Rat tissue concentrations of branched-chain 2-oxo acid dehydrogenase complex

Re-evaluation by immunoassay and bioassay

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A rabbit polyclonal antibody to purified ox kidney branched-chain oxo acid dehydrogenase complex was shown by a variety of techniques to be an antibody to the E2 (acyltransferase) component. Rocket immunoelectrophoresis showed that the antibody does not discriminate between phosphorylated (inactive) or dephosphorylated (active) complex, and the same technique is used to assay total branched-chain complex (sum of active and inactive forms) in rat liver and heart mitochondrial extracts. The values obtained in normal rats fed on normal diet were comparable with those obtained by spectrophotometric assay of the holocomplex reaction after conversion of inactive complex into active complex. The values obtained in liver mitochondria from rats fed on 0%-casein diet or starved for 48 h were comparable with those in rats fed on normal diet, whereas earlier studies using spectrophotometric assay had shown substantial decreases in rats fed on 0%-casein diet or starved for 48 h. It has been shown that conversion of inactive complex into active complex requires prolonged incubation (120 min) in the presence of ketoleucine (4-methyl-2-oxopentanoate; to inhibit branched-chain oxo acid dehydrogenase kinase) to effect complete conversion in mitochondria from rats fed on 0%-casein diet, or starved for 48 h, or made diabetic with alloxan. By this technique, total activity of the complex in rat liver mitochondria was unaffected by diet or diabetes. The effects of diet and diabetes to decrease the activity of branched-chain complex in rat liver are therefore apparently mediated wholly through inactivation of the complex by phosphorylation.

INTRODUCTION

In animals the rate of degradation of branched-chain amino acids is adjusted in relation to dietary intake. In rats the rate is apparently determined by the activity of the mitochondrial branched-chain 2-oxo acid dehydrogenase complex (branched-chain complex) (for review see Randle *et al.*, 1986). Branched-chain complex as purified contains multiple copies of two component enzymes, branched-chain dehydrogenase (E1) and branched-chain acyltransferase (E2), and requires dihydrolipoamide dehydrogenase (Pettit *et al.*, 1978). The complex is regulated by reversible phosphorylation of E1 catalysed by a mitochondrial kinase and phosphatase; phosphorylation inactivates it (Parker & Randle, 1978; Fatania *et al.*, 1981; Cook *et al.*, 1984; Damuni *et al.*, 1984). Phosphorylated complex is also re-activated without dephosphorylation by a protein (activator protein), which is either free E1 or an isoenzyme of E1. This is present in rat liver and kidney mitochondria, but it has not been detected in rat muscle mitochondria (Fatania *et al.*, 1982; Yeaman *et al.*, 1984; Espinal *et al.*, 1985).

In rats fed on normal diet, approx. 75% of the active form of branched-chain complex is in liver and kidney. The effect of low-protein diet is to decrease the activity of branched-chain complex in these tissues, and three mechanisms have been implicated. These are decreases in the concentration of total complex (sum of active and inactive forms), in the percentage of complex in the active

form, and in the activity of activator protein (Gillim *et al.*, 1983; Patston *et al.*, 1984). The decreased concentrations of total complex and of activator protein activity suggested the possibility of decreased synthesis or accelerated degradation of the E2 and/or E1 components of the complex. It was important to confirm the apparent decrease in total complex by other methods, and this has now been achieved by immunoassay with an antiserum to E2. The results of immunoassay failed to confirm the effect of low-protein diet to decrease the concentration of total complex in rat liver. This has been further substantiated by more stringent re-evaluation of the bioassay.

EXPERIMENTAL

Materials

Details relating to male albino Wistar rats (source, feeding, standard and special diets, induction of alloxan-diabetes) and sources of chemicals and biochemicals are as in Patston *et al.* (1984). Other biochemicals and immunological reagents were from Sigma (London) Chemical Co., Poole, Dorset, U.K. Nitrocellulose membranes (Schleicher and Schuell; 0.1 µm pore size) were from Anderman and Co., East Molesey, Surrey, U.K. Prestained M_r markers were from BRL Inc., Gaithersburg, MD, U.S.A.

Abbreviation used: PBS, phosphate-buffered saline (0.14 M-NaCl/2.7 mM-KCl/1.5 mM-KH₂PO₄/8.1 mM-Na₂HPO₄, pH 7.4).

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Mitochondria were isolated by methods given in Patston *et al.* (1984). Branched-chain complex was purified from ox kidney mitochondria by the method of Fatania *et al.* (1981), and the E1 and E2 components were resolved by the method of Pettit *et al.* (1978). Antibodies were produced in half-Sandylop-eared rabbits by six subcutaneous injections of ox kidney complex in an emulsion with Freund's complete adjuvant (200 µg of complex/rabbit). Booster injections of 200 µg of complex in PBS were given by ear vein after 2 and 4 weeks. Blood was collected 3 days after the final injection. Additional booster injections were given 3 days before further collections of blood (these were of E2 once it had been established that antisera were to E2). Serum was separated and stored at -20 °C.

Mitochondria

Incubation of mitochondria and preparation of extracts for assay of branched-chain complex, citrate synthase and activator protein were as described by Patston *et al.* (1984), except that the extraction buffer for activator protein was used throughout. Extracts for rocket immunoelectrophoresis were made by freezing and thawing (three times) in 30 mM-potassium phosphate/5 mM-EGTA/2 mM-dithiothreitol/5% (v/v) Triton X-100, pH 7.4.

Immunological methods

Ouchterlony diffusion gels were 1% (w/v) agarose in PBS with 5 mm-diameter wells. Rocket immunoelectrophoresis was by the method of Laurell (1972), using an LKB Multiphor flat-bed system, agarose gel containing 2% (v/v) Triton X-100, and 50–200 µl of antiserum per 12 ml of gel solution. Running buffer was 25 mM-diethylbarbituric acid/75 mM-Tris/0.5 mM-calcium lactate/2 mM-Na₂S₂O₃, pH 8.6. Electrophoresis was at a constant 10 V/cm for 16 h. After pressing between filter papers and washing with 0.1 M-NaCl, gels were dried and stained with Coomassie Blue.

SDS/polyacrylamide-gel electrophoresis (16 cm slabs) was as described by Espinal *et al.* (1985), with duplicate samples on each half of the gel (one half for Coomassie Blue staining; one half for Western blotting by the method of Towbin *et al.*, 1979). Prestained *M_r* markers were used if these were required on the Western blot. After transfer, nitrocellulose sheets were incubated in PBS/2% (w/v) bovine serum albumin to block protein-binding sites and incubated overnight with antiserum (0.1%, v/v) in PBS containing 1% serum albumin. Sheets were then washed either with PBS or with PBS/0.05% (w/v) Nonidet P-40, followed by 15 min of washing with four changes of PBS [treatment with Nonidet P-40 more effectively decreased non-specific staining (Burnette, 1981)]. Bound rabbit antibody was then stained by exposure to goat anti-rabbit IgG IgG-horseradish peroxidase, followed by H₂O₂/o-dianisidine.

Rocket immunoelectrophoresis was utilized to assay branched-chain complex in mitochondrial extracts, by using four standards (usually 2.5, 5, 12 and 24 munits of ox kidney complex) and two unknowns (usually 225 and 450 µg of mitochondrial protein) on each plate. The concentration of antiserum was typically 0.17% (v/v), but this was optimized for each batch in preliminary experiments. The amount of complex in mitochondrial

extracts was calculated from linear standard curves (ln[complex] against rocket height).

Analytical methods

Mitochondrial protein was assayed by the method of Gornall *et al.* (1949), branched-chain complex by the method of Lau *et al.* (1981), and activator protein and citrate synthase were assayed as in Patston *et al.* (1984). Citrate synthase was assayed routinely to check losses of mitochondria during aspiration of incubation medium after sedimentation. Simultaneous measurements of protein and citrate synthase in extracts established that the activity of citrate synthase averaged 120 units/g of mitochondrial protein in rat liver mitochondria and was unaffected by diet or alloxan-diabetes. If citrate synthase activity in individual mitochondrial extracts deviated from this value by more than 10%, then the activity of branched-chain complex was corrected to 120 munits of citrate synthase.

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

RESULTS AND DISCUSSION

Characterization of rabbit antiserum to ox kidney branched-chain complex

Ouchterlony diffusion gels gave single lines of precipitation between undiluted antiserum and ox kidney branched-chain complex (range 1.5–50 munits) or E2 component; no lines or precipitation were seen with E1 component or non-immune sera (results not shown). The specificity of the antiserum for E2 component was confirmed by Western blots of SDS/polyacrylamide gels of partially purified bovine kidney complex (tracks 1 and 2 of Fig. 1a), E1 component (tracks 3 and 4, Fig. 1a) and E2 component (tracks 5 and 6, Fig. 1a). The Western blots showed only cross-reaction with E2 subunit; there was no cross-reaction with E1α or E1β subunits or with any of the numerous minor contaminants revealed by Coomassie Blue in this preparation of complex (track 1, Fig. 1a).

The results of the immunotitration experiment, in which holocomplex activity was assayed in the supernatant after mixing branched-chain complex and antiserum and centrifuging immediately, showed that approx. 200 µl of antiserum/unit of complex was required for 50% inactivation and that 1 ml of antiserum/unit of complex gave complete inactivation. When [³²P]phosphorylated complex (205 munits; 1.9 nmol of ³²P/unit) and antiserum were incubated for 30 min at 0 °C and centrifuged (Eppendorf 3200; 2 min), 94% of protein-bound ³²P remained in the supernatant. When active (dephospho) branched-chain complex (10 munits) was incubated with 20 µl of antiserum and centrifuged, the supernatant was devoid of holocomplex activity. However, the supernatant gave 70% re-activation of 5 munits of phosphorylated branched-chain complex, indicating that free E1 was present (cf. Espinal *et al.*, 1985; Cook *et al.*, 1985).

These results indicate that the antiserum was specific for the E2 component of ox kidney complex, and they suggest that reaction of antibody with E2 led to dissolution of the complex and presumably to specific precipitation of E2.

Fig. 1. SDS/polyacrylamide-gel electrophoresis and Western blots of (a) partially purified ox kidney complex and (b) rat liver mitochondrial extracts

(a) Ox kidney branched-chain complex: track 1, Coomassie Blue; track 2, Western blot. E1 component of complex: track 3, Coomassie Blue; track 4, Western blot. E2 component of complex: track 5, Coomassie Blue; track 6, Western blot. (b) Rat liver mitochondrial extracts: tracks 7 and 9, Western blots (Nonidet P-40 treatment before staining in track 9). Tracks 8 and 10, prestained M_r markers: from the top, M_r values ($\times 10^{-3}$) were 200, 92.5, 68, 43 and 25.7.

Immunoassay of branched-chain complex (E2 component) in extracts of rat liver and heart mitochondria by rocket immunoelectrophoresis

Western blots after SDS/polyacrylamide-gel electrophoresis of rat liver mitochondrial proteins showed only one band cross-reacting with antiserum when Nonidet P-40 was used to decrease non-specific reaction (track 9, Fig. 1b). In the absence of Nonidet P-40 treatment, several minor bands were stained (track 7, Fig. 1b). Inclusion of prestained M_r markers (tracks 8 and 10, Fig. 1b) gave $M_r = 53078 \pm 2250$ for the major band in tracks 7 and 9 (mean $\pm$ S.E.M. by least-squares linear-regression analysis). This corresponds closely to $M_r = 52000$ reported for the E2 subunit of purified rat kidney complex by Odessey (1982).

The results of rocket immunoelectrophoresis of purified ox kidney complex (2.5–24 munits) are shown in tracks 1–4 of Fig. 2(a), together with a linear standard curve in Fig. 2(b). Results obtained with phosphorylated ox kidney complex were indistinguishable from those obtained with dephosphorylated complex (results not shown). This indicated that rocket immunoelectrophoresis could be used to assay total complex (sum of inactive and active forms). Satisfactory rockets were obtained with rat liver and heart mitochondrial extracts [tracks 5 and 6 in

Fig. 2(a) are for liver; results for heart were comparable, but are not shown]. Inclusion of two concentrations of extract showed for all assays that standards and extracts shared a common regression line (e.g. Fig. 2b). By reference to such standard curves (e.g. Fig. 2b), concentrations of total complex were computed as 9.3 ± 0.66 (15) (liver) and 6.1 ± 0.36 (6) (heart) (units/g of mitochondrial protein, means $\pm$ S.E.M. for numbers of observations in parentheses: normal rats, normal diet; see also Table 1). These values are strictly comparable with the highest estimates obtained in previous studies by spectrophotometric assay of the holocomplex reaction (cf. Patston *et al.*, 1984; Espinal *et al.*, 1985). However, by immunoassay the concentrations of total complex in liver mitochondria from rats fed on normal or 0% -casein diets did not differ significantly, whereas spectrophotometric assay (Patston *et al.*, 1984) had shown a 70% decrease in rats fed on 0% -casein diets relative to normal-diet controls. Likewise immunoassay failed to detect any decrease after 48 h starvation (see Table 1), whereas spectrophotometric assay had shown a decrease.

Three reasons for this discrepancy between immunoassay and spectrophotometric assay have been considered. One possibility is substantial cross-reaction of the antiserum with other mitochondrial proteins, including degradation products of E2. This is considered unlikely,

Fig. 2. Typical examples of (a) rocket immunoelectrophoresis of ox kidney complex and rat liver mitochondrial extracts and (b) linear regression with ox kidney standards

(a) Tracks 1–4, purified ox kidney branched-chain complex (24, 12, 5 and 2.5 munits respectively). Tracks 5 and 6 were rat liver mitochondrial extracts (450 and 225 μ g of protein respectively). The unmarked central tracks were blank. (b) Purified ox kidney branched-chain complex (●) (24.5, 12.3, 4.9 and 2.5 munits) and rat liver mitochondrial extract (○), giving estimates (units/g of mitochondrial protein) of 9.0 (450 μ g applied) and 10.4 (250 μ g applied).

Table 1. Branched-chain complex activities in rat liver mitochondria by immunoassay and spectrophotometric assay

Assays of total complex were performed on extracts prepared from mitochondria incubated as described in Fig. 3. Values are means of those obtained after 120 min of incubation. A correction for the blank rate in the absence of ketoleucine was obtained from mitochondria incubated without ketoleucine (correction was 18% of the rate with ketoleucine). Immunoassays (rocket immunoelectrophoresis) employed four standards (usually 2.5, 5, 12 and 24 munits of ox kidney complex) and two concentrations of mitochondrial extracts (usually 225 and 450 μ g of protein). The active form of branched-chain complex was assayed spectrophotometrically in extracts prepared from mitochondria isolated in the presence of 100 mM-NaF and 5 mM-ketoleucine from separate livers (cf. Patston *et al.*, 1984). Results are means $\pm$ S.E.M. for the numbers of livers are shown in parentheses. * $P < 0.001$ for effect of diet or diabetes. For other differences relating to effects of diet or diabetes, or for immunoassay versus spectrophotometric assay, $P > 0.05$.

Rats	Diet	Branched-chain complex (units/g of mitochondrial protein)			Active form (% of total)
		Immunoassay	Spectrophotometric assay		
			Total complex	Total complex	
Normal	Normal	9.3 ± 0.65 (15)	9.2 ± 0.12 (16)	3.1 ± 0.34 (18)	34 ± 2.80
Normal	0% casein	9.1 ± 0.85 (6)	8.9 ± 0.19 (8)	0.3 ± 0.03* (15)	3.4 ± 0.29*
Normal	Starved 48 h	11.8 ± 0.58 (4)	9.8 ± 0.61 (8)	0.3 ± 0.06* (8)	3.1 ± 0.48*
Alloxan-diabetic	Normal	—	10.0 ± 0.42 (12)	1.1 ± 0.26* (8)	11 ± 1.8*

because of the results obtained with Western blotting and because of the close agreement between spectrophotometric assay and immunoassay with mitochondrial extracts from normal rats fed on a normal diet. It was possible that there was a specific loss of E1 on 0% -casein diet and that the concentration of E2 is maintained. This is unlikely, however, because addition of free E1

(prepared from rat liver mitochondria or ox kidney complex) failed to increase branched-chain holocomplex activity in extracts of liver mitochondria from rats fed on 0% -casein diet. This left as a likely possibility failure to achieve complete conversion of inactive complex into active complex before spectrophotometric assay of total complex in the previous study (Patston *et al.*, 1984). In

that study pilot experiments of 40 min duration had suggested that 30 min of incubation of mitochondria in KCl medium sufficed to effect complete conversion of inactive complex into active complex.

The conversion of inactive complex into active complex in rat liver mitochondria has been re-investigated by prolonging incubation for up to 240 min and by inclusion of 5 mM-ketoleucine (4-methyl-2-oxopentanoate) (to inhibit branched-chain kinase). Livers of rats fed on 0%-casein diets contain a substantial quantity of triacylglycerol readily visible during isolation of mitochondria; mitochondria may therefore contain a substantial amount of endogenous respiratory substrate, and in consequence ATP may be available for the kinase reaction. As shown in Fig. 3(a), conversion of inactive complex into active complex in liver mitochondria from normal rats fed on normal diet was maximum in 20 min and remained unchanged for up to 180 min, and may have declined thereafter. With liver mitochondria from rats fed on 0%-casein diet, maximum conversion required 120 min of incubation and remained unchanged up to 180 min and may have declined thereafter. There was an apparent break in the curve at 20–30 min, with a subsequent slower rate of conversion. This may explain why pilot experiments with ketoleucine in the previous study (Patston *et al.*, 1984) suggested complete conversion by 30 min. In the absence of ketoleucine (the present work), conversion was even slower (results not shown). With the more prolonged incubation with ketoleucine in the present study there was no difference in total branched-chain complex activity in liver mitochondrial between normal rats fed on normal diets or 0%-casein diets or starved for 48 h, or between normal rats and alloxan-diabetic rats (see Table 1).

The activity of activator protein (free E1) was also assayed in mitochondria in these prolonged incubations because of the possibility that it may be present in a phosphorylated and inactive form, especially in rats fed on 0%-casein diet, where its activity is low (Patston *et al.*, 1984). The results are shown in Fig. 3(b). The activity of activator protein increased significantly between zero time and 30 min of incubation, but decreased substantially thereafter. The increase is assumed to result from dephosphorylation. The reason for the decline has not been established. Intramitochondrial inactivation seems unlikely, as holocomplex E1 activity was maintained for at least 180 min in the same experiment. The medium in which mitochondria had been incubated for 80 min contained activator protein, as shown by bioassay, but the activity was insufficient to account for the loss of activity in mitochondria (results not shown). It is suggested that activator protein (free E1) was lost from mitochondria to the incubation medium, but that recovery was incomplete because of inactivation in the incubation medium. This finding provides further evidence for the view that free E1 makes no contribution to the activity of dephosphorylated branched-chain complex in liver (cf. Espinal *et al.*, 1985). Comparable studies with heart mitochondria failed to reveal activator protein at any time point (results not shown; cf. Fatania *et al.*, 1982; Espinal *et al.*, 1985).

General discussion and conclusions

Heffelfinger *et al.* (1983) have described antisera to bovine liver branched-chain complex and have shown that rocket immunoelectrophoresis may detect complex

Fig. 3. Activities of (a) branched-chain complex and (b) activator protein (free E1) in liver mitochondria from rats fed on normal diet (O) or 0%-casein diet (●)

Rat liver mitochondria (6 mg of protein) were incubated at 30 °C in KCl medium (5 mM-ketoleucine); mitochondria were separated, frozen in liquid N₂, and extracts were prepared (48 mg of protein/ml) and assayed for branched-chain complex (10 or 20 μ l). Extracts were centrifuged for 90 min at 150000 g, and 40 μ l of supernatant was assayed for activator protein. Points are means of duplicate determinations on mitochondrial preparations from at least four livers. Values for branched-chain complex are not corrected for the blank rate in the absence of ketoleucine (see the legend to Table 1). Activities of activator protein were computed as $100/K_{0.5}$, where $K_{0.5}$ is the concentration of mitochondrial protein (mg/ml in the cuvette) required for 50% re-activation of 10 munits of phosphorylated complex (see Patston *et al.*, 1984).

in extracts of bovine and mouse liver mitochondria. We show here that an antiserum to the E2 component of bovine kidney complex may be used to assay total complex in rat heart and liver mitochondria. The results obtained were strictly comparable with those obtained by spectrophotometric assay of the holocomplex reaction after complete conversion of inactive complex into active complex. The reactions catalysed by E2 are apparently rate-limiting for the holocomplex reaction. In purified bovine kidney complex, addition of E2 increased the rate by approx. 50% (results not shown), whereas addition of E1 was without effect (Espinal *et al.*, 1985; also results not shown). Comparability between immunoassay of E2 and bioassay of total holocomplex may indicate that E2 is rate-limiting in the holocomplex reaction in liver mitochondria. The presence of excess (free) E1 further supports this view.

Based on mitochondrial concentrations of 100 mg of protein/g fresh wt. of liver or heart (Foden & Randle, 1978; Randle *et al.*, 1970) the tissue concentration of total complex (sum of active and inactive forms) may be computed as approx. 930 (liver) and 610 (heart) munits/g fresh wt. These are much higher than the values of 136–178 (liver) and 301–330 (heart) given by Wagenmakers *et al.* (1984a,b), and greater than values of approx. 650 (liver) and 300 (heart) given by Gillim *et al.* (1983). This apparent underestimation of total complex in liver could explain why the percentage of active complex was found to be greater by Gillim *et al.* (1983) and Wagenmakers *et al.* (1984a,b) than in the present study (for results of the present study see Table 1, column 6). A further and important factor may be our use of fluoride/ketoleucine to prevent interconversion during isolation of mitochondria for assay of the active form. Thus when liver mitochondria (fed rats) were isolated in the absence of fluoride/ketoleucine, the percentage of active complex (75%, see Fig. 1a) was greater than when mitochondria were isolated in their presence (34%; see Table 1) (see also Patston *et al.*, 1984). The percentage of active complex in liver mitochondria in the study by Patston *et al.* (1984) was greater than in the present study (55%, as compared with 34%). This is attributed to the higher estimate for total complex in the present study; the activities of the active form were comparable (see also Espinal *et al.*, 1985).

The present study has shown that, contrary to earlier findings, total branched-chain complex in rat liver mitochondria was not decreased by low-protein diet, starvation or alloxan-diabetes. The decreases reported in the earlier study by Patston *et al.* (1984) are shown to be due to failure to effect complete conversion of inactive complex into active complex before assay, and the likely reasons for this are given. Gillim *et al.* (1983) have also described a 70% decrease in total complex in livers of rats fed on a low-protein diet, but they utilized a rat liver cytosolic phosphoprotein phosphatase to convert inactive complex into active complex after extraction. The

decrease in total complex in the studies of Gillim *et al.* (1983) is not explained by our present findings.

We conclude that the substantial effects of low-protein diet, starvation and alloxan-diabetes to decrease hepatic branched-chain complex activity are due to inactivation of the complex by phosphorylation. In rats fed on a normal diet, approx. 70% of active complex is hepatic, and > 70% of the decrease occasioned by a low protein diet is also hepatic. The present study indicates therefore that these adaptive changes to dietary protein intake are mediated by reversible phosphorylation.

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