Regulation of pyruvate dehydrogenase activity through phosphorylation at multiple sites

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The enzymic activity of the mammalian pyruvate dehydrogenase complex is regulated by the phosphorylation of three serine residues (sites 1, 2 and 3) located on the E1 component of the complex. Here we report that the four isoenzymes of protein kinase responsible for the phosphorylation and inactivation of pyruvate dehydrogenase (PDK1, PDK2, PDK3 and PDK4) differ in their abilities to phosphorylate the enzyme. PDK1 can phosphorylate all three sites, whereas PDK2, PDK3 and PDK4 each phosphorylate only site 1 and site 2. Although PDK2 phosphorylates site 1 and 2, it incorporates less phosphate in site 2 than PDK3 or PDK4. As a result, the amount of phosphate incorporated by each isoenzyme decreases in the order PDK1 > PDK3 ≥ PDK4 > PDK2. Significantly, binding of the coenzyme thiamin pyrophosphate to pyruvate dehydrogenase alters the rates and stoichiometries of phosphorylation of the individual sites. First, the rate of phosphorylation of site 1 by all isoenzymes of kinase is decreased. Secondly, thiamin pyrophosphate markedly decreases the amount of phosphate that PDK1 incorporates in sites 2 and 3 and that PDK2 incorporates

in site 2. In contrast, the coenzyme does not significantly affect the total amount of phosphate incorporated in site 2 by PDK3 and PDK4, but instead decreases the rate of phosphorylation of this site. Furthermore, pyruvate dehydrogenase complex phosphorylated by the individual isoenzymes of kinase is reactivated at different rates by pyruvate dehydrogenase phosphatase. Both isoenzymes of phosphatase (PDP1 and PDP2) readily reactivate the complex phosphorylated by PDK2. When pyruvate dehydrogenase is phosphorylated by other isoenzymes, the rates of reactivation decrease in the order PDK4 ≥ PDK3 > PDK1. Taken together, results reported here strongly suggest that the major determinants of the activity state of pyruvate dehydrogenase in mammalian tissues include the phosphorylation site specificity of isoenzymes of kinase in addition to the absolute amounts of kinase and phosphatase protein expressed in mitochondria.

Key words: phosphorylation sites, pyruvate dehydrogenase kinase, pyruvate dehydrogenase phosphatase.

INTRODUCTION

The reaction catalysed by mitochondrial pyruvate dehydrogenase complex (PDC):

serves as an important link between two major metabolic pathways: glycolysis and the tricarboxylic acid cycle. It is generally believed that under aerobic conditions the rate of the pyruvate dehydrogenase reaction is primarily responsible for the overall rate of aerobic oxidation of carbohydrate fuels [1]. Like many other rate-limiting, flux-generating metabolic reactions, the step catalysed by PDC is tightly regulated. In mammals this regulation is achieved through a reversible phosphorylation cycle [2]. Mammalian mitochondria contain two dedicated enzymes, protein kinase [pyruvate dehydrogenase kinase (PDK)] [3] and phosphoprotein phosphatase [pyruvate dehydrogenase phosphatase (PDP)] [4], which catalyse the phosphorylation and dephosphorylation of the dehydrogenase (E1) component of the complex in a highly co-ordinated manner [1]. Phosphorylation inactivates the E1 component, thus rendering the entire complex inactive [3]. Phospho-PDC can be re-activated only through dephosphorylation catalysed by PDP [4]. The activities of both PDK and PDP seem to be highly regulated [1]. PDK is stimulated by products of the pyruvate dehydrogenase reaction (NADH and acetyl-CoA). The substrates [pyruvate, coenzyme thiamin

pyrophosphate (TPP) and, to some extent, NAD⁺ and CoA] inhibit the phosphorylation reaction. In contrast, the phosphatase activity is inhibited by NADH, the product of the pyruvate dehydrogenase reaction. Therefore when intra-mitochondrial concentrations of NADH and acetyl-CoA are high, PDC becomes phosphorylated and inactive, and, vice versa, accumulation of the substrates of the dehydrogenase reaction (pyruvate, NAD⁺ and CoA) signals the re-activation of PDC.

Pioneering studies conducted on native PDC isolated from heart [5] and kidney [6] mitochondria established that phosphorylation occurs on three serine residues (Ser-264, Ser-271 and Ser-203) of the α-chain of E1 (which is a heterotetramer of two E1α and E1β subunits). These serine residues were designated phosphorylation sites 1, 2 and 3 respectively [6]. Analysis of the phosphorylation and inactivation of the purified heart complex revealed that the relative initial rates of phosphorylation of the individual sites are in the order site 1 > site 2 > site 3 [5]. Furthermore, site 1 was the predominant inactivating site, with the contribution of site 2 being approx. 1.6 % over the range 1.5–90 % of complex inactivated [5]. On the basis of these observations, it was proposed that phosphorylation of site 2 is not primarily concerned with inactivation and that the phosphorylation of site 3 is non-inactivating. Further analysis of phosphorylation site occupancy led to the suggestion that the

Abbreviations used: E1, pyruvate dehydrogenase component of PDC; E2, dihydrolipoil acetyltransferase component of PDC; E3BP, dihydrolipoamide-dehydrogenase-binding protein component of PDC; PDC, pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase; PDP, pyruvate dehydrogenase phosphatase; S1,3E1PDC, PDC lacking phosphorylation site 2; S2,3E1PDC, PDC lacking phosphorylation site 1; S3E1PDC, PDC lacking phosphorylation sites 1 and 2; TPP, thiamin pyrophosphate.

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major function of site 2 and the only function of site 3 might be to inhibit reactivation by the phosphatase [7]. When the individual components of PDC became available as recombinant proteins, these conclusions were tested with mutants of the three phosphorylation sites of E1 [8]. It was found that phosphorylation of each site alone resulted in enzyme inactivation. However, much higher rates were observed for the phosphorylation of site 1 than for sites 2 and 3. Half-of-the-site reactivity during the phosphorylation of all three sites was also reported, meaning that only one site out of two per $\alpha_2\beta_2$ tetramer of E1 could be phosphorylated. This makes the maximal number of phosphoryl groups that could be incorporated in E1 not more than three. It was also concluded that the dephosphorylation reaction occurs randomly and there is no influence of one site on the rate of dephosphorylation of other sites [8].

Recently, an additional level of complexity in the regulation of mammalian PDC became apparent owing to the discovery of multiple isoenzymes of PDK (PDK1, PDK2, PDK3 and PDK4) [9,10] and PDP (PDP1 and PDP2) [11] that differ in their kinetic parameters, regulation and expression patterns [11,12]. The current study was undertaken to determine whether the isoenzymes of PDK show any preference for particular phosphorylation site(s), to improve our understanding of the physiological significance of isoenzymes in the regulation of PDC activity.

EXPERIMENTAL

Site-directed mutagenesis

Point mutations within the amino acid sequence of human E1 α subunit [13] were introduced by oligonucleotide-directed mutagenesis [14]. The sequences of mutagenic oligonucleotides were 5'-TAC CAC GGA CAC GCT ATG AGT CGA CC-3' for the Ser-264 $\rightarrow$ Ala mutant (site 1), 5'-GAC CCT GGA GTC GCT TAC CGT ACA C-3' for the Ser-271 $\rightarrow$ Ala mutant (site 2) and 5'-GGA ATG GGA ACG GCT GTT GAG AGA 3' for Ser-203 to Ala mutant (site 3) (Life Technologies, Rockville, MD, U.S.A.). Mutagenesis reactions were performed on double-stranded DNA of human E1 α [13] subcloned into pUC 19 with the ExSite™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). The reactions were set up essentially as recommended by the manufacturer. The mutations and the fidelity of the rest of the DNA species were confirmed by direct sequencing [15].

Expression and purification of the mutant enzymes

The construction of the expression vector for the human E1 component is described elsewhere [13]. Plasmids carrying both E1 α and E1 β cDNA species were identified by restriction analysis. Positive plasmids were co-transfected into BL21(DE3) cells (Novagen) with a pGroESL plasmid carrying the genes encoding the molecular chaperones GroEL and GroES under the control of isopropyl β -D-thiogalactoside-inducible promoter [the respective plasmid was obtained as a gift from Dr Anthony Gatenby (DuPont Central Research and Development, Wilmington, DE, U.S.A.)]. Double transformants were selected on yeast/tryptone agar containing ampicillin and chloramphenicol (50 μ g/ml each) [16]. Several individual colonies from each transformation were tested for their ability to produce significant amounts of soluble recombinant E1. Clones expressing the greatest amount of the soluble enzyme were used for further analysis.

The expression of the wild-type and mutant E1 was performed essentially as described previously [16]. Human E2–E3BP sub-

complex (consisting of the dihydrolipoyl acetyltransferase and the dihydrolipoamide-dehydrogenase-binding components of PDC) was expressed in *Escherichia coli*. It was reconstituted with wild-type or mutant E1 on ice for 30 min. The resulting PDC was purified by precipitation with polyethylene glycol (8000 Da) (Sigma, St Louis, MO, U.S.A.), gel filtration on Sepharose 4B (Amersham Pharmacia Biotech) and high-speed centrifugation [16]. All preparations of PDC analysed in this study had less than 5% holoenzyme (TPP-bound E1). The isoenzymes of PDK [9] and PDP [11] were expressed in *E. coli*. They were purified by chromatography with Talon™ metal-affinity resin (Clontech Laboratories, Palo Alto, CA, U.S.A.) [9,11]. All enzyme preparations used in this study were more than 90% pure, as judged by SDS/PAGE analysis followed by staining with Coomassie Blue.

Standard phosphorylation assay

The phosphorylation of PDC was determined by measuring the incorporation of [32 P]phosphate from [γ - 32 P]ATP into the E1 α subunit, essentially as described previously [12]. Phosphorylation reactions were set up at 37 °C in a final volume of 100 μ l containing 20 mM Tris/HCl, pH 7.8, 5 mM MgCl₂, 50 mM KCl, 1 mM 2-mercaptoethanol, 0.1 mg/ml BSA, 1.0 mg/ml PDC and 200 μ M ATP (specific radioactivity 200–500 c.p.m./pmol). The kinases were reconstituted with PDC before the assay. Reconstituted preparations were kept on ice for 30 min. The final protein concentration of the recombinant kinases in the assay cocktail was 10 μ g/ml. Phosphorylation reactions were initiated by the addition of ATP after equilibration of the reaction mixtures at 37 °C for 30 s. ATP was added in one-tenth of the total reaction volume. At the indicated times, 10 μ l aliquots of phosphorylation cocktail were withdrawn and quenched on Whatman 3MM filters presoaked in 20% (w/v) trichloroacetic acid/50 mM K₄P₂O₇/50 mM ATP. Protein-bound radioactivity was determined as described previously [12]. The effect of TPP on the rates of phosphorylation of PDC mutants lacking phosphorylation site 2 (S1,3E1PDC), phosphorylation site 1 (S2,3E1PDC) and phosphorylation sites 2 and 3 (S3E1PDC) by the individual isoenzymes of PDK was studied at a final TPP concentration of 50 μ M. During preliminary studies, the latter concentration was found to be sufficient to cause the maximal effect on the rates of phosphorylation of the respective mutants.

ATP-dependent inactivation assay

The general conditions for the ATP-dependent inactivation assay were identical with those of the phosphorylation assay except that (1) all reactions were performed at ambient temperature and (2) the amount of kinase protein used in the assay was halved. These modifications were necessary to decrease the rate of inactivation. The residual activity of PDC in the aliquots withdrawn from the phosphorylation cocktail was determined spectrophotometrically by following the production of NADH at 340 nm, as described elsewhere [17].

Phosphatase activity assay

Phosphorylated PDC was prepared under the conditions of the standard phosphorylation assay, with some modifications. Phosphorylation reactions were set up in a final volume of 100 μ l containing 20 mM Tris/HCl, pH 7.4, 5 mM MgCl₂, 50 mM KCl, 5 mM dithiothreitol and 100 μ M ATP. Reactions were initiated by the addition of PDC (final concentration 2 mg/ml), which was reconstituted with the appropriate isoenzyme of PDK at a molar ratio of 6 molecules of kinase per molecule of PDC.

Phosphorylation reactions were performed at 37 °C. After 60 min, glucose (final concentration 10 mM) and hexokinase (final concentration 5 units/ml) were added to each reaction and samples were incubated at room temperature for a further 15 min to scavenge ATP. The resulting preparations were divided in two halves; one was used to determine the rate of reactivation by isoenzyme PDP1 and the other was used to determine the rate of reactivation by PDP2. General conditions for phosphatase activity assay (final volume 100 μ l) were as follows: 20 mM Tris/HCl (pH 7.4)/10 mM $MgCl_2$ /50 mM KCl/5 mM dithiothreitol/1.0 mg/ml phospho-PDC. When the activity of PDP1 was assayed, the respective reactions were supplemented with $CaCl_2$ to a final concentration of 0.1 mM. Reactions were initiated by the addition of the appropriate isoenzyme of phosphatase (final concentration 5 μ g/ml) and performed at room temperature. At the times indicated in Figure 6, aliquots of dephosphorylation cocktail were withdrawn and quenched in 1 ml of cocktail for the determination of PDC activity. PDC activity was measured spectrophotometrically by following the production of NADH at 340 nm [17].

Analysis of kinetic data

Raw kinetic data were analysed with GraFit version 3 software (Erithacus Software Limited, Horley, Surrey, U.K.). Kinase activities in the ATP-dependent inactivation assay were expressed

Table 1 Kinetic parameters of wild-type E1 and mutant E1 components carrying substitutions of individual phosphorylation sites

Kinetic parameters were determined with the standard PDC activity assay [17]. E1 components were isolated in a complex with E2–E3BP as described in the Experimental section. Initial rates of pyruvate dehydrogenase reaction were determined at several concentrations of pyruvate (varied from 20 to 400 μ M) and TPP (varied from 0.05 to 20 μ M). Results are expressed as mean $\pm$ S.E.M.

Parameter	Wild-type PDC	S1,3E1PDC	S2,3E1PDC	S3E1PDC
Specific activity (units/mg)	16.2 $\pm$ 0.6	15.3 $\pm$ 0.8	9.4 $\pm$ 0.3	8.5 $\pm$ 0.5
K_m^{pyruvate} (μ M)	77.2 $\pm$ 3.5	70.5 $\pm$ 3.5	163.2 $\pm$ 6.2	115.5 $\pm$ 4.6
K_m^{TPP} (μ M)	0.09 $\pm$ 0.02	0.10 $\pm$ 0.01	1.62 $\pm$ 0.06	4.32 $\pm$ 0.21

as first-order rate constants calculated with the single exponential decay option. Rates of phosphorylation in the [32 P]phosphate incorporation assay were expressed as rate constants calculated with the rate option of GraFit 3.09 software. PDP-catalysed rates of phospho-PDC reactivation were also calculated with the rate option. The apparent K_m values for TPP and pyruvate were determined by measuring the initial rates of the pyruvate dehydrogenase reaction at various concentrations of TPP and pyruvate (matrix of 24 different conditions). The resulting

Figure 1 Time courses of phosphorylation of wild-type PDC by individual isoenzymes of PDK

Wild-type PDC was phosphorylated by isoenzyme PDK1 (A), PDK2 (B), PDK3 (C) or PDK4 (D). Phosphorylation reactions were performed in the absence (○) or in the presence (●) of 50 μ M TPP. General conditions for phosphorylation assay are described in the Experimental section.

matrices were analysed as a set to determine the respective kinetic parameters. PDC activity was determined spectrophotometrically essentially as described in [17]. Data in Table 1 and Figures 1, 3, 4, 5 and 6 represent the average of three determinations for one preparation of each protein analysed in this study. Comparable results were obtained for at least three other preparations of each protein.

Other procedures

The activity of PDC was determined as described previously [17]. SDS/PAGE was performed by the method of Laemmli [18]. Protein concentrations were determined as described by Lowry [19], with BSA as standard.

RESULTS

Kinetics of phosphorylation of wild-type PDC by individual isoenzymes of PDK

To investigate the kinetics of phosphorylation of the wild-type PDC by the individual isoenzymes of PDK, both substrate (PDC) and enzymes (kinases) were overproduced in a heterologous system and purified to near homogeneity. Purified kinases were reconstituted with PDC at the ratio of two kinase molecules per complex, corresponding to the enzyme-to-substrate ratio in native PDC [2]. As shown in Figure 1, the individual isoenzymes of PDK were markedly different in their abilities to phosphorylate wild-type PDC. The major differences were observed in the absolute amount of [32 P]phosphate incorporated by each kinase. After 30 min of incubation, PDK1 incorporated approx. 3.4 mol of phosphate per mol of E1. Under the same conditions, PDK2 phosphorylated PDC with a stoichiometry of 1.6 mol of phosphate per mol of E1, whereas PDK3 and PDK4 incorporated 2.8 and 2.6 mol of phosphate per mol of E1 respectively. It is generally believed that out of six phosphorylation sites available on each E1 component, on average only three sites can be phosphorylated by PDK, a phenomenon generally referred to as 'half-of-the-site' reactivity [8]. Our results, however, clearly demonstrate that some isoenzymes deviate from this general rule. The latter is especially apparent for PDK2, which incorporates much less phosphate into wild-type PDC than do the other isoenzymes.

Having analysed the time courses of phosphorylation of wild-type apo-PDC by the individual isoenzymes of PDK, we investigated the effect of TPP on the kinetics of the phosphorylation reaction (Figure 1). In the presence of 50 μ M TPP, the initial rates of phosphorylation were decreased 3.3-fold, 4.4-fold, 1.6-fold and 2.6-fold for PDK1, PDK2, PDK3 and PDK4 respectively. The corresponding stoichiometries of phosphorylation after 30 min of incubation were 2.0, 1.4, 2.4 and 2.0 mol of phosphate per mol of E1. These results indicate that TPP binding caused a change in the conformation of E1, which differentially affected the isoenzymes of PDK. TPP had the greatest effect on PDK1 (a 3.3-fold decrease in the initial rate of phosphorylation and a 1.7-fold decrease in the stoichiometry of phosphorylation). The inhibitory effect of TPP on PDK2 came about primarily as a result of a decrease in the initial rate of phosphorylation (4.4-fold). For PDK4, both the initial rate and the final stoichiometry of phosphorylation were affected by TPP. However, the absolute values of these changes for PDK4 were substantially lower than those for PDK1. Finally, PDK3 seemed to be much less affected by TPP: although it still showed some decrease in the rate and stoichiometry of phosphorylation when holo-PDC was used as a substrate, the magnitude of the effect was rather subtle (Figure 1).

Taken together, these results indicate that the individual isoenzymes of PDK differ in their abilities to phosphorylate apo-PDC and holo-PDC. The latter might reflect the differences in the specificity of each PDK isoenzyme for the individual phosphorylation sites. This also suggests that the changes in the conformation of E1 caused by TPP binding might differentially affect the site-specificity of the kinase isoenzymes. To test these hypotheses, we generated several mutants of E1 in which the individual phosphorylation sites were replaced by alanine residues and investigated the kinetics of phosphorylation of the mutant proteins.

Kinetic parameters of PDC carrying substitutions of individual phosphorylation sites of E1

To investigate the site specificity of PDK isoenzymes, we prepared three mutant forms of E1: S1,3E1 with Ser-271 (phosphorylation site 2) altered to Ala; S2,3E1 with Ser-264 (phosphorylation site 1) altered to Ala; and S3E1 with both Ser-264 and Ser-271 altered to Ala. Mutant E1 components were overexpressed in BL21(DE3) cells similarly to wild-type E1. They were purified after reconstitution with E2 (E2–E3BP subcomplex) expressed separately as described in [16]. The resulting preparations were identical with those of wild-type PDC in purity and subunit composition (results not shown). The kinetic parameters of all enzymes were characterized with the standard PDC assay [17]. As shown in Table 1, the substitution at phosphorylation site 1 affected the kinetics of the pyruvate dehydrogenase reaction, confirming the results of Korotchikina and Patel [8]. All preparations of S2,3E1PDC analysed in the present study consistently showed an approx. 2-fold increase in the apparent K_m for pyruvate and an almost 18-fold increase in the apparent K_m for TPP, with a concomitant approx. 40 % decrease in specific activity. The latter presumably reflects the location of site 1 at the interface of the α and β subunits of E1 at the substrate-binding channel of the active site [21]. In contrast, substitution at phosphorylation site 2 (S1,3E1PDC) had little, if any, effect on the kinetics of the pyruvate dehydrogenase reaction (Table 1). When both sites 1 and 2 were eliminated, the complex behaved similarly to S1,3E1PDC except that the apparent K_m for TPP was approx. 2.7-fold greater. Thus the kinetics of E1 mutant proteins generated in the present study was largely similar to those reported by others [8].

Phosphorylation of wild-type E1 and its mutants by individual isoenzymes of PDK

To investigate the phosphorylation of wild-type E1 and its mutants by individual isoenzymes of PDK, reconstituted preparations were subjected to phosphorylation under the conditions of the standard phosphorylation assay. After 30 min of incubation, aliquots of phosphorylation cocktail were withdrawn and quenched in SDS/PAGE loading buffer. Samples were separated by SDS/PAGE and phosphoproteins were detected by autoradiography (Figure 2). Preincubation with [γ - 32 P]ATP resulted in the phosphorylation of a single polypeptide with an approximate molecular mass of 43 kDa, corresponding to the molecular mass of the E1 α subunit [20]. All of the kinases were capable of phosphorylating sites 1 and 2, although the amount of [32 P]phosphate incorporated into site 2 by isoenzyme PDK2 was consistently lower for several preparations of PDK2 and S2,3E1PDC tested (Figure 2B). Under the conditions described, only PDK1 phosphorylated S3E1PDC (Figure 2A). We could not detect [32 P]phosphate incorporation into this mutant by any other kinase, even after a 72 h exposure to the X-ray film. Taking into account the sensitivity of this assay, the rate of

Figure 2 Autoradiographs showing the phosphorylation of wild-type (wt) and mutant E1 components by PDK1 (A), PDK2 (B), PDK3 (C) and PDK4 (D)

Wild-type and mutant E1 proteins were phosphorylated under the conditions of the standard phosphorylation assay. Proteins were separated by SDS/PAGE. Phosphoproteins were detected by autoradiography. Gels were exposed to the X-ray film for 30–60 min at room temperature without an intensifying screen. Lane 3 in (D) was underloaded in this experiment.

Figure 3 Stoichiometries of phosphorylation of mutant E1 components by individual isoenzymes of PDK

Phosphorylation reactions were performed under the conditions of the standard phosphorylation assay. Absolute amounts of [³²P]phosphate incorporated were determined with the filter assay as described in the Experimental section. Each experiment received the negative (minus kinase) control. Background counts were less than 0.5% of the kinase-catalysed incorporation.

phosphorylation of site 3 by PDK2, PDK3 and PDK4 is less than 1% of the rates of phosphorylation of sites 1 and 2.

To determine the stoichiometries of phosphorylation of E1 mutants by the individual isoenzymes of PDK, the reconstituted complexes were incubated with [γ -³²P]ATP for 40 min at 37 °C. The amounts of [³²P]phosphate incorporated were determined by filter assay. In accord with the results of the gel assay, PDK2, PDK3 and PDK4 showed little, if any, phosphorylation of S3E1PDC in excess of the background values (Figure 3).

Figure 4 Time courses of ATP-dependent inactivation of mutant E1 components by individual isoenzymes of PDK

S1,3E1PDC was phosphorylated and inactivated by PDK4 (○), S2,3E1PDC was phosphorylated and inactivated by isoenzyme PDK4 (●), and S3E1PDC was phosphorylated and inactivated by isoenzyme PDK1 (□). General conditions for ATP-dependent inactivation assay are described in the Experimental section. Residual PDC activity was determined spectrophotometrically by following the production of NADH at 340 nm.

S1,3E1PDC was phosphorylated by these kinases at a stoichiometry of approx. 1.6 mol of phosphate per mol of E1. PDK3 and PDK4 phosphorylated S2,3E1PDC at a stoichiometry of 1.6–1.7; PDK2 incorporated only 1 mol of phosphate per mol of E1 under the same conditions. PDK1 phosphorylated S1,3E1PDC and S2,3E1PDC at a stoichiometry of 2.8–2.9 and S3E1PDC at a stoichiometry of 1.4. It therefore seems that the individual isoenzymes of PDK are markedly different in the absolute amount of phosphate that each isoenzyme can incorporate into the particular phosphorylation site of PDC. Only PDK1 could phosphorylate all three sites of PDC; PDK3 and PDK4 phosphorylated sites 1 and 2 at comparable stoichiometries. However, neither phosphorylated site 3. Finally, PDK2 seemed to be able to phosphorylate site 1, like the other isoenzymes, but showed a lower capacity for phosphorylation of site 2, and did not phosphorylate site 3 appreciably.

Figure 5 Time courses of phosphorylation of mutant E1 components by individual isoenzymes of PDK

Phosphorylation reactions were performed with PDK1 (A), PDK2 (B), PDK3 (C) or PDK4 (D). Phosphorylation reactions were performed in the absence (○, □, △) or in the presence (●, ■, ▲) of 50 μ M TPP; ○, ●, phosphorylation of S1,3E1PDC; □, ■, phosphorylation of S2,3E1PDC; △, ▲, phosphorylation of S3E1PDC.

The contribution of the individual phosphorylation sites to the inactivation of PDC was characterized with an ATP-dependent inactivation assay. As shown in Figure 4, all three phosphorylation sites were inactivating, although the rates of inactivation seemed to be greatly different: site 1 was phosphorylated much faster than sites 2 and 3. For example, the first-order rate constant for the inactivation of S1,3E1PDC by PDK4 was approx. 0.25 min^{-1} , and for S2,3E1PDC this was 0.08 min^{-1} . The first-order rate constant for inactivation of S3E1PDC by PDK1 was approx. 0.02 min^{-1} (Figure 4). Thus, under conditions favouring the inactivation of PDC, the phosphorylation of site 1 largely accounted for the loss of PDC activity, whereas sites 2 and 3, although inactivating, could not contribute to the inactivation under physiological conditions, owing to the slower rates of phosphorylation.

Interestingly, the relative rates of inactivation of S1,3E1PDC and S2,3E1PDC by the individual isoenzymes of PDK were markedly different. As shown in Figure 4, PDK4 inactivated S1,3E1PDC 3-fold faster than S2,3E1PDC. For PDK3 the respective ratio was 10-fold (first-order rate inactivation constants 1.0 min^{-1} and 0.1 min^{-1}), although in this case it was difficult to obtain a reliable estimate, owing to an extremely fast inactivation of S1,3E1PDC. For PDK2 this ratio was even higher, almost 20-fold (first-order rate inactivation constants 0.2 min^{-1} and 0.01 min^{-1}). Finally, even after 60 min of incubation of S3E1PDC with PDK1 and MgATP^{2-} , the residual

activity of the respective complex was still approx. 20–30% of the control complex incubated in the absence of ATP (Figure 4). The latter might suggest that phosphorylation of site 3 does not completely inactivate PDC. However, this seems to be highly unlikely, because when the incubation times were extended beyond 60 min, there was a progressive decrease in the residual activity of S3E1PDC (results not shown), suggesting that phosphorylation site 3 was fully inactivating, similarly to sites 1 and 2.

Kinetics of phosphorylation of E1 mutants by individual isoenzymes of PDK: effect of TPP

The kinetics of phosphorylation of E1 mutant protein by individual isoenzymes of PDK was investigated with apo-PDC or holo-PDC as a substrate. As shown in Figure 5, there was a great difference in the relative rates of phosphorylation of E1 mutant proteins with apo-PDC. PDK2 phosphorylated S1,3E1PDC 19-fold faster than S2,3E1PDC. For PDK3, the corresponding rates differed by 6-fold, and for PDK4 only by 2.5-fold. For PDK1, which phosphorylates all three sites, the rate of phosphorylation of S1,3E1PDC was 2.7-fold greater than that of S2,3E1PDC and 2.4-fold greater than S3E1PDC, suggesting that PDK1 phosphorylated individual sites in the following order: site 1 > site 3 > site 2. These results indicate that, under conditions favouring the phosphorylation and inactivation of PDC, PDK1, PDK3 and PDK4 phosphorylated three or two

sites respectively. PDK2, in contrast, phosphorylated primarily one site (site 1).

To gain further insight into the molecular mechanisms underlying the effect of TPP, we characterized the kinetics of phosphorylation of E1 mutant proteins by individual isoenzymes of PDK with holo-PDC. As shown in Figure 5, the addition of TPP decreased the rate of phosphorylation of site 1 by PDK2, PDK3 and PDK4 (1.5-fold for PDK3 and approx. 2.3-fold for PDK2 and PDK4). The changes in the absolute amounts of phosphate incorporated by these kinases into S1,3E1PDC were not significant. Differences between these isoenzymes became apparent when we studied the phosphorylation of S2,3E1PDC (Figure 5). For PDK3 and PDK4, TPP primarily decreased the rate of phosphorylation of site 2 by 2.7-fold and 3.4-fold respectively. There was only a 22–25 % decrease in the absolute amount of phosphate incorporated with holo-S2,3E1PDC as substrate. In contrast, the effect of TPP on the phosphorylation of site 2 by PDK2 was associated with a significant (4.3-fold) decrease in the amount of phosphate incorporated. Therefore, with holo-PDC as substrate, PDK2 phosphorylated site 1 almost exclusively. Finally, TPP had a very strong effect on PDK1. For S2,3E1PDC and S3E1PDC there was a large decrease in the absolute amount of phosphate incorporated (4.6-fold and 5.6-fold respectively). This suggests that coenzyme binding made sites 2 and 3 largely unavailable for PDK1. When S1,3E1PDC was used in its holoenzyme form, there was a 1.5-fold decrease in the rate of phosphorylation, as well as a 1.6-fold decrease in the amount of phosphate incorporated. The latter probably reflects the protection of site 3 against phosphorylation exerted by TPP, along with a decrease in the rate of phosphorylation of site 1. Therefore, when holo-PDC is used as a substrate, PDK1 behaves very similarly to PDK2, largely because of the inhibitory effect of TPP on phosphorylation of sites 2 and 3. Thus, depending on which isoenzyme of PDK phosphorylates PDC, the phosphate content of PDC is different, reflecting the site specificity of a particular kinase. Furthermore, because the inhibitory effect of TPP is isoenzyme-specific, the phosphate content also reflects whether PDC is in its apoenzyme or holoenzyme form.

PDP1- and PDP2-mediated reactivation of wild-type PDC phosphorylated by different isoenzymes of PDK

Inactivation of PDC by any isoenzyme of PDK was correlated with the phosphorylation of site 1 (Figure 4). Thus, if the phosphorylation of additional serine residues (sites 2 and 3) has physiological significance, it might be important for the control of the dephosphorylation reaction catalysed by phosphatase. To test this hypothesis, we investigated the rates of phosphatase-mediated reactivation of wild-type PDC phosphorylated by individual isoenzymes of PDK. As shown in Figure 6, there was a clear correlation between the type of PDK isoenzyme used to phosphorylate PDC and the rate of reactivation of the respective complex. For both isoenzymes of phosphatase the rates of reactivation decreased in the order PDK2 $\geq$ PDK3 $\geq$ PDK4 $>$ PDK1. However, differences between PDP1 and PDP2 were also apparent. Both phosphatases reactivated PDC phosphorylated by PDK2 with similar rates. When PDC was phosphorylated by PDK4 or PDK3, PDP2 reactivated the respective complexes more slowly (1.6-fold and 2-fold respectively) than PDP1. When PDK1 was used for phosphorylation, this difference was greater (approx. 3-fold). Thus the rate of PDC reactivation by phosphatase probably reflects the amount of phosphate that was incorporated into PDC by a particular isoenzyme of kinase. It also seems that the phosphorylation of

Figure 6 Time courses of phosphatase-mediated reactivation of wild-type PDC inactivated through phosphorylation by individual isoenzymes of PDK

Wild-type PDC was phosphorylated by PDK1 (○), PDK2 (●), PDK3 (□) or PDK4 (■). The respective phosphorylated complexes were re-activated using isoenzyme PDP1 (A) or PDP2 (B). General conditions for reactivation assay are described in the Experimental section. PDC activity was determined spectrophotometrically by following NADH production at 340 nm.

sites 2 and 3 affects the ability of PDP2 to reactivate PDC more than PDP1.

DISCUSSION

Results reported here demonstrate clearly that the isoenzymes of PDK are markedly different in their abilities to phosphorylate PDC. PDK1 can incorporate up to 3.4 mol of phosphate per mol of E1, PDK3 and PDK4 can incorporate somewhat less than 3 mol/mol and PDK2 incorporates only 1.6 mol/mol (Figure 1). All kinases can phosphorylate the major inactivating site of PDC (site 1) equally well. Phosphorylation of sites 2 and 3, in contrast, is isoenzyme-specific. PDK1 phosphorylates both sites 2 and 3, whereas the other isoenzymes phosphorylate only site 2 (Figure 2). Although PDK2 is capable of phosphorylating site 2, it incorporates 40–45 % less phosphate into this site than PDK3 and PDK4 (Figure 3). The relative rates of phosphorylation of individual sites are also markedly different. PDK2 phosphorylates site 1 19-fold faster than site 2 (Figure 5); the corresponding ratios for PDK3 and PDK4 are 6.2 and 2.5 respectively. PDK1 phosphorylates sites 1 and 3 almost 3-fold faster than sites 2 and 3,

and 2.4-fold faster than site 3 alone, in the order site 1 > site 3 > site 2. Thus, although all isoenzymes of the kinase are similar with respect to the kinetics of phosphorylation of site 1, they differ in their abilities to phosphorylate the additional sites. This, in turn, markedly affects the rates of reactivation of phospho-PDC catalysed by phosphatase (Figure 6). Both isoenzymes of phosphatase (PDP1 and PDP2) reactivate phospho-PDC phosphorylated by PDK2 much more rapidly than phospho-PDC phosphorylated by other isoenzymes, in the order $\text{PDK2} > \text{PDK4} \geq \text{PDK3} > \text{PDK1}$, with PDP2 being affected more than PDP1 by the phosphorylation of additional sites.

So far, most of the studies *in vitro* on the regulation of PDC activity by phosphorylation have been performed with apo-complex, although in mitochondria PDC functions in a complex with TPP. Results presented here (Figures 1 and 5) clearly show that TPP is a physiologically significant regulator of phosphorylation reaction. Furthermore, although TPP decreases the rate of phosphorylation of site 1 by all isoenzymes, its effect seems to be isoenzyme-specific with regard to the phosphorylation of sites 2 and 3 (Figure 5). For PDK1 and PDK2, TPP affects primarily the absolute amount of phosphate that these kinases incorporate in sites 2 and 3 (PDK1) and site 2 (PDK2). For PDK3 and PDK4, TPP affects primarily the rate of phosphorylation of site 2. The extent of saturation of PDC by the co-enzyme might therefore be an important factor that controls the activity state (the percentage of enzyme in its active dephosphorylated form) of the complex in mitochondria. When PDC is not saturated with TPP, it is phosphorylated more completely than the fully saturated complex. As shown in Figure 6, the rate of phosphatase-catalysed reactivation of phospho-PDC decreases when the degree of PDC phosphorylation increases. As a result, the activity state of PDC, which reflects the relative rates of the phosphorylation and dephosphorylation reactions, is different for the unsaturated and fully saturated complexes. The phenomena should be the most apparent when phosphorylation reaction is catalysed by the highly TPP-sensitive isoenzyme PDK1 (Figure 1) and the dephosphorylation reaction is catalysed by PDP2 (Figure 6), which shows a strong dependence on the stoichiometry of PDC phosphorylation. The latter might be especially apparent under conditions associated with thiamin deficiency.

The physiological significance of the findings reported here can be fully understood if consideration is given to the tissue distribution of the isoenzymes of PDK and PDP [10–12]. In the normal fed state, only PDK2 is expressed ubiquitously. This isoenzyme is likely to mediate the effects of pyruvate, NADH and acetyl-CoA [11]. PDK1 and PDK3 have a rather limited tissue distribution. PDK1 is abundant in cardiac muscle, whereas PDK3 is abundant in testis [11]. The physiological significance of the latter isoenzymes has not yet been determined. PDK4 is present in several tissues, being abundant in heart and skeletal muscle [17]; it is inducible and is involved in the long-term regulation of PDC activity. Under conditions such as starvation [17], administration of high-fat diet [22], chemically induced diabetes [17,23] or hyperthyroidism [24], the mitochondrial content of PDK4 increases severalfold in most tissues. Finally, although the tissue distribution of the isoenzymes of PDP has not been studied systematically, the available results strongly suggest that the Ca^{2+} -sensitive PDP1 is the major isoenzyme in skeletal muscle and brain, whereas the Ca^{2+} -insensitive PDP2 is the major isoenzyme in liver and adipose tissue [11]. Heart muscle contains both PDP1 and PDP2 (M. F. Allard, personal communication). Thus many mammalian tissues contain different combinations of PDK and PDP isoenzymes, for example PDK2, PDK4 and PDP2 in liver and adipose tissue; PDK2,

PDK4 and PDP1 in skeletal muscle; PDK2 and PDP1 in brain; PDK1, PDK2, PDK4, PDP1 and PDP2 in heart. The results presented here show that PDK isoenzymes differ in their abilities to phosphorylate the additional phosphorylation sites on E1, which largely determines the rates of reactivation of PDC by the isoenzymes of phosphatase and therefore must have a considerable impact on the activity state of PDC in various tissues. Under metabolic conditions favouring phosphorylation (high concentrations of NADH and acetyl-CoA) in tissues primarily expressing PDK2, PDC is readily inactivated. However, when conditions favouring phosphorylation change to the conditions favouring dephosphorylation (high concentrations of pyruvate, NAD^+ and CoA), PDC is reactivated rather rapidly. In tissues expressing other PDK isoenzymes, PDC is phosphorylated to a greater extent and consequently is reactivated much more slowly. This hypothesis is corroborated by studies *in vivo* [25] showing that, after acute starvation (6 h), hepatic PDC is readily reactivated on refeeding. In contrast, prolonged starvation (24–48 h) results in much slower rates of reactivation. In normal circumstances, phosphorylation of PDC in liver is catalysed by PDK2. Evidence from several laboratories strongly suggests that prolonged starvation is associated with the overexpression of isoenzyme PDK4 [17,23,26], which, as shown in Figure 1, can phosphorylate PDC much more completely than PDK2. Furthermore, PDC phosphorylated by PDK2 can be readily reactivated by PDP2 (Figure 6), which is abundantly present in hepatocytes [11]. In contrast, PDC phosphorylated by PDK4 is reactivated by PDP2 much more slowly. Therefore, after prolonged starvation, PDC should be reactivated more slowly than after acute starvation, as shown by Holness and Sugden [25]. This strongly suggests that the different site-specificity of isoenzymes of kinase might be one of the major determinants of the activity state of pyruvate dehydrogenase in mammalian tissues.

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