Multi-site phosphorylation of branched-chain 2-oxoacid dehydrogenase complex within mitochondria isolated from rat liver, kidney and heart

Kenneth G. Cook, Rowena Lawson and Stephen J. Yeaman*

Department of Biochemistry, The University, Newcastle upon Tyne NE1 7RU, England

Received 10 October 1983

The α subunit of the E_1 component of branched-chain 2-oxoacid dehydrogenase complex becomes rapidly phosphorylated in rat liver, kidney and heart mitochondria incubated in the presence of succinate and [^{32}P]phosphate. Peptide mapping of tryptic digests of the phosphorylated α subunit indicates that 3 distinct sites are phosphorylated, as has been reported previously by us for phosphorylation in vitro of highly purified complex.

Branched-chain 2-oxoacid dehydrogenase complex *Multi-site phosphorylation* *Mitochondria*

1. INTRODUCTION

The mitochondrial branched-chain 2-oxoacid dehydrogenase complex catalyses a rate-limiting step in the oxidation of the essential amino acids leucine, isoleucine and valine. It is now clear, from studies with whole tissue, isolated mitochondria, mitochondrial extracts and purified enzymes that the activity of the enzyme is regulated by covalent phosphorylation (review [1]). The branched-chain 2-oxoacid dehydrogenase complex is inactivated by phosphorylation of the α subunit of the E_1 component [2] by a protein kinase which co-purifies with the complex [3–5]. Phosphorylation of the α subunit has also been shown to occur within mitochondria isolated from rat liver [6,7], kidney [2,6] and heart [6].

We have reported previously [8] that when highly purified branched-chain 2-oxoacid dehydrogenase complex from bovine kidney is incubated with [γ-^{32}P]ATP-Mg, three distinct sites on the α subunit become phosphorylated. In this communication we present evidence that these same sites on the enzyme become phosphorylated within intact mitochondria isolated from rat liver, kidney and heart.

* To whom correspondence should be addressed

2. MATERIALS AND METHODS

Female Porton-Wistar rats (body weight approximately 300 g) were allowed free access to food and water. They were killed by stunning and cervical dislocation. [^{32}P]Phosphate was from Amersham International. Trypsin (TPCK-treated) was from Worthington. Branched-chain 2-oxoacid dehydrogenase and pyruvate dehydrogenase complexes were purified as in [5,9]. All other chemicals and biochemicals were of the highest available purity and were from BDH Chemicals or Sigma.

Mitochondria were prepared at 4°C from rat liver, kidney and heart. Liver and kidney were disrupted by homogenisation in 5 vols 0.25 M sucrose, 10 mM Tris–HCl 1 mM EGTA, 2 mg/ml bovine serum albumin (pH 7.4) in a Potter-Elvehjem homogeniser. Heart was homogenised in the same buffer using a Polytron PT10S. Following centrifugation at 500 × g for 3 min, the resultant supernatant was spun at 8500 × g for 10 min. The mitochondrial pellet was washed twice with the same buffer (minus serum albumin) and then re-suspended in 130 mM KCl, 10 mM Tris–HCl (pH 7.4) at a protein concentration of 15–30 mg/ml. Mitochondria were used on the day of preparation and showed respiratory control ratios of approx-

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

imately 2.5 with succinate as substrate.

Mitochondria (final protein concentration ≃ 10 mg/ml) were incubated at 30°C in 130 mM KCl, 10 mM Tris–HCl (pH 7.4) in the presence of 2 mM dichloroacetate (to inhibit pyruvate dehydrogenase kinase), 0.5 mM [^{32}P]phosphate (1000 dpm/ϱmol), 10 mM succinate. At intervals following addition of succinate, aliquots (15 μl) were withdrawn and the protein precipitated in ice-cold 10% trichloroacetic acid. The precipitate was collected by centrifugation at 10000 × g for 2 min in an Eppendorf microfuge. The pellet was washed twice with ice-cold H_2O and then redissolved by standing overnight in 15 μl 0.7 M Tris–HCl (pH 6.8) containing 10% (w/v) SDS and 50% (v/v) glycerol. Before electrophoresis the samples were diluted 2-fold with H_2O, β-mercaptoethanol was added to a final concentration of 0.5 M and the samples boiled for 2 min.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was carried out as in [10]. The running gel contained 10% (w/v) acrylamide and the stacking gel 5%. After running, gels were dried down and autoradiographed without prior fixing or staining.

Slices containing ^{32}P-labelled polypeptides were cut from the dried gel and transferred to Eppendorf centrifuge tubes. They were chopped finely and crushed then extracted using 50 mM triethanolamine–HCl (pH 8.0), containing 1% (w/v) SDS, 0.05 mM EDTA, 0.1 mM DTT. The extraction mixture was heated at 65°C for 15 min then centrifuged. The supernatant was removed and the gel pellet extracted a further two times. The pooled supernatants contained 85–90% of the radioactivity in the original slices.

Protein extracted from gels was precipitated by 20% trichloroacetic acid and collected by centrifugation in an Eppendorf microfuge. Essentially all the radioactivity was recovered in the pellet which was then washed once with acetone and twice with diethyl ether. The pellet was resuspended then incubated overnight at 20°C in 200 μl of 0.2 M ammonium bicarbonate containing 100 μg trypsin. Insoluble material was then removed by centrifugation. Over 90% of the radioactivity was recovered in the supernatant which was lyophilised and subjected to high voltage electrophoresis at pH 1.9 as in [8].

Branched-chain 2-oxoacid dehydrogenase activity in mitochondria was assayed as in [5] except that no additional dihydrolipoyl dehydrogenase was added and the assay was carried out in the presence of 2 mM KCN, 1% (v/v) Triton X-100 and 10 mM NaF. Following addition of mitochondrial incubation (50 μl) to the cuvette, the background rate of NADH production was measured and then the reaction was initiated by addition of the substrate 3-methyl-2-oxybutyrate.

3. RESULTS

When fresh mitochondria from rat liver, kidney and heart were incubated with [^{32}P]phosphate and succinate, two major phosphorylated polypeptides were detected by SDS-PAGE and autoradiography

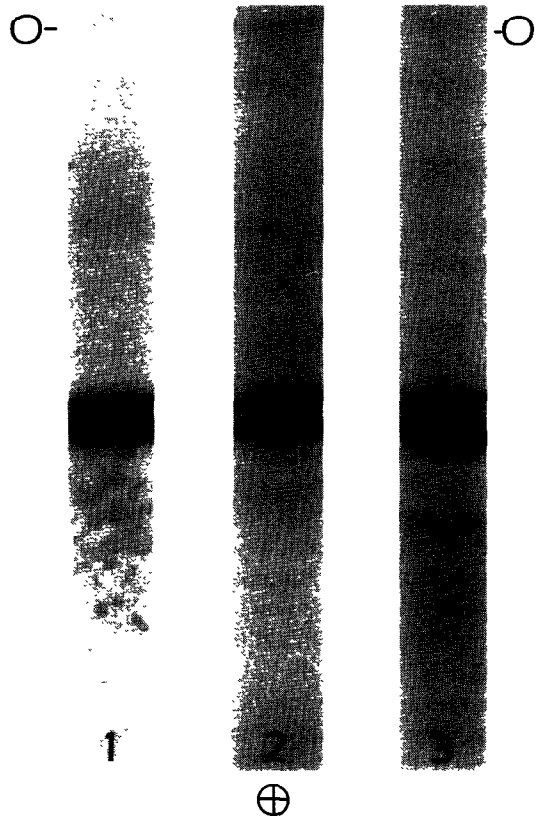

Fig.1. Autoradiograph of SDS-PAGE of mitrochondrial extracts following incubation of mitochondria with [^{32}P]phosphate and succinate. Aliquots were withdrawn 10 min after start of incubation and processed as described in section 2. Lane 1, liver mitochondria; lane 2, kidney mitochondria; lane 3, heart mitochondria. (○) indicates the origin.

(fig.1). These have subunit M_r values of approximately 46000 and 41000 and it has been shown previously that they correspond to the α subunits of the E_1 components of the branched-chain 2-oxoacid dehydrogenase complex and pyruvate dehydrogenase complex, respectively [6,7]. Addition of dichloroacetate was necessary to limit labelling of the pyruvate dehydrogenase and enable phosphorylation of the branched-chain 2-oxoacid dehydrogenase to be visualised. The identification of these polypeptides is supported by the observation that they co-migrate with the α subunits of the respective purified complexes phosphorylated in vitro using [γ-^{32}P]ATP-Mg (not shown). With some preparations a minor phosphorylated polypeptide of $M_r \approx 36000$ was observed. The identity of this polypeptide is not known.

Under the conditions employed, maximum labelling of these polypeptides was observed within 2 min of the addition of succinate and the extent of labelling did not vary significantly during further incubation. Using liver and kidney mitochondria, incubation with succinate led to approximately 50% decrease in the observed activity of the branched-chain 2-oxoacid dehydrogenase. No

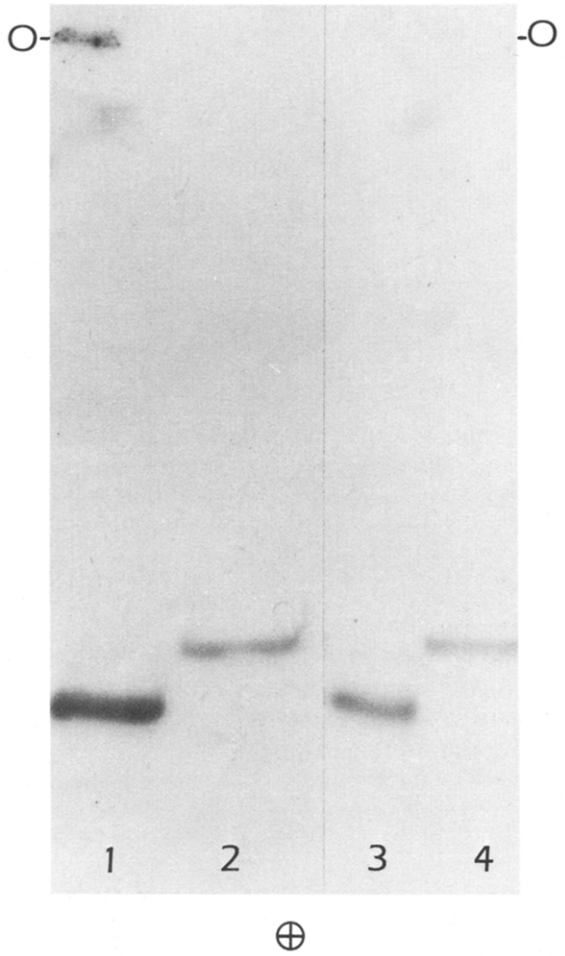

Fig.2. Autoradiograph of SDS-PAGE of phosphorylated samples of branched-chain 2-oxoacid dehydrogenase and pyruvate dehydrogenase. Samples in lanes 1 and 2 were phosphorylated in intact liver mitochondria, the two phosphorylated polypeptides then separated by SDS-PAGE, extracted from gel and re-subjected to SDS-PAGE. Lane 1, α-subunit of pyruvate dehydrogenase phosphorylated in mitochondria. Lane 2, α subunit of branched-chain 2-oxoacid dehydrogenase phosphorylated in mitochondria. Lane 3, bovine heart pyruvate dehydrogenase phosphorylated in vitro. Lane 4, bovine kidney branched-chain 2-oxoacid dehydrogenase phosphorylated in vitro. (○) indicates the origin.

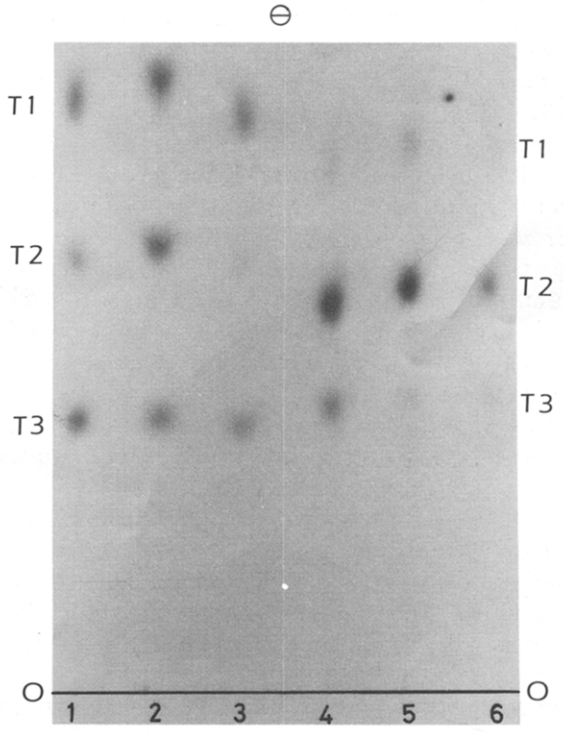

Fig.3. Autoradiograph of electrophoretogram (pH 1.9) of tryptic digests of polypeptides phosphorylated within intact mitochondria. Lanes 1–3 are digests of branched-chain 2-oxoacid dehydrogenase, lanes 4–6 are pyruvate dehydrogenase. Lanes 1 and 4, liver mitochondria; lanes 2 and 5, kidney mitochondria; lanes 3 and 6, heart mitochondria.

activity measurements were carried out using heart mitochondria.

The two phosphorylated polypeptides can be sufficiently resolved by SDS-PAGE to allow each to be recovered from the gel without significant contamination by the other polypeptide. This is shown in fig.2.

Analysis of the pattern of tryptic phosphopeptides obtained following digestion of the polypeptides shows the presence of the three phosphopeptides in both the branched-chain 2-oxoacid dehydrogenase and pyruvate dehydrogenase preparations (fig.3). The mobilities of the phosphopeptides during electrophoresis correspond closely to those of the phosphopeptides from purified complexes phosphorylated in vitro using [γ-^{32}P]ATP-Mg and the respective endogenous kinases [8,11]. In addition the detection of these three tryptic phosphopeptides has been reported previously for pyruvate dehydrogenase phosphorylated in mitochondria and in intact fat cells [12,13].

4. DISCUSSION

The data presented here indicate that the branched-chain 2-oxoacid dehydrogenase complex is phosphorlyated within intact mitochondria at three sites on the α subunit of the E_1 component. Such multi-site phosphorylation has been reported previously by us using purified enzyme with endogenous kinase activity [8]. This has subsequently been confirmed by others [14]. In our earlier studies, inactivation of the complex was shown to correlate closely with incorporation of phosphate into the phosphopeptide T1 [8]. In the present work no attempt has been made to correlate phosphorylation of the individual sites with control of the activity of the complex. Development of the methods reported here for determining radioactivity in the different phosphorylation sites should now make this feasible. However, the amount of radioactivity in each phosphopeptide is not necessarily an indication of the total amount of phosphate present at that site. For this to be the case conditions must first be worked out which cause complete dephosphorylation of all three sites prior to addition of [^{32}P]phosphate and succinate to the mitochondrial incubation. It is noteworthy therefore from fig.3 that in addition to peptide T1, significant radioactivity is also incorporated into peptides T2 and T3, indicating that there is significant turnover of phosphate at all three sites.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council, UK. R.L. is the recipient of a Research Studentship from the Science and Engineering Research Council, UK. We thank Drs I.C. West and H.S.A. Sherratt for advice on the preparation and treatment of mitochondria and thank Mr P.M. Brown for technical assistance.

REFERENCES

[1] Randle, P.J., Fatania, H.R. and Lau, K.S. (1983) Mol. Asp. Cell Regul. 3, in press.
[2] Odessey, R. (1980) FEBS Lett. 121, 306–308.
[3] Fatania, H.R., Lau, K.S. and Randle, P.J. (1981) FEBS Lett. 132, 285–288.
[4] Odessey, R. (1982) Biochem. J. 204, 353–356.
[5] Lawson, R., Cook, K.G. and Yeaman, S.J. (1983) FEBS Lett. 157, 54–58.
[6] Hughes, W.A. and Halestrap, A.P. (1981) Biochem. J. 196, 459–469.
[7] Patel, T.B. and Olson, M.S. (1982) Biochemistry 21, 4259–4265.
[8] Cook, K.G. Lawson, R.L. and Yeaman, S.J. (1983) FEBS Lett. 157, 59–62.
[9] Stanley, C.J. and Perham, R.N. (1980) Biochem. J. 191, 147–154.
[10] Laemmli, U.K. and Favre, M. (1973) J. Mol. Biol. 80, 575–599.
[11] Yeaman, S.J., Hutcheson, E.T., Roche, T.E., Pettit, F.H., Brown, J.R., Reed, L.J., Watson, D.C. and Dixon, G.H. (1978) Biochemistry 17, 2364–2370.
[12] Hughes, W.A. and Denton, R.M. (1978) Biochem. Soc. Trans. 6, 1228–1230.
[13] Hughes, W.A., Brownsey, R.W. and Denton, R.M. (1980) Biochem. J. 192, 469–481.
[14] Lau, K.S., Phillips, C.E. and Randle, P.J. (1983) FEBS Lett. 160, 149–152.