DIRECT EVIDENCE FOR THE INACTIVATION OF BRANCHED-CHAIN OXO-ACID DEHYDROGENASE BY ENZYME PHOSPHORYLATION

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Received 6 October 1980

1. Introduction

The branched-chain 2-oxo-acid dehydrogenase (BCOAD) from mitochondria of several different rat tissues is inactivated by ATP and can be reactivated by incubation in Mg^{2+}-containing buffers [1–4]. Reactivation is blocked by NaF [1]. This activation/inactivation cycle of BCOAD also occurs in bovine and porcine kidney mitochondria [1]. Work carried out on the system from skeletal muscle mitochondria has shown that inactivation requires the cleavage of the γ-phosphate group of ATP and that modification is covalent. The non-metabolized ATP analog, p[NH]ppA, can block the inhibitory effect of ATP when added prior to ATP addition, but cannot reverse the inhibition of the inactivated dehydrogenase [1]. These and other data raise the possibility that BCOAD may be regulated by enzyme phosphorylation. This hypothesis is supported by the finding [1] that various procedures which separate the enzyme from its mitochondrial environment (e.g., detergent treatment, ammonium sulfate precipitation and freeze–thawing) do not alter the degree of inhibition induced by ATP in the mitochondrial preincubation. These experiments suggested the feasibility of labelling the enzyme with ^{32}P and purifying it.

2. Experimental

The assay mixture consisted of: potassium phosphate (pH 7.2, 0.1 M); $MgCl_2$ (0.25 mM); TPP (0.2 mM); NAD (1.0 mM); CoASH (0.2 mM); amino-oxyacetate (0.2 mM); lipoic dehydrogenase (3 U/ml) (pig heart, Sigma). When mitochondria were to be assayed Triton X-100 (1 mg/ml) was also present. The reaction was initiated by addition of enzyme followed by addition of (4-methyl)-2-oxopentenoate (1 mM) or (3-methyl)-2-oxobutyrate (1 mM) and the rate of increase of NADH fluorescence was recorded for 12 s at 37°C in a Farrand Fluorometer. Freshly prepared NADH was used as a standard. From these data the initial rate was calculated as nmol/min (mU). Mitochondrial protein was assayed densitometrically [5]. Protein in other fractions was assayed using a dye binding protein assay kit (BioRad Lab.). Both methods gave equivalent measurements to the Lowry protein assay.

Mitochondria from ~100–200 g rat kidney was prepared as in [6] with slight modifications. This procedure yielded 10–15 mg mitochondrial protein/g tissue. To purify BCOAD, mitochondria were diluted to ~5 mg/ml with buffer 1: Tris–HCl (0.1 M, pH 7.8); sucrose (0.125 M); EGTA (2.5 mM); $MgCl_2$ (6.8 mM); amino-oxyacetate (0.2 mM); FCCP (0.5 μM); KCl (50 mM); DTT (1 mM). The mixture was incubated at 37°C for 20 min to maximally activate BCOAD as in [1] and then centrifuged for 15 min at 10 000 × *g*. The pellet was resuspended at ~30 mg/ml in: potassium phosphate (20 mM, pH 6.5); TPP (0.2 mM); DTT (1 mM); dialyzed horse serum (1%); then was frozen in liquid nitrogen and thawed with tap water 3 times. The mixture was made 0.05 M in NaCl and centrifuged at 15 000 × *g* for 30 min in a Sorvall GSA rotor. Subsequent purification was a scaled down modification of the procedures in [7,8]. The mitochondrial extract was adjusted to pH 6.4 with 5% acetic acid and $MgCl_2$ added to 10 mM. While stirring, a 50% (w/v) solution of polyethylene glycol (PEG) was added to 3% final conc. After stirring for an addi-

Abbreviations: p[NH]ppA, adenylyl 5′-[B, γ imido]diphosphate; EGTA, ethane dioxybis (ethylamine) tetraacetate; DTT, dithiothreitol; PEG, polyethylene glycol; BCOAD, branched-chain oxo-acid dehydrogenase (EC 1.2.4.4); FCCP, carbonylcyanide *p*-trifluoromethoxyphenyl hydrazone; BSA, bovine serum albumin; TPP, thiamine pyrophosphate; MOPS, 2-(*N*-morpholino) propane sulfonate; PDC, pyruvate dehydrogenase complex (EC 1.2.4.1 + EC 2.3.1.12 + EC 1.6.4.3)

tional 10 min, the mixture was centrifuged at 15 000 X *g* for 10 min. The precipitate was resuspended in buffer 2: potassium–MOPS (50 mM, pH 7.0); TPP (0.2 mM); $MgCl_2$ (1 mM); DTT (2 mM); horse serum (1%); and frozen overnight at –80°C. The following day, the mixture was thawed and spun at 20 000 X *g* for 15 min. The pellet was resuspended in a small volume of the same buffer without serum and made 0.2 M in NaCl. The suspension was centrifuged at 20 000 X *g* for 15 min and the clear supernatant was adsorbed to a column of hydroxyapatite (BioRad) (0.5 X 6 cm) equilibrated with 10 mM potassium phosphate (pH 7.0). The column was washed with 6 vol. 0.14 M potassium phosphate (pH 7.0). This procedure removed most of the protein and all of the pyruvate and 2-oxoglutarate dehydrogenase activities. BCOAD was eluted using a gradient of potassium phosphate (pH 7.0) from 0.14–0.35 M. The enzyme activity eluted 0.22–0.28 M phosphate.

3. Results and discussion

As shown in table 1, BCOAD was purified 1800-fold over the mitochondrial pellet. If one corrects for the slow inactivation of BCOAD which occurs in dilute solutions (unpublished), the specific activity of the BCOAD is 9.9 U/mg (somewhat lower the bovine kidney enzyme which was assayed at 30°C [8]). To determine whether BCOAD could be phosphorylated, rat kidney mitochondria were prepared as in section 2. After preincubation for 20 min to activate BCOAD, one aliquot was incubated with 0.5 mM [γ-^{32}P]ATP (114 μCi/mol) in buffer 1 (containing NaF (50 mM), oligomycin (5 μg/ml) without FCCP). Another aliquot was incubated in buffer without NaF or ATP. After 20 min, mitochondria were pelleted and purified as described except that 1% horse serum and NaF (50 mM) were present in all buffers. The fractions were combined, and layered on a hydroxyapatite column and eluted as described. Table 2 shows that the % inhibition of the enzyme is maintained throughout the purification. A substantial fraction of the ^{32}P cochromatographed with BCOAD activity. This peak is devoid of any PDC activity. The amount of ^{32}P incorporated into BCOAD was 0.32 nmol/U. Values of 0.3–0.8 nmol/U have been obtained in 4 expt. Assuming spec. act. 11.8 U/mg [8], 1–2.5 mol P is incorporated per 250 000 X *g*. (M_r 250 000 is the smallest active fraction of BCOAD observed [7].) In addition, this ratio is in the range of ^{32}P incorporation of PDC by PDC kinase (0.46 nmol/U [9]).

Table 1
Purification of the branched chain 2-oxo-acid dehydrogenase

Fraction	Protein (mg)	Total act. (units)	Spec. act. (units/mg)	Recovery (%)
Mitochondria	2601	7.8	0.003	100
Freeze–thaw supernatant	368	4.8	0.013	62
PEG precipitate	14.7	3.6	0.247	46
Buffer 2: NaCl supernatant	5.8	3.0	0.523	38
Hydroxyapatite eluant	0.20	1.1	5.6	14

BCOAD was purified from rat kidney mitochondria as described. The dehydrogenase was assayed using 2-oxo-3-methyl butyrate (1 mM)

Table 2
Purification of ATP-inhibited BCOAD

Fraction	–ATP (mu/mg)	+ATP (mu/mg)	Inhibition (%)
Mitochondria	12.4	7.0	43
Freeze–thaw supernatant	61.6	23.9	61
PEG pellet	456.0	187.0	59

One aliquot of rat kidney mitochondria were preincubated with 0.25 mM [γ-^{32}P]ATP and NaF (+ATP) and one was incubated without further additions (–ATP) as described. Each aliquot was purified separately to the PEG precipitate stage

To verify the phosphorylation of BCOAD, the peak from the hydroxyapatite column was concentrated and electrophoresed on 1.5% agarose gel in Tris–acetate (50 mM, pH 8.0)/DTT (0.5 mM). One lane was stained with Coomassie blue G250 and autoradiographed. A duplicate sample was stained for BCOAD activity ([10], modified by using assay buffer and iodonitrotetrazolium). A densitometric scan of BCOAD activity (fig.1) shows a large single peak that has the identical R_F value as bovine BCOAD (purified as in [8]). PDC (gift from Dr T. E. Roche) runs more slowly (R_F = 0.225). The autoradiograph of the same gel shows that a major peak of ^{32}P runs coincident with BCOAD. No radioactivity appears over the region where PDC would be found.

The concentrated effluent from the hydroxyapatite column was also subjected to SDS–polyacrylamide electrophoresis. Although a number of bands are apparent of the stained gel (fig.2), the peaks belonging to BCOAD were identified by an identically run sample of purified bovine BCOAD. (Most of the addi-

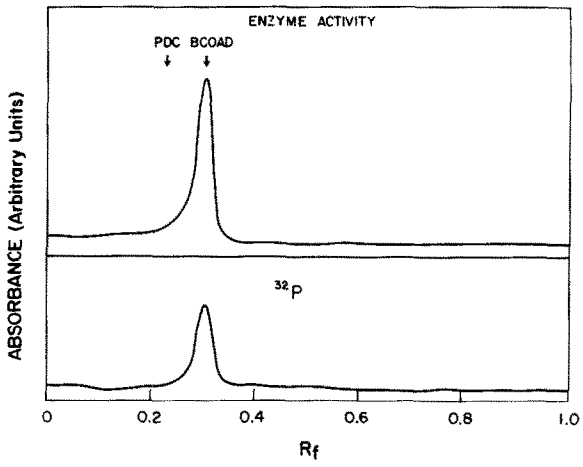

Fig.1. Electrophoresis of the hydroxyapatite peak of BCOAD on a 1.5% Agarose gel. The samples were run for 6 h in Tris–acetate (50 mM, pH 8.0)/DTT (0.5 mM). After staining and autoradiography A_{525} was measured. Arrows indicate the positions of PDC (pig heart) and BCOAD (bovine kidney) run in parallel lanes.

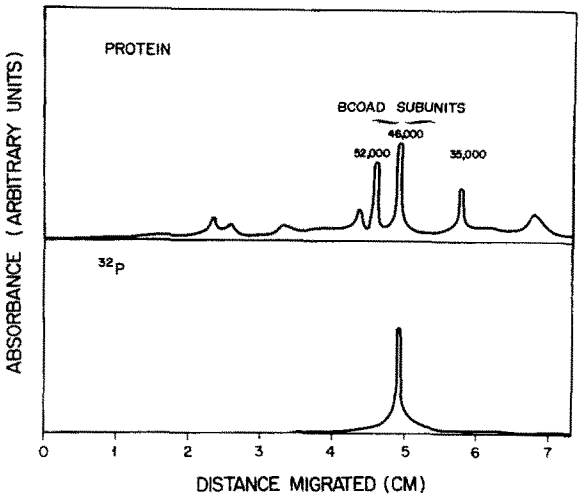

Fig.2. SDS–polyacrylamide gel electrophoresis of BCOAD after hydroxyapatite chromatography. 8% SDS gels were run for 3 h [14]. The bands corresponding to BCOAD were identified by running a parallel sample of purified bovine kidney BCOAD.

tional peaks probably arise from serum protein which was added to the extracts during purification to prevent proteolysis [8].) An autoradiogram of the same gel showed one single sharp peak of 46 000 M_r corresponding to a major protein subunit of the BCOAD decarboxylase dimer [8].

These data strongly suggest that BCOAD is phosphorylated by ATP in agreement with [3,4,11,13]. However, in [7,8] phosphorylation or inhibition of purified enzyme by ATP was not observed. These findings suggest that unlike PDC kinase, the kinase activity toward BCOAD does not copurify with enzyme activity. The purified BCOAD from rat is also not phosphorylated or inhibited by ATP (unpublished). However, inhibition is readily observed in crude extracts [1,4] and these experiments indicate that phosphorylation of BCOAD is also observed when crude extracts are used.

In summary, when rat BCOAD is preincubated with [γ-^{32}P]ATP and purified by hydroxyapatite chromatography, ^{32}P is associated with the enzyme activity. Agarose gel electrophoresis gives rise to a single peak of radioactivity which comigrates with the peak of BCOAD activity and is distinct from PDC. On SDS gels, a single band of radioactivity is found associated with the 46 000 M_r subunit of BCOAD decarboxylase. These findings directly demonstrate that BCOAD can be phosphorylated. Since it has been shown that BCOAD can be inactivated by ATP, it is most likely that inhibition of BCOAD by ATP is mediated by enzyme phosphorylation.

Acknowledgements

I thank Mr Paul Erba and Mr Gary King for their tireless and expert technical assistance and Dr T. E. Roche for invaluable discussions. This work was supported by a Publich Health Service Biomedical Research Support Award 5 SO7 RR05431-18.

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