Calcium plays an important role in the regulation of hepatic branched-chain 2-oxo acid dehydrogenase activity

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1. INTRODUCTION

The activity of the branched-chain 2-oxo acid dehydrogenase complex (EC 1.2.4.4) is regulated by end-product inhibition and therefore subject to the NAD/NADH and CoA/branched-chain acyl CoA ratios in the cell [1–4]. Regulation by energy-linked metabolites such as ATP has also been reported [5–9]. Presumably the enzyme is phosphorylated by a kinase with concomitant loss in activity and activated by a phosphatase which removes the phosphate group.

The activation–deactivation cycle has been especially studied in rat heart and skeletal muscle – tissues in which the enzyme mainly exists in the inactive form. In these tissues the active complex is formed by incubating mitochondria without respiratory substrates or by incubation with uncouplers [6]. Addition of ATP leads to an inactivation of the complex [8].

As, in contrast to muscle mitochondria, the dehydrogenase complex of freshly prepared liver mitochondria was only detected in the active form, little response to experimental manipulation was observed [8]. Inactivation by ATP was shown in [9]. Here we show that dietary treatment with a synthetic amino acid diet leads to inactivation of the hepatic branched-chain 2-oxo acid dehydrogenase complex which can be reactivated by incubation in Krebs-Ringer phosphate buffer. In the presence of Ca^{2+}, reactivation can be blocked and the activated enzyme can be inactivated which in turn can be prevented by the prior addition of 4-methyl-2-oxo-pentanoate. The role of calcium in mediating branched-chain 2-oxo acid dehydrogenase activity suggests a potential cellular control mechanism.

2. EXPERIMENTAL

Male Sprague Dawley rats (130–150 g) were fed a commercial synthetic amino acid diet (C 1011, Altromin, Lage) for 6 days. At the end of the feeding period the animals were sacrificed, the liver was quickly excised and homogenized in ice-cold Krebs-Ringer phosphate buffer (KRP-buffer, pH 7.6) to give a 15% homogenate. The buffer contained: 123 mM NaCl, 4.9 mM KCl, 1.24 mM $MgSO_4$, 16 mM Na_2HPO_4 and 5.5 mM glucose.

Liver mitochondria were isolated as in [10,11] with minor modifications. Rat liver was homogenized in 5 vol. 0.25 M sucrose/10 mM Tris–HCl buffer/0.25 mM EDTA and centrifuged at 1500 × *g* for 5 min in a Heraeus Christ centrifuge. The supernatant fraction was centrifuged at 10 000 × *g* to sediment a crude mitochondrial pellet. The resulting supernatant used in the experiments was called 'supernatant'.

Incubation was at 37°C for 20 min in final vol. 2 ml KRP-buffer containing 1 ml homogenate or 1 ml mitochondrial suspension. Additions to the incubation mixture are given in the legends. Branched-chain 2-oxo acid dehydrogenase activity was assayed by measuring decarboxylation of 4-methyl-2-oxo [1-^{14}C]pentanoate as in [12]. The final reaction mixture contained in 0.5 ml: NAD (6.68 mM), CoA (3.32 mM), 4 methyl-2-oxo [1-^{14}C]-pentanoate (5 mM, 222 dpm/nmol), NaH_2PO_4 (pH 6.8) (16.6 mM), $MgCl_2$ (3.34 mM), $CaCl_2$ (3.34 mM) EDTA (0.166 mM), Na_2CO_3 (3.34 mM), NaF (25 mM) and 0.1 ml of the preincubated homogenate or 0.1 ml mitochondrial suspension.

Activity is expressed as μmol CO_2 . g tissue^{-1} . h^{-1} ± SEM. Protein concentration was determined by a modification of the method in [13].

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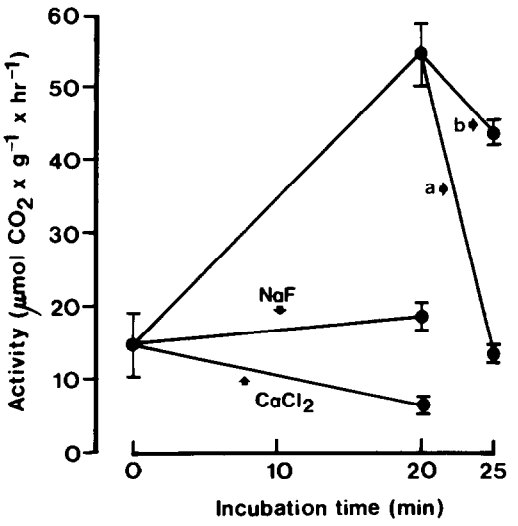

Fig.1. Activation of branched-chain 2-oxo acid dehydrogenase by incubation of liver homogenate and inhibition of activation by NaF and $CaCl_2$. Liver homogenate was incubated for 20 min in KRP-buffer at 37°C, then assayed for branched-chain 2-oxo acid dehydrogenase activity. Inhibition of activation was shown by adding NaF (25 mM) and $CaCl_2$ (0.25 mM) at time 0 to the incubation mixture. To some samples $CaCl_2$ (0.1 mM) was added after 20 min incubation either (a) alone or (b) together with 4-methyl-2-oxopentanoate (1 mM), the sequence being 2-oxo acid followed by $CaCl_2$. After 5 min of further incubation these samples were assayed for branched-chain 2-oxo acid dehydrogenase activity. Each point represents the mean ± SEM from 5–7 determinations.

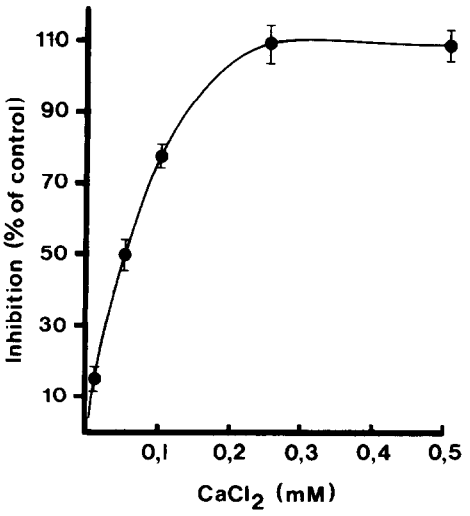

Fig.2. Inhibition of activation of hepatic branched-chain 2-oxo acid dehydrogenase by various levels of $CaCl_2$. $CaCl_2$ at 0.01–0.5 mM was added to rat liver homogenate which was incubated in KRP-buffer (pH 7.6) at 37°C. After 20 min incubation the samples were assayed for branched-chain 2-oxo acid dehydrogenase activity. Each point represents the mean ± SEM from 5–7 determinations.

3. RESULTS AND DISCUSSION

Feeding a synthetic amino acid diet (17%) results in low hepatic branched-chain 2-oxo acid dehydrogenase activity (12.5 ± 3.1) compared to values observed when feeding a laboratory chow containing intact protein (83.3 ± 7) [14].

Activation of inactive 2-oxo acid dehydrogenase complex from rats fed the amino acid diet was demonstrated by incubating rat liver homogenate in KRP-buffer for 20 min at 37°C (fig.1). There was a 4–5-fold increase in activity relative to unincubated liver homogenate indicating that ~80% of the enzyme exists in the inactive form. Working with rat heart, muscle and kidney mitochondria other authors also noted [5,8] an increase in activity upon incubation of mitochondria. They suggested that branched-chain 2-oxo acid dehydrogenase activity would be restored by action of a phosphoprotein phosphatase which dephosphorylates and thus activates the enzyme.

NaF (25 mM), an inhibitor of the phosphoprotein phosphatases [15], prevents reactivation of the complex nearly completely (fig.1) but has no effect on the activated enzyme (fig.2). Inhibition of activation was also observed when incubating rat liver homogenate with various concentrations of $CaCl_2$ (fig.2). $CaCl_2$ not only blocked activation but also inactivated active enzyme completely when added after 20 min preincubation (fig.1). This effect can be nearly abolished when the substrate 4-methyl-2-oxopentanoate (1 mM) is added at the same time but prior to $CaCl_2$ (fig.1). Protection against inactivation by the substrate might occur by inhibition of the kinase as has been shown for the pyruvate dehydrogenase complex [16].

EGTA (2.5 mM) in the incubation mixture also prevents inhibition by $CaCl_2$, it even stimulates activity when $CaCl_2$ is not added, possibly by removing endogeneous Ca^{2+} (fig.3). 2,4-Dinitrophenol,

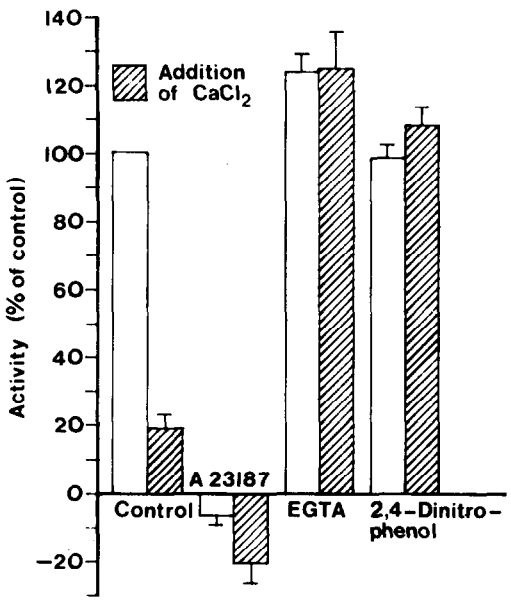

Fig.3. Effect of various compounds on hepatic branched-chain 2-oxo acid dehydrogenase activity. 2,4-Dinitrophenol (75 μM), the ionophore A23187 (4.75 μM) or EGTA (2.5 mM) were added to rat liver homogenate which was incubated for 20 min in KRP-buffer (pH 7.6) at 37°C. $CaCl_2$ (0.1 mM) was added after 20 min and the incubation mixture was further incubated for 5 min. The samples were assayed for branched-chain 2-oxo acid dehydrogenase activity. Each value is the mean ± SEM from 3–6 determinations.

an uncoupler of oxidative phosphorylation, shows no effect on the activation of the enzyme complex (fig.3). Yet when uncoupler is present and $CaCl_2$ is added after 20 min preincubation no inactivation by $CaCl_2$ occurs.

As 2,4-dinitrophenol prevents mitochondrial ATP synthesis, accelerates ATP hydrolysis and inhibits Ca^{2+} uptake by mitochondria [17] one could speculate that the energy state of the mitochondria is linked to the inactivation caused by calcium. Further evidence that Ca^{2+} is involved in the regulation of the enzyme is given by the observation that the ionophore A23187 in amounts of 4.5×10^{-6} M (fig.4) leads to complete inactivation of the enzyme. Ionophore A23187 has been used to dissipate Ca^{2+} gradients across a variety of membranes and to simulate Ca^{2+}-dependent phenomena in cells. The interpretation of the ionophore's action is complicated since in addition to effects upon plasma-membrane Ca^{2+} transport and its lack of membrane specificity it releases Ca^{2+} from isolated mitochondria [18]. If under our incubation conditions treatment with the ionophore leads to an efflux of Ca^{2+} from the mitochondria, and if a subsequent rise of cytosolic Ca^{2+} occurs, Ca^{2+} may bind to a cytosolic protein which in turn could affect regulation of branched-chain 2-oxo acid dehydrogenase activity. The suggestion that the Ca^{2+}-dependent regulation might occur via a non-mitochondrial factor is supported by the data in fig. 4. Incubating isolated liver mitochondria does not lead to an increase in enzyme activity.

Only when adding the 'supernatant' to the mitochondria an activation of the branched-chain 2-oxo

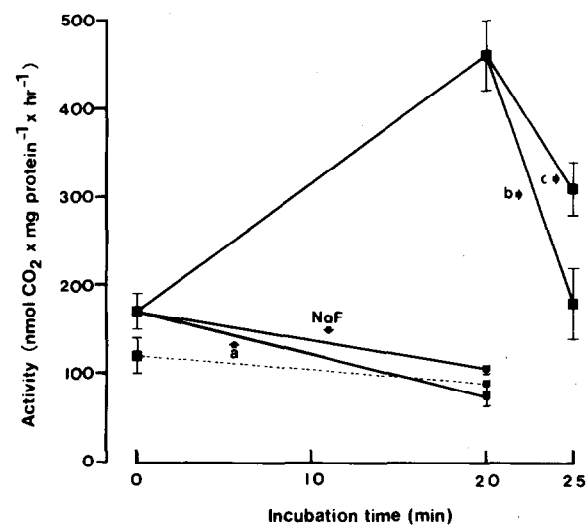

Fig.4. Activation of branched-chain 2-oxo acid dehydrogenase by incubation of isolated liver mitochondria to which 'supernatant' was added and inhibition of activation by $CaCl_2$ (0.1 mM) and NaF (25 mM). The samples (---, mitochondria; ——, mitochondria + supernatant) were incubated in KRP-buffer (pH 7.6) at 37°C for 20 min. Inhibition of activation was shown by adding NaF (25 mM) at the time 0 to the incubation mixture. $CaCl_2$ (0.1 mM) was added: (a) at the beginning; (b) after 20 min of incubation; (c) after 20 min together with 4-methyl-2-oxopentanoate, the sequence being 2-oxo acid (1 mM) followed by $CaCl_2$ (0.1 mM). When $CaCl_2$ was added after 20 min the samples were incubated for further 5 min. At the end of the incubation time all samples were assayed for branched-chain 2-oxo acid dehydrogenase activity. Each point represents the mean ± SEM from 3–6 determinations.

acid dehydrogenase complex occurs. As the enzyme activity measured in the 'supernatant' only accounts for 10% of the mitochondrial activity, and as this decarboxylating activity decreases during incubation, the observed activation of the mitochondrial enzyme cannot be explained by the negligible 'supernatant' activity. Evidently a compound of the 'supernatant' is responsible for the activation of the mitochondrial enzyme complex.

As observed in experiments with rat liver homogenate, $CaCl_2$ not only blocks activation of the mitochondrial enzyme achieved by the addition of the supernatant but also inactivates active enzyme when added after 20 min incubation. 4-Methyl-2-oxopentanoate partially prevents the Ca^{2+}-induced inactivation. These data are consistent with the possibility that the observed phenomena are brought about by a Ca^{2+}-dependent non-mitochondrial compound, which may play an important role in the regulation of branched-chain 2-oxo acid dehydrogenase activity. The chemical nature of this compound is under investigation.

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REFERENCES

[1] Danner, D.J., Lemmon, S.K. and Elsas, L.J. (1978) Biochem. Med. 19, 27–38.
[2] Parker, P.J. and Randle, P.J. (1978) FEBS Lett. 90, 183–186.
[3] Parker, P.J. and Randle, P.J. (1978) Biochem. J. 171, 751–757.
[4] Pettit, F.H., Yeaman, S.J. and Reed, L.J. (1978) Proc. Natl. Acad. Sci. USA 75, 4481–4485.
[5] Parker, P.J. and Randle, P.J. (1978) FEBS Lett. 95, 153–156.
[6] Parker, P.J. and Randle P.J. (1980) FEBS Lett. 112, 186–190.
[7] Odessey, R. and Goldberg, A.L. (1979) Biochem. J. 178, 475–489.
[8] Odessey, R. (1980) Biochem. J. 192, 155–163.
[9] Lau, K.S., Fatania, H.R., Randle P.J. (1981) FEBS Lett. 126, 66–70.
[10] Schneider, W.C. (1948) J. Biol. Chem. 176, 259–276.
[11] Cooper, C. and Lehninger, A.L. (1956) J. Biol. Chem. 219, 489–506.
[12] Hauschildt, S. and Brand, K. (1980) J. Nutr. 110, 1709–1716.
[13] Bensadoun, A., Weinstein, D. (1976) Anal. Biochem. 79, 241–250.
[14] Hauschildt, S., Lüthje, J. and Brand, K. (1981) J. Nutr. 111, 2188–2194.
[15] Linn, T.C., Telley, J.W., Pettit, F.H., Hucho, F., Randall, D.D. and Reed, L.J. (1972) Arch. Biochem. Biophys. 148, 326–342.
[16] Lin, T.C., Pettit, F.H., Hucho, F. and Reed, L.J. (1969) Proc. Natl. Acad. Sci. USA 64, 227–234.
[17] Lehninger, A.L., Carafoli, G. and Rossi, R.S. (1967) Adv. Enzymol. Rel. Areas Mol. Biol. 29, 259–320.
[18] Reed, P.W. and Lardy, H.A. (1972) J. Biol. Chem. 247, 6970–6977.