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Phosphorylation of Additional Sites on Pyruvate Dehydrogenase Inhibits its Re-activation by Pyruvate Dehydrogenase Phosphate Phosphatase

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The phosphorylation of sites additional to an inactivating site inhibits the formation of active pig heart pyruvate dehydrogenase complex from inactive pyruvate dehydrogenase phosphate complex by pig heart pyruvate dehydrogenase phosphate phosphatase.

Mammalian pyruvate dehydrogenase complexes (EC 1.2.4.1+EC 2.3.1.12+EC 1.6.4.3+pyruvate dehydrogenase kinase) exist as active (dephosphorylated) and inactive (phosphorylated) complexes. These are interconverted by pyruvate dehydrogenase kinase (utilizing $MgATP^{2-}$) and pyruvate dehydrogenase phosphate phosphatase (Linn *et al.*, 1969). In rat heart, the proportion of active complex is decreased by alloxan-induced diabetes, starvation and oxidation of lipid fuels (Wieland *et al.*, 1971*a*,*b*). Activation of the kinase reaction by high concentration ratios of acetyl CoA:CoA, NADH:NAD^+ and ATP:ADP provides only a partial explanation for these findings (Kerbey *et al.*, 1976, 1977). There is evidence for at least one further mechanism in heart mitochondria of diabetic rats. This results in impaired conversion of inactive complex into active complex by the phosphatase after addition of pyruvate or dichloroacetate (inhibitors of the kinase reaction) (Kerbey *et al.*, 1976, 1977).

Pig heart pyruvate dehydrogenase complex is inactivated by incorporation of approx. 0.45 nmol of P/unit of enzyme (1 unit of enzyme forms 1 μmol of NADH/min at 30°C). Further incorporation up to a total of 1.25 nmol of P/unit can occur without further change in activity (Sugden & Kerbey, 1978). This is consistent with three sites of phosphorylation in the complex, only one of which is required for inactivation as reported for the bovine kidney complex (Davis *et al.*, 1977). Evidence is now given that these additional phosphorylations interfere with the formation of active complex by the phosphatase reaction.

Experimental

Pyruvate dehydrogenase complex (free of phosphorylated complex and phosphatase) and pyruvate dehydrogenase phosphate phosphatase (free of phosphorylated complex) were prepared from pig heart [Cooper *et al.* (1974) and Severson *et al.* (1974) respectively]. The complex was more than 95% pure by sodium dodecyl sulphate/polyacrylamide disc gel electrophoresis (results not shown). The sources of other materials were as given by Cooper *et al.* (1974) or Kerbey *et al.* (1976).

For phosphorylation, pyruvate dehydrogenase complex (10 units) was incubated at 30°C in 1 ml of 20 mM-potassium phosphate/2 mM-dithiothreitol/10 mM-EGTA, pH 7.0. For preparation of partially phosphorylated complex $MgCl_2$ was added to 0.2 mM. Then 2–10 nmol additions of [γ-^{32}P]ATP (10 mM, 60 μCi/μmol) were made at 6 min intervals until activity of the complex had decreased to about 2.5% of the initial value (direct assay by the method of Cooper *et al.*, 1974). Incorporation of ^{32}P was measured by the method of Corbin & Reimann (1974). Fully phosphorylated complex was prepared by incubation with 1 mM-$MgCl_2$ and 0.5 mM-[γ-^{32}P] ATP for 30 min. After addition of 50 μl of 0.2 M-EDTA, pH 7.0, the incubation mixtures were dialysed separately against 3 × 2 litre of 10 mM-potassium phosphate / 1 mM - EDTA / 0.1 mM - dithiothreitol, pH 7.0, for 68 h. Trichloroacetic acid-soluble ^{32}P radioactivity was less than 0.5% of total.

The pyruvate dehydrogenase phosphate phosphatase reaction was followed in incubations made at 30°C with 70 μl of phosphatase and 140 μl of 0.1 M-Tris/HCl/40 mM-$MgCl_2$/2 mM-dithiothreitol/20 mM-EGTA/19.8 mM-$CaCl_2$, pH 7.5 (Ca^{2+} approx. 10 μM). The reactions were initiated with 70 μl of pyruvate dehydrogenase [^{32}P]phosphate prepared as above (equivalent to 0.7 unit of pyruvate dehydrogenase). Samples (10 or 20 μl respectively) were taken at intervals and assayed for pyruvate dehydrogenase activity (see above) or trichloroacetic acid-soluble ^{32}P (Denton *et al.*, 1972).

Results and Discussion

Fig. 1(*a*) shows the rate of formation of active pyruvate dehydrogenase complex by the phosphatase reaction. The initial rate with partially phosphorylated complex as substrate was approx. 3 times that with fully phosphorylated complex as substrate. This

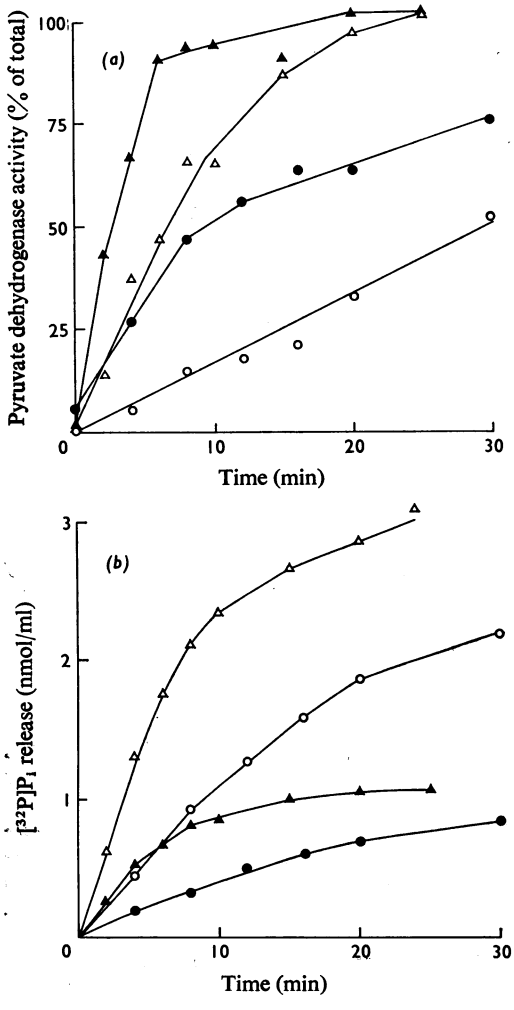

Fig. 1. *Rate of formation of (a) active pyruvate dehydrogenase complex and (b) [^{32}P]P_i from pyruvate dehydrogenase phosphate by pyruvate dehydrogenase phosphate phosphatase*

For details of incubation and methods of analysis see the Experimental section. Details of pyruvate dehydrogenase [^{32}P]phosphate were: ●, 0.42nmol of P/unit and 1:4 diluted phosphatase; ○, 1.14nmol of P/unit and 1:4 diluted phosphatase; ▲, 0.42nmol of P/unit and undiluted phosphatase; △, 1.14nmol of P/unit and undiluted phosphatase. Each point is the mean of observations from two separate incubations. In each incubation, the concentration of pyruvate dehydrogenase phosphate was equivalent to 2.5 units of dehydrogenase/ml.

was seen at two different phosphatase concentrations (ratio 1:5) ($P<0.01$, lower phosphatase concentration; $P<0.05$, higher phosphatase concentration). Fig. 1(*b*) shows the rate of formation of acid-soluble

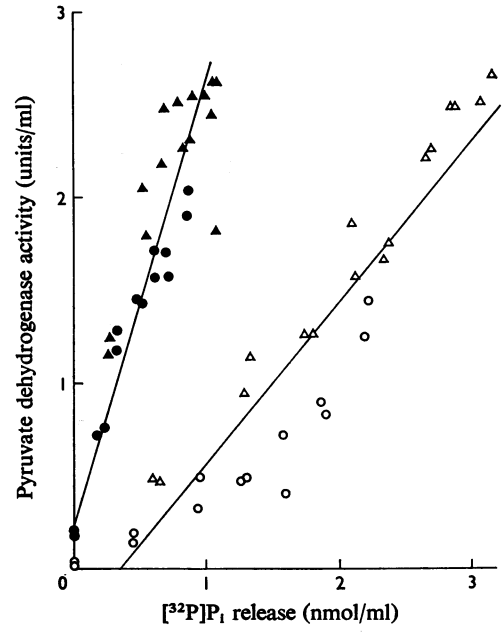

Fig. 2. *Relationship between concentrations of active pyruvate dehydrogenase and [^{32}P]P_i formed from pyruvate dehydrogenase phosphate by pyruvate dehydrogenase phosphate phosphatase*

Data plotted are from Figs. 1(*a*) and 1(*b*). For details of incubation and use of symbols see legend to Fig. 1. Linear-regression lines were assigned by least-squares analysis.

^{32}P. The initial rate with fully phosphorylated complex was approx. 2.3 times that with partially phosphorylated complex at either concentration of phosphatase. The higher phosphatase concentration gave complete recovery of pyruvate dehydrogenase activity with either substrate. The phosphatase preparation contained pyruvate dehydrogenase activity. This was subtracted from the pyruvate dehydrogenase activity measured (the correction was always less than 5%). The initial ^{32}P concentrations in the two preparations are shown in Fig. 1. Similar results were obtained with another preparation of substrates and a preparation of ox heart phosphatase (results not shown).

Fig. 2 shows the relationship between the concentrations of active dehydrogenase complex and trichloroacetic acid-soluble ^{32}P formed in incubations with phosphatase. Linear-regression analysis gave values (mean±S.E.M. in nmol of acid-soluble ^{32}P/unit of dehydrogenase) of 0.41±0.04 ($r=0.91$) and 1.16±0.08 ($r=0.94$) for partially and fully phosphorylated complexes respectively. There is some suggestion of curvature with the fully phosphorylated complex and the lower phosphatase concentration, but the deviations from linearity were not statistically significant.

The partially or fully phosphorylated dehydrogenase complexes gave only one band of radioactivity on sodium dodecyl sulphate/polyacrylamide disc gel electrophoresis. This corresponded to the α-subunit of the decarboxylase (EC 1.2.4.1) (results not shown). The concentration of ^{32}P in the fully phosphorylated dehydrogenase complex was 2.83 times that in the partially phosphorylated complex. This is consistent with a total of three sites of phosphorylation, and inactivation of the dehydrogenase complex by phosphorylation of only one site, as described for the bovine kidney complex by Davis *et al.* (1977). Although the existence of three sites of phosphorylation in the pig heart complex has yet to be shown by amino acid sequence, we shall assume for the purposes of discussion that the pig heart complex is analogous to the bovine kidney complex. Subject to this assumption, the present study shows that multi-site phosphorylation of the pig heart complex inhibits re-activation of the complex by pyruvate dehydrogenase phosphate phosphatase. It is known that all three tryptic phosphopeptides from the bovine kidney complex are dephosphorylated by bovine kidney phosphatase (Davis *et al.*, 1977). However, the effect of multi-site phosphorylations on the rate of formation of active (dephosphorylated) complex by the phosphatase has not been described before. The development of a technique, described here, for preparation of pyruvate dehydrogenase phosphate of a defined extent of phosphorylation and inactivation has enabled the present study to be undertaken. The results of this study suggest a mechanism whereby the kinase reactions may regulate re-activation of the complex by the phosphatase reaction. The present study does not distinguish between various models (random or sequential) for the release of phosphate from the fully phosphorylated complex. It should be noted that multi-site phosphorylations have already been implicated in the regulation of the dephosphorylation of phosphorylase kinase and glycogen synthetase (for review, see Cohen, 1976).

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