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Purification of rat kidney branched-chain oxo acid dehydrogenase complex with endogenous kinase activity

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A method was devised to purify branched-chain oxo acid dehydrogenase (BCOAD) from rat kidney which retains endogenous kinase activity. Incorporation of ^{32}P into purified enzyme parallels the time course of enzyme inhibition by ATP. Phosphorylation occurs on a serine residue(s) of the 46000-mol.wt. subunit of the enzyme complex. Endogenous phosphatase activity is not present after purification, and added pyruvate dehydrogenase phosphate phosphatase does not re-activate BCOAD or liberate ^{32}P from previously labelled enzyme. These results demonstrate that BCOAD can be regulated by an endogenous protein kinase and that the phosphorylation-cycle enzymes regulating BCOAD appear to be distinct from those associated with pyruvate dehydrogenase complex.

BCOAD catalyses the rate-limiting step in the oxidation of the branched-chain amino acids in many tissues, including heart, skeletal muscle, kidney and adipose tissue (Odessey & Goldberg, 1979; Goodman, 1978; Dawson & Hird, 1967). Work in my (Odessey, 1980*a*,*b*) and other laboratories (Parker & Randle, 1978, 1980; Hughes & Halestrap, 1981) have shown that in isolated mitochondria this enzyme can be inhibited by an ATP-dependent phosphorylation. Several factors that alter enzyme activity have reciprocal effects on ^{32}P-labelling of the enzyme. By analogy with the PDH complex, it has been suggested that phosphorylation is mediated by an enzyme-associated protein kinase. However, previous purifications of this multienzyme complex have failed to detect phophorylating activity (Pettit *et al.*, 1978) or ATP-induced inhibition (Danner *et al.*, 1979). Neither PDH kinase nor the catalytic subunit of cyclic AMP-dependent protein kinase can stimulate ^{32}P incorporation into purified enzyme (Pettit *et al.*, 1978). These results suggest that if a BCOAD-specific kinase exists, it must be lost or degraded during isolation. To test this hypothesis, co-purification of enzyme and kinase activity was attempted.

Materials and methods

Purification

Digitonin-washed mitochondria were prepared as described previously (Odessey, 1980*b*). After removal of the digitonin, the mitochondrial pellet was washed once with 0.5mM-dithiothreitol (100ml/g of protein), followed by one wash with 20mM-potassium phosphate buffer (pH6.5) containing 0.2mM-thiamin pyrophosphate, 1mM-dithiothreitol and 1% dialysed horse serum. The pellet was resuspended in potassium phosphate buffer (30mg/ml) and frozen at −80°C. To purify BCOAD, the mitochondria were thawed and centrifuged (19000*g*, 30min). The supernatant was adjusted to pH6.6 with acetic acid (if necessary) and polyethylene glycol was slowly added with stirring (4°C) to a final concentration of 3.2% (v/v). After 10min the mixture was centrifuged (19000*g*, 10min) and the pellet resuspended at approx. 4 units/ml in kinase assay buffer [50mM-Tris/HCl, 10mM-potassium phosphate, 1mM-dithiothreitol, 0.5mM-$MgCl_2$, 0.2mM-thiamin pyrophosphate, pH7.4 (when prepared at 37°C)]. The solution was clarified by centrifugation (19000*g*, 15min). The supernatant was layered on a hydroxyapatite (Bio-Rad) column (1cm × 30cm) and washed with 2vol. of 0.14M-potassium phosphate (pH7.0)/1mM-dithiothreitol to remove most of the protein, including all PDH activity (Odessey, 1980*b*). BCOAD was eluted, with a 120ml linear gradient of phosphate (0.14–0.35M), at 0.21–0.28M. The fraction (0.24–0.28M) exhibiting high kinase activity (see the Results section) was centrifuged in a Beckman 60 Ti rotor at 114000*g* for 4h. The pellet was resuspended in kinase assay buffer (5mg/ml) and clarified by centrifugation in a Beckman Microfuge for 10min.

Assays

BCOAD was assayed as described previously

Abbreviations used: BCOAD, branched-chain oxo acid dehydrogenase (EC 1.2.4.4); PDH, pyruvate dehydrogenase complex (EC 1.2.4.1 + EC 2.3.1.12 + EC 1.6.4.3).

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(Odessey, 1980*b*). To measure ATP inhibition, 2.5 nmol of ATP was added to kinase assay buffer and the reaction was started by addition of enzyme (final vol. 25 μl). After 5 min the reaction was terminated by transfer of the mixture to ice-cold tubes containing 20 nmol of adenosine 5'-[β,γ-imido]triphosphate (Odessey, 1980*a*). BCOAD activity was assayed immediately.

To measure phosphorylation, the incubation mixture described above also contained [γ-^{32}P]ATP (2500 c.p.m./pmol). The reaction was terminated by spotting a sample of the mixture on ITLC strips (Fisher Scientific) previously spotted with a solution containing 20% (v/v) trichloracetic acid, 1 mM-ATP and 8 mM-potassium phosphate (Huang & Robinson, 1976; DePaoli-Roach *et al.*, 1979). The strips were developed in 5% trichloroacetic acid containing 0.2 M-KCl. The radioactivity remaining at the origin was cut out and counted in a Beckman 8000 liquid-scintillation counter (Beckman EP scintillant). Blanks of the incubation mixture without enzyme were run in triplicate and subtracted from the experimental values. Blanks were less than 5% of the maximum amount of labelled enzyme.

To measure dephosphorylation, a sample of enzyme was incubated with [^{32}P]ATP as described above and the reaction terminated with adenosine 5'-[β,γ-imido]triphosphate. Excess ATP and other small molecules were removed by centrifuging the mixture through columns (0.5 cm × 4 cm) of Sephadex G-25 (Pharmacia Fine Chemicals) equilibrated with kinase assay buffer (Lawrence & Larner, 1978). Control experiments showed that this procedure removed 95% of the ATP, and over 85% of the added BCOAD could be recovered in the column effluent. Enzyme solution (0.1 vol.) was incubated in either modified kinase assay buffer (0.5 mM-Mg^{2+}, 50 μM-Ca^{2+}) or modified KCl medium (Parker & Randle, 1980) (5 mM-Mg^{2+}, 0.1 mM-Ca^{2+}) at 37°C for periods up to 60 min. Phosphatase activity was measured as an increase in BCOAD activity and/or a decrease in acid-precipitable radioactivity.

Protein was assayed with a protein assay kit (Bio-Rad), with bovine serum albumin as a standard. This method gave values equivalent to those obtained by the Lowry procedure on purified BCOAD.

For phosphoamino acid analysis, a sample of ^{32}P-labelled BCOAD was hydrolysed under nitrogen in 6 M-HCl at 100°C for 60 min. The solution was evaporated to dryness, resuspended in water, and the procedure repeated to remove traces of HCl. The sample was then spotted on paper and electrophoresed at 2.5 kV for 1 h in 0.062 M-pyridine acetate (pH 3.5). Standards of phosphoamino acids were also run. After drying, the electrophoretogram was radioautographed.

All biochemicals were of the highest grade available from Sigma. [γ-^{32}P]ATP was obtained from New England Nuclear.

Results and discussion

The introduction of washing the digitonin-treated mitochondria with hypo-osmotic solutions resulted in the solubilization of 100% of the BCOAD activity after a single freezing and thawing. Furthermore, the BCOAD activity in the freeze–thaw extract was inhibited 95% by ATP (Table 1). However, further purification by precipitation with 50%-satd. $(NH_4)_2SO_4$ or by 3% polyethylene glycol in the presence of 10 mM-$MgCl_2$ caused the loss of ATP-inhibiting activity. These results suggest that previous failures to demonstrate BCOAD kinase activity (Pettit *et al.*, 1978; Danner *et al.*, 1979) were probably due to the omission of the digitonin washing step during mitochondrial isolation and/or the use of $(NH_4)_2SO_4$ or polyethylene glycol (in the presence of high Mg^{2+}) as precipitants. The loss of kinase activity may be due to enzyme inactivation, since remixing the supernatant (with or without the

Table 1. *Purification of BCOAD and associated kinase activity*

The preparation was from about 340 g of rat kidney. A unit is defined as 1 μmol of NADH formed/min at 37°C in the presence of excess dihydrolipoyl dehydrogenase and with 3-methyl-2-oxobutyrate as substrate. '+/− ATP' means ratio of activity after incubation with ATP (0.1 mM) for 5 min to that before incubation (see the Materials and methods section).

Fraction	Protein (mg)	Total activity (units)	Specific activity (units/mg)	+/− ATP
Freeze–thaw extract	580	45.1	0.078	0.05
Polyethylene glycol precipitate	65.5	38.8	0.59	0.06
Total hydroxyapatite peak	22.1	33.6	1.46	—
Low kinase (0.21–0.24 M)	9.9	13.6	1.23	0.53
High kinase (0.24–0.28 M)	12.2	20.0	1.64	0.11
Ultracentrifuge pellet	6.1	19.3	3.19	0.12

removal of the precipitant) failed to restore the ability to inhibit BCOAD by ATP. If, however, the freeze–thaw extract was adjusted to pH 6.6 (in the absence of $MgCl_2$), slow addition of polyethylene glycol to a final concentration of 3.2% precipitated 85% of the BCOAD activity and resulted in a 7.5-fold purification (Table 1). BCOAD could still be inhibited 94% by ATP (Table 1). Hydroxyapatite chromatography further purified the enzyme and separated it from pyruvate dehydrogenase (Odessey, 1980b). The use of a longer column and a less-steep phosphate gradient than used previously (Odessey, 1980*b*) revealed that some kinase activity can be lost from the complex at this step; a leading fraction of BCOAD activity (eluted at 0.21–0.24 M) was inhibited by about 50% by ATP, whereas the latter part of the peak (0.24–0·28 M) was inhibited by 90% and accounted for 60% of the total activity. Subsequent ultracentrifugation further purified the enzyme in this fraction and preserved kinase activity (Table 1).

Although the specific activity of the purified BCOAD is somewhat lower than previously observed (Odessey, 1980*b*; Pettit *et al.*, 1978), on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis only three bands are apparent (Fig. 1) and correspond to the decarboxylase dimer (37000 and 46000 mol.wt.) and the transacylase (52000 mol.wt.) (Pettit *et al.*, 1978). Furthermore, incubation of the purified enzyme with [γ-^{32}P]ATP for 5 min caused intense labelling of the 46000-mol.wt. band exclusively. This is also the subunit phosphorylated when the labelling is performed with intact mitochondria (Odessey, 1980*b*). The failure to observe a band corresponding to the endogenous kinase suggests that this component may be a small fraction of the total protein.

To demonstrate the relationship between phosphorylation and activity, purified enzyme (20 munits) was incubated with [^{32}P]ATP (0.1 mM) in kinase assay buffer. Inhibition occurred rapidly, with a half-time ($t_{\frac{1}{2}}$) of approx. 1.5 min. Concomitantly, ^{32}P was incorporated in the enzyme protein with a $t_{\frac{1}{2}}$ of 1.5 min (Fig. 2). Thus ^{32}P incorporation into BCOAD mirrored enzyme inhibition. Maximum incorporation corresponded to 0.72 nmol/unit. Acid hydrolysis and high-voltage electrophoresis of the labelled enzyme (see the Materials and methods section) showed that ^{32}P is incorporated exclusively into serine residues (Fig. 3). Calculation of the degree of 46000-mol.wt.-subunit phosphorylation must await precise knowledge of the molecular weight and subunit stoichiometry of the complex.

Although the BCOAD complex, purified as described above, retains endogenous kinase activity, phosphatase activity (in the presence or absence of Ca^{2+}) is not observed. Negative results were also obtained with freeze–thaw extracts and BCOAD prelabelled and purified as described previously (Odessey, 1980*b*). It therefore appears that endogenous phosphatase activity [which has only been observed in intact mitochondrial preparations (Odessey, 1980*a*; Parker & Randle, 1980; Hughes & Halestrap, 1981)] is either easily dissociated or degraded during an early stage of purification. Although some of these properties superficially resemble those of the regulatory enzymes of the PDH complex, purified pig heart or ox liver PDH phosphate

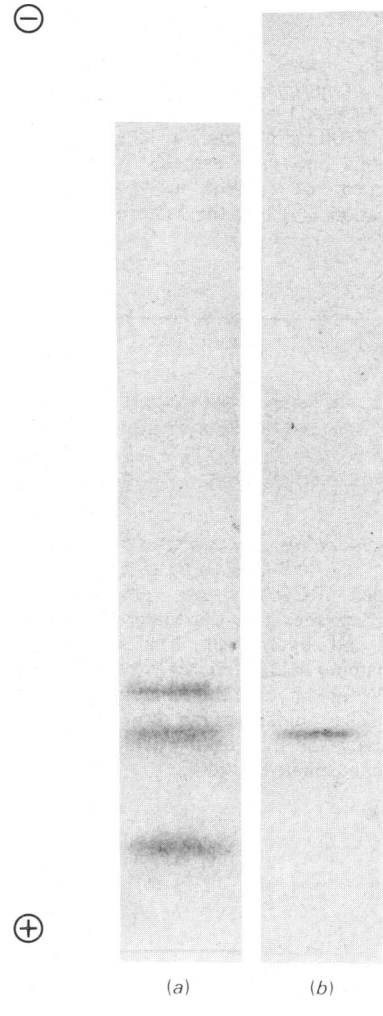

Fig. 1. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of ^{32}P-labelled BCOAD*

Slab gels (8% acrylamide) containing sodium dodecyl sulphate were run for 4 h as described previously (Odessey, 1980*b*). Track (*a*) is stained for protein with Coomassie Brilliant Blue. Track (*b*) is a radioautograph of the same gel.

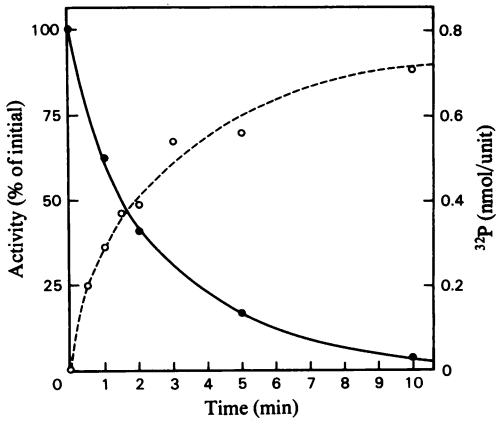

Fig. 2. *Time course of ATP inhibition and enzyme phosphorylation of BCOAD*

Purified BCOAD was incubated with [γ-^{32}P]ATP (0.1 mM; 2400 c.p.m./pmol) in kinase assay buffer. At various intervals samples were taken for measurement of enzyme activity (●) and ^{32}P incorporation (○) (see the Materials and methods section).

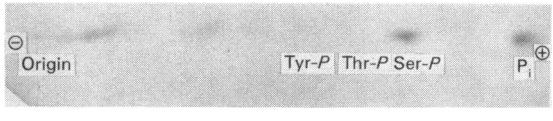

Fig. 3. *High-voltage electrophoresis of hydrolysate of ^{32}P-labelled BCOAD*

^{32}P-labelled BCOAD was prepared, dialysed through Sephadex (see the Materials and methods section) and hydrolysed. The hydrolysate and phosphoamino acid standards were electrophoresed at 2.5 kV for 1 h (see the Materials and methods section). The radioautograph shows the presence of radioactivity and the position of the phosphoamino acid and phosphate markers.

phosphatase (gift from Dr. T. E. Roche) failed to activate the enzyme or liberate ^{32}P when incubated with modified kinase assay buffer or modified KCl medium. Pyruvate dehydrogenase phosphate could be re-activated in either buffer. Although Parker & Randle (1980) have reported re-activation with PDH phosphatase, other investigators (Hughes & Halestrap, 1981) have also failed to reproduce their findings. In addition, Pettit *et al.* (1978) have reported that PDH kinase does not phosphorylate the BCOAD complex. It is therefore likely that the enzymes controlling the phosphorylation of BCOAD are distinct from those regulating PDH.

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References

Danner, D. J., Lemmon, S. K., Besharse, J. C. & Elsas, L. J. (1979) *J. Biol. Chem.* **254**, 5522–5526

Dawson, A. J. & Hird, F. J. R. (1967) *Arch. Biochem. Biophys.* **122**, 426–433

DePaoli-Roach, A. A., Roach, P. J. & Larner, J. (1979) *J. Biol. Chem.* **254**, 12062–12068

Goodman, H. M. (1978) *Am. J. Physiol.* **233**, E97–E103

Huang, K.-P. & Robinson, J. C. (1976) *Anal. Biochem.* **72**, 593–599

Hughes, W. A. & Halestrap, A. P. (1981) *Biochem. J.* **196**, 459–469

Lawrence, J. C. & Larner, J. (1978) *J. Biol. Chem.* **253**, 2104–2113

Odessey, R. (1980*a*) *Biochem. J.* **192**, 155–163

Odessey, R. (1980*b*) *FEBS Lett.* **121**, 306–308

Odessey, R. & Goldberg, A. L. (1979) *Biochem. J.* **178**, 475–489

Parker, P. J. & Randle, P. J. (1978) *FEBS Lett.* **95**, 153–156

Parker, P. J. & Randle, P. J. (1980) *FEBS Lett.* **112**, 186–190

Pettit, F. H., Yeaman, S. J. & Reed, L. J. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4881–4885