Effects of low-protein diet and starvation on the activity of branched-chain 2-oxo acid dehydrogenase kinase in rat liver and heart

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The activity of branched-chain 2-oxo acid dehydrogenase kinase was 3-fold greater in extracts of heart mitochondria than in extracts of liver mitochondria from rats fed on normal diet. Feeding rats on a 0%-casein diet for 10 days increased the activity of branched-chain kinase 4-fold in extracts of liver mitochondria and in branched-chain dehydrogenase complex purified from such extracts; starvation (48 h) was without effect. In extract of heart mitochondria, kinase activity was increased 2-fold by feeding on 0%-casein diet and 1.5-fold by 48 h of starvation.

INTRODUCTION

The branched-chain 2-oxo acid dehydrogenase complex (branched-chain complex) of animal tissues catalyses the irreversible oxidative decarboxylation of branched-chain 2-oxo acids to branched-chain acyl-CoA. The activity of the complex determines the rate of degradation and the dietary requirement for branched-chain amino acids. The branched-chain complex is regulated by reversible phosphorylation, catalysed by a specific branched-chain dehydrogenase (branched-chain) kinase and phosphatase; phosphorylation of the α -subunit of the E1 component leads to inactivation (for review see Randle *et al.*, 1986). Phosphorylated branched-chain complex can also be re-activated without dephosphorylation by a protein, termed activator protein, which has been detected in liver and kidney, but not in heart or skeletal-muscle mitochondria, and which is either the free E1 component of the complex or an isoenzyme of it (Fatania *et al.*, 1982; Yeaman *et al.*, 1984; Espinal *et al.*, 1985).

In rats fed on normal diets, approx. 35% of branched-chain complex in liver is in the active form, whereas only 5% is in the active form in heart muscle. When rats are fed on 0%-casein diet (10 days for maximum effect) or starved for 48 h, the percentage of active complex in liver is decreased to 3% but that in heart shows no significant change. The concentration of total complex is unchanged in both tissues. [Earlier studies by Gillim *et al.* (1983) and Patston *et al.* (1984) suggested that total complex was decreased by low-protein diet, but this decrease was apparently due to failure to effect complete conversion of inactive complex into the active complex before assay (Patston *et al.*, 1986)]. These findings suggest the possibility that the rate of the kinase reaction is increased and/or that the rate of the phosphatase reaction is decreased in rat liver by 0%-casein diet or starvation. It seemed possible that similar mechanisms might contribute to the lower percentage of active complex in heart as compared with liver. In the present study we show that branched-chain kinase activity in heart is greater than that in liver in rats fed on normal diet, and that 0%-casein diet increases branched-chain kinase activity in both tissues.

EXPERIMENTAL

Materials

Details relating to male Wistar rats (source, feeding, normal and 0%-casein diet) and sources of chemicals, biochemicals, [γ - 32 P]ATP, Centriflo cones, Sephacryl S-300 and X-ray film are as in Kerbey *et al.* (1979), Patston *et al.* (1984) and Espinal *et al.* (1985).

Mitochondrial extracts; purified rat liver branched-chain complex

The usual method of preparation of liver and heart mitochondrial was modified by the use of digitonin, to disrupt and remove lysosomes, and by inclusion of proteinase inhibitors in the extraction buffer, to prevent proteolysis of branched-chain complex. Livers (four to eight rats) were disrupted in a Teflon/glass Potter/Elvehjem homogenizer (0.1 g of tissue/ml), and hearts (eight to ten rats) were disrupted in a Polytron homogenizer (50 mg of tissue/ml), in sucrose medium (0.25 M-sucrose/5 mM-Tris/2 mM-EGTA, pH 7.5). The homogenate was centrifuged at 600 *g* for 10 min, the supernatant was collected and the pellets were re-extracted and centrifuged. The pooled supernatants were centrifuged at 10000 *g* for 10 min. The mitochondrial pellets were taken up in the same medium (4 mg of protein/ml) and incubated for 2 min at 0 °C with digitonin (0.4 mg/ml). The incubation mixture was centrifuged at 600 *g* for 5 min to precipitate digitonin, and the supernatant was centrifuged at 10000 *g* for 10 min. Mitochondria were taken up and incubated at 30 °C for 30 min in KCl medium containing 10 μ M-carbonyl cyanide *m*-chlorophenylhydrazone (rats fed on normal diet or starved for 48 h) or for 2 h in medium containing 10 μ M-carbonyl cyanide *m*-chlorophenylhydrazone and 5 mM-ketoleucine (4-methyl-2-oxopentanoate) (rats fed on 0%-casein diet). These conditions effect complete conversion of inactive complex into active complex (Patston *et al.*, 1986). After incubation, mitochondria were separated by centrifuging at 10000 *g* for 20 min, washed (twice) by centrifugation and resuspension, and the pellets were frozen in liquid N₂.

(washing was shown to effect complete removal of ketoleucine, which is an inhibitor of branched-chain kinase). Extraction of mitochondria (50 mg of protein/ml) was carried out by freezing and thawing (three times) in 50 mM-potassium phosphate/10 mM-EGTA/2 mM-dithiothreitol/1 mM-benzamidine/1 mM-phenylmethanesulphonyl fluoride/0.3 mM-tosyl-lysylchloromethane/2% (v/v) ox serum, pH 7.5. The extracts were centrifuged at 36000 *g* for 20–30 min, and branched-chain complex and kinase activities were assayed in the supernatant as described below.

In some experiments the extract supernatants were concentrated, by using Centrifo cones, to approx. 0.5–1 unit of branched-chain complex/ml, and approx. 1.5 units was loaded on to a Sephacryl S300 column (bed volume 180 ml) equilibrated with 30 mM-potassium phosphate/5 mM-EGTA/2 mM-dithiothreitol, pH 7.5. The column was run at a flow rate of 0.3 ml/min. Branched-chain complex was eluted in the void volume (typically 35 ml) and recovered in a total volume of approx. 15 ml. Fractions containing branched-chain complex were concentrated (5-fold) in Centrifo cones, and complex and kinase activities were assayed as described below.

Highly purified branched-chain complex from extracts of liver mitochondria was prepared by the method of Fatania *et al.* (1981), but with omission of the final precipitation at pH 6.4. SDS/polyacrylamide-gel electrophoresis (15 cm × 14 cm slabs) was carried out by the method of Laemmli (1970) and autoradiographs were prepared as described by Lau *et al.* (1981).

Assay of branched-chain complex and kinase activities

Branched-chain complex was assayed as described by Patston *et al.* (1984). For assays for kinase activity by ATP-dependent inactivation, branched-chain complex was incubated at a final concentration of 25 munits/ml in 30 mM-potassium phosphate/5 mM-EGTA/2 mM-dithiothreitol/10 mM-MgCl₂, pH 7.5, at 30 °C (or at 10 °C or 20 °C if necessary). Reaction was initiated with ATP (final concn. 0.5 mM), and samples (corresponding to 10 munits of complex) were taken at specified time intervals (usually 15, 30, 45 and 60 s) for assay of branched-chain complex activity at 30 °C. Kinase activities are presented as the apparent first-order rate constants (min⁻¹) calculated by least-squares linear-regression analysis.

In experiments where inactivation and [³²P]phosphorylation of the complex were studied, [³²P]ATP was added (final concn. 0.5 mM) at a specific radioactivity of 635 d.p.m./pmol. Incorporation of ³²P into branched-chain complex was measured by the method of Corbin & Reimann (1974). ATPase activity in mitochondrial extracts was assayed with [³²P]ATP as described by Cooper *et al.* (1974).

One unit of enzyme forms 1 μmol of products/min at 30 °C.

RESULTS AND DISCUSSION

Effect of diet on the activity of rat liver and rat heart mitochondria branched-chain dehydrogenase kinase

In studying the activity of branched-chain kinase in mitochondrial extracts, the following essential criteria were fulfilled. Complete conversion of inactive complex

Fig. 1. SDS/polyacrylamide-gel electrophoresis of purified rat liver branched-chain complex

(a) Coomassie-Blue-stained gels of (left to right) branched-chain complex, 35, 70 and 140 munits, and M_r ($\times 10^{-3}$) markers: 66, 45, 36, 29, 24, 20.1, 14.2. Computed M_r values for branched-chain complex, 49 700, 45 500, 34 700. (b) Autoradiograph of SDS/polyacrylamide-gel electrophoresis. Branched-chain complex (100 munits in 250 μl) incubated for 1, 2, 3 or 4 min with 0.5 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 2084 d.p.m./mol (complex from rats fed on normal diet) or 1095 d.p.m./pmol (complex from rats fed on 0% casein diet). Incubation was terminated by adding trichloroacetic acid (final concn. 10%) and centrifuging, pellets were washed (five times) by resuspension and centrifugation, and taken up in SDS, neutralized (NaOH, universal indicator), and all were loaded for electrophoresis. Tracks (left to right); 0% casein diet (1, 2, 3, 4 min), normal diet (1, 2, 3, 4 min). For further details see the Experimental section.

into active complex was effected in mitochondria before extraction and assay. This entailed different conditions of incubation for mitochondria from rats fed on normal diets as compared with rats fed on 0% casein diet (Patston *et al.*, 1986). Control experiments with mitochondria from rats fed on normal diets showed that the different conditions of incubation had negligible effects on branched-chain kinase activity (results not shown). ATPase activity of mitochondrial extracts were negligible ($< 4\%$ hydrolysis of ATP/min at 30 °C) at the extract concentration used (25 munits of branched-chain complex/ml). Oligomycin, which had no significant effect on ATPase or branched-chain kinase activity, was not used (results not shown). Purified branched-chain complex had zero ATPase activity. Branched-chain complex in extracts was stable and showed no loss of activity when incubated at 30 °C for up to 7 min in the absence of ATP (results not shown). Use of digitonin to remove lysosomes had no effect on branched-chain kinase activity (results not shown). There was no interference by branched-chain phosphatase in kinase assays in mitochondrial extracts; thus NaF (50 mM), which inhibits the phosphatase reaction (Patston *et al.*,

Table 1. Effect of 0%-casein diet (for 10 days) or starvation (48 h) on the activity of branched-chain dehydrogenase kinase in rat liver and heart

Branched-chain kinase was assayed by the rate of ATP-dependent inactivation of branched-chain complex (25 munits/ml in the incubation) with extracts of mitochondria (extracts), or extracts subjected to gel filtration on Sephacryl S300 (GF extracts), or with purified complex. Assays of branched-kinase activity were carried out at 30 °C, except where the apparent first-order rate constant is $> 4 \text{ min}^{-1}$, when assays were at 10 °C. The latter values were corrected to 30 °C by the Q_{10} . For further details see the Experimental section. Each extract is derived from mitochondria prepared from four to eight livers or eight to ten hearts. The number of assays is shown in square brackets and the number of preparations (extracts or purified complex) is shown in parentheses. Results are means $\pm$ S.E.M. for preparations (not assays), except for single preparations, where mean $\pm$ S.E.M. is for assays. * $P < 0.001$, † $P < 0.01$ for effect of diet; ‡ $P < 0.01$ for difference from liver; § $P < 0.05$ for effect of gel filtration; for other differences $P > 0.05$.

Diet	Preparation	Branched-chain kinase activity, apparent first-order rate constant (min^{-1}) for:	
		Liver	Heart
Normal	Extract	1.32 ± 0.10 [40] (12)	3.35 ± 0.12 † [6] (3)
	GF extract	1.46 ± 0.02 [8] (2)	—
	Purified complex	1.30 ± 0.11 [13] (2)	—
Starved (48 h)	Extract	1.14 ± 0.06 [21] (6)	5.63 ± 0.45 †† [7] (3)
	GF extract	1.27 ± 0.02 [3] (1)	—
	Purified complex	1.23 ± 0.13 [12] (2)	—
0%-casein (10 days)	Extract	5.02 ± 0.31 * [25] (6)	6.01 ± 0.34 * [3] (1)
	GF extract	6.71 ± 0.48 *§ [3] (1)	—
	Purified complex	5.46 ± 0.14 † [7] (2)	—

1984), had no effect on kinase activity (results not shown). With some mitochondrial extracts, kinase activities were too high for accurate assay at 30 °C. Kinase assays were therefore performed at 10 °C or at 20 °C and corrected to 30 °C by the Q_{10} , which was 1.96 ± 0.03 (normal diet) and 2.05 ± 0.17 (0%-casein diet) (means $\pm$ S.E.M. for four observations at 10 °C, 20 °C and 30 °C; $P > 0.05$ for effect of diet). The results of SDS/polyacrylamide-gel electrophoresis of the highly purified branched-chain complex are shown in Fig. 1. The three major bands correspond in M_r to E2 (50000), El α (45500) and El β (34700); there was remarkably little contamination by other protein bands.

Feeding rats on 0%-casein diet for 10 days resulted in an approx. 4-fold increase in the activity of branched-chain kinase in liver mitochondrial extracts, in branched-chain complex partially purified from mitochondrial extracts by gel filtration, and in branched-chain complex purified to near homogeneity (Table 1, measured by rate of ATP-dependent inactivation). There was an approx. 20–30% increase in branched-chain kinase activity after gel filtration of mitochondrial extracts, presumably owing to removal of inhibitors of the kinase such as thiamin pyrophosphate and ADP (Table 1). The activity of branched-chain kinase in purified rat liver branched-chain complex was not significantly different from that in rat liver mitochondrial extracts. In contradistinction to the effects of feeding 0%-casein diet for 10 days, starvation (48 h) had no effect on the activity of branched-chain kinase in rat liver mitochondrial extracts or after gel filtration of extracts or purification of the complex (Table 1).

In rats fed on normal diets, the activity of branched-chain kinase in extracts of heart mitochondria was approx. 3 times that of liver. Both starvation (48 h) and feeding 0%-casein diet for 10 days increased kinase activity in the heart by approx. 50%.

The specific activity of fully re-activated complex in mitochondrial extracts averaged (all experiments) 11.8 munits/mg of mitochondrial protein for liver and 9.5 munits/mg of mitochondrial protein from heart. These values are somewhat higher than those obtained previously (cf. Espinal *et al.*, 1985; Patston *et al.*, 1986), owing to loss of other proteins with digitonin.

[^{32}P]Phosphorylation and inactivation of branched-chain complex

It was considered important to confirm that the increased rate of ATP-dependent inactivation of branched-chain complex from livers of rats fed on a 0%-casein diet for 10 days was correlated with a higher rate of incorporation of ^{32}P from [γ - ^{32}P]ATP into the complex. To this end, purified liver branched-chain complex from rats fed on normal diets or 0%-casein diets for 10 days was incubated at a concentration of 400 munits/ml with 0.5 mM-[γ - ^{32}P]ATP (635 d.p.m./pmol) at 10 °C, and the rates of incorporation of ^{32}P into branched-chain complex were measured. The rate of incorporation of ^{32}P into liver branched-chain complex was increased in rats fed on 0%-casein diet as compared with normal diet (Fig. 2). The ratio of initial rats (0%-casein diet/normal diet) was 3.86 ± 0.26 (mean $\pm$ S.E.M. for three pairs of observations). This is comparable with the ratio based on rats of ATP-dependent inactivation (Table 1). It was important in experiments with ^{32}P to exclude the possibility of incorporation of ^{32}P into the El α subunit of pyruvate dehydrogenase complex. To this end, samples of [^{32}P]phosphorylated complex were taken at 1, 2, 3 and 4 min of incubation at 10 °C and subjected to SDS/polyacrylamide-gel electrophoresis, and autoradiographs were prepared. The autoradiographs (Fig. 1b) showed only a single band, corresponding to the El α subunit of branched-chain complex. They also show qualitatively the difference in incorporation between normal and

Fig. 2. Effect of 0%-casein diet on [^{32}P]phosphorylation of purified rat liver branched-chain complex by branched-chain kinase

Branched-chain complex purified from livers of rats fed on normal diet (●) or 0%-casein diet for 10 days (○) was incubated (400 munits/ml) with 0.5 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (635 d.p.m./pmol), and samples (25 μl) were taken on to Whatman 3MM paper squares for assay for protein-bound ^{32}P (Corbin & Reimann, 1974). Incorporations were corrected for blank radioactivity by separate additions to paper squares of equivalent amounts of branched-chain complex and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in 10% trichloroacetic acid at 0 °C. Results are means $\pm$ S.E.M. for quadruplicate runs. The initial rates of [^{32}P]phosphorylation (means $\pm$ S.E.M.; pmol of protein-bound ^{32}P /min per ml of incubation) were 76 ± 5.2 (normal diet) and 280 ± 13.6 (0%-casein diet) ($P < 0.001$ for difference).

0%-casein diet. The El α subunit of rat liver pyruvate dehydrogenase is readily resolved under these conditions (it migrates faster; Lau *et al.*, 1981).

General discussion and conclusions

The present study has shown that the 10-fold decrease in percentage of active branched-chain complex observed in liver after 10 days of 0%-casein diet (Patston *et al.*, 1986) is associated with a 4-fold increase in the activity of branched-chain kinase as assayed either by ATP-dependent inactivation of the complex or by [^{32}P]phosphorylation of its El α subunit with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (the present study). Time-course experiments showed that 8–10 days of 0%-casein diet are required for maximum decrease in percentage of active complex (Patston *et al.*, 1984). Pilot experiments showed that a similar period is required for maximum increase in branched-chain kinase activity (results not shown). The increase in kinase activity induced by 0%-casein diet is stable to isolation of mitochondria, 2 h of incubation of mitochondria with carbonyl cyanide *m*-chlorophenylhydrazone plus ketoleucine, gel filtration of mitochondrial extract, and extensive purification of branched-chain

complex. The mechanism is not known, but likely mechanisms may include an increase in the concentration of branched-chain kinase in the complex or a stable form of activation, e.g. covalent modification. The absence of any obvious effect of gel filtration of mitochondrial extracts on the increase in kinase activity induced by 0%-casein diet would appear to exclude the participation of a dissociable effector of lower M_r . The results suggest that this stable increase in kinase activity may contribute to the decrease in percentage of active complex, but do not exclude the participation of other mechanisms, e.g. activator protein (Patston *et al.*, 1984; Espinal *et al.*, 1985). Similar considerations may apply to the 7-fold difference in percentage of active complex of liver as compared with heart in rats fed on normal diet (Patston *et al.*, 1984, 1986), where the activity of branched-chain kinase in heart is 3-fold greater than that of liver (the present study). The activity of branched-chain kinase in heart was also increased by 0%-casein diet and by starvation, but only by 50%. This increase in kinase activity is not associated with any demonstrable decrease in percentage of active complex (Patston *et al.*, 1984), presumably because only 5% of complex in heart is active in rats in normal diet.

An interesting further discovery is that starvation (48 h), which also leads to a 10-fold decrease in percentage of active complex in liver (Patston *et al.*, 1986), does not result in a stable increase in branched-chain kinase activity (the present study). No changes in kinase activity were observed in extracts, in gel-filtered extract or in purified complex. Therefore the mechanism of the effect of starvation must differ from that of 0%-casein diet, e.g. by some other mechanism of kinase activation, or by inhibition of branched-chain phosphatase, or both.

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