets, the complex has been reported to be nearly 100% active with freeze-clamped liver [6,7,9,12], but 25% [10], 34% [11], 55% [4], and 99% [12] active by mitochondrial isolation procedures. The discrepancies in the literature are even greater with 48 h-starved rats: about 100% active form is obtained with freeze-clamping procedure [6,7,9,12], whereas 3% [11], 10% [4] and 99% [14] active form have been reported with mitochondrial procedures. The present study determined BCODH activity state in liver of normal fed and starved rats by the different procedures and explored some of the potential mechanisms for the different results. Partial explanations are given for some of the differences in published BCODH activity states.

MATERIALS AND METHODS

Materials

3-Methyl-2-oxo[1-¹⁴C]butanoate (0.32 mCi/mmol) was produced from [1-¹⁴C]valine obtained from Research Product International Corp., Mount Prospect, IL, U.S.A. [15]. All other biochemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A. The broad-specificity phosphoprotein phosphatase was prepared as described previously [5]. Rat liver BCODH was purified by a modification of the basic procedure [1] described in detail elsewhere [16].

Abbreviation used: BCODH, branched-chain 2-oxo acid dehydrogenase.

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Tissue and mitochondria preparation

Male Wistar rats [body wt. 175–220 g; fed on Purina rodent laboratory chow diet 5001 (minimum 23% protein) or 48 h-starved] were decapitated, livers were rapidly removed and about one-third was freeze-clamped at liquid-N₂ temperature within 10 s and stored at –70 °C. Mitochondria were immediately isolated [17] from another one-third of the liver in ice-cold 0.25 M-sucrose (adjusted to pH 7.4 with Tris; 'mitochondria A' in Tables 1 and 2) and from the remaining one-third in ice-cold 0.25 M-sucrose/5 mM-Tris/HCl (pH 7.4)/2 mM-EGTA/0.1 M-NaF/5 mM-4-methyl-2-oxopentanoate ('mitochondria B' in Tables 1 and 2). The latter medium corresponds to that described by Patston *et al.* [4] to protect the phosphorylation state of the enzyme complex during mitochondrial isolation. Protein was determined by the biuret method [18] or a modified Lowry method [19]. Bovine serum albumin solutions were standardized for protein determination by their A_{279} [20].

Preparation of tissue and mitochondrial extracts

BCODH of tissues was extracted as described previously [7]. Freeze-clamped liver was powdered in liquid N₂, suspended in 5 vol. (v/w) of ice-cold extraction buffer [50 mM-Hepes, pH 7.4, 3 mM-EDTA, 2 mM-dithiothreitol, 0.1 mM-2-chloro-4-methylpentanoate, 1 mM-N- α -tosyl-L-lysylchloromethane, trypsin inhibitor from egg white (0.1 mg/ml), 0.5 μ M-leupeptin, 0.5 μ M-*aprotinin*, 1% (v/v) Triton X-100 and 2% (v/v) bovine serum] and homogenized with a Polytron PT-10 homogenizer for 15 s at setting 4. Part of this extract was incubated with the phosphatase to obtain total BCODH activity. For expressed activity, the extract was made with the same extraction buffer supplemented with 50 mM-KF and assayed directly. Mitochondrial pellets were homogenized in the same buffer with or without 50 mM-KF and then frozen in liquid N₂. The mitochondrial extract without KF was also preincubated with phosphatase for determination of total activity, whereas the extract with KF was assayed directly.

Enzyme assays

BCODH activity was assayed radiochemically by decarboxylation of 3-methyl-2-oxo[1-¹⁴C]butanoate or spectrophotometrically as described previously [5]. The assay system contained 30 mM-potassium phosphate, pH 7.5, 2 mM-MgCl₂, 0.4 mM-thiamin pyrophosphate, 0.4 mM-CoA, 2 mM-dithiothreitol, 3 mM-NAD⁺, 0.1% Triton X-100 and 3 units of dihydrolipoamide reductase/ml. For the decarboxylation assay, 1.5 ml Eppendorf tubes containing assay buffer and extracts (final volume 0.4 ml) were placed in mini-scintillation vials, which contained 1 ml of 1.2 M-KOH, and preincubated for 5–10 min at 30 °C. Reactions were initiated with 1 mM-3-methyl-2-oxo[1-¹⁴C]butanoate (150 c.p.m./nmol), and vials were sealed with rubber serum caps. After 10 min incubation at 30 °C, 0.5 ml of 2 M-acetic acid/1% (w/v) SDS was injected to terminate the reaction. CO₂ was collected by incubation at 40 °C for 3 h. The Eppendorf tubes were removed and 0.3 ml of 33% (v/v) phenethylamine in methanol (to wash KOH from the centre well) and 5 ml of xylene-based scintillation fluid were added to each vial for determination of radioactivity. This assay was found to be linear,

with respect to both time and quantity of extract. Citrate synthase was assayed by the procedure described by Shepherd & Garland [21].

Determination of mitochondrial respiratory control ratio and swelling

Mitochondria (4 mg of protein) were added to 2 ml of 0.25 M-sucrose/0.75 mM-Tris/0.25 mM-EDTA/60 mM-KCl/10 mM-potassium phosphate (pH 7.4)/10 mM-MgCl₂/5 mM-glutamate/5 mM-malate (pH 7.4) in the sample chamber of a YSI model 53 Biological Oxygen Monitor. State-3 respiration was induced by the injection of 0.4 μ mol of ADP. Mitochondrial water content was determined gravimetrically [22]; swelling was monitored by changes in A_{520} as a function of time [22].

RESULTS

A procedure for extraction and assay of BCODH in freeze-clamped rat liver has been reported previously by this laboratory [7,9]. Freeze-clamping liver at liquid-N₂ temperature was used to preserve the activity state as it existed *in vivo*. The freeze-clamped tissue was extracted in the presence of Triton X-100 to solubilize the intramitochondrial enzyme complex, EDTA to inhibit endogenous phosphatase(s), 2-chloro-4-methylpentanoate to inhibit BCODH kinase, and a variety of proteinase inhibitors to minimize proteolysis. Including fluoride in the extraction buffer was reported previously to be without effect on the activity state of the liver enzyme from chow- and low-protein-fed rats [7]. This was also found to be the case with freeze-clamped liver from starved animals in the present study. Activity of BCODH extracted and assayed without fluoride was 1.47 ± 0.08 units/g wet wt.; activity extracted and assayed with fluoride (50 mM and 25 mM respectively) was 1.42 ± 0.09 units/g wet wt. (mean $\pm$ S.E.M.; $n = 4$). This strongly suggests that EDTA and low temperature are sufficient to inhibit phosphatase activity during extraction of freeze-clamped tissue. We also 'spiked' liver from a 48 h-starved rat with ³²P-labelled BCODH (purified from rat liver and phosphorylated with [γ -³²P]ATP). As reported previously for liver from chow-fed rats [7], no significant phosphatase activity as measured by [³²P]P_i release was detected during the extraction/assay procedures used by this laboratory. As yet another check on our procedures, we 'spiked' frozen liver powders from 48 h-starved rats with purified rat liver BCODH_A (completely active, dephosphorylated, form of the enzyme) as well as BCODH_B (enzyme inactivated by incubation with ATP); 1 unit of BCODH activity (or incipient activity, for BCODH_B) was added per g of liver tissue before extraction. With liver supplemented with BCODH_A, recovery of the exogenous enzyme was complete without phosphatase treatment (1.31 ± 0.14 versus 2.30 ± 0.12 units/g wet wt.; $n = 3$). No significant increase in basal activity was found in extracts of liver supplemented with BCODH_B (1.31 ± 0.14 versus 1.43 ± 0.08 units/g wet wt.; $n = 3$). As expected, subsequent treatment with phosphatase had no effect on non-supplemented and BCODH_A-supplemented extracts, but greatly increased enzyme activity in BCODH_B-supplemented extracts (2.32 ± 0.12 units/g wet wt.; $n = 3$). The point of all these control experiments, as well as those given in [7], is that discrepancies in reported BCODH activity state between methods using freeze-

Table 1. BCODH activity in freeze-clamped rat liver and isolated mitochondria

Mitochondria A were isolated in 0.25 M-sucrose (pH 7.4 with Tris). Mitochondria B were isolated in 0.25 M-sucrose/5 mM-Tris/HCl/0.1 M-NaF/2 mM-EGTA/5 mM-4-methyl-2-oxopentanoate (pH 7.4). When BCODH was measured in isolated mitochondria, tissue BCODH activities (units/g wet wt.) were calculated by multiplying mitochondrial BCODH activity (munits/mg of mitochondrial protein) by tissue mitochondrial content (mg of protein/g wet wt.) (see Table 2). Columns headed by '– Pase' refer to activities measured before phosphatase treatment. Columns headed by '+ Pase' refer to activities measured after phosphatase treatment. Activities were determined radiometrically and are given as means $\pm$ S.E.M.: * $P < 0.05$ by two-tailed Student's t test, comparing freeze-clamped liver with isolated mitochondria.

	Mitochondrial BCODH activity (munits/mg of protein)		Tissue BCODH activity (units/g wet wt.)	
	– Pase	+ Pase	– Pase	+ Pase
Normal rats ($n = 12$)				
Freeze-clamp liver	–	–	1.04 ± 0.05	1.04 ± 0.05
Mitochondria A	20.7 ± 0.5	20.9 ± 0.4	1.07 ± 0.04	1.09 ± 0.04
Mitochondria B	9.2 ± 0.6	11.3 ± 0.5	$0.76 \pm 0.04^*$	0.95 ± 0.04
Starved rats ($n = 6$)				
Freeze-clamp liver	–	–	1.42 ± 0.05	1.43 ± 0.09
Mitochondria A	21.5 ± 1.1	21.8 ± 1.0	1.33 ± 0.06	1.34 ± 0.05
Mitochondria B	7.7 ± 0.8	13.2 ± 1.2	$0.68 \pm 0.06^*$	1.15 ± 0.06

Table 2. Citrate synthase activity and calculated mitochondrial content of liver

Mitochondria A and B were isolated as described in Table 1. Citrate synthase activities in freeze-clamped liver were 13.5 ± 0.1 units/g wet wt. for normal fed rats ($n = 12$) and 13.5 ± 0.5 units/g wet wt. for 48 h-starved rats ($n = 6$). Mitochondrial content was calculated by dividing citrate synthase activity in freeze-clamped rat liver (units/g wet wt.) by citrate synthase activity (units/mg of protein) in either mitochondria A or B. All values are means $\pm$ S.E.M.: * $P < 0.05$ by paired Student's t test, comparing mitochondria A with mitochondria B.

	Citrate synthase activity (units/mg of protein)	Mitochondrial content of tissue (mg/g wet wt.)
Normal rats ($n = 12$)		
Mitochondria A	0.25 ± 0.01	51.8 ± 1.7
Mitochondria B	$0.16 \pm 0.01^*$	$83.4 \pm 3.2^*$
Starved rats ($n = 6$)		
Mitochondria A	0.22 ± 0.01	62.0 ± 2.1
Mitochondria B	$0.15 \pm 0.01^*$	$89.1 \pm 4.0^*$

clamping [6,7,9,12] and mitochondrial isolation [4,10,11] for livers from starved rats cannot be due to inadequate inhibition of phosphatase activity during extraction of freeze-clamped tissue.

Experiments were conducted to compare BCODH activity in isolated mitochondria with that of whole tissue extracts. Both EDTA and fluoride were used in the freeze-clamping method to inhibit phosphatase activity. For normal fed and starved rats, no significant change in BCODH activity with phosphatase treatment was observed with freeze-clamped liver homogenates, indicating, as reported previously [6,7,9,12], that the enzyme in livers of these animals was in the active state (Table 1). BCODH activity of mitochondria isolated in the

absence of fluoride and 4-methyl-2-oxopentanoate was not different from that found in the freeze-clamped tissue (Table 1). Mitochondrial BCODH activity was normalized to tissue activity by multiplying the mitochondrial BCODH activity (units/mg of mitochondrial protein) by the liver mitochondrial content (mg of mitochondrial protein/g wet wt., based on citrate synthase activity; see Table 2). However, the enzyme activity of mitochondria isolated in the presence of fluoride and 4-methyl-2-oxopentanoate was only 73% (for normal fed rats) and 48% (for starved rats) of the activity found in tissue extracts. Phosphatase treatment increased the activity to 91% and 80% of the activities obtained for tissue extracts (Table 1), suggesting that inactivation of the enzyme was due, at least in part, to the phosphorylation of the enzyme complex during the process of mitochondrial isolation in the presence of fluoride and 4-methyl-2-oxopentanoate. No effect of phosphatase treatment was found with extracts of mitochondria isolated in the absence of fluoride and 4-methyl-2-oxopentanoate.

BCODH activities of mitochondria isolated from normal fed and starved rats in media with or without fluoride and 4-methyl-2-oxopentanoate vary over a wide range ([4,10,11]; see the results above). In order to study the effect of fluoride and 4-methyl-2-oxopentanoate on enzyme activity, mitochondria isolated from liver of chow-fed rats in the absence of these agents were incubated with different concentrations of fluoride and 4-methyl-2-oxopentanoate (Fig. 1). Incubations were carried out at 0 °C to approximate to conditions during mitochondrial isolation. Incubation without additions at 0 °C caused only a slight decrease in BCODH activity (Fig. 1). Similar results were obtained in the presence of KCl. However, incubation with fluoride and 4-methyl-2-oxopentanoate led to a time-dependent inactivation of the enzyme complex (Fig. 1). Inhibition of the enzyme complex occurred more rapidly and to a greater extent when these agents were present at higher concentrations (Fig. 1). Similar results were obtained when KF was

Fig. 1. Inactivation of BCODH by incubation of mitochondria prepared from normal fed rats in the presence of fluoride and 4-methyl-2-oxopentanoate

Mitochondria (2 mg of protein/ml) isolated from normal fed rat liver ($n = 3$) were incubated at 0 °C in 0.25 M-sucrose/20 mM-Hepes/KOH (pH 7.4), and different concentrations of KF and 4-methyl-2-oxopentanoate. At each time point, 1 ml of mitochondrial suspension was removed, the mitochondria were pelleted and the enzyme activity was determined spectrophotometrically. Error bars are S.E.M. ●, No addition, or 100 mM-KCl; ○, 50 mM-KF + 2 mM-4-methyl-2-oxopentanoate; ▲, 100 mM-KF + 5 mM-4-methyl-2-oxopentanoate.

replaced in the above experiment with NaF (results not shown). Furthermore, the rate and extent of inactivation were greater with mitochondria from starved-rat liver (Fig. 2). When extracts of these mitochondria were incubated with phosphatase at 30 °C for 30 min, BCODH activity increased from 55% to 80% of the initial activity, whereas incubation of the same extract at 30 °C without phosphatase led to further inactivation (Fig. 2). To try to differentiate the effects of fluoride and 4-methyl-2-oxopentanoate on BCODH activity, mitochondria were incubated with each of these agents separately. Although less than with the combination, a marked decrease in enzyme activity was caused by both agents (Fig. 2).

The water content (or degree of swelling) of mitochondria from normal fed rats was monitored in two ways. Gravimetric analysis showed greater water content of mitochondria isolated in the presence of fluoride and 4-methyl-2-oxopentanoate than in their absence (4.00 ± 0.32 and $2.03 \pm 0.13 \mu\text{l}$ of water/mg of mitochondrial protein respectively, for three different mitochondrial preparations; $P < 0.01$). The mitochondrial inner membrane is permeable to alkali-metal acetates and other weak acid ions, and the rate of decrease in A_{520} corresponds to the volume change of mitochondria [22]. Turbidity changes at 520 nm of mitochondrial suspensions in three different salt solutions were measured in the present study. NaCl solution (0.1 M) was used as control. The A_{520} at zero time for the mitochondria isolated in the presence of fluoride and 4-methyl-2-oxopentanoate was 35% lower (Fig. 3), confirming gravimetric analysis that the volume of these mitochondria was substantially greater than that of the mitochondria isolated without these agents. The absorbance decrement in 0.15 M-sodium acetate was initially

Fig. 2. Inactivation of BCODH by incubation of mitochondria prepared from starved rats with NaF and/or 4-methyl-2-oxopentanoate

Mitochondria (2 mg of protein/ml) were isolated from 48 h-starved rats ($n = 3$) and incubated at 0 °C in 0.25 M-sucrose/20 mM-Hepes/KOH (pH 7.4), without addition (●), or with 100 mM-NaF (□) or 5 mM-4-methyl-2-oxopentanoate (■) or both (○). Extracts of mitochondria incubated with both NaF and 4-methyl-2-oxopentanoate were subsequently incubated with (▲) or without (Δ) broad-specificity phosphatase at 30 °C. BCODH activity was assayed as given in Fig. 1.

Fig. 3. Study of swelling characteristics of mitochondria A and mitochondria B

Mitochondria (1.1 mg of protein), isolated as described in Table 1, were added to 3 ml of different salt solutions. The A_{520} was measured at room temperature: 1A, 1B, in 0.1 M-NaCl (pH 7.4); 2A, 2B, in 0.1 M-NaF (pH 7.4); 3A, 3B, in 0.15 M-sodium acetate (pH 7.4).

0.06/min for mitochondria isolated without fluoride and 4-methyl-2-oxopentanoate, and 0.02/min for mitochondria isolated with these agents (Fig. 3). A similar

difference in rate of swelling for the two mitochondrial preparations was observed for 0.1 M-NaF solution. The swollen initial state of mitochondria isolated with fluoride and 4-methyl-2-oxopentanoate readily explains the difference in swelling rate between the two mitochondrial preparations. The presence of fluoride and 4-methyl-2-oxopentanoate in the media during isolation of mitochondria also caused lower respiratory control ratios (3.55 ± 0.12 versus 6.57 ± 0.25 ; $n = 5$, $P < 0.05$). Citrate synthase activities per mg of mitochondrial protein were also decreased significantly (Table 2). Furthermore, citrate synthase was released at a greater rate from mitochondria incubated on ice in sucrose media supplemented with 0.1 M-KF and 5 mM-4-methyl-2-oxopentanoate (7.8 ± 0.2 versus $5.1 \pm 0.2\%$ released in 3 h; $n = 3$, $P < 0.05$). Thus mitochondria isolated or incubated in the presence of fluoride and 4-methyl-2-oxopentanoate were functionally damaged because of swelling, which may have promoted other alterations as well.

Liver mitochondrial content was calculated by dividing citrate synthase activity in freeze-clamped rat liver by the citrate synthase activity of mitochondrial preparations from fed and starved rats (Table 2). Higher apparent mitochondrial content (mg of mitochondrial protein/g wet wt.) was obtained when calculations were based on the lower citrate synthase activities of the damaged mitochondria isolated in fluoride- and 4-methyl-2-oxopentanoate-supplemented medium.

DISCUSSION

Discrepancies in the literature exist with regard to the activity state of BCODH under different dietary conditions. For the chow-fed and 48 h-starved rat, almost 100% of the hepatic enzyme has been reported to be in the active form by this laboratory [6–9] and by Wagenmakers *et al.* [12,14]. However, studies by Patston *et al.* [4,11] and Espinal *et al.* [10], using an isolated-mitochondria method, showed that the enzyme complex was only 25–55% active in liver from normal fed rats, and the active form decreased to 10% or less with 48 h starvation. Block *et al.* [13] reported 69% active form in rats fed on chow diet, with the assay being carried out by a procedure that included exposure of intact mitochondria to fluoride. The explanation for the different results for BCODH activity state obtained with the freeze-clamped rat liver method and the isolated-mitochondria procedures must lie either with overestimation of the expressed activity by the former procedure, owing to inadequate inhibition of phosphoprotein phosphatases, or with underestimation of the expressed activity by the latter procedure, owing to inadequate inhibition of the kinase. The objective has been to maintain the phosphorylation state of BCODH *in vivo* by inhibiting both the kinase and phosphatase activities equally. A change in activity of one without an equal change in the other will result in a phosphorylation state different from that *in vivo*. The current study provides further evidence that the phosphatase is not active during the extraction of freeze-clamped tissues and enzyme assay used in this laboratory. Indeed, the lack of any effect of fluoride, plus the recovery experiments described here and previously [7], seem to eliminate the possibility of artifacts in our extraction and assay procedure for BCODH.

Fluoride and 4-methyl-2-oxopentanoate, inhibitors of BCODH phosphatase and kinase respectively, have been routinely included in the mitochondrial isolation medium in an attempt to prevent interconversion of the active and inactive forms of the enzyme [4,10,11]. Patston *et al.* [4] reported that no inactivation of BCODH occurred during storage of heart mitochondria in fluoride and 4-methyl-2-oxopentanoate. Yet the present study showed that incubation of isolated liver mitochondria with fluoride and 4-methyl-2-oxopentanoate caused a time-dependent decrease in BCODH activity. Tissue differences may explain this discrepancy, because heart BCODH is routinely reported to be largely in the inactive form [4,12], and therefore is not subject to further inactivation in the presence of fluoride and 4-methyl-2-oxopentanoate. Inactivation was greater with higher fluoride and 4-methyl-2-oxopentanoate concentrations. Also, in a direct experimental comparison with the same liver, freeze-clamped tissue and mitochondria isolated without fluoride and 4-methyl-2-oxopentanoate gave the same values (100% active), whereas mitochondria isolated in the presence of fluoride and 4-methyl-2-oxopentanoate gave a lower percentage active form. Patston *et al.* [4] drew the conclusion that, without fluoride and 4-methyl-2-oxopentanoate, the phosphatase activity was greater than the kinase activity, resulting in an increase in percentage active form of BCODH. The present data suggest, however, that the presence of these agents increases the phosphorylation state of BCODH, apparently because the phosphatase was inhibited more by fluoride than the kinase was inhibited by 4-methyl-2-oxopentanoate. Inability to inhibit the kinase to the same extent as the phosphatase may be explained on the basis that 4-methyl-2-oxopentanoate can only effect partial inhibition of kinase activity [23]. The observation that exogenous phosphatase treatment partially restores enzyme activity (Table 1 and Fig. 2) is strong evidence for greater inhibition of endogenous phosphatase activity relative to kinase activity. Nevertheless, the effects of fluoride and 4-methyl-2-oxopentanoate on the activity of BCODH are more complex than their apparent direct effects on the activities of the phosphatase and kinase. The lower respiratory control ratio, leakage of citrate synthase, and the evidence for mitochondrial swelling, indicate that the integrity of mitochondria prepared with fluoride and 4-methyl-2-oxopentanoate was poor. Indeed, the phosphorylation state of BCODH has been shown [24] to increase with mitochondrial swelling. Furthermore, Hughes & Halestrap [24] found that apparent phosphorylation (measured by [32 P]P_i incorporation) of BCODH in mitochondria was greatly decreased by 4-methyl-2-oxopentanoate, although at high concentrations the activity of the complex was inexplicably diminished as well. We also noted a similar inhibitory effect of high concentrations of 4-methyl-2-oxopentanoate on mitochondrial BCODH activity (Fig. 2), an effect that may be an important component in understanding the decreased total BCODH activities reported by laboratories that use high 4-methyl-2-oxopentanoate concentrations during mitochondrial isolation. Incubation of purified BCODH with 2-oxo acids in the absence of CoA and NAD⁺ results in a time-dependent, non-reversible, inactivation of BCODH [1,25]. Whether the same phenomenon occurs in intact mitochondria is not known. Nevertheless, it is evident that using both

kinase and phosphatase inhibitors at high concentrations with intact mitochondria leads to a loss of BCODH activity.

Wagenmakers *et al.* [12,14] obtained activity states for liver BCODH that agree well with those published from this laboratory. This provides a paradox, because their strategy has been essentially the same as that used by Patston *et al.* [4]. Homogenates containing intact mitochondria were prepared in two different media: one designed to allow subsequent activation of the complex by the endogenous BCODH phosphatase, and the other to preserve the activity state of the enzyme. The last medium consisted of 0.25 M-sucrose, 10 mM-Tris/HCl (pH 7.4), 2 mM-EDTA, 50 mM-NaF and 5 mM-ADP. The combination of EDTA and ADP was present to inhibit the kinase, and fluoride to inhibit the phosphatase. In our hands, however, this medium proved no better than the fluoride-plus-4-methyl-2-oxopentanoate-supplemented media used by Patston *et al.* [4]. Some 30% less BCODH was found in hepatic mitochondria isolated from 48 h-starved rats in the media of Wagenmakers *et al.* [12,14] compared with mitochondria A of the present study (15.7 ± 0.1 and 22.3 ± 0.2 munits/mg of protein respectively; $n = 3$, $P < 0.05$). Furthermore, we found a significant loss of BCODH activity (27%) when mitochondria A prepared from 48 h-starved rats were incubated for 3 h at 0 °C in a medium supplemented with ADP, EDTA and NaF. It is problematic whether the addition of EDTA and ADP to the outside of mitochondria can provide effective kinase inhibition. EDTA probably does not penetrate the mitochondrial inner membrane, and it removes only limited amounts of endogenous Mg^{2+} [26]. ADP is only a competitive inhibitor of BCODH kinase, and the K_i is relatively high [1,3]. The procedure used by Wagenmakers *et al.* [12,14] may give correct values for BCODH activity state, because homogenates were rapidly prepared and assayed immediately, without isolating mitochondria. This feature of their assay may minimize the loss of BCODH activity that we observe when mitochondria are isolated or stored at 0 °C in media supplemented with kinase/phosphatase inhibitors.

It should be noted that we, as well as Patston *et al.* [4], believe it is important to disrupt mitochondria before assay of BCODH activity. Wagenmakers *et al.* [12,14] and also Paul & Adibi [27] tried to determine the activity state of the enzyme from flux measurements carried out with intact mitochondria. This aspect of their procedures, plus the use of low primary substrate concentrations (0.1 mM- and 0.2 mM-4-methyl-2-oxopentanoate, respectively) explain why the reported values are less than 15% of the values reported here. Sub-saturating concentrations of substrates, accumulation of inhibitory products, and differences in matrix pH and ionic composition are complications known to occur and difficult to control with intact mitochondria. Although uncontrolled factors can sometimes cancel each other out, we believe that latent enzyme activities as well as the activity state of BCODH are best assayed in disrupted mitochondria.

The total activity of BCODH in rat liver estimated in our recent studies by the freeze-clamped-liver method (1.0–1.5 units/g wet wt.) ([7,9] and the present study) agrees with those obtained with homogenates by Block *et al.* [13] (1.3 units/g wet wt., corrected to 30 °C). The original estimates of rat liver BCODH activity from our

laboratory [6] were low (0.6 unit/g wet wt.), presumably because loss of enzyme occurred during a centrifugation step used to concentrate the complex before spectrophotometric assay and perhaps also because of irreversible inactivation by 4-methyl-2-oxopentanoate. Using 4-methyl-2-oxopentanoate rather than 3-methyl-2-oxobutanoate as primary substrate, Patston *et al.* [4,11] have estimated that the liver contains 0.82–0.93 unit of BCODH/g wet wt. Correcting the latter values for the 35% greater V_{max} of the rat liver enzyme with 3-methyl-2-oxobutanoate [16] gives values (1.1–1.3) in good agreement with other work noted above. This agreement is more apparent than real, however, when the data used for the calculation of total tissue BCODH content are compared. Patston *et al.* [4,11] used BCODH specific activity of 6.8–9.3 munits/mg of mitochondrial protein plus (apparently) 100–121 mg of mitochondrial protein/g wet wt. to calculate BCODH activities in whole liver tissue. In contrast, as presented in Tables 1 and 2, we used BCODH activities of 21 munits/mg of mitochondrial protein and liver mitochondrial content of 52 mg/g for chow-fed rats and 62 mg/g for 48 h-starved rats. Although the final products of multiplying these numbers are remarkably similar, the discrepancies in values obtained for mitochondrial BCODH activity and mitochondrial content are large between our laboratories. Correcting for the greater activity of BCODH with 3-methyl-2-oxobutanoate will only partially explain the discrepancy in mitochondrial BCODH specific activities. Estimates of the mitochondrial content of the liver certainly vary significantly in the published literature. Leighton *et al.* [28] reported 52.5 mg of mitochondrial protein/g wet wt., based on measurements of electron micrographs and assay of enzyme markers; Scholz & Bucher [29] found 60 mg/g, based on cytochrome *a* and glutamate dehydrogenase activity; Schollmeyer & Klingenberg [30] calculated 72 mg/g on the basis of cytochrome *c*, 78 mg/g on the basis of cytochrome *a*; and Williamson *et al.* [31] have used a value of 68 mg/g. These values stand in contrast with the 96 mg of mitochondrial protein/g wet wt. reported by Foden & Randle [32], also based on citrate synthase measurements. Furthermore, Patston *et al.* [11] appear to have used a mitochondrial content of rat liver in the range of 100–121 mg/g wet wt., based again on citrate synthase. We show in the present study that overestimation of mitochondrial content can be caused by damage to mitochondria induced by fluoride and 4-methyl-2-oxopentanoate. Furthermore, it is questionable whether citrate synthase is a suitable marker for BCODH recovery. This enzyme is relatively small in size (M_r 100 000 [33]) and may escape the damaged mitochondrion more readily than the BCODH complex ($M_r > 2 \times 10^6$ [1]). Corrected for most literature values for mitochondrial content of liver, the total tissue BCODH activities reported by Patston *et al.* [4,11] would appear to be low because of underestimation of the specific activity of the enzyme in isolated mitochondria.

The greater effect observed in the present study of fluoride and 4-methyl-2-oxopentanoate on BCODH activity state in mitochondria from starved rats compared with that in chow-fed rats may explain why Patston *et al.* [4,11] and Espinal *et al.* [10] found the enzyme to be less active in the liver of the starved rat. This may reflect difference in degree of mitochondrial swelling/damage between these two nutritional states. A relatively poor

respiratory control ratio for mitochondria isolated from 48 h-starved rats has been reported previously by our laboratory [34].

The biologically important conclusion from this work is that rat liver BCODH is about 100% active in chow-fed rats. The activity state is not affected by 48 h starvation. Another important point is that methods for BCODH activity determination that include fluoride and 4-methyl-2-oxopentanoate to maintain the phosphorylation state *in vivo* within intact mitochondria create a complex system in which the phosphorylation state is not preserved and the total activity of the enzyme may be decreased.

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