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The regulation of branched-chain 2-oxo acid dehydrogenase of liver, kidney and heart by phosphorylation

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1. Incubation of mitochondria from heart, liver and kidney with [^{32}P]phosphate allowed ^{32}P incorporation into two intramitochondrial proteins, the decarboxylase α-subunit of the pyruvate dehydrogenase complex (mol.wt. 42000) and a protein of mol.wt. 48000. 2. This latter protein incorporated ^{32}P more slowly than did pyruvate dehydrogenase, was not precipitated by antibody to pyruvate dehydrogenase and showed behaviour distinct from that of pyruvate dehydrogenase towards high-speed centrifugation and pyruvate dehydrogenase phosphate phosphatase. 3. ^{32}P incorporation into the protein was greatly diminished by the presence of 0.1 mM-4-methyl-2-oxopentanoate, but enhanced by pyruvate (1 mM), hypo-osmotic treatment of mitochondria and, under some conditions, by uncoupler. 4. The activity of branched-chain 2-oxo acid dehydrogenase was assayed in parallel experiments. Under appropriate conditions the enzyme was inhibited when ^{32}P incorporation was increased and activated when incorporation was decreased. The data suggest that the 48000-mol.wt. phosphorylated protein is identical with the decarboxylase subunit of branched-chain 2-oxo acid dehydrogenase and that this enzyme may be controlled by a phosphorylation–dephosphorylation cycle akin to that for pyruvate dehydrogenase. 5. Strict correlation between activity and ^{32}P incorporation was not observed, and a scheme for the regulation of the enzyme is proposed to account for these discrepancies.

The metabolism of the branched-chain amino acids valine, isoleucine and particularly leucine may play an important role in the regulation of protein synthesis and breakdown (see Fulks *et al.*, 1975; Buse & Reid, 1976; Goldberg & Chang, 1978; Chua *et al.*, 1979). The primary event in the catabolism of these three amino acids is their cytoplasmic transamination to the respective keto acids followed by transport of these keto acids into the mitochondria. Intramitochondrial oxidative decarboxylation to the relevant CoA derivative (see Meister, 1965; Odessey & Goldberg, 1979) is catalysed by a single multienzyme complex, the branched-chain 2-oxo acid dehydrogenase, which, like pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase, utilizes lipoamide and thiamin pyrophosphate as cofactors (Parker & Randle, 1978*a*,*b*; Pettit *et al.*, 1978; Danner *et al.*, 1979). As a reaction involving a considerable decrease in Gibbs energy, like that catalysed by pyruvate dehydrogenase, it represents a committed step in the catabolic pathway and thus is a likely candidate for regulation. Indeed, the enzyme from liver, kidney and heart is susceptible to similar end-product inhibition by CoA derivatives and NADH to that observed with pyruvate dehydrogenase (Parker & Randle, 1978*a*,*b*; Denton *et al.*, 1975). Moreover, there is indirect evidence to suggest that the enzyme from heart and skeletal muscle may be inhibited by phosphorylation (Parker & Randle, 1978*c*, 1980; Odessey & Goldberg, 1979). However, direct demonstration of phosphate incorporation into the enzyme complex has not been reported, and there is no evidence for the control of the enzyme from kidney and liver by phosphorylation (Parker & Randle, 1978*c*, 1980; Pettit *et al.*, 1978).

In the present paper we show that in isolated liver, kidney and heart mitochondria [^{32}P]phosphate may be incorporated into an intramitochondrial protein of mol.wt. 48000 that has the properties of a subunit of the branched-chain 2-oxo acid dehydrogenase

Abbreviation used: SDS, sodium dodecyl sulphate.

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complex. It is demonstrated that an increase in phosphorylation may lead to inhibition, whereas dephosphorylation may activate the enzyme. However, a strict correlation of ^{32}P incorporation with enzyme activity is not always observed. Various effectors of the phosphorylation system are described and related to a proposed scheme for the regulation of the enzyme.

A preliminary account of some of these results has been published (Hughes & Halestrap, 1980).

Experimental

Materials

Female albino Wistar rats (body wt. 225–275 g) were allowed free access to food and water and were killed by decapitation.

Unless otherwise stated the sources of all chemicals, biochemicals and radiochemicals were as given in Halestrap (1975) and Hughes *et al.* (1980).

Antiserum was raised in a sheep against purified pig heart pyruvate dehydrogenase complex by Dr. R. J. Mayer, University of Nottingham, Nottingham, U.K. (titre 15 units/ml of antiserum). For details of the specificity of the antibody and its general use see Hughes *et al.* (1980).

Preparation of mitochondria

Mitochondria from rat heart, liver and kidney cortex were prepared in 300 mM-sucrose/2 mM-EGTA/10 mM-Tris/HCl buffer, pH 7.5, as described previously (Halestrap, 1975). For kidney-cortex and heart mitochondria tissue disruption was achieved by using a Polytron PT 10 homogenizer (10 s, setting 3) and the extraction medium was supplemented with 10 mg of bovine serum albumin/ml. All mitochondrial preparations used showed respiratory control ratios of greater than 3 with succinate as substrate.

Incubation and extraction of mitochondria

Incubations of mitochondria both for ^{32}P incorporation into mitochondrial proteins and for enzyme-activity measurements were conducted in parallel experiments with identical media and conditions.

Mitochondria (2–4 mg of protein) were added to 0.5 ml of medium containing $MgCl_2$ (1 mM), potassium phosphate (0.5 mM), potassium succinate (5 mM), potassium EGTA (1 mM), Tris/HCl (20 mM) and KCl to give the required osmolarity (see the legends to Figures and Tables); the pH was 7.4 and the temperature 37°C. Where present, [^{32}P]phosphate was at a specific radioactivity of 200–500 c.p.m./pmol, and other additions were made as indicated. Incubation was continued for 15 min with shaking to maintain oxygenation. For most ^{32}P-labelling experiments incubation was terminated by addition of trichloroacetic acid (final concn. 10%, w/v) and separation of the denatured protein by centrifugation. The protein pellet was then washed once with 1 ml of 0.2 M-NaCl. For measurement of enzyme activity and some ^{32}P-radioactivity measurements where ^{32}P-labelled native protein was required, the incubation mixture was transferred to plastic centrifuge tubes and the mitochondria were rapidly sedimented by centrifugation at 9000 ***g*** for 30 s in an Eppendorf 3200 centrifuge. The supernatant was immediately removed, and the pellet was frozen in liquid N_2 and stored at −70°C. Control experiments demonstrated that neither ^{32}P incorporation nor the activities of branched-chain 2-oxo acid dehydrogenase and pyruvate dehydrogenase were appreciably altered during this separation procedure. The pellet was subsequently extracted for enzyme assay, antibody treatment and other studies by using 250 μl of medium containing potassium phosphate (100 mM), potassium EDTA (5 mM), KF (20 mM), dithiothreitol (3 mM), rat serum (100 μl/ml) and Triton X-100 (0.5%, w/v). To complete mitochondrial disruption, freezing and thawing were performed twice. The activities of branched-chain 2-oxo acid dehydrogenase and pyruvate dehydrogenase remained stable for several hours at 0°C in this medium.

Enzyme assays

Citrate synthase and pyruvate dehydrogenase activities and mitochondrial protein concentrations were assayed as described previously (Cooper *et al.*, 1974). Citrate synthase was used as a routine correction for any variation in extraction efficiency between experiments. Branched-chain 2-oxo acid dehydrogenase was assayed spectrophotometrically at 37°C essentially as described by Parker & Randle (1978*c*). The medium contained 30 mM-potassium phosphate, 3 mM-2-mercaptoethanol, 2 mM-$MgCl_2$, 0.4 mM-CoA, 0.4 mM-thiamin pyrophosphate, 1 mM-NAD^+ and 2 mM-KCN. After addition of mitochondrial extract (100 μl to 2 ml of medium), the background rate of NADH production was measured before addition of 0.25 mM-3-methyl-2-oxobutyrate to initiate the enzyme reaction. The initial rate of absorbance change was used to calculate enzyme activity, progressive inhibition of the reaction occurring after 1–2 min as described by Parker & Randle (1978*c*). Similar reaction rates were observed when 3-methyl-2-oxopentanoate was used as substrate, but the curve in the trace was more pronounced, perhaps reflecting more effective end-product inhibition (Parker & Randle, 1978*a*,*b*). Addition of excess lipoamide dehydrogenase, as suggested by Pettit *et al.* (1978), was without effect on the assay.

The maximal activities of the enzyme, obtained after incubation of mitochondria with 4-methyl-

2-oxopentanoate (0.1mM) or uncoupler at high osmolarity (see below), were (in munits/mg of protein at 37°C) for liver mitochondria 6.7±0.4 (34), for kidney mitochondria 11.7±2.1 (9) and for heart mitochondria 4.7±0.6 (9). All values are expressed as the means±S.E.M. for the numbers of separate mitochondrial preparations shown in parentheses. These values are in good agreement with those obtained by others (Pettit *et al.*, 1978; Parker & Randle, 1978*c*, 1980; Danner *et al.*, 1979), when the temperature of the assay is taken into account.

Separation and detection of [^{32}P]phosphoproteins

Routine separation of proteins was performed as described previously (Hughes *et al.*, 1980) by using discontinuous SDS/polyacrylamide-gel electrophoresis in 1cm tracks on 10% (w/v) slab gels (14cm×14cm) with the solutions described by Laemmli (1970). Samples for analysis, whether acid-precipitated proteins or antibody pellets, were dissolved in stacking-gel buffer, containing SDS (40mg/ml), sucrose (200mg/ml), Bromophenol Blue (0.2mg/ml) and 100mM-2-mercaptoethanol, by heating at 100°C for 5min. Electrophoresis was performed at 20°C for 2–3h at 40mA/slab.

After electrophoresis, the gel was washed for 1h in 1M-trichloroacetic acid in 50% (v/v) methanol to precipitate proteins in the gel. Protein was detected by staining with Coomassie Blue, and the gels were laid on cellophan and dried on boards under vacuum. The dried gels were exposed to Kodak Kodirex KT X-ray film for 2–7days. If an increased sensitivity was required, intensifier screens were used in combination with Kodak X-Omat X-ray film (Laskey & Mills, 1977). The radioautographs were scanned at 630nm with a Gilford spectrophotometer linked to a Hewlett Packard 9845S computer. Exposure of the film was such that peak heights did not exceed an absorbance of 1.0. The peak height and area were measured from a base-line, which was taken as the lowest reading of absorbance on that track.

Expression of results

The incorporation of ^{32}P into mitochondrial proteins is presented as densitometric scans of radioautographs representative of at least three similar experiments. Exact areas for the peak identified as branched-chain 2-oxo acid dehydrogenase cannot be calculated, since there is incomplete resolution from pyruvate dehydrogenase phosphate. Similar representative results are given for enzyme-activity measurements, since variation in activity between experiments makes combining of results difficult. Trends within experiments were reproducible, however, as shown in Table 1.

Results and discussion

Mitochondrial phosphorylation experiments

Incubation of mitochondria from heart, liver or kidney with [^{32}P]phosphate and succinate labelled two intramitochondrial proteins of molecular weights 42000 and 48000, which were resolved on SDS/polyacrylamide-gel electrophoresis (Fig. 1). With heart mitochondria and to a smaller extent those from liver and kidney, the 48000-mol.wt. protein was clearly resolved only when mitochondria were also exposed to pyruvate [scans (ii) and (iii) in Figs. 1(*a*), 1(*b*) and 1(*c*)] to activate pyruvate dehydrogenase and thus diminish its ^{32}P incorporation (Cooper *et al.*, 1974). The presence of pyruvate also appeared to enhance labelling of the 48000-mol.wt. protein in all three types of mitochondria (Fig. 1). Pyruvate was added in all the experiments described below unless otherwise stated. This allowed clear resolution of the 48000-mol.wt. peak in all three mitochondrial preparations (Fig. 1*d*), although the extent of resolution was somewhat variable [e.g. scan (ii) in Fig. 1(*a*) compared with scan (ii) in Fig. 1(*d*)]. In kidney mitochondria an additional intramitochondrial phosphorylated protein of mol.wt. 80000 was observed that was not present in other mitochondrial preparations (Fig. 1*b*).

In all three mitochondrial preparations addition of 4-methyl-2-oxopentanoate (0.1–0.5mM) to the incubation greatly decreased or abolished the labelling of the 48000-mol.wt. protein while having little or no effect on the labelling of pyruvate dehydrogenase (Fig. 1). This specific effect of 4-methyl-2-oxopentanoate, a substrate of the branched-chain 2-oxo acid dehydrogenase, suggests that the identity of the 48000-mol.wt. protein might be that of a phosphorylated subunit of the enzyme. In addition, it is known that the decarboxylase subunit has a molecular weight of about 46000 (Pettit *et al.*, 1978). Further experiments were conducted to confirm this identification.

The branched-chain 2-oxo acid dehydrogenase is located exclusively within the mitochondria (Odessey & Goldberg, 1979), and it was necessary to demonstrate that labelling of the 48000-mol.wt. protein was solely intramitochondrial. This was achieved by demonstrating that neither atractyloside nor the additions of extramitochondrial glucose and hexokinase diminished the extent of labelling. These two additions should have diminished [^{32}P]ATP leakage from the mitochondria while utilizing any ATP that did leak out, thus preventing any extramitochondrial protein phosphorylation. Furthermore addition of extramitochondrial ADP (which allows synthesis of [^{32}P]-ATP by oxidative phosphorylation followed by its export) gave additional protein phosphorylations quite distinct from the two observed in the absence

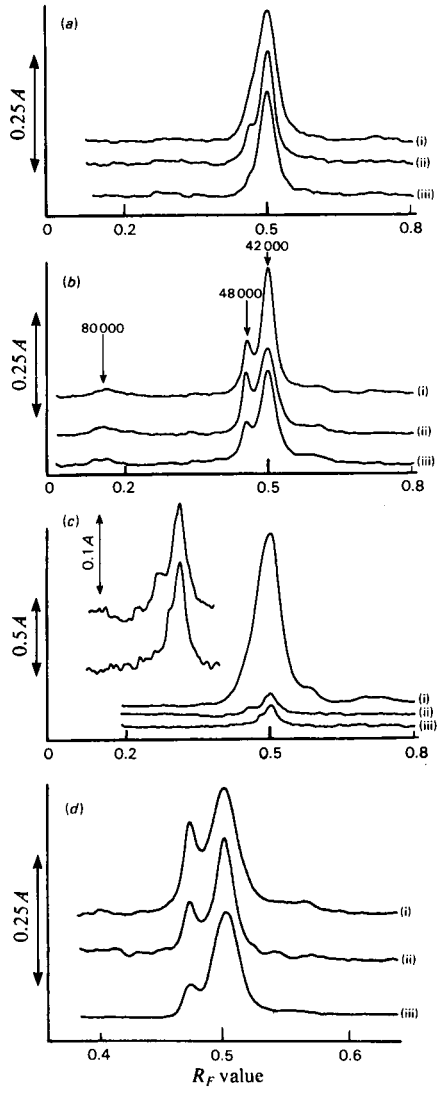


Fig. 1. *Densitometric traces of the radioautographs locating the labelled proteins of liver, kidney and heart mitochondria previously incubated with* $^{32}P_i$

Preparation and incubation of (*a*) liver mitochondria, (*b*) kidney mitochondria and (*c*) heart mitochondria were as described in the Experimental section. In each case mitochondria were incubated for 15 min in the presence (ii and iii) or in the absence (i) of 1mM-pyruvate. In (iii) 0.5mM-4-methyl-2-oxopentanoate was also added. In all cases proteins from equivalent samples were separated by SDS/polyacrylamide-slab-gel electrophoresis. The dye front (Bromophenol Blue) was allowed to migrate 10cm. (*d*) Results from a similar but separate experiment in which kidney (i), liver (ii) and heart (iii) mitochondria were incubated in the presence of pyruvate as in condition (ii) of (*a*), (*b*) and (*c*) and the proteins were resolved on the same gel. In (*b*) the molecular weights of the three peaks, determined by using bovine serum albumin (mol.wt. 68000), lactate dehydrogenase (mol.wt. 36500) and lysozyme (mol.wt. 14500) as standards, are given. In (*c*) the insert shows the two major peaks on an expanded (×5) absorbance scale.

of ADP (results not shown). After solubilization of the mitochondrial proteins with Triton X-100 as described in the Experimental section, centrifugation for 20min at room temperature in a Beckman Airfuge (100000*g* approx.) sedimented both pyruvate dehydrogenase (>90%) and branched-chain 2-oxo acid dehydrogenase (>80%) activities, and both radioactive proteins were also found almost totally in the pellet. Shorter centrifugation (5min) was sufficient to sediment most (>80%) of the pyruvate dehydrogenase activity but little (<30%) of the branched-chain 2-oxo acid dehydrogenase activity, and this was reflected in the differential behaviour of the two ^{32}P-labelled proteins (Fig. 2*b*). The parallel behaviour of the ^{32}P-labelled proteins and enzyme activities strengthens the evidence that the 42000-mol.wt. and 48000-mol.wt. proteins are the phosphorylated subunits of pyruvate dehydrogenase and the branched-chain 2-oxo acid dehydrogenase respectively, and is consistent with the known molecular weights and hydrodynamic properties of the two complexes (Hayakawa *et al.*, 1966; Barrera *et al.*, 1972; Linn *et al.*, 1972; Pettit *et al.*, 1978).

Incubation of mitochondrial extracts with specific antibody to pyruvate dehydrogenase led to precipitation of all the 42000-mol.wt. protein but none of the 48000-mol.wt. protein (Fig. 2*c*). Parallel experiments showed that antibody precipitated and inactivated pyruvate dehydrogenase, but was without effect on branched-chain 2-oxo acid dehydrogenase activity. Similarly incubation of mitochondrial extracts with pyruvate dehydrogenase phosphate phosphatase activated pyruvate dehydrogenase and all radioactivity was lost from the 42000-mol.wt. protein (Fig. 2*a*). Little or no decrease in the 48000-mol.wt. peak was observed, however, and there was no appreciable activation of branched-chain 2-oxo acid dehydrogenase. This contrasts with the data of Parker & Randle (1980), who observed activation of the enzyme with pyruvate dehydrogenase phosphate phosphatase, but these workers incubated their system in the absence of Triton X-100 and fluoride, which were used in the present experiments.

Thus the behaviour of this 48000-mol.wt. phosphoprotein of heart, liver and kidney mitochondria is distinct from that of the 42000-mol.wt. subunit of pyruvate dehydrogenase. The evidence suggests that it is a phosphorylated subunit of the branched-chain 2-oxo acid dehydrogenase. Furthermore two-dimensional gel electrophoresis (isoelectric focusing followed by SDS/polyacrylamide-gel electro-

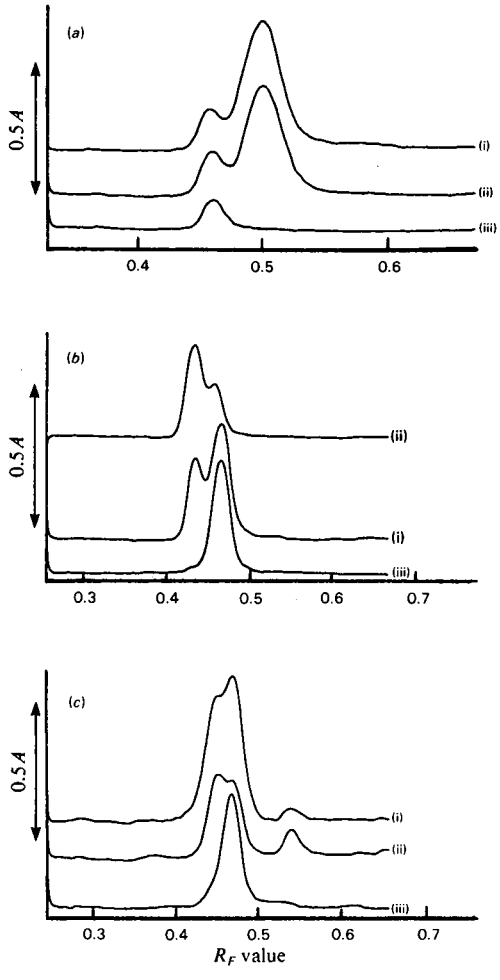


Fig. 2. *Densitometric traces of the radioautographs locating mitochondrial [^{32}P]phosphoproteins after various treatments*

Extracts of heart (*a*) and liver (*b* and *c*) mitochondrial pellets, derived from intact mitochondria previously incubated for 15 min with $^{32}P_i$ as described in the Experimental section, were treated as follows. (*a*) The extract was incubated at 30°C for 20 min alone (i), or with 25mM-$MgCl_2$ and 1mM-$CaCl_2$ in the absence (ii) or in the presence (iii) of pyruvate dehydrogenase phosphate phosphatase (1munit/ml). (*b*) The extract (i) was centrifuged at 120000*g* at room temperature for 5min in a Beckman Airfuge centrifuge, then the supernatant (ii) and pellet (iii) fractions were analysed. (*c*) The extract was treated with Triton X-100 (1%, w/v) and then centrifuged at 9000*g* for 5min. A sample of this supernatant (i) was incubated at 30°C for 30min with 50μl of anti-(pyruvate dehydrogenase) serum/ml, and after centrifugation at 9000*g* for 5min the supernatant (ii) and pellet (iii) fractions were collected. In all cases proteins from equivalent samples were separated by SDS/polyacrylamide-gel electrophoresis. The dye front (Bromophenol Blue) was allowed to migrate 10cm.

phoresis) showed that the labelled protein was identical in all three types of mitochondria and the same as the 48000-mol.wt. protein labelled in mitochondria of intact adipocytes (Hughes *et al.*, 1980).

Relationships between activity and phosphorylations of branched-chain 2-oxo acid dehydrogenase

Studies were conducted to discover the optimal conditions of ^{32}P-labelling of the 48000-mol.wt. protein in an attempt to correlate activity and ^{32}P incorporation into the enzyme. A variety of incubation conditions for the mitochondria were used to monitor any effect on the activity of the extracted branched-chain 2-oxo acid dehydrogenase complex.

The time course of ^{32}P incorporation into the 48000-mol.wt. protein is shown in Fig. 3. It is apparent that the rate of labelling is lower than that of pyruvate dehydrogenase, and labelling does not reach complete equilibrium even after 20min incubation at 37°C. Incubation for longer than 20 min

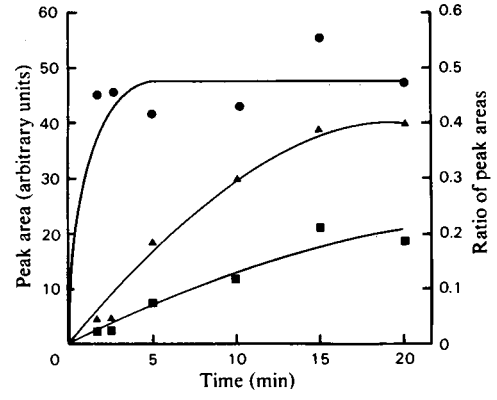


Fig. 3. *Time course of ^{32}P incorporation into rat liver mitochondrial pyruvate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complexes*

Preparation and incubation of mitochondria with $^{32}P_i$ and subsequent resolution by SDS/polyacrylamide-gel electrophoresis were as described in the Experimental section. Liver mitochondria were incubated for the appropriate time and the incubation was terminated by precipitation with 10% (w/v) trichloroacetic acid. The approximate peak area on the radioautograph scan of each phosphoprotein was calculated by using the computer graphics of a Hewlett Packard 9845S and assuming the peak obeyed a normal distribution. Values are given in arbitrary units, ■ representing branched-chain 2-oxo acid dehydrogenase, ● pyruvate dehydrogenase and ▲ the ratio of the two areas. The time course of the change in ratio shows a rate of change significantly greater ($P < 0.001$) than zero when analysed by linear regression. The experiment shown represents one of two identical experiments producing similar results.

at this temperature proved to be inadvisable, since leakage of adenine nucleotides out of the mitochondria occurred and considerable mitochondrial deterioration was observed. For routine experiments a 15 min incubation proved most suitable. It is important to stress that ^{32}P incorporation does not reach a steady state under these conditions, and this imposes constraints on the interpretation of parallel ^{32}P-labelling and activity measurements, as indicated below. Any condition that slows either the kinase or phosphatase responsible for the phosphorylation–dephosphorylation cycle may increase the time taken to reach a steady state, and so further complicate interpretation of such parallel experiments.

In Fig. 4 it is shown that the extent of labelling of both proteins could be greatly enhanced by increasing the mitochondrial volume, brought about by decreasing the osmotic support of the incubation medium. Conversely, contraction of mitochondria decreased the labelling. Labelling of the 48000-mol.wt. protein was greatly diminished by 4-methyl-2-oxopentanoate (0.1 mM) at all osmolarities. The results shown were obtained with sucrose used as an osmotic support, but similar results were observed with KCl (results not shown). The data suggest either that the phosphatase responsible for dephosphorylation of each protein is activated by a decrease in mitochondrial volume or that the kinase is inhibited. An effect on the phosphatase might be expected if, as with pyruvate dehydrogenase, the kinase is an integral part of the complex and is exposed to a constant concentration of the substrate, whereas the phosphatase is a separate entity whose activity could be subject to substrate concentrations (Denton *et al.*, 1975).

Under similar conditions the effects of changing the osmolarity on the activity of branched-chain 2-oxo acid dehydrogenase were studied in the presence and in the absence of uncoupler and 4-methyl-2-oxopentanoate (Fig. 5). It has already been stated that pyruvate (1 mM) was present throughout to make the experiment comparable with the ^{32}P-labelling experiments, and, as indicated below, this also serves to diminish the basal enzyme

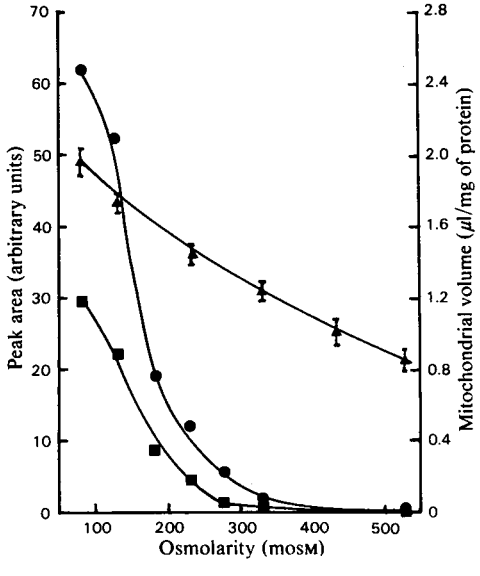


Fig. 4. *Effect of osmolarity on the labelling of pyruvate dehydrogenase and branched-chain 2-oxo acid dehydrogenase with $^{32}P_i$ in intact liver mitochondria*

Incubations of mitochondria, separation of phosphoproteins by electrophoresis and measurement of peak areas were performed as described in the Experimental section and in the legend to Fig. 3. Incubations of mitochondria with $^{32}P_i$ were for 15 min and the osmolarity was varied with sucrose. Mitochondrial volumes (▲) were measured in a separate experiment as described by Halestrap & McGivan (1979) and are shown as the means ± S.E.M. for four separate observations. Peak areas for pyruvate dehydrogenase (●) and branched-chain 2-oxo acid dehydrogenase (■) are given in arbitrary units.

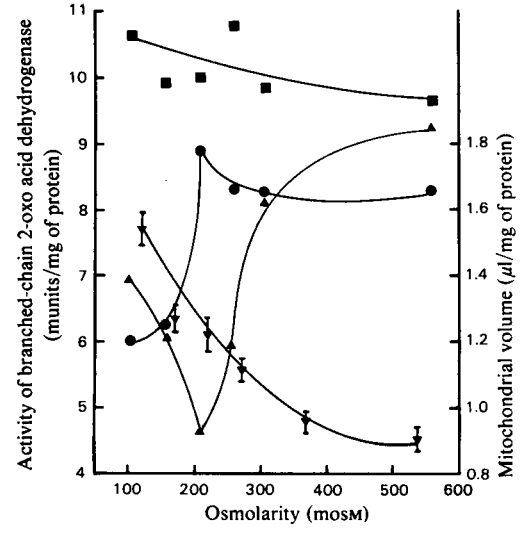


Fig. 5. *Activity of branched-chain 2-oxo acid dehydrogenase in liver mitochondria incubated at different osmolarities*

Rat liver mitochondria were incubated for 15 min in the absence (●) and in the presence of 0.1 mM-4-methyl-2-oxopentanoate (■) or 2 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (▲) as described in the Experimental section. Pyruvate (1 mM) was present throughout and the osmolarity was varied with KCl. Extraction and measurement of enzyme activities and determination of mitochondrial volumes (measured in the absence of further additions) (▼) are described in the Experimental section and the legend to Fig. 4.

activity. As the osmolarity increased the basal enzyme activity also increased, and to a limited degree this correlated with the decrease in labelling observed in Fig. 4. The presence of 4-methyl-2-oxopentanoate (0.1 mM) led to activation at all osmolarities, in parallel with a decease in ^{32}P incorporation. The effect of uncoupler on the activity of branched-chain 2-oxo acid dehydrogenase was complex. As the osmolarity increased from 100 to 200 mosM the activity of the enzyme decreased, whereas at higher osmolarities the activity began to increase again. It is worth noting that pyruvate dehydrogenase was fully activated by uncoupler under all these conditions. Two important points emerge from these studies. Firstly, under iso-osmotic conditions (about 300 mosM) little if any activation of branched-chain 2-oxo acid dehydrogenase is observed in the presence of 4-methyl-2-oxopentanoate (0.1 mM) or uncoupler. This may explain the inability of Parker & Randle (1978*c*, 1980) to demonstrate control of the liver and kidney enzyme by phosphorylation and dephosphorylation. Secondly, uncoupler, which might be expected to activate the enzyme by decreasing mitochondrial ATP and so decreasing phosphorylation, only does so under conditions of high osmolarity, and may even inhibit at low osmolarity. Under these latter conditions ^{32}P-labelling experiments (Fig. 6) show that ^{32}P incorporation into pyruvate dehydrogenase is abolished whereas that into the 48000-mol.wt. protein is actually increased. Again ^{32}P incorporation and activity measurements correlate, albeit in an unexpected way. Fig. 6 also demonstrates that dichloroacetate decreases ^{32}P incorporation into both proteins, and this also correlates with an increase in branched-chain 2-oxo acid dehydrogenase activity (see Table 1). Similar results (Table 1) could be observed with heart and kidney mitochondria at low osmolarity (150 mosM).

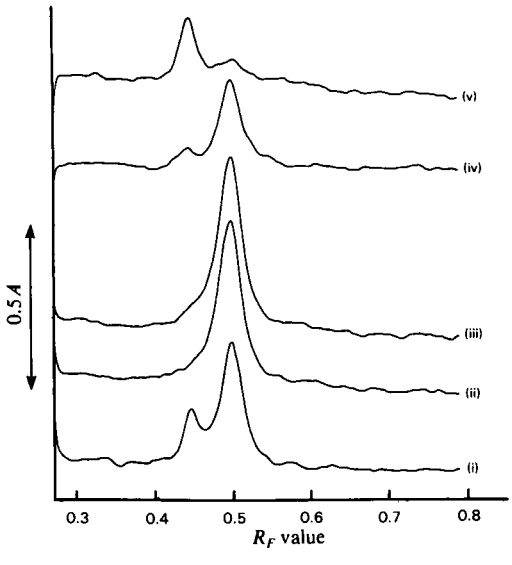


Fig. 6. *Densitometric traces of the radioautographs locating rat liver mitochondrial [^{32}P]phosphoproteins derived from intact mitochondria incubated with $^{32}P_i$ and various effectors*

Preparation and incubation of liver mitochondria were as described in the Experimental section. Intact mitochondria were incubated for 15 min in the absence (i) or in the presence of 0.1 mM- (ii) or 0.5 mM- (iii) 4-methyl-2-oxopentanoate, with 1 mM-dichloracetate (iv) or with 2 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (v). In each case proteins from the acid-precipitated pellet were separated by SDS/polyacrylamide-slab-gel electrophoresis. The dye front (Bromophenol Blue) was allowed to migrate 10 cm.

The activating effect of 4-methyl-2-oxopentanoate (0.1 mM) on branched-chain 2-oxo acid dehydrogenase activity agrees well with the data of Parker & Randle (1978*c*, 1980) for heart mitochondria. However, we were unable to detect an effect of 3-methyl-2-oxobutyrate (0.1 or 0.5 mM) on either enzyme activity or ^{32}P-labelling, whereas Parker & Randle (1978*c*) do report activation of the heart enzyme by this effector. In addition, when higher concentrations of the 4-methyl-2-oxopentanoate were used (0.2–0.5 mM) results became extremely variable, and frequently inhibition of activity was seen, especially at high osmolarity (see Table 1). Thus, in liver mitochondria incubated at 150 mosM, 0.1 mM-4-methyl-2-oxopentanoate caused an activation of the enzyme to $165 \pm 16\%$ of the control value (mean $\pm$ S.E.M. for 19 separate incubations) whereas at 300 mosM the activity was decreased to $74 \pm 3\%$ ($n = 10$) by 0.5 mM-4-methyl-2-oxopentanoate. For kidney mitochondria the changes in activity as percentages of the control values were $143 \pm 13\%$ ($n = 6$) and $74 \pm 2\%$ ($n = 4$) respectively, and for heart mitochondria $234 \pm 70\%$ ($n = 5$) and $65 \pm 6\%$ ($n = 4$) respectively. However, at all concentrations of 4-methyl-2-oxopentanoate, ^{32}P incorporation into the 48000-mol.wt. protein was greatly diminished.

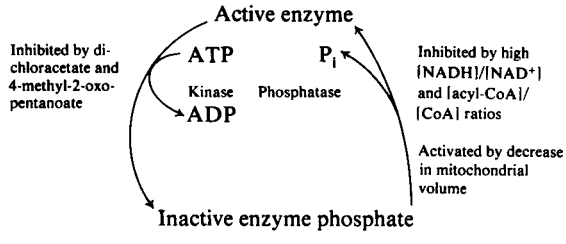
Proposed scheme

To account for the observed results a scheme (Scheme 1) can be proposed and further tested. The fundamental propositions of the scheme are that the kinase is inhibited by 4-methyl-2-oxopentanoate and dichloroacetate, but is relatively insensitive to inhibition by ADP in comparison with pyruvate dehydrogenase kinase (Linn *et al.*, 1969). The phosphatase activity is assumed to be low, and to be inhibited by conditions favouring end-product inhibition. In this respect the activity of the phosphatase may differ from the activity of pyruvate dehydro-

Table 1. *Activity of branched-chain 2-oxo acid dehydrogenase in liver, kidney and heart mitochondria incubated under various conditions*

Mitochondria were incubated with the additions shown for 15 min and then extracted for measurement of enzyme activities as described in the Experimental section. Osmolarity was altered with KCl. All values were corrected for variable extraction efficiency by using citrate synthase activities as described in the Experimental section. For liver mitochondria three experiments are shown on different preparations of mitochondria to demonstrate the variation in actual activities but similarity of trends between experiments. Similar additional experiments performed on kidney and heart mitochondria also showed variation in actual values but similarity of trends between experiments. The uncoupler used was carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

	Activity of branched-chain 2-oxo acid dehydrogenase (munits/mg of protein) in mitochondria from				
	Liver			Kidney	Heart
Additions to incubation mixture	Expt. 1	Expt. 2	Expt. 3	Expt. 1	Expt. 1
I 150 mosM + pyruvate (1.0 mM)					
None	6.4	3.5	4.4	5.5	1.11
4-Methyl-2-oxopentanoate (0.1 mM)	7.5	9.1	8.4	8.0	1.34
4-Methyl-2-oxopentanoate (0.5 mM)	—	—	8.1	8.1	1.61
Dichloroacetate (1.0 mM)	7.3	5.4	7.3	7.2	1.71
Uncoupler (2.0 μM)	4.9	4.1	5.3	4.5	0.16
L-Carnitine (1.0 mM)	—	4.8	5.4	—	—
Uncoupler (2.0 μM) + L-carnitine (1.0 mM)	5.4	—	—	5.8	—
Uncoupler (2.0 μM) + acetyl-DL-carnitine (1.0 mM)	4.3	—	—	1.1	—
3-Methyl-2-oxobutyrate (0.1 mM)	5.7	—	—	6.5	1.14
3-Methyl-2-oxobutyrate (0.5 mM)	6.4	—	—	6.8	1.06
No pyruvate	7.7	—	—	9.5	4.06
II 550 mosM + pyruvate (1.0 mM)					
None	9.4	3.7	4.8	4.0	0.47
4-Methyl-2-oxopentanoate (0.5 mM)	3.4	3.0	4.6	1.1	0.26
III No incubation	6.5	7.4	5.8	6.3	<0.1



Scheme 1. *Proposed mechanisms for regulating the phosphorylation state of the branched-chain 2-oxo acid dehydrogenase complex*

The kinase is postulated to be an integral part of the enzyme complex and the phosphatase a separate entity.

genase phosphatase towards pyruvate dehydrogenase, whose end products, with the possible exception of NADH (Pettit *et al.*, 1975), have no observed effect on dephosphorylation (Pettit *et al.*, 1975; Denton *et al.*, 1975). Pyruvate dehydrogenase kinase, on the other hand, is activated under conditions of end-product inhibition, and a change in the conformation of pyruvate dehydrogenase is a likely explanation of this effect (Pettit *et al.*, 1975; Cooper *et al.*, 1975; Cate & Roche, 1978). Such a conformational change of the branched-chain 2-oxo acid dehydrogenase by end-product inhibition could similarly affect the affinity of the phosphorylated form of this enzyme towards its phosphatase. To account for the effects of osmolarity on ^{32}P incorporation and enzyme activity, it is proposed that the phosphatase is a separate entity from the enzyme complex, as discussed above.

The activation of enzyme activity by 0.1 mM-4-methyl-2-oxopentanoate may be explained by inhibition of the kinase allowing a rise in the concentration of active dephosphorylated complex. However, inhibition of activity observed at high concentrations of 4-methyl-2-oxopentanoate may be explained by the metabolism of the acid to the branched-chain acyl-CoA derivative, which is known to accumulate in mitochondria under similar conditions (Williamson *et al.*, 1979; Walajtys-Rode *et al.*, 1979). Such conditions of end-product inhibition would lead to inhibition of the phosphatase and hence to inhibition of enzyme activity, as well as inhibition of the kinase by 4-methyl-

2-oxopentanoate (diminished ^{32}P-labelling seen). Incorporation of ^{32}P would be far displaced from a true steady state under these conditions, and could account for the decreased enzyme activity accompanied by decreased ^{32}P incorporation.

The inhibitory effects of uncoupler under some conditions might be explained by activation of pyruvate dehydrogenase, causing an elevation of acetyl-CoA through increased pyruvate oxidation. This would decrease the free CoA concentration and thus inhibit the phosphatase. The kinase would continue to function, since it is relatively insensitive to inhibition by ADP. As the osmolarity increases and the volume decreases, enhanced phosphatase activity (see above) would allow activation of the enzyme to be observed.

To test this hypothesis further, additional experiments reported in Table 1 were performed. It can be seen that in the presence of pyruvate, which would be expected to increase acetyl-CoA at the expense of free CoA, the enzyme is considerably less active than in the absence of pyruvate. Addition of uncoupler in the presence of pyruvate caused an inhibition of the enzyme that could be partially relieved by L-carnitine (1mM). Addition of acetyl-DL-carnitine in place of L-carnitine had the reverse effect. Since carnitine might be expected to liberate free CoA but acetylcarnitine to utilize it to produce acetyl-CoA, the results are consistent with inhibition of branched-chain 2-oxo acid dehydrogenase under conditions of low free CoA concentrations. Table 1 also demonstrates that high concentrations of 4-methyl-2-oxopentanoate were most effective at causing inhibition at high osmotic strength. It might be expected that branched-chain acyl-CoA derivatives would exist at a higher concentration under conditions of matrix condensation, thus causing greater phosphatase inhibition.

The proposed inhibitory effects of increased branched-chain acyl-CoA concentrations and diminished free CoA concentrations on the phosphatase responsible for dephosphorylation of the branched-chain 2-oxo acid dehydrogenase were tested as follows. Mitochondria (liver, heart or kidney) were incubated for 10min with succinate and pyruvate, at which point 4-methyl-2-oxopentanoate (0.5mM) was added. After a further 10min incubation little or no loss of ^{32}P present in the 48000-mol.wt. protein occurred when compared with incubations terminated at 10min. Yet under these conditions incorporation of ^{32}P into the protein is greatly diminished if 4-methyl-2-oxopentanoate is present throughout the incubation, as demonstrated in Fig. 1. This confirms that the phosphatase is extremely inactive under these conditions and that Scheme 1 may allow understanding of the relationship between activity and phosphorylation of branched-chain 2-oxo acid dehydrogenase.

General conclusions

The data reported in this paper demonstrate that, when incubated under suitable conditions, and especially with hypo-osmotic buffers in the presence of pyruvate, liver, heart and kidney mitochondria phosphorylate an intramitochondrial protein of mol.wt. 48000 in addition to pyruvate dehydrogenase. The protein can be distinguished from pyruvate dehydrogenase by its different behaviour towards pyruvate dehydrogenase phosphate phosphatase, antibody to pyruvate dehydrogenase and high-speed centrifugation. The phosphorylation of the protein is greatly inhibited by the presence of 4-methyl-2-oxopentanoate (0.1mM), which suggests that it may be identified as the branched-chain 2-oxo acid dehydrogenase. This enzyme is known to be exclusively mitochondrial (see Odessey & Goldberg, 1979) and to have a decarboxylase subunit of mol.wt. about 48000 (Pettit *et al.*, 1978). The molecular weight and hydrodynamic properties of the enzyme complex (Pettit *et al.*, 1978; Danner *et al.*, 1979) are also compatible with the sedimentation of the phosphorylated protein after high-speed centrifugation for 20min in a Beckman Airfuge. Data from various laboratories have provided indirect evidence for inactivation of this enzyme in heart mitochondria by phosphorylation, which may be inhibited by 4-methyl-2-oxopentanoate (Parker & Randle, 1978*c*, 1980; Odessey & Goldberg, 1979). We have confirmed these observations, and demonstrated the parallel activation of the enzyme and inhibition of ^{32}P incorporation into the 48000-mol.wt. protein. Parker & Randle (1978*c*, 1980) have suggested that the enzymes from kidney and liver mitochondria are not regulated in this manner. Clearly the data in the present paper refute this suggestion. Furthermore the results indicate that the branched-chain 2-oxo acid dehydrogenase dephosphorylation–phosphorylation system differs in some respects from the pattern observed for pyruvate dehydrogenase. To account for the type of behaviour seen with uncoupler, high concentrations of 4-methyl-2-oxopentanoate and different osmotic strengths, a scheme was put forward and tested experimentally (see above for discussion of Scheme 1).

The proposed scheme for the regulation of the branched-chain 2-oxo acid dehydrogenase not only accounts for the data in the present paper, but also explains many published observations on the metabolism of branched-chain amino acids and 2-oxo acids in mitochondria and intact tissue preparations. Thus the inhibitory effects of branched-chain acylcarnitines, pyruvate and uncoupler and the activating effect of carnitine on oxidation of branched-chain 2-oxo acids by intact heart, liver and skeletal-muscle mitochondria (Bremer & Davis, 1978; Van Hinsbergh *et al.*, 1979) all comply with

the proposed scheme. Similarly, in the perfused heart the activation of leucine and branched-chain 2-oxo acid oxidation by dichloroacetate and the inhibition by pyruvate and ketone bodies (Buffington *et al.*, 1979; Sans *et al.*, 1980*a*,*b*) can be explained by the model. Dichloroacetate could act by inhibition of the kinase, and pyruvate and ketone bodies could cause inhibition of the phosphatase by decreasing the free CoA. Lund (1978) has suggested that free CoA may also be important in regulating leucine catabolism in the liver. Williamson *et al.* (1979) reached similar conclusions by showing that pyruvate and fatty acids inhibited 4-methyl-2-oxobutyrate oxidation by hepatocytes, but stressed that end-production inhibition by NADH may be more important. Measurement of short-chain and medium-chain acyl-CoA showed that the branched-chain 2-oxo acid dehydrogenase was the site of inhibition. In freeze-clamped liver we have been unable to observe any appreciable activity of the dehydrogenase, suggesting that *in vivo* the enzyme may be largely inhibited. This correlates with the enhanced labelling of the protein seen in intact fat-cells (Hughes *et al.*, 1980) and liver cells (A. Vargas & A. P. Halestrap, unpublished work) when compared with isolated mitochondria.

Whether the enzyme can be regulated hormonally through changes in phosphorylation similarly to pyruvate dehydrogenase remains to be established. Frick & Goodman (1980) have reported some activation of the enzyme by insulin in adipose tissue through a decrease in the K_m of the enzyme for 2-oxo acids rather than by an increase in $V_{max.}$. A large stimulation by insulin of leucine and 4-methyl-2-oxopentanoate oxidation by starved rat hindquarters has also been reported (Hutson *et al.*, 1980), although slight inhibition was observed in fed animals. Starvation also appears to cause an increase in the activity of the enzyme in skeletal muscle and kidney (Goldberg & Odessey, 1972; Adibi *et al.*, 1974), and induction of the liver enzyme is caused by amino acids (Wohlhueter & Harper, 1970). However, these effects are long-term effects and may represent an increase in total enzyme activity rather than changes in the phosphorylation state of the enzyme (Wohlhueter & Harper, 1970).

Both leucine and 4-methyl-2-oxopentanoate cause an increase in protein synthesis and a decrease in protein degradation in heart and muscle (see Chua *et al.*, 1979). Thus any regulation of the branched-chain 2-oxo acid dehydrogenase, the rate-limiting step in muscle leucine catabolism (Odessey & Goldberg, 1979), could have far-reaching consequences in protein metabolism. The demonstration of a phosphorylation–dephosphorylation cycle of the enzyme in all tissues studied provides an obvious mechanism for such regulation.

Note added in proof (received 8 January 1981)

Since the writing of the present paper there have been three reports in the literature that support the results presented here. Odessey (1980*a*) has provided further indirect evidence for a regulatory phosphorylation–dephosphorylation cycle of branched-chain 2-oxo acid dehydrogenase activity from liver, kidney and heart mitochondria. In agreement with our own observations, he found that the enzyme from kidney and liver was almost fully active (dephosphorylated) in isolated mitochondria, whereas that from heart was largely in the inactive (phosphorylated) form. Inactivation of the enzyme required the splitting of the γ-phosphate group of ATP. He has subsequently shown that ^{32}P may be incorporated into the kidney enzyme from [γ-^{32}P]-ATP (Odessey, 1980*b*). Waymack *et al.* (1980) have demonstrated a substantial and rapid decrease in branched-chain 2-oxo acid dehydrogenase in the perfused heart exposed to pyruvate. This effect required the transport of pyruvate into the mitochondria and supports the inhibitory effects of pyruvate observed in our own experiments.

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