# The effect of phosphorylation on pyruvate dehydrogenase

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**Abstract Phosphorylation of the pyruvate dehydrogenase component (E1) of the muscle pyruvate dehydrogenase complex (PDC) by E1-kinase inhibits substrate conversion both in oxidative and non-oxidative reactions. Circular dichroism spectra were used to monitor the effect of phosphorylation on the following stages of the process: holoform formation from apo-E1 and thiamine pyrophosphate (TPP), substrate binding and active site deacetylation. It has been shown that phosphorylation of E1 reduces its affinity for TPP and prevents holo-E1 interaction with pyruvate. Phosphorylated and dephosphorylated PDC convert 2-hydroxyethyl-TPP in similar ways involving half of their active sites; all active sites of E1 function in the presence of deacetylating agents. The data obtained suggest that the phosphorylation and substrate binding sites interact with each other.**

*Key words:* Pyruvate dehydrogenase; Protein kinase; Phosphorylation; Circular dichroism

## 1. Introduction

The pyruvate dehydrogenase complex (PDC), turning pyruvate to acetyl CoA, is crucial to metabolism since it catalyzes an irreversible reaction at the junction of the glycolytic pathway with the Krebs cycle. PDC from eucaryotes comprises three catalytic components (E1, E2, and E3) as well as component X essential to the E2–E3 interaction [1,2]. The E1 component catalyzes the rate-limiting reaction, namely, thiamine pyrophosphate (TPP)-dependent decarboxylation of pyruvate to produce 2-hydroxyethyl-TPP (HETPP) and reductive acetylation of lipoic acid residues covalently bound to E2. Two regulatory enzymes, kinase and phosphatase, phosphorylate-dephosphorylate E1, thereby inactivating-reactivating the whole complex [1]. Three serine residues in the $\alpha$ subunit of tetrameric E1 molecule $(\alpha\beta)_2$ have been shown to undergo modification, and their role and relative contribution to the inactivation process have been examined. E1 has been shown to be completely inactivated by phosphorylation of only one site per enzyme molecule [3–5]. The mechanism by which phosphoserine residue formation inhibits the catalytic activity of E1 is not clear. It is believed that the modification of the enzyme by phosphorylation blocks HETPP-producing reactions [6].

In this work the effect of regulatory phosphorylation on holoenzyme complex formation, substrate binding and active center deacetylation was studied using spectral measurements [7,9]. It has been shown that E1 phosphorylation, which decreases its affinity for TPP, does not preclude holo-E1 formation but inhibits its interaction with pyruvate.

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## 2. Experimental

PDC was isolated from pigeon breast muscle according to Jagannathan and Schweet [10], with some modifications. The PDC activity was estimated from the rate of NADH production in the presence of CoA [11]. E1 activity was determined with an artificial electron acceptor (2,6-dichlorophenolindophenol) as described previously [12]. Pyruvate consumption due to non-oxidative action of PDC was measured as follows: PDC (native or phosphorylated by endogenous E1-kinase with ATP) was incubated in the presence of excess TPP and pyruvate. Aliquotes were taken in time and the residual level of the substrate was determined from NADH decrease in the presence of lactate dehydrogenase. E1-kinase was assayed by following PDC inactivation in the presence of ATP. The incubation medium contained 0.1 M potassium phosphate buffer, pH 7.0, 0.5–2 mg/ml PDC, 0.1 M $MgCl_2$ and varied amounts of ATP. Aliquots were withdrawn to be assayed for the enzyme activity. Circular dichroism (CD) spectra were recorded on a CNRS-Russel-Jouan Dichrograph 111 as described earlier [7,9]. The data obtained were expressed in the form of the ratio $\Delta\varepsilon/c$, where $\Delta\varepsilon$ is the differential dichroic absorbance, expressed in optical scale units; $c$ is the protein concentration in mg/ml. Acetoin was assayed by the method of Westerfeld [13].

## 3. Results

Fig. 1A shows the time course of the E1-kinase action on the pyruvate:NAD oxidoreductase activity of PDC in the presence of different concentrations of ATP. Changes in the E1 activity measured with an artificial electron acceptor were similar (not shown). Besides its oxidative function, PDC catalyzes TPP-dependent non-oxidative side reactions to produce acetaldehyde and products of acyloin condensation [10,14]. The time course of pyruvate consumption by PDC in the absence of CoA and NAD is shown in Fig. 1B (curve 1). PDC activity is decreased at increasing ATP concentration (curves 2–5). The effect of ATP on the amount of acetoin produced by PDC is shown in Fig. 1C. This reaction is also inhibited by E1-kinase. Thus, modification of PDC by phosphorylation inhibits all modes of its activity requiring the action of the E1 component.

It was of interest in this connection to investigate the effect of pyruvate on enzyme inactivation by phosphorylation. Fig. 2 shows that enzyme inactivation is more substantial in the presence of pyruvate (compare curves 1 and 2–4). Hydroxypyruvate, a convertible substrate analog, produces a similar effect, whereas pyruvamide (a decarboxylation-resistant competitive inhibitor and an allosteric activator of PDC [11]) partially protects the enzyme from inactivation by phosphorylation (curve 5).

The effect of phosphorylation on different steps of the E1 action was studied by spectral analysis. We have shown earlier that a tryptophan residue of apo-E1 forms a charge transfer complex (CTC) with TPP during holo-E1 reconstruction. This is well reflected by a negative CD spectral Cotton effect with a maximum at 330 nm [7–9]. Fig. 3 shows that the spectral characteristics of PDC saturated with TPP are similar to those of the isolated E1 [9].


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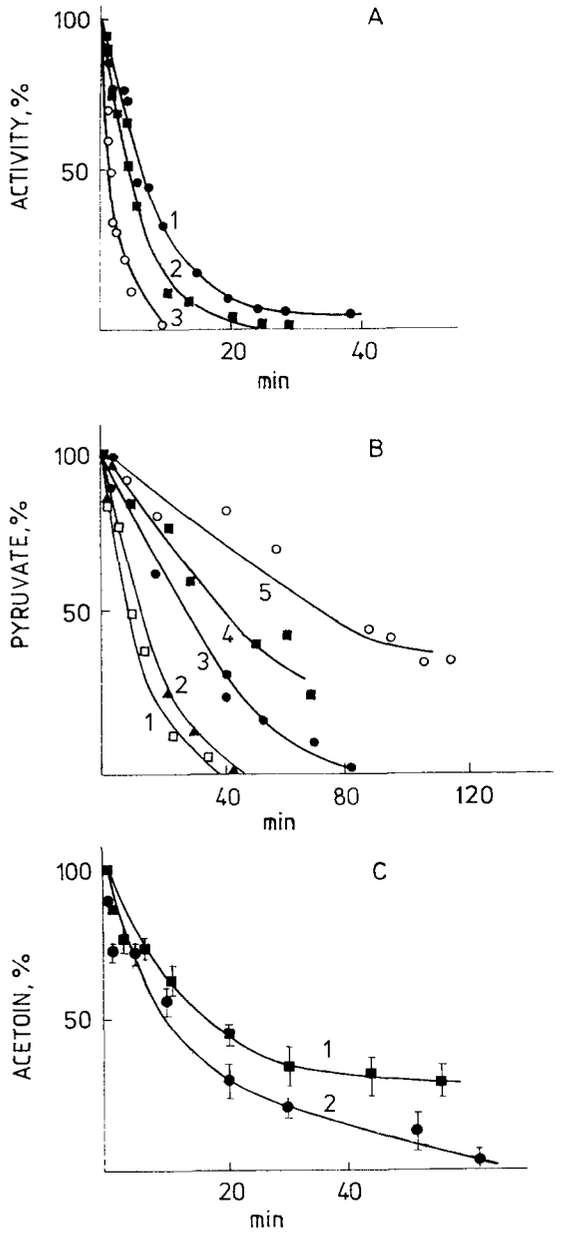


Fig. 1. The effect of ATP on the time course of PDC activity assayed by the rate of NADH generation (A), non-oxidative pyruvate consumption (B), and acetoin production (C). Conditions: (A) 0.6 mg/ml PDC, 0.05 (1), 0.1 (2) or 0.44 (3) mM ATP; (B) 1.53 mg/ml PDC, 0 (1), 0.04 (2), 0.05 (3), 0.4 (4) or 1.92 (5) mM ATP; (C) 2.83 mg/ml PDC, 0.29 (1) or 4.6 (2) mM ATP.

Addition of pyruvate to the holoform leads to a decrease in the CTC band intensity until its full diappearance (Fig. 4A). The effect of pyruvate on the protein spectrum can be explained by disturbance of the electronic interaction in the dono-acceptor pair, the indolyl group of tryptophan and the thiazol ring of TPP, due to substrate binding to the 2-carbanion of TPP. Fig. 4B (curve 1) shows changes in the CTC spectral band intensity at 330 nm on addition of TPP to the phospho-PDC with the residual activity of 8%. It is apparent that protein phosphorylation does not prevent CTC formation, but saturation of phospho-PDC with the coenzyme requires one order of magnitude higher concentrations of TPP. These data show that PDC phosphorylation by endogenous E1-kinase does not prevent TPP binding to the active sites of phospho-E1, but decreases the strength of the interaction. Fig. 4B (curve 2) shows that the spectral amplitude due to CTC for phospho-PDC-TPP is not affected by pyruvate, suggesting that the substrate is not bound to the active sites of phosphorylated holo-E1.

The interaction of E1 (whether isolated or within PDC) with HETPP reveals the half-of-the-sites reactivity [15]. Fig. 4C, (curve 1) shows that the phosphorylated PDC also uses only half of its active sites to interact with the HETPP. The amplitude of the CTC band increases twofold upon addition of CoA (curve 2) or DTT (not shown). When the deacetylating reagent and HETPP are added to the apoenzyme simultaneously, the amplitude of the CTC band in the spectra is indicative of the holoform formation at both active sites of E1 (curve 3).

## 4. Discussion

The regulation of muscle PDC by means of phosphorylation–dephosphorylation of the $\alpha$ subunit of E1 was described earlier [8]. Kinetic investigations show that phosphorylation inhibits pyruvate conversion both in oxidative and non-oxidative reactions (Fig. 1). This means that the formation of phosphoserine in the E1 blocks an early step of enzyme catalysis.

Spectral studies showed that phosphorylation of PDC does not prevent the formation of the 'correct' holo-E1, but changes the spectral effect caused by its interaction with pyruvate. This suggests that phosphorylation of E1 prevents substrate binding to the active site. These results are consistent with the data reported earlier [6] in that phosphorylation of PDC influences the formation of HETPP via the mainstream reaction. However, the spectral analysis used to detect separate stages of the enzyme action allowed one to identify holo-E1–substrate adduct formation as the step blocked by phosphorylation.

E1 tetramer $(\alpha\beta)_2$ has been found to be a functional dimer exhibiting cooperativity during its interaction with HETPP [15]. According to the mechanism proposed, HETPP undergoes oxidative conversion to form an acetylthioenzyme with

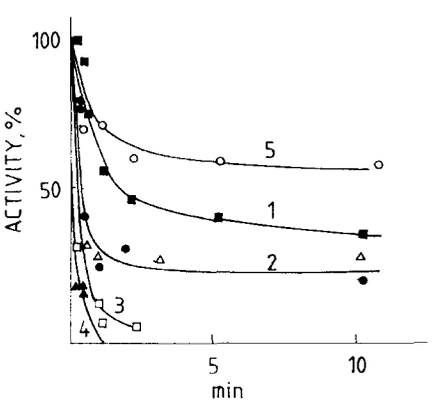


Fig. 2. The effects of pyruvate and its analogs on PDC (0.33 mg/ml) inactivation by endogenous E1-kinase in the presence of 0.19 mM ATP. Additions: ■, none; ●, □, ▲, 0.25, 0.37 and 0.61 mM pyruvate, respectively; △, 3.9 mM hydroxypyruvate; ○, 1.5 mM pyruvamide.

free TPP at the active site. Enzyme acetylation–deacetylation is presumed to be a rate-limiting step in cooperative catalysis [15].

We have shown that phospho-PDC can interact with HETPP to form CTC only in half of the active sites (Fig. 4C). All active sites of phospho-E1 become involved in HETPP conversion only after being deacetylated in the presence of CoA or DTT. Similar results have been reported earlier for dephospho-E1 [9]. This means that: (i) E1 phosphorylation does not preclude its ability to convert the already formed HETPP to produce the acetylated enzyme with free TPP at the active site; (ii) phosphoserine formation in the regulatory site does not affect subunit interactions ensuring the cooperativity of HETPP conversion by functional dimers of E1 within PDC.

In view of the dependency of the holoenzyme–pyruvate interactions on phosphorylation, it was of interest to examine the reverse relationship: how pyruvate affects the rate of E1 inactivation by kinase. We show here that pyruvate in the concentrations used does not prevent phosphorylation, rather, it stimulates kinase. Hydroxypyruvate, when used as a substrate, produced a similar effect, in contrast to pyruvamide, which bears no carboxylic group and hence cannot be converted by PDC. The stimulating effect of pyruvate on the E1-kinase seems to be indirect and related to the formation of acetyldihydrolipoate residues, effective accelerators of kinase reactions [5]. The protective effect of pyruvamide, which displays an affinity for E1 similar to that of the substrate and simulates holoenzyme-substrate complex formation, suggests that the substrate is effective, when bound. The partial protection from phosphorylation (inactivation) of the enzyme provided by this analog does not rule out that the phosphorylation and substrate binding sites are close to each other. Competition for the positively charged guanidinium group of the arginine residue, which binds the pyruvate carboxylate [16], may block substrate binding to the active sites of phosphorylated E1.

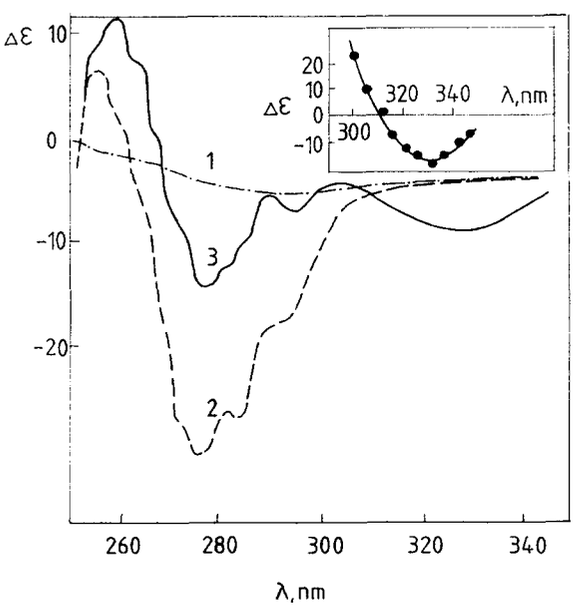


Fig. 3. TPP-induced changes in the CD spectra of PDC. (1), baseline; PDC, 2 mg/ml in the absence (2) and in the presence (3) of 10.2 $\mu$M TPP. The difference spectrum is shown in the inset.

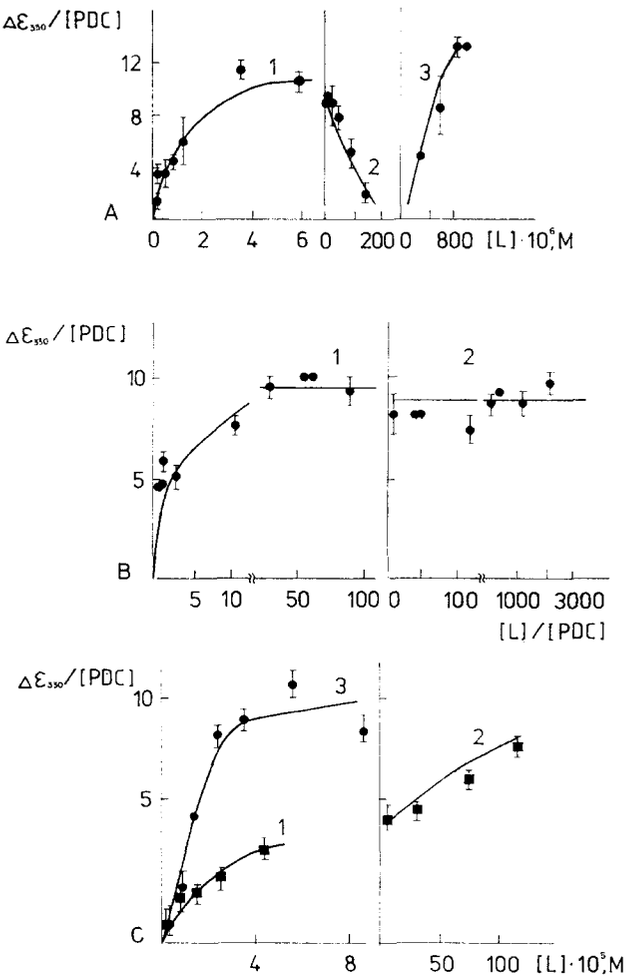


Fig. 4. The effects of various ligands (L) on the intensity of the CTC band of PDC (1.87 mg/ml). A, dephospho-PDC, and B, phospho-PDC after interactions with TPP (1), pyruvate (2) or CoA (3) added consecutively; C, phospho-PDC with added HETPP (1), CoA (1 mM) added after HETPP (2), HETPP added to phospho-PDC plus 1 mM CoA (3).

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