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Chemical modification of the essential arginine residues of pyruvate dehydrogenase prevents its phosphorylation by kinase

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Abstract The mechanism of regulatory phosphorylation of the pyruvate dehydrogenase component (E1) of muscle pyruvate dehydrogenase complex was studied. Chemical modification of the arginine residues essential for substrate binding was shown to prevent incorporation of ^{32}P from [γ-^{32}P]ATP into E1 catalyzed by kinase and to exclude completely the interaction of holo-E1 with pyruvate. It is proposed that negatively charged phosphoseryl residues may compete with pyruvate for the active site arginine and thereby block the substrate binding.

Key words: Pyruvate dehydrogenase; Kinase; Arginine

1. Introduction

The activity of animal pyruvate dehydrogenase complex (PDC) is regulated by a phosphorylation-dephosphorylation cycle carried out by endogenous regulatory enzymes: kinase and phosphatase. The target of this covalent modification is pyruvate dehydrogenase (E1) – the first component of PDC. E1 catalyzes the thiamine pyrophosphate (TPP)-dependent decarboxylation of pyruvic acid to produce 2-hydroxyethyl-TPP (HETPP), and reductive acetylation of lipoamide residues attached to E2, the dihydrolipoamide acetyltransferase [1]. E1 exists as a tetramer $(\alpha\beta)_2$, having two active sites [2]. Phosphorylation occurs at 3 seryl residues (Ser-264, Ser-271, Ser-203) located on the α subunit. It has been shown that phosphorylation of Ser-264 closely is correlated with major inactivation of the enzyme [3,4].

The mechanism of this effect is largely unknown. It was found that phosphorylation of pig heart E1 inhibited reactions leading to formation of HETPP [5]. Spectral studies used to monitor the separate steps of the enzyme action showed that pigeon breast muscle phospho-E1 was able to bind TPP in the active conformation to form a charge transfer complex, to convert HETPP in a half-of-the-site manner, but could not interact with the substrate pyruvate [6]. It was suggested that a negatively charged phosphoseryl residue may compete with pyruvate for the active site arginyl residue and thereby block the substrate binding. The aim of this work was to prove this hypothesis.

2. Materials and methods

PDC was isolated from pigeon breast muscle according to Jagannathan and Schweet [7], with some modifications. Resolution of PDC into components, namely E1 and E2 tightly binding protein X and kinase (E2-X-kinase), was carried out according to [8]. The PDC and E1 activities were measured as described previously [9,10]. Kinase was assayed by following enzyme inactivation and phosphorylation in the presence of [γ-^{32}P]ATP. Aliquots were removed and either assayed for PDC (E1) activity or spotted on 2×2 cm Whatman 3MM paper and, following further treatment, counted for protein-bound radioactivity according to [11]. Protein was determined by the method of Bradford [12]. Arginine residues were modified with 2,3-butanedione, phenylglyoxal (PG) and 4-OH-3-NO_2-PG as described earlier [13,14]. Unreacted diketone reagents were removed by the gel-filtration technique. Kinase action in these experiments was controlled after combining native or modified E1 with saturating concentrations of the E2-X-kinase fraction. Circular dichroism spectra were recorded on a CNRS-Russel-Jouan Dichrograph 111 as described earlier [2].

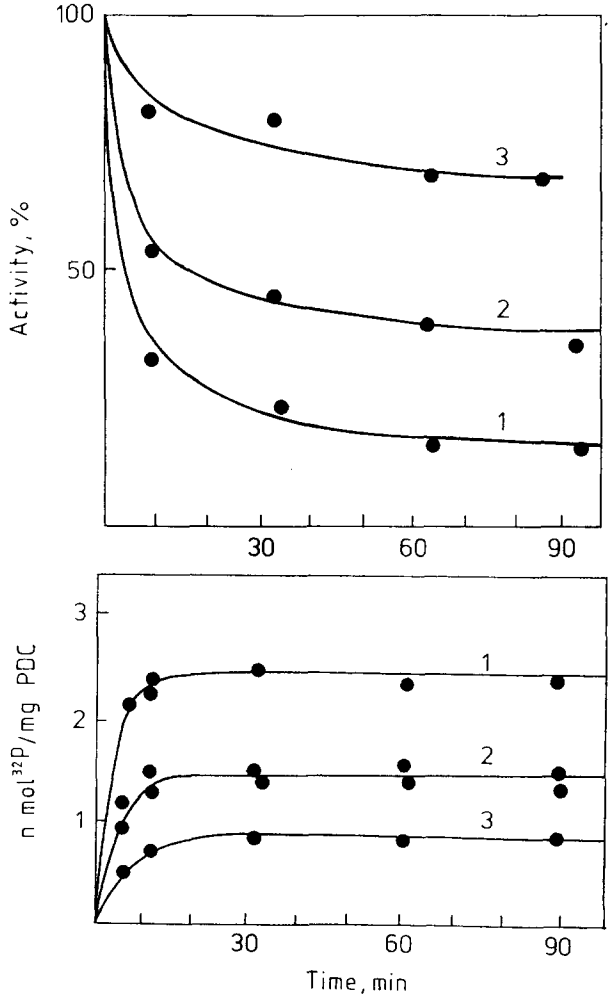

Fig. 1. Effect of puryvate on the time course of PDC inactivation (upper) and ^{32}P incorporation (lower) under the action of endogenous E1 kinase. Concentrations: 1.46 mg/ml PDC and 0.5 mM [γ-^{32}P]ATP, 80000 cpm/nmol (1); as (1) plus 2.5 mM (2) or 5 mM (3) puryvate.

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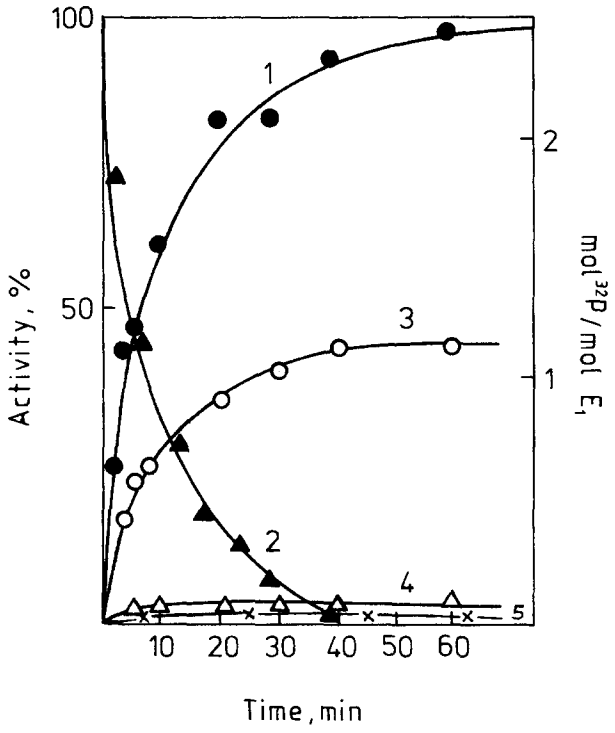

Fig. 2. Effect of chemical modification of E1 arginine residues on its phosphorylation by kinase. Concentrations: E1, 0.32 mg/ml; E2-X-kinase, 0.64 mg/ml, and [γ-^{32}P]ATP, 0.5 mM, 80000 cpm/nmol. Curves 1,2, phosphorylation and inactivation of the intact E1, respectively; curves 3,4, ^{32}P incorporation into E1 modified by 2,3-butanedione to residual activity of 35 and 4%, respectively; curve 5, phosphorylation of E1 modified at two arginine residues per mole with 4-OH-3-NO_2-PG.

3. Results and discussion

Fig. 1 shows the correlation between the extent of phosphorylation and PDC activity changing under the action of ATP-dependent kinase. The addition of pyruvate in increasing concentrations essentially decreases the extent of enzyme inactivation and phosphorylation. Such substrate analogs as pyruvamide and acetylphosphinate also displayed protective effects in similar experiments (not shown). Thus, pyruvate and its analogs saturating E1 prevent enzyme inactivation and phosphorylation under the action of protein kinase. This suggests that protection can be achieved at the level of E1 active sites and be connected with the binding of ligands. A specific site on the protein essential for phosphorylation of the regulatory center can be somewhat involved in the formation of the holoenzyme-substrate (analog) complex. Such a site could be a arginine residue located in the E1 active center in the region of the holo-E1 interaction with pyruvate [13]. In this case any modification of the functionally important arginine residue should prevent not only substrate binding, but also phosphorylation of the serine residue performed by the protein kinase in the closely located regulatory site

In order to confirm or disprove this hypothesis we studied the effect of chemical modification of the essential arginine residues on the ability of E1 to bind pyruvate or be a substrate for the ATP-dependent protein kinase.

Fig. 2 (curve 1) shows that more than 2 moles of ^{32}P are incorporated per E1 molecule during enzyme inactivation in a control assay containing unmodified E1 in the system (E1+E2-X-kinase). The extent of phosphorylation decreases up to 1 mol of ^{32}P per mol of E1 after modification of the arginine residues by 2,3-butanedione up to 35% of the residual activity (curve 3). Practically no incorporation of ^{32}P into the protein was detected after further E1 inactivation up to 4% of the residual activity (curve 4). It is possible that the first level of inactivation correlates with arginine residue modification in one of the two E1 active sites and blockage of the phosphorylation reaction on one of the two α subunits of $(\alpha\beta)_2$ tetramer. The second level of inactivation can be associated with modification of the arginine residues in both active sites. This prevents alternating phosphorylation of serine residue on the second α subunit of the E1 tetramer.

Previously, the number of essential E1 arginine residues was determined with the use of the chromophore reagent 4-OH-3-NO_2-PG and found to be two per enzyme molecule [14]. Fig. 2 (curve 5) shows that the enzyme modified by two arginine residues with 4-OH-3-NO_2-PG is not able to be phosphorylated. Hence E1 modified at the essential arginine residues completely loses the ability to serve as a substrate for the kinase.

Furthermore, we studied the effect of modification of the arginine residues on the ability of E1 to bind pyruvate. For this purpose E1 was modified at two arginine residues, then TPP was added followed by pyruvate. The binding of the ligands was controlled by the quantitative registration of charge transfer complex band. Fig. 3 shows that the modified E1 binds TPP with the same affinity as the native enzyme, but is not able to bind pyruvate. This is verified by the absence of any changes in the charge transfer complex band spectrum after the addition of pyruvate.

Thus, we have shown that the ability of E1 to bind pyruvate or its susceptibility to phosphorylation at the regulatory sites depends on the arginine residues being intact. Interaction of the functionally important arginine residues with chemical reagents or substrates prevents phosphorylation of serine residues in regulatory sites. This provides evidence for the close location of the substrate binding and regulatory sites. The binding of pyruvate by the guanidinium group of arginine in the active site probably neutralizes the local positive charge essential for orientation of the terminal ATP phosphate participating in the action of kinase. On the other hand, phos-

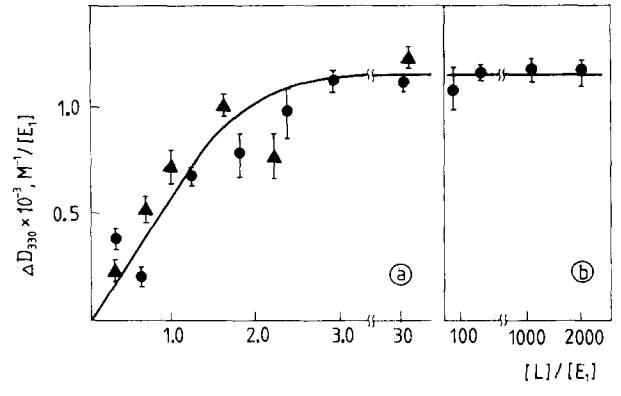

Fig. 3. Effect of chemical modification of E1 arginine residues on the intensity of the charge transfer complex band during enzyme saturation by TPP (a), and TPP+pyruvate (b). Concentration of E1 was 1.9 mg/ml; (▲) intact enzyme and (●) modified at two arginine residues per mole, respectively.

pho-Ser formation in the regulatory site prevents pyruvate binding in the E1 active site as shown previously [6].

The results obtained allow one to conclude that E1 inactivation upon phosphorylation may be due to competition between the negatively charged phosphorine group and pyruvate carboxylic group for the positive charge of the arginine guanidinium group, located on the joint of the active and regulatory sites.

The interaction of the substrate binding sites and phosphorylation sites has previously been suggested for isocitrate dehydrogenase [15] and branched chain α-ketoacid dehydrogenase [16]. Recently, the inactivation of the phosphorylation site mutants of human E1 has shown that phosphorylation of each of three sites leads to enzyme inactivation [17]. The mechanism we propose could explain the inactivation by phosphorylation of one of the three sites (probably Ser-264, site 1). Phosphorylation of sites 2 and 3 may affect the activity of E1 in different ways.

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