Rapid purification of bovine kidney branched-chain 2-oxoacid dehydrogenase complex containing endogenous kinase activity

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Branched-chain 2-oxoacid dehydrogenase complex has been purified to near homogeneity by a simple, rapid procedure. The final product contains endogenous kinase activity capable of phosphorylating and inactivating the complex. Phosphorylation continues after complete inactivation, indicating the possibility of several phosphorylatable sites.

Branched-chain 2-oxoacid dehydrogenase complex *Rapid purification* *Phosphorylation* *Inactivation* *(Bovine kidney cortex)*

1. INTRODUCTION

Mitochondria of mammalian tissues contain 3 distinct 2-oxoacid dehydrogenase multi-enzyme complexes which catalyse the oxidative decarboxylation of pyruvate, 2-oxoglutarate and the branched chain 2-oxoacids. Each complex consists of 3 enzyme components, a 2-oxoacid dehydrogenase (E_1), a dihydrolipoyl transacylase (E_2) and a dihydrolipoyl dehydrogenase (E_3) which catalyse consecutive steps in the overall reaction [1].

Branched-chain 2-oxoacid dehydrogenase complex oxidises the 2-oxoacids derived from the essential amino acids leucine, isoleucine and valine. Studies have been done on the enzyme complex from a variety of tissues, including kidney, liver, heart, skeletal muscle and adipose tissue (reviewed [2]). Evidence was first obtained by Parker and Randle [3] that the activity of the enzyme is regulated by covalent phosphorylation, the enzyme being inactivated by phosphorylation. This has been substantiated by various studies using perfused heart [4,5], isolated mitochondria [6] and enzyme preparations [7–9].

The enzyme complex was first purified to apparent homogeneity from bovine kidney mitochondria in [10]. However, unlike the pyruvate dehydrogenase complex which is also regulated by phosphorylation, the final product did not contain endogenous kinase activity. Subsequently, methods have been reported for the purification from bovine kidney [8] and rat kidney [9] mitochondria of branched-chain 2-oxoacid dehydrogenase complex associated with a kinase activity capable of phosphorylating and inactivating the complex.

Traditionally, methods for purification of the 2-oxoacid dehydrogenase complexes have involved preparation of mitochondria, disruption of these mitochondria and subsequent purification of the complexes from the mitochondrial extract. Stanley and Perham [11] first reported a method for purification from bovine heart of the pyruvate and 2-oxoglutarate dehydrogenase complexes which involves disruption of tissue in the presence of Triton X-100 and subsequent purification of the enzymes from the whole extract, without the need to prepare mitochondria.

Here, we report a procedure for the rapid, large-scale purification, to apparent homogeneity, of the branched-chain 2-oxoacid dehydrogenase complex from bovine kidney cortex, without the require-

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ment of isolating mitochondria. The final product contains an endogenous kinase, which in the presence of ATP-Mg, phosphorylates the α-subunit of the E_1 component, inactivating the complex against its 3 branched-chain 2-oxoacid substrates.

2. MATERIALS AND METHODS

Branched-chain 2-oxoacids and antifoam A concentrate were from Sigma; Triton X-100 and polyethylene glycol 6000 from BDH Chemicals; hexokinase (yeast) from Boehringer; hydroxylapatite HTP from BioRad Labs. [γ-^{32}P]ATP was synthesised as in [12], or purchased from Amersham International.

Branched-chain 2-oxoacid dehydrogenase complex was assayed at 30°C in the presence of excess dihydrolipoyl dehydrogenase [10]. One unit of enzyme catalyses the formation of 1 μmol NADH/min. Protein was determined by a scaled-down version of the Bradford [13] or Lowry method [14] following precipitation of the protein using trichloroacetic acid.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS–PAGE) was done essentially as in [15]. The running gel contained 10% (w/v) acrylamide and the stacking gel 5%. Staining was with Coomassie brilliant blue R and the gels were dried prior to autoradiography. Scanning of the gels and autoradiographs was done using a Joyce Loebl Chromoscan 3.

Correlation of phosphorylation with inactivation was carried out as in [16]. Branched-chain 2-oxoacid dehydrogenase complex was incubated at 20°C in buffer C containing 10 mM $MgCl_2$; 0.5 mM [γ-^{32}P]ATP and 4 μg oligomycin B/ml. At intervals aliquots were removed and phosphorylation in these aliquots was terminated by addition of 0.1 vol. 1 M glucose and 0.01 vol. 10 mg hexokinase/ml. The aliquots were then assayed for branched-chain 2-oxoacid dehydrogenase activity, protein-bound [^{32}P]phosphate [17] and analysed by SDS–polyacrylamide gel electrophoresis and autoradiography.

Buffer A consists of 50 mM potassium phosphate, 2 mM EDTA, 3% (v/v) Triton X-100, 1 mM benzamidine, 1 mM PMSF, 0.1 mM DTT (pH 7.5); buffer B is 30 mM potassium phosphate, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM benzamidine, 1 mM PMSF, 1 mM DTT (pH 7.3); buffer C is 30 mM sodium phosphate, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM benzamidine, 1 mM PMSF, 1 mM DTT (pH 7.3).

3. RESULTS

3.1. *Purification of branched-chain 2-oxoacid dehydrogenase complex*

Ox kidney was obtained within 10 min of slaughter and transported on ice to the laboratory. All subsequent operations were carried out at 4°C. Cortex (~250 g) was removed, finely chopped and then homogenised in a blender for 3 min in 2 vol. buffer A containing antifoam A (0.5 ml/l). After homogenisation an equal volume of buffer A was added and the homogenate was centrifuged at 26000 × *g* for 15 min. The resulting supernatant had pH ~7.3.

To this supernatant was added 0.12 vol. polyethylene glycol (35%, w/v), the mixture was stirred for 30 min, and the resultant precipitate was collected by centrifugation at 30000 × *g* for 15 min. The precipitate was resuspended in buffer B (1/3 initial vol.), resuspension being completed by treating the sample with a Polytron PT10S at setting 2 for 20 s. The mixture was stirred for 30 min (PEG I) and then clarified by centrifugation at 30000 × *g* for 30 min (PEG I clarified). This first precipitation step is crucial as it removes most of the 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase complexes, the remainder of these complexes being removed at subsequent stages of the purification procedure.

To the clarified solution was then added 0.12 vol. polyethylene glycol and after stirring for 30 min the precipitate was again collected by centrifugation at 30000 × *g* for 15 min. The precipitate was resuspended in buffer B (1/20 initial vol.), and homogenised, stirred (PEG II) and clarified (PEG II clarified) as above.

The clarified material was applied to hydroxylapatite (2.6 × 6 cm) pre-equilibrated in buffer B. This was washed extensively with buffer B containing 200 mM potassium phosphate until the absorbance at 280 nm of the eluant decreased to zero. The branched-chain 2-oxoacid dehydrogenase complex was then eluted using buffer B containing 350 mM potassium phosphate. Fractions contain-

ing enzyme activity were pooled and further purified and concentrated by centrifugation at 180000 × *g* for 3.5 h. The final pellet was carefully resuspended in buffer C.

A typical purification is summarised in table 1. Because of the high levels of Triton X-100 and the presence of competing activities it is not possible to determine accurately the branched-chain 2-oxoacid dehydrogenase activity in the 1st supernatant. The whole procedure can be completed within 1.5 working days. A convenient stopping point at the end of day 1 is to load the PEG II clarified fraction onto hydroxylapatite and to wash the column overnight with buffer B containing 200 mM potassium phosphate. All centrifugation steps (except the 180000 × *g* spin) can be carried out in one 6 × 300 ml rotor (e.g., Beckman JA-14). The procedure can be scaled up several fold without any significant increase in preparation time. If larger quantities of tissue are being processed it is convenient to use a larger-capacity rotor and lower gravitational forces (e.g., 10000 × *g*) to obtain the 1st supernatant fraction. No changes in the subsequent purification steps are necessary.

3.2. *Characterisation of the purified complex*

Analysis of the final material by SDS–polyacrylamide gel electrophoresis indicates that the complex is essentially homogeneous (fig.1). The 3 protein bands corresponding to E_2 and the α- and β-subunits of E_1, constitute >90% of the stainable protein, the remainder being several minor bands of higher-M_r. In agreement with [8,10] the purified complex is devoid of the E_3 component.

The specific activity of the final material, using 3 methyl 2-oxobutyrate as substrate, was ~3.4 U/mg, this value ranging from 1.5–5.0 for several different preparations of enzyme. This is lower than the highest reported value of 11.8 [10]. The reasons for this difference are not clear although one possibility is that homogenisation in the presence of Triton causes some irreversible inactivation of the complex. The purified complex is active against all 3 branched-chain 2-oxoacids, namely 3-methyl 2-oxobutyrate, 4-methyl 2-oxopentanoate and 3-methyl 2-oxopentanoate in the ratio 2:1.4:1.0 and also has some activity (0.06) against pyruvate but is completely devoid of activity against 2-oxoglutarate.

A key feature of the purification method described here is that the purified complex contains an endogenous kinase activity. In the presence of ATP-Mg this phosphorylates the α-subunit of the E_1 component (fig.1). Under these conditions no other component becomes phosphorylated. Phosphorylated is accompanied by loss of activity against all 3 branched-chain 2-oxoacids, the 3 activities being lost concurrently (fig.2). As judged by the time required to half-inactivate the complex, there is no significant loss of kinase activity from the complex during the purification procedure.

Fig.2 indicates that phosphorylation continues after complete inactivation of the complex is achieved. One possible explanation of this is the

Table 1

Purification of branched-chain 2-oxoacid dehydrogenase complex

Fraction	Vol. (ml)	Total act. (U)	Protein (mg)	Spec. act. (U/mg)	Yield (%)
1st Supernatant	980	–	21560	–	–
PEG I	300	128	3300	0.039	100
PEG I clarified	265	68	1670	0.041	53
PEG II	60	63	670	0.094	49
PEG II clarified	51	38	280	0.136	30
Hydroxylapatite	27.5	33	25.6	1.29	26
180000 × *g* pellet	2.2	23	6.8	3.38	18

Starting material was 194 g kidney cortex. Activity was measured using 3-methyl 2-oxobutyrate as substrate. Protein was determined as in [13], except in the 1st supernatant fraction where, because of the presence of Triton X-100, the modified Lowry method [14] was used

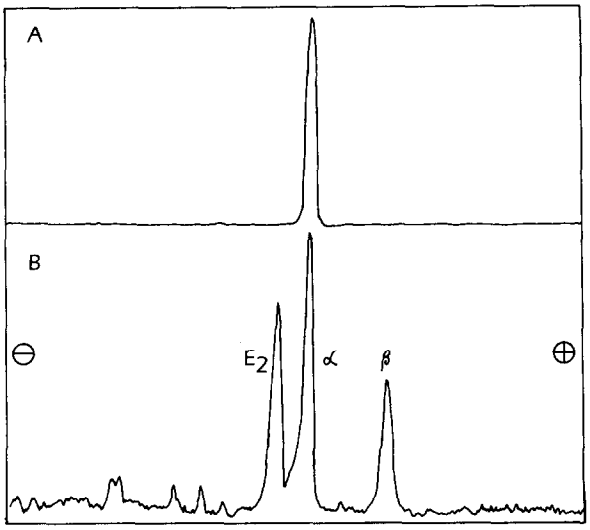

Fig.1. SDS–polyacrylamide gel electrophoresis of purified branched-chain 2-oxoacid dehydrogenase complex: (A) densitometric scan of autoradiograph of sample phosphorylated using [γ-^{32}P]ATP-Mg; (B) densitometric scan of protein stain of sample.

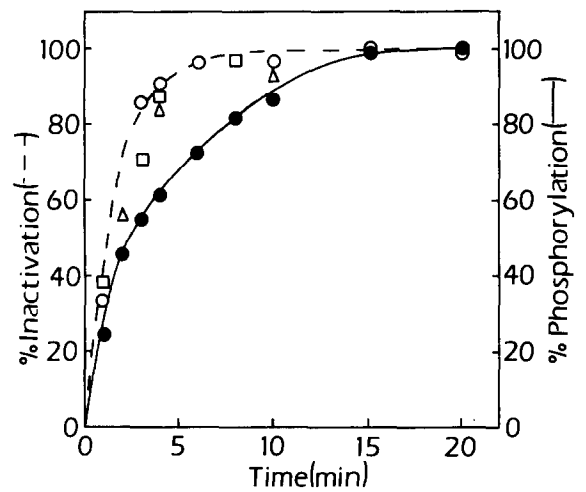

Fig.2. Phosphorylation and inactivation of purified branched-chain 2-oxoacid dehydrogenase complex: purified complex (10 U/ml) in buffer C was incubated with [γ-^{32}P]ATP-Mg (3.5×10^4 cpm/nmol) and treated as in section 2. Inactivation using as substrate: 3-methyl 2-oxobutyrate (○); 4-methyl 2-oxopentanoate (△); 3-methyl 2-oxopentanoate (□); and protein-bound phosphate (●). The maximum phosphorylation value corresponds to ~5 nmol [^{32}P]phosphate/mg protein, representing ~0.8 mol phosphate/mol α-subunit.

presence of several phosphorylation sites on the enzyme, inactivation resulting from the rapid phosphorylation of one such site. Evidence in support of multi-site phosphorylation comes from high-voltage electrophoresis of tryptic digests of phosphorylated complex, which indicates the presence of 3 distinct phosphopeptides [19].

4. DISCUSSION

The simple purification scheme reported here has several advantages over procedures. A major advantage over the method in [10] is that the final preparation contains intrinsic kinase activity capable of phosphorylating and inactivating the branched-chain 2-oxoacid dehydrogenase complex. In the final preparation only one polypeptide, namely the α-subunit of the E_1 component, can be phosphorylated. This makes the method extremely useful for obtaining purified enzyme to study, in detail, the regulation of the complex by phosphorylation. The second advantage is that the purification method is rapid, allowing purified enzyme to be prepared within 1.5 working days, and is capable of being scaled-up several fold. The yield of >11 U activity/100 g tissue compares favourably with the highest yield (4.1 U/100 g) reported in [10]. A method for obtaining highly purified complex from mitochondrial extracts with intrinsic kinase activity was reported in [8]. The specific activity of the final product was not determined but the yield of 0.9 U/100 g cortex is 10-fold lower than reported here. In [18], a method has been reported for purification of the complex from Triton extracts of rabbit liver. However, the method is considerably slower than that reported here and the level of kinase in the final complex is very low, incubation for 80 min at 37°C being necessary to achieve complete inactivation.

The availability, in large amounts from a convenient source, of highly-purified complex with endogenous kinase activity will now allow detailed investigation of the number of phosphorylation sites and their possible effect on enzyme activity.

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