Short Communications

Stimulation by Calcium Ions of Pyruvate Dehydrogenase Phosphate Phosphatase

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In a number of mammalian tissues (heart, liver, kidney, fat-cells and brain), pyruvate dehydrogenase activity (EC 1.2.4.1) has been shown to be regulated by a phosphorylation–dephosphorylation cycle (Linn *et al.*, 1969*a,b*; Wieland & Siess, 1970; Jungas, 1971; Coore *et al.*, 1971; Siess *et al.*, 1971). Phosphorylation of the dephosphorylated (active) form of the enzyme by an ATPMg^{2+} -requiring kinase tightly bound to the enzyme complex leads to the formation of pyruvate dehydrogenase phosphate, which is inactive; conversion of pyruvate dehydrogenase phosphate into active pyruvate dehydrogenase is catalysed by a phosphatase, which is activated by Mg^{2+} and is less tightly bound to the complex than is the kinase.

Insulin action on rat epididymal fat-cells activates pyruvate dehydrogenase by increasing the proportion of the active form to that of the inactive phosphorylated form (Jungas, 1971; Coore *et al.*, 1971). During experiments designed to ascertain whether insulin acted through inhibition of pyruvate dehydrogenase kinase or activation of pyruvate dehydrogenase phosphate phosphatase it was noticed that the use of EGTA* in the isolation of fat-cell mitochondria led to a diminution in the activity of the phosphatase in extracts of mitochondria. We have found since that the Mg^{2+} -requiring pyruvate dehydrogenase phosphate phosphatase from pig heart, pig kidney cortex and rat epididymal fat-cell mitochondria is markedly activated by Ca^{2+} (0.1–10 μM).

Methods

Pyruvate dehydrogenase phosphate phosphatase activity was assayed by the release of $[\text{}^{32}\text{P}]\text{P}_i$ from pig heart pyruvate dehydrogenase $[\text{}^{32}\text{P}]\text{phosphate}$ and in a number of experiments by the associated increase in pyruvate dehydrogenase activity, which was measured as described by Coore *et al.* (1971). Incubations were made at 30°C in a total volume of 60 μl containing pyruvate dehydrogenase phosphate (equivalent to 0.15–0.30 unit of pyruvate dehydrogenase) or pyruvate dehydrogenase $[\text{}^{32}\text{P}]\text{phosphate}$ (equivalent to 0.15–0.30 unit of pyruvate dehydrogenase; 70–280 nCi of ^{32}P) and other additions given

*Abbreviation: EGTA, ethanedioxybis(ethylamine)-tetra-acetate.

in the text and Table 1 in 20 mM-potassium phosphate buffer, pH 7.0, containing 5 mM-2-mercaptoethanol (phosphate–mercaptoethanol buffer). Samples were taken for assay of pyruvate dehydrogenase activity or $[\text{}^{32}\text{P}]\text{P}_i$ at 0, 5 and 10 min. For the assay of $[\text{}^{32}\text{P}]\text{P}_i$, samples (20 μl) were added to 100 μl of 1% (w/v) bovine plasma albumin in Beckman 152 Microfuge tubes and protein was precipitated at 0°C with 100 μl of 12% (w/v) trichloroacetic acid. After a freezing and thawing to aggregate the precipitated protein, the tubes were centrifuged for 5 min and samples (100 μl) of supernatant assayed for radioactivity by liquid-scintillation spectrometry. Reaction rates were constant over this time-period and proportional to the amount of heart pyruvate dehydrogenase phosphate phosphatase or mitochondrial extract added; there was close correspondence between amounts of pyruvate dehydrogenase and $[\text{}^{32}\text{P}]\text{P}_i$ released. Trichloroacetic acid-soluble ^{32}P radioactivity in samples taken at zero time corresponded to less than 2% of the pyruvate dehydrogenase $[\text{}^{32}\text{P}]\text{phosphate}$ added.

The effects of Ca^{2+} were investigated by using CaEGTA buffers. The concentrations of free Ca^{2+} and Mg^{2+} were calculated from the association constants given by Portzehl *et al.* (1964) by using a computer program provided by Dr. P. J. England.

Mitochondria were prepared by using methods given by Martin & Denton (1970) (fat-cells) and Chappell & Hansford (1969) (rat liver and pig kidney cortex). Extracts were made by freezing and thawing three times in phosphate–mercaptoethanol buffer. Pig heart pyruvate dehydrogenase, pyruvate dehydrogenase phosphate and pyruvate dehydrogenase phosphate phosphatase were prepared as follows. Pyruvate dehydrogenase and pyruvate dehydrogenase phosphate were extracted by freezing and thawing (three times) a pig heart mitochondrial fraction prepared by the method of Sanadi *et al.* (1952). The phosphorylated form was then converted into active pyruvate dehydrogenase by incubation at 30°C with 10 mM- MgCl_2 in 20 mM-phosphate–mercaptoethanol buffer, pH 7.0 (complete in 30 min, tested by pyruvate dehydrogenase assay). Pyruvate dehydrogenase was then precipitated by addition of saturated $(\text{NH}_4)_2\text{SO}_4$ to 35% (v/v) at pH 7.0. The precipitate was redissolved in phosphate–mercaptoethanol

buffer (to 20 units/ml) and dialysed overnight against 20vol. of phosphate-mercaptoethanol buffer. The solution was adjusted first to pH 6.1 and then to pH 5.6 with 10% (v/v) acetic acid, and the precipitates were discarded. The supernatant, containing approx. 60% of the pyruvate dehydrogenase activity, was adjusted to pH 7 with KOH (10M and 1M) and centrifuged for 2h at 200000g. The supernatant, which was free of active pyruvate dehydrogenase, constituted the pyruvate dehydrogenase phosphate phosphatase used in these studies. The pellet of pyruvate dehydrogenase was redissolved in phosphate-mercaptoethanol buffer and freed of the phosphatase by further centrifugation at 200000g. The yield was approx. 20–30% of the initial activity, the specific activity being approx. 5 units/mg of protein. Pyruvate dehydrogenase phosphate (unlabelled or ^{32}P -labelled) was prepared by incubation of pyruvate dehydrogenase (approx. 20 units) with 50–100nmol of ATPMg^{2-} or $[\gamma\text{-}^{32}\text{P}]\text{ATPMg}^{2-}$ (100–150 μCi) at 30°C (total volume 1ml); conversion was better than 99% complete in 1h. The reaction mixture was freed of ATP by dialysis for 60–72h against three changes of 0.5–1 litre of phosphate-mercaptoethanol

buffer. The preparations contained less than 1% of active pyruvate dehydrogenase; the specific radioactivities of pyruvate dehydrogenase ^{32}P phosphate were 1–2 μCi /unit equivalent of pyruvate dehydrogenase and there was less than 2% of trichloroacetic acid-soluble ^{32}P radioactivity. The incorporation corresponded to 0.45–0.6nmol of phosphate/unit of pyruvate dehydrogenase.

Results and discussion

Partially purified pig heart pyruvate dehydrogenase phosphate phosphatase hydrolysed pyruvate dehydrogenase ^{32}P phosphate at a rate of approx. 0.75%/min in the presence of 16mm- Mg^{2+} under the conditions described in the legend to Table 1. The K_m for Mg^{2+} was approx. 10mm. As shown in column 1 of Table 1, addition of EGTA or EDTA lowered the activity to 0.09%/min. With EGTA and Mg^{2+} (12mm) the activity increased fourfold. A further increase was observed with CaEGTA buffers yielding Ca^{2+} concentrations from 0.011 to 9 μM ; at this latter Ca^{2+} concentration the activity was six times that in the absence of added Ca^{2+} . As shown by the first and

Table 1. *Effects of Ca^{2+} and Mg^{2+} concentrations on the activity of pyruvate dehydrogenase phosphate phosphatase from pig heart, pig kidney cortex, rat epididymal fat-cells and rat liver mitochondria*

Pyruvate dehydrogenase phosphate phosphatase activity was assayed by the release of $^{32}\text{P}\text{P}_i$ from pyruvate dehydrogenase ^{32}P phosphate measured after 5 and 10min of incubation at 30°C in 20 μl samples of incubation mixture. The incubation medium was 60 μl of 20mm-potassium phosphate buffer, pH 7.0, containing 5mm-mercaptoethanol, pyruvate dehydrogenase ^{32}P phosphate (equivalent to 0.15–0.30 unit of pyruvate dehydrogenase; 70–280nCi of ^{32}P) and other additions as detailed. The results are the mean of three separate experiments in each case. The concentrations of Ca^{2+} and Mg^{2+} in EGTA buffers were calculated from association constants for CaEGTA and MgEGTA given by Portzehl *et al.* (1964). Tube 1 contained 1mm-EDTA; tubes 1–6 and 8, 10mm-EGTA; tubes 2–7, 16mm- MgCl_2 ; CaCl_2 concentrations were zero in tubes 1 and 2, 0.5mm in tube 3, 4mm in tube 4, 8mm in tube 5, 9.75mm in tubes 6 and 8, and 0.5mm in tube 7.

		Activity (% of initial pyruvate dehydrogenase phosphate hydrolysed/min)							
		1	2	3	4	5	6	7	8
	Tube no. ...								
	Concn. of Ca^{2+} (μM) ...	0*	0*	0.011	0.15	0.94	9	500	9
	Concn. of Mg^{2+} (mm) ...	0*	12	12.3	13.5	15	16	16	0
Tissue	Preparation								
Pig heart	Partially purified pyruvate dehydrogenase phosphate phosphatase	0.09	0.33†	0.39	0.51	0.92	2.13	1.40	0.10
Pig kidney cortex	Mitochondrial extract	0.03	0.33†	0.42	0.67	1.37	3.00	3.78	—
Rat epididymal fat-cells	Mitochondrial extract	0.10	0.33†	0.37	0.46	0.59	0.94	1.07	—
Rat liver	Mitochondrial extract	—	0.33†	0.36	0.36	0.40	0.49	0.73	—

* These incubations contained no added CaCl_2 or MgCl_2 , but unknown amounts of Ca^{2+} and Mg^{2+} were presumably present in the reagents used.

† To facilitate comparison between preparations, rates have been adjusted to give 0.33% of initial pyruvate dehydrogenase phosphate hydrolysed/min in tube 2; the actual rates under these conditions varied between 0.19 and 0.75%.

last columns of Table 1, Ca^{2+} ($9\mu\text{M}$) was not effective in the absence of added Mg^{2+} . In separate experiments (not shown) it was found that increasing the Ca^{2+} concentration from 0.011 to $9\mu\text{M}$ not only increased the V_{max} just over fourfold but also decreased the K_m value for Mg^{2+} from 9.46 ± 1.46 to $4.49 \pm 1.21\text{ mM}$ (means $\pm$ S.E.M., based on observations at six concentrations of Mg^{2+} between 0 and 32 mM). In further experiments the activity of pig heart phosphatase, as assayed by the formation of active pyruvate dehydrogenase, was increased sevenfold on raising the concentration of Ca^{2+} from 0.011 to $9\mu\text{M}$.

Similarly, Ca^{2+} in the presence of 16 mM- MgCl_2 stimulated pyruvate dehydrogenase phosphate phosphatase activity in extracts of mitochondria from pig kidney cortex. As shown in Table 1, the activity was increased sevenfold on raising the Ca^{2+} concentration from 0.011 to $9\mu\text{M}$, and this increase was also seen in experiments in which the formation of active pyruvate dehydrogenase was assayed.

The activity of pyruvate dehydrogenase phosphate phosphatase in extracts of fat-cell mitochondria was trebled on the addition of $9\mu\text{M}$ - Ca^{2+} in the presence of 16 mM- MgCl_2 (see Table 1). Since it was found that the K_m for Mg^{2+} was 2.2 mM (rather lower than that of the pig heart enzyme), the effects of Ca^{2+} were also studied in the presence of 2 mM- Mg^{2+} ; again activity was approximately trebled on the addition of $9\mu\text{M}$ - Ca^{2+} .

In contrast with the other preparations studied, the pyruvate dehydrogenase phosphate phosphatase activity in rat liver mitochondrial extracts was rather insensitive to Ca^{2+} concentration, and effects were readily detected only at concentrations above $9\mu\text{M}$.

Further studies, preferably on pure preparations of pyruvate dehydrogenase phosphate phosphatase, are required to elucidate the exact relationship between the activation by Mg^{2+} and that by Ca^{2+} . For the moment, it appears that Ca^{2+} activates only when Mg^{2+} is present and that the effects of the two cations are not interchangeable. The extreme sensitivity to Ca^{2+} makes it difficult to ascertain whether or not the enzyme shows an absolute requirement for Ca^{2+} . The range of concentrations that were effective is similar to that involved in activation of the adenosine triphosphatase activity of the cardiac actomyosin-tropomyosin-troponin system (see Katz, 1970), skeletal-muscle phosphorylase kinase (Brostrom

et al., 1971) and glycerol phosphate dehydrogenase activity of blowfly mitochondria (Hansford & Chappell, 1967).

Our findings suggest the possibility of a physiological role of Ca^{2+} in the regulation of pyruvate dehydrogenase phosphate phosphatase and hence in the intramitochondrial conversion of pyruvate dehydrogenase phosphate into the active form. Other known mechanisms include inhibition of pyruvate dehydrogenase kinase by pyruvate and ADP (Linn *et al.*, 1969b) and perhaps Ca^{2+} (R. M. Denton & P. J. Randle, unpublished work). Physiological conditions involving the regulation of the inter-conversion of pyruvate dehydrogenase phosphate and active pyruvate dehydrogenase include the actions of hormones (insulin, adrenaline, corticotrophin) on fat-cells (Jungas, 1971; Coore *et al.*, 1971) and the interactions of glucose with fatty acids and ketone bodies in rat heart (Wieland *et al.*, 1971).

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- Brostrom, C. O., Hunkeler, F. L. & Krebs, E. G. (1971) *J. Biol. Chem.* **246**, 1961
 Chappell, J. B. & Hansford, R. G. (1969) in *Subcellular Components* (Birnie, G. D. & Fox, S. M., eds.), p. 43, Butterworths, London
 Coore, H. G., Denton, R. M., Martin, B. R. & Randle, P. J. (1971) *Biochem. J.* **124**, 115
 Hansford, R. G. & Chappell, J. B. (1967) *Biochem. Biophys. Res. Commun.* **27**, 686
 Jungas, R. L. (1971) *Metabolism* **20**, 43
 Katz, A. M. (1970) *Physiol. Rev.* **50**, 63
 Linn, T. C., Petit, F. H. & Reed, L. J. (1969a) *Proc. Nat. Acad. Sci. U.S.* **62**, 234
 Linn, T. C., Petit, F. H., Hucho, F. & Reed, L. J. (1969b) *Proc. Nat. Acad. Sci. U.S.* **64**, 227
 Martin, B. R. & Denton, R. M. (1970) *Biochem. J.* **117**, 861
 Portzehl, H., Caldwell, P. C. & Ruegg, J. C. (1964) *Biochim. Biophys. Acta* **79**, 581
 Sanadi, D. R., Littlefield, J. W. & Bock, R. M. (1952) *J. Biol. Chem.* **197**, 851
 Siess, E., Wittman, J. & Wieland, O. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 447
 Wieland, O. & Siess, E. (1970) *Proc. Nat. Acad. Sci. U.S.* **65**, 947
 Wieland, O., Funcke, H. V. & Löffler, G. (1971) *FEBS Lett.* **15**, 295