Purification and properties of a protein activator of phosphorylated branched-chain 2-oxo acid dehydrogenase complex

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The protein activator of phosphorylated branched-chain 2-oxo acid dehydrogenase complex was purified > 1000-fold from extracts of rat liver mitochondria; the specific activity was > 1000 units/mg of protein (1 unit gives half-maximum re-activation of 10 munits of phosphorylated complex). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis gave two bands (M_r 47 700 and 35 300) indistinguishable from the α - and β -subunits of the branched-chain dehydrogenase component of the complex. On gel filtration (Sephacryl S-300), apparent M_r was 190 000. This and other evidence suggests that activator protein is free branched-chain dehydrogenase; this conclusion is provisional until identical amino acid composition of the subunits has been demonstrated. Activator protein (i.e. free branched-chain dehydrogenase) was inhibited (up to 30%) by NaF, whereas branched-chain complex was not inhibited. There was no convincing evidence for interconvertible active and inactive forms of activator protein in rat liver mitochondria. Activator protein was detected in mitochondria from liver (ox, rabbit and rat) and kidney (ox and rat), but not in rat heart or skeletal-muscle mitochondria. In rat liver mitochondrial extracts, branched-chain complex sedimented with the mitochondrial membranes, whereas activator protein remained in the supernatant. Activator protein re-activated phosphorylated (inactive) particulate complex from rat liver mitochondria, but it did not activate dephosphorylated complex. Liver and kidney, but not muscle, mitochondria apparently contain surplus free branched-chain dehydrogenase, which is bound by the complex with lower affinity than is the branched-chain dehydrogenase intrinsic to the complex. It is suggested that this functions as a buffering mechanism to maintain branched-chain complex activity in liver and kidney mitochondria.

The mitochondrial branched-chain 2-oxo acid dehydrogenase complex (no EC numbers) (subsequently termed 'branched-chain complex') of animal tissues is inactivated by phosphorylation of the α -chain of the branched-chain dehydrogenase component and re-activated by dephosphorylation. These reactions are catalysed by an intrinsic kinase and a mitochondrial phosphatase respectively (Fatania *et al.*, 1981; Odessey, 1982; Lau *et al.*, 1982; Fatania *et al.*, 1983; Damuni *et al.*, 1984). Phosphorylated complex (but not dephosphorylated complex) is also re-activated within seconds, but without dephosphorylation, by a protein

(activator protein) present in a high-speed-supernatant fraction of rat liver and kidney mitochondria (Fatania *et al.*, 1982; Yeaman *et al.*, 1984). Activator protein was not detected in a comparable fraction of rat heart or skeletal-muscle mitochondria, and its activity in liver and kidney mitochondria is decreased by low-protein diet, starvation or (in liver only) alloxan-diabetes (Fatania *et al.*, 1982; Patston *et al.*, 1984). The purification and properties of activator protein are described here.

Experimental

Materials and methods

Sephadex and Sephacryl S-300 were from Pharmacia (G.B.) Ltd., Hounslow, Middx., U.K.;

Abbreviation used: SDS, sodium dodecyl sulphate.

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DEAE-cellulose (DE52) was from Whatman, Maidstone, Kent, U.K.; Protein-Pak DEAE-5PW column was from Waters Associates, Northwich, Cheshire, U.K.; Amicon Centriflo ultrafiltration membrane cones were from Amicon Corp., Danvers, MA, U.S.A. Branched-chain complex was purified from ox kidney mitochondria by the method of Fatania *et al.* (1981). The transacylase core and the branched-chain dehydrogenase component were separated from the complex by gel filtration on Sephacryl S-300 by using conditions given by Pettit *et al.* (1978). Mitochondria were isolated from rat tissues in sucrose medium, and mitochondrial incubations were made in KCl medium as described by Patston *et al.* (1984). Mitochondrial extracts and high-speed-supernatant fractions as sources of activator protein were prepared by freeze/thaw (50 mg of protein/ml) in buffer A [30 mM-potassium phosphate/10 mM-benzamide/1 mM-phenylmethanesulphonyl fluoride/5 mM-dithiothreitol/5 mM-EGTA/1% (v/v) ethanol (pH 7.4)] with addition of 1% (v/v) ox serum (as described by Patston *et al.*, 1984). In some experiments mitochondria were isolated in the presence of 100 mM-NaF/5 mM-ketoleucine (4-methyl-2-oxopentanoate), and high-speed-supernatant fractions were prepared in the presence of 100 mM-NaF as shown in the Tables or the text (see Patston *et al.*, 1984). Sources of rats and other materials are given by Patston *et al.* (1984).

Branched-chain complex was assayed spectrophotometrically by methods given in Lau *et al.* (1981) (mitochondrial extracts) and Fatania *et al.* (1982) (purified complex). One unit of enzyme

forms 1 μ mol of NADH/min at 30°C. Activator protein was assayed by the method of Fatania *et al.* (1982); one unit of activator protein (by definition) gives half-maximum re-activation of 10 munits of phosphorylated complex (see Patston *et al.*, 1984). Protein was estimated by the biuret method (albumin standards; Gornall *et al.*, 1949) or with Coomassie Blue (γ -globulin standards; Bradford, 1976). SDS/polyacrylamide-gel electrophoresis was as described by Lau *et al.* (1981), except that 15 cm $\times$ 14 cm gels were used. Where necessary, protein samples for electrophoresis were concentrated and/or freed of potassium salts by ultrafiltration (CF25 Centriflo cones).

Results and discussion

Purification of activator protein

Routinely preparations used five to ten livers from 250–350 g rats, yielding approx. 30 mg of mitochondrial protein/g fresh wt. of liver and 20 units of activator protein/g fresh wt. of liver in the 150 000 g-supernatant fraction of the mitochondrial extract. Preparation of the supernatant fraction resulted in approx. 4-fold purification and 50% loss of activity (Table 1, step 2). Activator protein was then concentrated by $(\text{NH}_4)_2\text{SO}_4$ fractionation, being precipitated between 20 and 50% saturation in 80% yield with 3-fold purification (Table 1, step 3). The precipitate was taken up in Buffer A and applied to a 1 ml column of DEAE-cellulose (DE52) equilibrated with 30 mM-potassium phosphate/5 mM-dithiothreitol/5 mM-EGTA (pH 7.5) and eluted with a linear gradient of 0.05–0.5 M-NaCl in the same buffer. In

Table 1. Purification of protein activator of phosphorylated branched-chain 2-oxo acid dehydrogenase complex

For further details of individual steps in fractionation see the Experimental section or the text. Steps 4a–4d were alternative fractionation procedures which followed step 3. A unit of activator protein produces half-maximum re-activation of 10 munits of phosphorylated branched-chain complex. Protein was estimated by the biuret method (steps 1–3) or with Coomassie Blue (steps 4a–4d). Results are means $\pm$ s.e.m. for the numbers of preparations in parentheses.

Step	Fraction	Activity (units/mg of protein)	Mean recovery (%)		Mean purification (fold)	
			Step	Overall	Step	Overall
1	Mitochondrial extract	1.1 $\pm$ 0.05 (12)	—	100	—	1
2	150 000 g supernatant	4.5 $\pm$ 0.6 (26)	46	46	4.1	4.1
3	20–50%-sat.-(NH_4) ₂ SO ₄ precipitate	12.3 $\pm$ 0.5 (8)	80	37	2.7	11.2
4a	DE52 eluate					
	Overall	217 $\pm$ 66 (4)	68	25	18	197
	Most active fraction	667 $\pm$ 220 (2)	18	7	54	606
4b	H.p.l.c. (DEAE-5PW) eluate					
	Overall	500 $\pm$ 110 (8)	55	20	41	455
	Most active fraction	1149 $\pm$ 211 (8)	18	7	93	1044
4c	Sephadex G-100 (superfine) eluate	12.3 (1)	50	18	1	—
4d	Sephadex G-150 eluate	12.3 (1)	32	12	1	—

some experiments gel filtration on a 130 ml column of Sephadex G-150 equilibrated with buffer A preceded DE52 chromatography (step 4d, Table 1). Activator protein was eluted from DE52 at approx. 0.3M-NaCl in 70% yield with 20-fold purification. Overall yield and purification were 25% and 200-fold respectively (Table 1, step 4a). The most active fraction was obtained in 7% yield with 600-fold purification. SDS/polyacrylamide-gel electrophoresis (with seven standards in the M_r range 66000–14200) showed three bands of M_r 72050, 48600 and 38900. On gel filtration of the $(\text{NH}_4)_2\text{SO}_4$ fraction on Sephadex G-100 (superfine grade) (step 4c, Table 1) or G-150 (step 4d, Table 1) (bed volumes 130 ml and 185 ml respectively), the elution volumes were: Blue Dextran 42.5 ml, activator protein 36–48 ml (G-100, superfine) or Blue Dextran 62–65 ml, activator protein 73–92 ml (G-150). Recovery was poor and no purification was achieved (Table 1, steps 4c and 4d). The results suggested an apparent M_r for activator protein in the range 100000–300000. The possibility that activator protein was the branched-chain oxo acid dehydrogenase component of the complex was suggested by the subunit M_r of 48600 and 38900. At this point further progress was rendered unlikely, mainly because of low yield of activator protein and because of loss of activity during storage of dilute solutions from DE52 chromatography (temperatures ranged from -80°C to room temperature). Attempts to purify activator protein from the $(\text{NH}_4)_2\text{SO}_4$ fraction by poly(ethylene glycol) fractionation, chromatography on CM-cellulose, hydroxyapatite, Affigel Blue, Matrex Red or Matrex Blue were either unsuccessful or showed no advantage over DE52 (results not shown).

With the availability of high-performance liquid chromatography on a Protein-Pak DEAE-5PW column, attempts at purification from the $(\text{NH}_4)_2\text{SO}_4$ fraction were resumed, and these were successful. The most active fractions obtained with a linear gradient of 0.2–0.5M-KCl in 30 mM-potassium phosphate/5 mM-dithiothreitol/5 mM-EGTA (pH 7.4) resulted in 90-fold purification and 18% yield. In eight successive preparations overall purification in the most active fraction averaged approx. 1000-fold, overall yield 7% and average specific activity approx. 1000 units/mg of protein (see Table 1, step 4b). Some preparations achieved approx. 5000-fold purification and 5000 units/mg of protein, but, in view of errors involved in protein estimations of $<1\mu\text{g}$, it seemed appropriate to emphasize the overall average of eight preparations. On SDS/polyacrylamide-gel electrophoresis the most active fractions showed only two bands, of M_r 47668 ± 338 and 35326 ± 178 (means $\pm$ S.E.M. for seven observations, with seven

Fig. 1. Subunit M_r of activator protein by SDS/polyacrylamide-gel electrophoresis

(a) SDS/polyacrylamide-gel electrophoresis of activator protein purified by h.p.l.c. (outer tracks) and of M_r markers [centre track (left to right), M_r = 66000, 45000, 36000, 29000, 24000, 20100, 14200]. Coomassie Blue stain. (b) Reflectance scan (Chromoscan 3: Joyce Loeb) of photograph of gel track for activator protein.

standard proteins of M_r range 66000–14200) (see Fig. 1). Ox kidney branched-chain complex on the same gel showed three bands, M_r 53000 ± 550 , 47464 ± 450 and 35100 ± 390 , corresponding to the acyltransferase subunit and the α - and β -subunits of the branched-chain oxo acid dehydrogenase component respectively. The M_r values of purified activator protein were indistinguishable from those of the α - and β -subunits of the branched-chain oxo acid dehydrogenase component of the complex.

The apparent M_r of activator protein [$(\text{NH}_4)_2\text{SO}_4$ fraction, 50 or 100 units] was estimated by gel filtration on Sephacryl S-300 (130 ml) calibrated with five standards (M_r 670000, 158000, 44000, 17000 and 1350). Least-squares linear-regression analysis [$\log M_r$ against (elution volume – void volume)] gave $r = -0.999$, slope -0.05 ± 0.0009 (mean $\pm$ S.E.M.) and intercept (void-volume M_r) of $1.43 \times 10^6 \pm 672$. Duplicate runs gave apparent M_r values (activator protein) of 189010 and 191438. Therefore activator protein is unlikely to be a dimer of the two dissimilar subunits, but could be a tetramer (estimated M_r 166000). The M_r of the branched-chain dehydrogenase component of purified complex has not been reported.

If activator protein is the branched-chain dehydrogenase component of the complex, then

activator protein should restore complex activity to the acyltransferase in the presence of lipoamide dehydrogenase. This has been shown by spectrophotometric assay with activator protein purified by high-performance liquid chromatography (steps 1–3 and 4b in Table 1); 2.5 units of activator protein gave rise to 2.94 ± 0.15 munits of branched-chain complex (mean $\pm$ s.e.m. for four assays).

With phosphorylated branched-chain complex (0.33 unit/ml; prepared with 50 μ M-ATP), ATP-dependent inactivation of activator protein (3.3 units/ml; purified by high-performance liquid chromatography) was demonstrable after addition of ATP to 0.5 mM. The reaction was pseudo-first-order, and the apparent first-order rate constant (least-squares linear-regression analysis, $r = -0.957$) was $0.046 \pm 0.005 \text{ min}^{-1}$, $t_{1/2} = 15 \pm 1.6 \text{ min}$ (mean $\pm$ s.e.m. for ten observations). The corresponding rate constant for inactivation of branched-chain complex alone under otherwise identical conditions was $0.045 \pm 0.004 \text{ min}^{-1}$, $t_{1/2} = 15.4 \text{ min}$ ($P > 0.05$ for difference from activator protein). The slow rate of inactivation was due to the presence of approx. 0.3 M-KCl ($t_{1/2}$ without KCl added was 1.1 min for complex alone).

Properties of activator protein

Tissue distribution: effects of diet and of the phosphorylation state of the branched-chain complex. Activator protein has been demonstrated in high-speed-supernatant fractions prepared from extracts of mitochondria from rabbit, ox or pig liver

and ox kidney (results not shown), rat kidney (Patston *et al.*, 1984) and rat liver (Fatania *et al.*, 1982; Patston *et al.*, 1984; Tables 1 and 2).

No activator protein was detected in rat heart or skeletal-muscle mitochondria, irrespective of whether branched-chain complex was predominantly in the phosphorylated (inactive) form or wholly in the dephosphorylated (active) form. The results shown in Table 2 are typical of many. All values are shown as zero because no activation of phosphorylated branched-chain complex was seen in any assay. Assays were performed with high-speed-supernatant fraction equivalent to 1.5–2 mg of mitochondrial protein; the lower limit of detection of activator protein was approx. 20 units/g of mitochondrial protein or <3% of the activity in liver mitochondria. The s.e.m. is based on the three rate measurements involved in each assay (phosphorylated complex alone; supernatant alone; both). In many assays the combined rate was slightly lower than the sum of the individual rates, but the difference was not statistically significant ($P > 0.05$). The experiments included muscle mitochondria made without proteinases, because Nagarse and trypsin inactivate branched-chain complex in mitochondria (Patston *et al.*, 1984); proteinases could inactivate activator protein in mitochondria.

Is activator protein inactivated by phosphorylation in vivo? Activator protein undergoes ATP-dependent inactivation in the presence of phosphorylated branched-chain complex (Yeaman *et al.*, 1984; see above). The active form of branched-

Table 2. Activities of branched-chain 2-oxo acid dehydrogenase complex and of activator protein in mitochondria from rat heart, skeletal muscle and liver

Mitochondria were prepared from tissues of rats fed on a standard diet, and extracts were prepared for assay of branched-chain complex and of activator protein as described in the Experimental section. Mitochondrial incubations were in KCl medium (2 mg of mitochondrial protein/ml) for 30 min at 30°C (no substrate) or in KCl medium containing 5 mM-2-oxoglutarate/0.5 mM-L-malate for 10 min with aeration at 5 min. Results are means $\pm$ s.e.m.; n is the number of mitochondrial samples assayed from a single preparation (heart, skeletal muscle) or 12 preparations each from five or ten rats (liver). A unit of branched-chain complex forms 1 μ mol of NADH/min at 30°C. A unit of activator protein produces half-maximum re-activation of 10 munits of phosphorylated branched-chain complex.

* $P < 0.001$ for effect of no-substrate incubation. For other differences within experiments (tissues) $P > 0.05$.

Tissue	Method	Incubation	n	Activity (units/g of mitochondrial protein) for:	
				Branched-chain complex	Activator protein
Heart	Polytron	None (as made)	4	1.05 ± 0.15	0 ± 0
		No substrate	8	$6.91 \pm 0.15^*$	0 ± 0.10
Heart	Nagarse	None (as made)	4	0.50 ± 0.08	0 ± 0.2
		No substrate	4	$1.25 \pm 0.01^*$	0 ± 0.2
		2-Oxoglutarate/L-malate	4	0.02 ± 0.01	0 ± 0.2
		No substrate	4	0.43 ± 0.04	0 ± 0
Hind-limb muscle	Polytron	None (as made)	4	$1.42 \pm 0.08^*$	0 ± 0.06
		No substrate	4	—	0 ± 0.08
Liver	Trypsin	None (as made)	4	—	720 ± 96
		None (as made)	12	—	792 ± 57
		No substrate	12	10.60 ± 0.50	792 ± 57

Table 3. Activity of branched-chain 2-oxo acid dehydrogenase complex and of activator protein in rat liver mitochondria; effect of diet and of NaF

Rat liver mitochondria (normal rats) were prepared in sucrose medium either without (normal preparation) or with 100 mM-NaF/5 mM-ketoleucine (NaF/ketoleucine). Mitochondria prepared without NaF/ketoleucine were incubated for 30 min at 30°C in KCl medium to effect conversion of inactive complex into active complex (see Patston *et al.*, 1984). Extracts were prepared in buffer A and assayed for branched-chain complex. A portion of the extract was centrifuged for 2 h at 150 000g without (normal preparation) or with addition of NaF to 100 mM (NaF), and the supernatant was assayed for activator protein. Units are defined in Table 2. Results are means $\pm$ S.E.M.; the numbers of assays (on mitochondria from three livers) are shown in parentheses. * $P < 0.01$, † $P < 0.05$ for effect of NaF/ketoleucine and/or NaF; ‡ $P < 0.01$ for effect on 2-oxoglutarate/L-malate; § $P < 0.01$ for effect of diet. For other differences $P > 0.05$.

Rats	Mitochondrial preparation medium (and incubation)	Mitochondrial supernatant	Activity (units/g of mitochondrial protein)	
			Branched-chain complex	Activator protein
Fed, standard diet	Normal (no substrate)	Normal	10.60 $\pm$ 0.50 (6)	792 $\pm$ 57 (24)
	NaF/ketoleucine (not incubated)	NaF	2.70 $\pm$ 0.05* (6)	394 $\pm$ 58* (6)
Fed, standard diet	Normal (no substrate)	—	12.00 $\pm$ 0.88 (4)	—
	Normal (no substrate) isolated with NaF	—	12.18 $\pm$ 0.93 (4)	—
Starved, 48 h	Normal (no substrate)	Normal	5.57 $\pm$ 0.23 (14)	337 $\pm$ 15 (5)
	Normal (no substrate)	NaF	5.57 $\pm$ 0.23 (14)	187 $\pm$ 28* (5)
	NaF/ketoleucine (not incubated)	Normal	0.29 $\pm$ 0.03* (14)	160 $\pm$ 17* (5)
	NaF/ketoleucine (not incubated)	NaF	0.29 $\pm$ 0.03* (14)	103 $\pm$ 10*† (4)
Starved, 48 h	Normal (no substrate)	Normal	4.27 $\pm$ 0.53 (6)	203 $\pm$ 28 (6)
	Normal (5 mM-2-oxoglutarate/0.5 mM-L-malate)	Normal	2.40 $\pm$ 0.18‡ (8)	207 $\pm$ 28 (8)
Zero-casein diet	Normal (no substrate)	Normal	0.13 $\pm$ 0.05§ (4)	7 $\pm$ 0.9§ (6)
	NaF/ketoleucine (not incubated)	Normal	0.40 $\pm$ 0.07§ (5)	4 $\pm$ 0.6§ (6)

chain complex in tissues may be assayed in mitochondria isolated in the presence of NaF and ketoleucine, which inhibit branched-chain phosphatase and kinase respectively. Total complex (sum of active and inactive forms) may be assayed in mitochondria isolated in the absence of these inhibitors after incubation in KCl medium for 30 min to effect conversion of inactive complex into active complex (Patston *et al.*, 1984). Application of this technique to assay activator protein appeared to show that only a proportion (approx. 50%) of activator protein was in the active form in liver mitochondria of either fed or starved rats; the proportions of branched-chain complex in the active form in the same experiments were about 25% and 5% respectively (Table 3; cf. line 1 with 2 and line 5 with 7). When NaF was added to the extract to 100 mM during preparation of the 150 000g-supernatant fraction, activator-protein activity fell to about 30% (starved rats; cf. line 5 with 8 in Table 3). At first sight these results suggested interconvertible active and inactive forms of activator protein, but there were a number of puzzling features. In mitochondria in which inactive complex had been converted into active complex by no-substrate incubation for 30 min, subsequent inclusion of NaF in extraction buffer

during preparation of the supernatant fraction still lowered activator-protein activity by 50% (Table 3; cf. line 5 with 6). Because of depletion of ATP, phosphorylation in such extracts is perhaps unlikely. When mitochondria were isolated without NaF/ketoleucine and complex activity was lowered by incubation with 2-oxoglutarate/L-malate, activator-protein activity was not decreased when extracts were prepared and centrifuged in the absence of NaF (Table 3, cf. line 9 with 10). Moreover, the estimates of the apparent proportion of activator protein in a active form in Table 3 are minimum estimates. In other experiments (not shown) the proportion in starved rats was 70–80%.

Assay of branched-chain complex is not influenced by NaF (Patston *et al.*, 1984). As shown in Table 3 (lines 3 and 4), branched-chain complex activity in mitochondria in which inactive complex had been converted into active complex was not changed when mitochondria were kept for 10 min at 0°C with 100 mM-NaF before sedimentation and extraction. When activator protein [5.7 units of (NH₄)₂SO₄ fraction] was added to extracts of liver mitochondria from 48 h-starved rats isolated in the presence of NaF/ketoleucine, no reactivation of inactive branched-chain complex

was seen. The activities (units of branched-chain complex/g of mitochondrial protein; means $\pm$ S.E.M. for six observations) were 1.95 ± 0.06 and 1.95 ± 0.07 in the absence and in the presence of activator protein respectively. The expected activity with 5.72 units of activator protein was approx. 5.03 units of complex/g of mitochondrial protein. In experiments with phosphorylated ox kidney complex, re-activation by activator protein was inhibited by 0.7 mM- or 1.4 mM-NaF (this range of concentrations encompasses the estimated concentration of NaF in the assay with extracts of mitochondria isolated in 100 mM-NaF). The degree of inhibition depended on the amount of activator protein, being approx. 20% at 0.5–1 unit/ml and 30% below 0.5 unit/ml. We conclude that the decrease in activator-protein activity occasioned by isolation of mitochondria in NaF/ketoleucine (approx. 50%; cf. line 1 with 2, 5 with 6, 5 with 7 and 6 with 8 in Table 3) may be attributed, at least in part, to inhibition by NaF. There is no conclusive evidence in these experiments for inactivation of activator protein by phosphorylation in liver mitochondria *in vivo* or *in vitro*. In other experiments it was shown that activator protein is not released from mitochondria by NaF/ketoleucine, and that NaF/ketoleucine had no effect on the recovery of activator protein during preparation of the 150 000 g-supernatant fraction (results not shown).

These observations are of especial importance for two reasons. Firstly it is likely that the actions of the two forms of branched-chain dehydrogenase (i.e. activator protein and branched-chain dehydrogenase intrinsic to the complex) differ because one is inhibited, albeit modestly, by NaF and the other is not. Secondly it is unlikely that activator protein activates inactive complex in extracts of mitochondria isolated in the presence of NaF/ketoleucine. Therefore the concentrations of active complex in tissues measured by isolation of mitochondria in the presence of NaF/ketoleucine are likely to be close to the true concentrations (cf. Patston *et al.*, 1984).

In rats fed on zero-casein diet the activities of activator protein and of branched-chain complex in liver mitochondria were very low under all conditions (Table 3, lines 11 and 12). It would appear that free branched-chain dehydrogenase and branched-chain complex were essentially absent from these mitochondria.

Mitochondrial location of branched-chain complex and of activator protein. Activator protein is present and may be assayed in extracts of rat liver mitochondria without high-speed centrifugation to remove branched-chain complex. Branched-chain complex (active form) was first assayed and then 10 munits of phosphorylated complex added. The

activity of activator protein was computed from the increment in branched-chain complex activity after addition of phosphorylated complex. In extracts prepared from liver mitochondria of fed rats, activities (in a typical experiment) were (means $\pm$ S.E.M. for eight observations) branched-chain complex 4.53 ± 0.3 units/g of mitochondrial protein; activator protein 1160 ± 29 units/g of mitochondrial protein. The activity of activator protein in non-centrifuged extracts was greater than in the high-speed-supernatant fraction prepared from them (720 ± 96) (see also Table 1). When particulate material was removed by centrifugation at 33 000 g for 5 min, the supernatant fraction was devoid of branched-chain complex activity. It was shown by resuspension and assay that branched-chain complex sedimented with the particulate fraction, although recovery was incomplete (approx. 35%; results not shown). Activator protein in the supernatant fraction (1009 ± 67 units/g of mitochondrial protein) was recovered in full after low-speed centrifugation. These observations suggest that activator protein may be located in the mitochondrial matrix (or possibly in the intermembrane space) and branched-chain complex in the mitochondrial inner membrane. Branched-chain complex extracted with Triton X-100 is not sedimented at 30 000 g for 5 min (results not shown). Location in the mitochondrial inner membrane is assumed because the mitochondrial complex utilizes intramitochondrial coenzymes.

Substrate for activator protein. In first describing activator protein (Fatania *et al.*, 1982), evidence was given that it activates phosphorylated branched-chain complex, but not dephosphorylated complex. The assumption was made that a modest (approx. 10%) activation of purified ox kidney branched-chain complex was due to phosphorylated complex present *in vivo* and co-purified with the non-phosphorylated complex. Further evidence in support of this assumption was given in a study of branched-chain dehydrogenase phosphatase by Fatania *et al.* (1983). As shown below, rat liver activator protein [(NH₄)₂SO₄ fraction] activated phosphorylated branched-chain complex in extracts prepared from rat heart mitochondria (Nagarse method) which had been incubated for 15 min at 30°C with 5 mM-2-oxoglutarate/0.5 mM-L-malate. There was no activation of dephosphorylated complex in extracts prepared from rat heart mitochondria which had been incubated in KCl medium (no substrate) for 15 min at 30°C. Branched-chain complex activities (units/g of mitochondrial protein; means $\pm$ S.E.M. for six observations) were: 2-oxoglutarate/L-malate 0.16 ± 0.05 (no activator protein) and 1.91 ± 0.07 ($P < 0.001$ for effect of activator protein); no-substrate incubation 2.69 ± 0.21 (no

activator protein) and 2.16 ± 0.13 ($P > 0.05$ for effect of activator protein).

It was important to show that branched-chain complex associated with the particulate fraction of rat liver mitochondrial extracts is inactivated by ATP and that the inactive complex so formed is reactivated by activator protein. Extracts (31 mg of protein/ml) were prepared (in buffer A) from liver mitochondria of fed rats (standard diet) which had been incubated for 30 min at 30°C to effect conversion of inactive complex into active complex (8.37 ± 0.11 units/g of mitochondrial protein; mean $\pm$ S.E.M. for four observations). The particulate fraction was separated (mitochondrial protein concentration 2 mg/ml; 33000g for 20 min) and resuspended to the original volume (buffer A). Branched-chain complex activity was 2.78 ± 0.08 units/g of mitochondrial protein (recovery 33%). For ATP inactivation the suspension was diluted with 2 vol. of 30 mM-potassium phosphate/5 mM-EGTA/2 mM-dithiothreitol (pH 7.4), and oligomycin B (to 50 μ g/ml) and $MgCl_2$ (to 1 mM) were added. Reaction was initiated at 30°C by addition of ATP to 0.5 mM. After 10 min of incubation with ATP ('+ATP'), activity fell to <10% of the control without ATP ('-ATP'). Addition of 1 unit of activator protein to the cuvette (high-speed-supernatant fraction from the same mitochondrial extract) resulted in complete re-activation of inactive complex. There was no re-activation of complex incubated without ATP [ratio of activities with activator protein (+ATP/-ATP) was 1.05 ± 0.05 ; mean $\pm$ S.E.M. of ratio for six observations].

General discussion and conclusion

Yeaman *et al.* (1984) have presented evidence that activator protein [$(NH_4)_2SO_4$ fraction] may be the free branched-chain dehydrogenase component of the branched-chain 2-oxo acid dehydrogenase complex. Their evidence comprised: restoration of complex activity to the acyltransferase core by activator protein plus lipoamide dehydrogenase; inactivation after incubation with ATP and phosphorylated branched-chain complex; inactivation after incubation with [γ - ^{32}P]-ATP and acyltransferase kinase, associated with ^{32}P incorporation into a protein of M_r 46000; and inhibition of activator protein by thiamin thiothiazolone pyrophosphate. Our own observations with respect to the first two of these lines of evidence are similar, with $(NH_4)_2SO_4$ fraction and with purified activator protein. It seemed desirable to achieve purification before concluding that activator protein is free branched-chain dehydrogenase. We have shown here that activator protein purified to apparent homogeneity displays two subunits on SDS/polyacrylamide-gel electrophore-

sis, with M_r values which are indistinguishable from those of the α - and β -subunits of branched-chain dehydrogenase. We conclude that activator protein displays all of the obvious properties of the free branched-chain dehydrogenase component of the complex. For reasons which are discussed below, this conclusion is provisional until such time as the primary structures of the individual subunits of activator protein and of the branched-chain dehydrogenase component of the complex are known to be identical. In the absence of such evidence we prefer to continue to use the term 'activator protein'.

The results of the present study provide further evidence (cf. Fatania *et al.*, 1982, 1983) that activator protein does not activate dephosphorylated complex. This has now been shown with purified bovine kidney complex, with fully dephosphorylated complex in extracts of rat heart mitochondria which lack activator protein, and with particulate complex of liver mitochondria which has been separated from activator protein by low-speed centrifugation. It follows, therefore, that in purified bovine complex and rat liver complex separated from activator protein, the activity of the branched-chain dehydrogenase component is not rate-limiting in the holocomplex reaction. Because saturating concentrations of substrates and of the added lipoamide dehydrogenase were used in assaying the holocomplex reaction, rate-limitation is presumably imposed by one of the elementary reactions involving the transacylase. Therefore, if activator protein is the free branched-chain dehydrogenase component, it is surplus to requirement for the dephospho-holocomplex reaction in liver and kidney mitochondria. Activator protein is apparently not present in heart and skeletal-muscle mitochondria, and in particular we have failed to detect it in extracts prepared from mitochondria in which inactive complex was converted into active complex by no substrate incubation. The possibility that it is present in a phosphorylated form, but is not dephosphorylated under these conditions, is considered unlikely, because of the lack of convincing evidence for an inactive form of activator protein in liver mitochondria. These findings do not support the suggestion by Yeaman *et al.* (1984) that failure to detect activator protein in heart mitochondria is due to the presence of inactive branched-chain complex in the mitochondrion. Evidence to the contrary was summarized in a review which they cite (Randle *et al.*, 1984).

Activator protein has been separated from the holocomplex in bovine kidney and rat liver mitochondrial extracts by precipitation of the complex at 150000g. It has also been separated from the complex in the absence of Triton X-100 by low-

speed centrifugation of rat liver mitochondrial extracts; 90s of centrifugation in an Eppendorff 3200 centrifuge suffices to yield a clear supernatant fraction containing all of the activator protein and none of the holocomplex (results not shown). Activator protein has also been demonstrated in mitochondrial extracts without centrifugation. This leads to the conclusions that activator protein may be located in the mitochondrial matrix (or possibly the intermembrane space), that branched-chain complex may be located in the mitochondrial inner membrane, and that the affinity of branched-chain holocomplex for activator protein is much less than the affinity for branched-chain dehydrogenase intrinsic to the holocomplex. This could suggest, for example, two categories of binding site for the branched-chain dehydrogenase in the holocomplex with different affinities. Alternatively activator protein might be structurally different in some way from branched-chain dehydrogenase intrinsic to the complex, resulting in lower affinity for the complex, e.g. as a result of gene duplication and mutation in one of a pair of genes. Further support for different actions of free branched-chain dehydrogenase (activator protein) and branched-chain dehydrogenase intrinsic to the complex is provided by the studies with NaF. These findings do not support the suggestion (Yeaman *et al.*, 1984) that activator protein is branched-chain dehydrogenase that has fallen from the complex during high-speed centrifugation.

It has been confirmed in the present study that activator protein is present in liver and kidney mitochondria, but not in heart and skeletal-muscle mitochondria (cf. Fatania *et al.*, 1982), and that activator protein and total holocomplex activity are decreased in liver mitochondria by 48h starvation and by zero-casein diet (cf. Patston *et al.*, 1984). Therefore some mechanism is required to explain why surplus branched-chain dehydrogenase may be produced in liver and kidney, but not in heart and skeletal muscle in rats fed on a normal diet, and to explain co-ordinate decreases in the overall concentrations of branched-chain complex and of activator protein (free branched-chain dehydrogenase) in livers and kidneys of rats fed on a low-protein diet or starved for 48h. Possibilities may include differences in the co-ordinated regulation of the genes for the component enzymes of the holocomplex as between liver and kidney and muscles, or expression of more than one gene for branched-chain dehydrogenase in liver and kidney, but not in muscles.

The function of free branched-chain dehydrogenase in liver and kidney mitochondria is envisaged as a buffering mechanism which

maintains holocomplex activity in these tissues, but not in muscles. Whether it may act by maintaining the activity of phosphorylated complex or by limiting phosphorylation of branched-chain dehydrogenase intrinsic to the complex is not known. The evidence for this working hypothesis is limited currently to the strong correlation between the activities of branched-chain complex and of activator protein in mitochondria from different tissues and in liver mitochondria from rats fed on different diets. The hypothesis is amenable to investigation, but, in view of the particulate nature of mitochondrial branched-chain complex, it is desirable to perform the studies with this preparation. The problems to be resolved include the recovery of full activity in this fraction and interference from associated ATPase(s). It would appear to be premature to conclude (Yeaman *et al.*, 1984) that it is difficult to envisage a regulatory role for free branched-chain dehydrogenase; one such role is envisaged here. The mechanism by which activator protein re-activates phosphorylated branched-chain complex is not known, but evidence given by Fatania *et al.* (1982) would suggest that it does not require exchange of activator protein (free branched-chain dehydrogenase) for phosphorylated branched-chain dehydrogenase intrinsic to the holocomplex.

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