Resolution and reconstitution of bovine kidney branched-chain 2-oxo acid dehydrogenase complex

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Branched-chain 2-oxo acid dehydrogenase complex was resolved into component E1 and E2-kinase subcomplex by gel filtration in the presence of 1M-NaCl. Essentially all the original activity of the complex can be regained after reconstitution of the component enzymes, reassociation being a rapid process. The specific activities of E1 and E2 were 25.1 and 19.0 units/mg respectively. Non-phosphorylated active E1 has an approx. 6-fold higher affinity for E2 than does phosphorylated E1. The components of the branched-chain 2-oxo acid dehydrogenase complex do not cross-react with the respective components from the pyruvate dehydrogenase complex. The significance of these results and of the tight association of the kinase with E2 are discussed.

Branched-chain 2-oxo acid dehydrogenase complex is a multienzyme complex catalysing the committed step in the breakdown of the essential amino acids leucine, isoleucine and valine. This complex is analogous to the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes, and comprises an acyltransferase core (E2) around which are arranged the 2-oxo acid dehydrogenase (E1) and dihydrolipoamide dehydrogenase (E3) components. In each complex the enzymes act in sequence to complete the oxidative decarboxylation of their respective substrates. As with the pyruvate dehydrogenase complex, the E1 component of the branched-chain 2-oxo acid dehydrogenase complex consists of two polypeptides, termed α and β . The activity of the complex can be controlled by phosphorylation/dephosphorylation of the α -subunit of the E1 component by a kinase intrinsic to the complex (Fatania *et al.*, 1981; Odessey, 1982; Lawson *et al.*, 1983). The kinase phosphorylates two sites closely grouped on the α -subunit, and the amino acid sequence surrounding these sites has been determined, inactivation correlating closely with phosphorylation of one site (Cook *et al.*, 1984). The amino acid sequence surrounding this site shows a distinct homology with

the corresponding phosphorylation site which controls activity of pyruvate dehydrogenase complex (Cook *et al.*, 1983).

Resolution of the pyruvate dehydrogenase complex (Linn *et al.*, 1972) has led to intensive studies of the subunit composition (Barrera *et al.*, 1972), respective dissociation and reassociation constants for the component enzymes (Brandt *et al.*, 1983), inter- and intra-core movements of the E1 components (Cate *et al.*, 1980), and the effect and mode of phosphorylation by the fixed intrinsic kinase (Brandt & Roche, 1983). This has yielded a wealth of information regarding the reaction mechanism of the complex and its mode of control by phosphorylation.

In contrast, much less is known about details of the organization of the branched-chain 2-oxo acid dehydrogenase complex. In this paper we report methods for resolution of the branched-chain 2-oxo acid dehydrogenase complex into its component enzymes and reconstitution of catalytically active complex from the individual components. Preliminary evidence is given of preferential binding of the non-phosphorylated active form of the E1 component to the E2 core.

We have investigated the possibility of cross-reaction with purified components of the pyruvate dehydrogenase complex, and the implications of the tight association of the kinase with E2 are also discussed.

Abbreviation used: SDS, sodium dodecyl sulphate.

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Materials and methods

All the materials used were of the highest available purity and were as described previously (Lawson *et al.*, 1983). AcA 34 gel-filtration matrix was purchased from LKB. Dihydrolipoamide dehydrogenase (E3) was from Boehringer.

Branched-chain 2-oxo acid dehydrogenase was purified and assayed as described by Lawson *et al.* (1983). Complex was phosphorylated as described in Lawson *et al.* (1983), with [γ - 32 P]ATP (200 c.p.m./pmol). Phosphorylated complex was resolved into its separate components, without termination of phosphorylation, when the activity of the complex had decreased to less than 0.5% of its initial value. The E1 component was obtained in the phosphorylated form by resolution of phosphorylated complex on AcA 34 as described in Fig. 1. The purified phosphorylated E1 (E1b) showed less than 0.5% of the activity of the non-phosphorylated form (E1a), with a phosphate incorporation of 0.91 mol/mol of α -subunit. By SDS/polyacrylamide-gel electrophoresis and high-voltage electrophoresis of tryptic digests of the complex, [32 P]phosphate incorporation was shown to be into only the two phosphorylation sites shown previously (Cook *et al.*, 1984) to be present on the α -subunit (results not shown).

Pyruvate dehydrogenase complex was purified as described by Stanley & Perham (1980) from frozen bovine heart. The complex was resolved into its component enzymes by the same procedures as described in Fig. 1 for the branched-chain 2-oxo acid dehydrogenase complex. The initial complex used had a specific activity of 14.0 units/mg. The resolved E2 component showed a specific activity of 17.5 units/mg when assayed in a reconstituted system. The resolved E1 peak also contained the E3 component and showed a specific activity of 13.0 units/mg when assayed in a reconstituted system. For this study no attempt was made to resolve the E1 and E3 components further.

SDS/polyacrylamide-gel electrophoresis and autoradiography were carried out as described by Lawson *et al.* (1983).

Results

We have previously developed a rapid method for the purification of bovine kidney branched-chain 2-oxo acid dehydrogenase complex from Triton extracts of kidney cortex (Lawson *et al.*, 1983). The highly purified complex consists of E1, E2 and an intrinsic kinase. E3, as reported previously (Pettit *et al.*, 1978; Fatania *et al.*, 1981) is lost on purification. The purified complex can now be resolved into E1 and E2 by gel filtration on

AcA 34 at pH 7.0 in the presence of 1 M-NaCl (Fig. 1). E2 has a high M_r , of over 1×10^6 , and is eluted in the void volume, whereas E1 is smaller and included in the matrix, being eluted with K_{av} = 0.28. Analysis by SDS/polyacrylamide-gel electrophoresis of both pooled components (Fig. 2) shows the absence of any major impurities and confirms (Pettit *et al.*, 1978) that E2 consists of a single polypeptide and E1 contains two non-identical polypeptides, termed α and β . After elution, each peak can be concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation, E2 being collected at 15–25% and E1 at 35–50% saturation. After concentration and dialysis for a minimum of 6 h into 50% (v/v) glycerol/50 mM-Tris/HCl/1 mM-dithiothreitol/0.1 mM-EDTA/0.1 mM-EGTA, pH 7.0, the samples can be stored at -20°C for at least 2 months (longest period tested) without appreciable loss of enzyme activity.

In experiments using NADH production by the complete complex as a means of monitoring reconstitution, it was found that reassociation is an

Fig. 1. Resolution of the branched-chain 2-oxo acid dehydrogenase complex on AcA 34

Purified branched-chain 2-oxo acid dehydrogenase complex (5 mg) from bovine kidney was preincubated for 30 min at room temperature in 1 M-NaCl/5 mM-dithiothreitol/0.1 mM-EDTA/0.1 mM-EGTA/30 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.0. The sample (400 μl) was then applied to an AcA 34 column (1.5 cm $\times$ 42 cm) equilibrated at 4°C in the same buffer. Chromatography was carried out at 4°C . Flow rate was 8.0 ml/h, and 2.3 ml fractions were collected. Samples (10 μl) were assayed for E2 activity (■) in the presence of excess E3 (5 μg) and excess purified E1 component (3 μg). Additional samples (10 μl) were assayed for E1 activity (○) in the presence of excess E3 (5 μg) and excess purified E2 component (3 μg). ●, A_{280} (protein).

Fig. 2. Analysis by SDS/polyacrylamide-gel electrophoresis of the resolved components of the branched-chain 2-oxo acid dehydrogenase complex

Samples were prepared as described in the Materials and methods section. (a) Purified bovine kidney branched-chain 2-oxo acid dehydrogenase complex; (b) E3 subunit (from Boehringer); (c) resolved E2 component; (d) resolved E1 component.

extremely rapid process. NADH formation can be detected immediately (less than 3 s) after the addition of the separate components into a cuvette containing all the essential substrates and cofactors required for activity. With a ratio of 1.2 μg of E1 to 1.0 μg of E2 (with 5 μg of E3), a specific activity of 8.5 units/mg could be achieved (results not shown). This compares reasonably well with a specific activity of 11.8 units/mg obtained for the purified complex (Pettit *et al.*, 1978). By using excess E2 (3 μg) to assay E1 activity and excess E1 (5 μg) to assay E2 (Fig. 1), specific activities of 25.1 and 19.0 units/mg were obtained for E1 and E2 respectively. This also compares well with values that one would expect from calculations using the known specific activity of the whole complex. These results suggest that essentially all the activity of the original complex can be regained by reconstitution of the component enzymes.

The purified E1 component can also reconstitute with phosphorylated branched-chain 2-oxo acid dehydrogenase complex to regain all the initial

Fig. 3. Relative activity of reconstituted complex at different ratios of E1a and E1b

A constant amount (6 μg) of purified E2 was preincubated for 10 min at 20°C in 100 μl of 30 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4/1$ mM-dithiothreitol/0.1 mM-EDTA/0.1 mM-EGTA, pH 7.0, with various ratios of active non-phosphorylated E1 (E1a) to inactive phosphorylated E1 (E1b). The total amount of E1 remained constant at 180 μg , which meant that, at any given ratio, there was sufficient E1a to reconstitute the available acyltransferase (E2) totally. Samples (40 μl) were then taken for assay in the presence of 5 μg of E3 (●). The continuous line represents the theoretical activity assuming equal binding affinities of E1a and E1b.

activity of the complex. Addition of E1 directly into an assay cuvette containing phosphorylated complex allows the immediate regain of enzyme activity, as indicated by the formation of NADH (results not shown). It is not yet known whether E1 acts by displacing phosphorylated E1 from the E2 core or whether it acts by binding to unoccupied sites on E2.

Purified E1 can be obtained in its phosphorylated form (E1b) by resolution of phosphorylated branched-chain 2-oxo acid dehydrogenase complex on AcA 34 in the presence of 1 M-NaCl, by using the same procedures as described for the non-phosphorylated complex (see the Materials and methods section). By using various ratios of E1a:E1b in reconstitution experiments with E2 and E3, we found that the active non-phosphorylated form of E1 (E1a) has a greater affinity for the E2 core than does E1b (Fig. 3). At a 1:1 ratio of E1b:E1a, 50% of the E1 available to bind to the E2 core is in the inactive phosphorylated form. However, the complex reconstituted under these conditions is almost 90% active (Fig. 3). Similarly

50% activity is observed at a 8.5:1.5 ratio of E1b:E1a, indicating that E1a has an approx. 6-fold greater affinity than E1b for the E2 core.

The activity of branched-chain 2-oxo acid dehydrogenase complex is controlled by phosphorylation/dephosphorylation at a specific site on the α -subunit of the E1 component (Cook *et al.*, 1983), phosphorylation being mediated by a kinase intrinsic to the complex. This branched-chain 2-oxo acid dehydrogenase kinase is present in the highly purified preparations of branched-chain 2-oxo acid dehydrogenase complex that were used in the resolution of the complex. A significant observation was that, after separation of the components, the kinase is found associated with the E2 core and not with its substrate E1. Fig. 4 shows the result of an incubation of E2-kinase subcomplex with E1a substrate in the presence of [γ - 32 P]ATPMg. The autoradiograph of an SDS/polyacrylamide gel shows that the α -subunit of the E1 component becomes phosphorylated when incubated in the presence of E2-kinase. In the control incubation without E2-kinase subcomplex, the E1a remains non-phosphorylated. This implies that, like the pyruvate dehydrogenase complex, the kinase is tightly bound to the E2 core.

The mechanism of action and subunit composition of pyruvate and branched-chain 2-oxo acid dehydrogenase complexes are similar. The complexes both have a dehydrogenase (E1), an acyltransferase (E2), and appear to use the same dihydrolipoyl dehydrogenase (E3). Although pyruvate dehydrogenase complex does not utilize branched-chain 2-oxo acids, branched-chain 2-oxo acid dehydrogenase complex will oxidize pyruvate, but has a high K_m for it (Pettit *et al.*, 1978). This gives rise to the possibility of 'cross-reaction' *in vitro* between the subunits of the two complexes, where pyruvate dehydrogenase E1 component may catalyse the oxidation of pyruvate utilizing the E2 component of the branched-chain 2-oxo acid dehydrogenase complex, and vice versa. We investigated this possibility using the resolved components from both complexes. E1 components from both complexes were incubated with the E2 component from the other complex. By monitoring the NADH produced by the 'reconstituted' system, we have found that the enzyme components of the two complexes do not interact with each other to any significant extent (Table 1). The E2 component of the branched-chain 2-oxo acid dehydrogenase complex did substitute to a small extent for the E2 of the pyruvate dehydrogenase complex in an incubation with the E1 component of pyruvate dehydrogenase, with pyruvate as substrate. However, the activity in this case was less than 0.5% of that in an identical incubation with E2 from the pyruvate dehydrogenase complex (Table 1).

Fig. 4. Autoradiograph after SDS/polyacrylamide-gel electrophoresis, showing phosphorylation of E1 by E2-kinase subcomplex

For this, 15 μ g of purified E1 component and 25 μ g of E2-kinase subcomplex (a), and 15 μ g of E1 component only (b), were incubated for 1 h at 20°C in 30 mM- Na_2HPO_4 /NaH $_2$ PO $_4$ /1 mM-dithiothreitol/0.1 mM-EDTA/0.1 mM-EGTA/0.5 mM-[γ - 32 P]ATP (17 c.p.m./pmol)/2 mM-MgCl $_2$, pH 7.0, in a total volume of 50 μ l. The incubations were terminated by addition of 1 vol. of 2-fold concentrated sample buffer (Laemmli & Favre, 1973). SDS/polyacrylamide-gel electrophoresis and autoradiography were performed as described in the Materials and methods section.

Discussion

We have previously reported a rapid purification scheme for bovine kidney branched-chain 2-oxo acid dehydrogenase complex containing an intrinsic kinase (Lawson *et al.*, 1983). We have now further resolved the component enzymes of the complex into E2-kinase and E1. (Fig. 2). The resolved enzymes still retain their respective catalytic activities and rapidly reassociate to form

Table 1. Cross-reactivity of the components of pyruvate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complexes

Components were purified as described in the text. Ep denotes an enzyme component of the pyruvate dehydrogenase complex; Eb denotes an enzyme component of the branched-chain 2-oxo acid dehydrogenase complex. Assays contained 5 µg of dihydrolipoyl dehydrogenase (E3) and the indicated substrate (pyruvate or 3-methyl-2-oxobutyrate). The component enzymes were incubated, in the indicated combinations, in 10 µl of 30 mM-Na₂HPO₄/NaH₂PO₄/0.1 mM-EDTA/0.1 mM-EGTA/1 mM-dithiothreitol, pH 7.3, for 5 min before assay.

Substrate	Activity (munits)	
	3-Methyl-2-oxobutyrate	Pyruvate
Elp (1.3 µg) + E2b (0.96 µg)	0	0.1
Elp (1.3 µg) + E2b (9.60 µg)	0	1.1
E1b (1.1 µg) + E2p (1.0 µg)	0	0
E1b (11 µg) + E2p (10.0 µg)	0	0
Elp (1.3 µg) + E2p (1.0 µg)	0	17.0
E1b (1.1 µg) + E2b (0.96 µg)	17.5	3.0

complete active complex (Table 1). Resolution of branched-chain 2-oxo acid dehydrogenase complex has been reported previously (Pettit *et al.*, 1978). However those authors made no reference to reconstitution of the components, nor to regain of enzyme activity. In our hands, the procedure described in that paper for the resolution of the complex yields an inactive E1 component (results not shown).

The resolution of active components will allow further studies of the organization of the complex, as has been reported for the pyruvate dehydrogenase system (Cate *et al.*, 1980; Brandt *et al.*, 1983; Brandt & Roche, 1983). Determination of the dissociation constant for the dihydrolipoyl dehydrogenase (E3) may shed some light into why this component is lost in most methods of purification of the complex (Pettit *et al.*, 1978; Fatania *et al.*, 1981; Lawson *et al.*, 1983).

A greater affinity of the active non-phosphorylated form of the dehydrogenase (E1) component for the E2 core has also been reported for pyruvate

dehydrogenase complex (Pratt *et al.*, 1979; Wu & Reed, 1984). The results here indicate this may also be true of branched-chain 2-oxo acid dehydrogenase complex.

The association of the kinase with the E2 core suggests that, like the pyruvate dehydrogenase complex (Linn *et al.*, 1972; Brandt & Roche, 1983), the kinase is tightly bound to the E2 core. Phosphorylation of the E1 components may thus take place at specific sites on the surface of E2, requiring intra-core movements of E1. This requires further investigation.

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