Site Specificity of Four Pyruvate Dehydrogenase Kinase Isoenzymes toward the Three Phosphorylation Sites of Human Pyruvate Dehydrogenase*

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Activity of the mammalian pyruvate dehydrogenase complex is regulated by phosphorylation-dephosphorylation of three specific serine residues (site 1, Ser-264; site 2, Ser-271; site 3, Ser-203) of the α subunit of the pyruvate dehydrogenase (E1) component. Phosphorylation is carried out by four pyruvate dehydrogenase kinase (PDK) isoenzymes. Specificity of the four mammalian PDKs toward the three phosphorylation sites of E1 was investigated using the recombinant E1 mutant proteins with only one functional phosphorylation site present. All four PDKs phosphorylated site 1 and site 2, however, with different rates in phosphate buffer (for site 1, PDK2 > PDK4 > PDK1 > PDK3; for site 2, PDK3 > PDK4 > PDK2 > PDK1). Site 3 was phosphorylated by PDK1 only. The maximum activation by dihydrolipoamide acetyltransferase was demonstrated by PDK3. In the free form, all PDKs phosphorylated site 1, and PDK4 had the highest activity toward site 2. The activity of the four PDKs was stimulated to a different extent by the reduction and acetylation state of the lipoyl moieties of dihydrolipoamide acetyltransferase with the maximum stimulation of PDK2. Substitution of the site 1 serine with glutamate, which mimics phosphorylation-dependent inactivation of E1, did not affect phosphorylation of site 2 by four PDKs and of site 3 by PDK1. Site specificity for phosphorylation of four PDKs with unique tissue distribution could contribute to the tissue-specific regulation of the pyruvate dehydrogenase complex in normal and pathophysiological states.

The mammalian multi-enzyme pyruvate dehydrogenase complex (PDC)1, which plays an important role in carbohydrate metabolism, catalyzes the oxidative decarboxylation of pyruvic acid with formation of CO₂, acetyl-CoA, and NADH. PDC is composed of multiple copies (per complex) of three catalytic components, pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2), and dihydrolipoamide dehydrogenase (E3); a binding protein, referred to as E3-binding protein (E3BP); and two regulatory enzymes, pyruvate dehydrogenase kinase (PDK) and phospho-pyruvate dehydrogenase phosphatase (1, 2). E1 is a thiamine pyrophosphate-requiring αβ2 heterotrimer with two active sites that interact with each other during catalysis (3, 4). There are three specific serine residues in the α subunit of E1 that are subject to ATP-dependent phosphorylation and inactivation by PDK (5). Phospho-pyruvate dehydrogenase phosphatase dephosphorylates these three serine residues and reactivates PDC (1).

PDK exists as the multiple isoenzymes in several organisms, four isoenzymes of PDK in humans (PDK1, PDK2, PDK3, PDK4), three in rodents (PDK1, PDK2, PDK4 (PDK3 was not cloned, but its existence is not excluded)), two in plants (two in Zea mays and one in Arabidopsis thaliana), one in nematodes (Ascaris suum and Caenorhabditis elegans), and one in fruit fly (6–11). Although PDKs phosphorylate specific serine residues of the E1α subunit, they show very little amino acid sequence similarity with eukaryotic Ser/Thr protein kinases. PDKs were suggested to belong to the ATPase/kinase superfamily (composed of bacterial histidine protein kinase, DNA gyrase, and molecular chaperone Hsp90) based on similarity of their catalytic domains (12).

PDKs are bound to the PDC core through the lipoyl domains of E2, and this interaction is important for their efficient catalytic function (13). PDK activity is enhanced through changes in the status of the lipoyl domains from oxidized to reduced and acetylated forms, which in turn depend on the NADH/NAD⁺ and acetyl-CoA/CoA ratios (14). Mammalian PDK isoenzymes differ in their catalytic activity, responsiveness to the modulators such as NADH and acetyl-CoA, and tissue-specific expression (15). PDK1 is present mostly in heart, whereas PDK2 is found in most tissues. PDK3 is predominantly expressed in tests, whereas heart and skeletal muscle have the highest amount of PDK4 (6, 15).

The E1 component has six potential phosphorylation sites (3 sites per α subunit) namely, site 1 (Ser-264), site 2 (Ser-271), and site 3 (Ser-203) (5). The rates of phosphorylation of the three sites in mammalian E1 were shown to be different (4.6-fold higher for site 1 than for site 2 and 16-fold higher for site 1 than for site 3) with PDK preparations (containing more than one isoenzyme) purified from mammalian tissues (16). We showed recently that the mechanism of inactivation appears to be different for phosphorylation of each of the three sites, i.e. phosphorylation of site 1 affects E1 interaction with its substrates, especially lipoyl moieties of E2, whereas phosphorylation of site 3 interferes with thiamine pyrophosphate binding (17). Because phosphorylation of any one site nearly completely renders E1 (and hence PDC) inactive, the presence of the three phosphorylation sites in E1 remains an enigma. It has been...
suggested that hyperphosphorylation of E1 may play a role in regulation of PDC in certain pathological conditions (18, 19). The existence of the four isoenzymes in mammalian tissues raises an intriguing question about their specificity toward the three phosphorylation sites of mammalian E1. The availability of recombinant human mutant E1s with only one functional phosphorylation site, as well as E1-S264A and E1-S264E, has provided us the unique opportunity to examine the specificity of PDK isoenzymes toward each phosphorylation site. Our findings show that four PDK isoenzymes have different specificity toward the three phosphorylation sites. Site 3 was phosphorylated by PDK1 only. In contrast, the stimulation of PKDs toward reduction and acetylation of the lipoyl moieties of E2 is not site-specific. Presence of glutamate at site 1 as a phosphorylation mimic did not interfere with phosphorylation of sites 2 and 3 by PKDs.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ni-nitrolotriacetate-agarose was obtained from Qiagen. The protein assay reagent was from Bio-Rad. [γ-32P]ATP was from ICN Biomedicals, Costa Mesa, CA. pPDKi, pPDK2, pPDK3, and pPDK4 expression vectors and *Escherichia coli* strain BL21 with pDH2E3BP and E3BP were generously provided by Dr. Robert A. Harris of Indiana University School of Medicine, Indianapolis, IN.

**Protein Expression and Purification**—A coexpression vector for the wild-type E1 (pQE-9–6HEL6/E1β) carrying coding sequences of both human E1α and E1β cDNAs was constructed previously in our laboratory (20). Several mutant human E1s constructed previously (16, 17) were used in this study. E1-S2A/S3A, with serines at site 2 (Ser-271) and site 3 (Ser-293) replaced with alanine leaving only serine at site 1 (Ser-264) available for phosphorylation; E1-S1A/S3A, with serines at sites 1 and 3 replaced with alanine leaving only site 2 for phosphorylation; E1-S1A/S2A, with serines at sites 1 and 2 replaced with alanine leaving only site 3 for phosphorylation. E1-S1A and E1-S1E mutants in which Ser-264 (site 1) was replaced with alanine and glutamate, respectively, were also available (17) to study phosphorylation of site 2 (and 3) in the presence of modified site 1. Recombinant wild-type and mutant human E1s were overexpressed in *E. coli* M15 cells and purified using Ni-nitrolotriacetate-agarose chromatography as described previously (20). Recombinant human E2-E3BP was overexpressed and purified from BL21 cells harboring pDH2E3BP as described previously (17). Recombinant human E3 was overexpressed and purified with >96% purity as described by Liu et al. (21). Recombinant rat PDK1, rat PDK2, human PDK3, and rat PDK4 were overexpressed and purified individually from BL21 cells transformed with pPDK expression vector to 90–95% purity according to Bowker-Kinley et al. (15) with some modifications as described previously (17).

**Determination of PDK Activity and Kinetic Parameters**—Activity of PKDs, using either E1 alone or E1 reconstituted into PDC by measuring the incorporation of [γ-32P]ATP into E1. Wild-type and mutant E1s (40–125 μg) were incubated in the absence or presence of E2-E3BP (46 μg) plus E3 (10–20 μg) with 100–500 μM [γ-32P]ATP (200–1000 cpm/μmol) and a specific PDK isozyme (0.05–0.1 μg) at 30 °C. Reactions were performed in the following different buffer systems: (i) phosphate buffer (20 mM potassium phosphate, 1 mM MgCl₂, 2 mM dithiothreitol (16, 22)) and (ii) MOPS-K⁺ buffer (50 mM MOPS, 60 mM KCl, pH 7.5, 20 mM potassium phosphate buffer, pH 7.0, 0.1 mM EDTA, 1 mM MgCl₂, 2 mM dithiothreitol (16, 13)) and (iii) MOPS-K⁺ buffer (50 mM MOPS, 60 mM KCl, pH 7.5, 20 mM potassium phosphate buffer, pH 7.5, 0.05 mM EDTA, 1 mM MgCl₂, 1 mM dithiothreitol (13)). Reactions were stopped at different time intervals by applying aliquots (20 μl) on paper discs (VWR brand, 238 blotting paper) presoaked in 10% trichloroacetic acid with 10 mM pyrophosphate. Discs were incubated in 10% trichloroacetic acid with 10 mM pyrophosphate for 1 h, washed with 10% trichloroacetic acid (3 × 20 min) followed by ethanol wash (2 × 5 min), and dried, and the radioactivity of the dried filters was measured by a Beckman LS 6500 scintillation counter.

In kinetic studies of PKDs, PDC was reconstituted from its catalytic components E1/E2-E3BP/E3 as follows: 30 E1 tetramers/1 E2 60-mer/12 E3-Gus. The amount of E3 was increased to 24 E3 dimers in experiments when PDK activity was measured in the absence or presence of NADH and acetyl-CoA to ensure rapid oxidation, reduction, or reduction/acetylation of the lipoyl groups of E2 in the presence of E3. Saturation concentrations of E1 and ATP for each PDK isoenzyme were determined in preliminary experiments prior to measuring activity. The amount of E1 was increased from 40 to 60 μg to measure activity and kinetic parameters of PDK1 for site 2 and site 3 (apparent \(K_m\) values for sites 2 and 3 were higher for PDK1; see Table II) and of PDK2, PDK3, and PDK4 for site 3 in the presence of E2-E3BP. The amount of E1 was increased to 75–125 μg to reach saturating concentration when free E1 was used as a substrate in the absence of other PDC components (apparent \(K_m\) for E1 was increased to 125 μg of E2-E3BP; results not shown). The concentrations of ATP used were as follows: 300 μM for PDK1 and PDK2, 100 μM for PDK3, and 500 μM for PDK4 (see apparent \(K_m\) values for ATP in Table II). To study the stimulation of PDK by reduction and acetylation/acetylation of the lipoyl moieties of E2, PDC was incubated for 5 min with the following: (i) NADH and ATP with 50 μM CoA (oxidized lipoyl moieties), (ii) NADH/NAD⁺ = 3/1 (600 μM/200 μM) with 50 μM CoA (reduced lipoyl moieties), and (iii) NADH/NAD⁺ = 3/1 (600 μM/200 μM) with 50 μM acetyl-CoA (reduced and acetylated lipoyl moieties) prior to starting PDK reaction by the addition of [γ-32P]ATP (14, 15).

Kinetic parameters of PKDs were determined using phosphate buffer and PDC (with oxidized lipoyl moieties of E2) by varying the concentrations of E1 from 0.02 to 3.89 μM and ATP from 1.5 to 200 μM. Apparent \(K_m\) values were determined instead of apparent \(V_{max}\) values for E1 as a substrate, because the interaction of PKDs with E1 was cooperative. To calculate \(K_m\) for E1, \(v_0 - E_1\) data were fitted to the Hill equation as follows: \(v_0/E_1 = (r_{max}/S^n) + S^n\). Apparent \(K_m\) values for ATP were calculated from the double reciprocal plots. \(K_m\) values were calculated based on molecular weights of PDK isozymes as determined from SDS-polyacrylamide gel electrophoresis and taking into account the extension of the His tag used for affinity purification.

Under the assay conditions used the velocity of the reaction was linear with respect to both time and PDC concentrations used. Reactions were followed for 30 to 20 min depending on the PDK activity. Triplicate-quadruplicate determinations were averaged for each data point. Negative controls (without PDK or E1) were subtracted from all experimental data. One unit of PDK activity is defined as 1 μmol of [32P]P incorporated in E1 per min at 30 °C. Specific activity of PKD is expressed as milliunits per mg of PDK. Correlation coefficients in all kinetic experiments were at least 95%. Protein was measured by the Bradford method using bovine serum albumin as the standard (23).

**RESULTS**

**Activities of Four Isoenzymes of PKDs with Only One Functional Phosphorylation Site in Mutant E1 in the Presence of E2**—To determine activities of four PKD isoenzymes toward the three phosphorylation sites of human E1, double site mutant E1s were used with alanine substitutions of two of the three phosphorylation sites as follows: E1-S2A/S3A with site 1 only, E1-S1A/S3A with site 2 only, and E1-S1A/S2A with site 3 only. The first striking difference among the behavior of four PDK isoenzymes was that only PDK1 phosphorylated site 3. Fig. 1 demonstrates that PDK1 was able to phosphorylate three sites in wild-type E1 (Fig. 1A) and site 3 only in the mutant E1-S1A/S2A (Fig. 1B), whereas PDK2, PDK3, and PDK4 incorporated only 2 mol of phosphoryl groups per mole of wild-type tetrameric E1 (Fig. 1A) and did not phosphorylate site 3 in the mutant E1-S1A/S2A (Fig. 1B). It should be noted that because of the half-of-the-site reactivity of E1 phosphorylation (24), maximally only three of potentially six available sites are modified under standard experimental conditions in the wild-type tetrameric E1.

Table I compares activities of PKDs toward the three phosphorylation sites of mutant E1s reconstituted with E2-E3BP and E3 and toward free mutant E1s in phosphate buffer and MOPS-K⁺ buffer. The results obtained in the presence of E2 are discussed first. For site 1 PDK2 had the highest activity, followed by PDK4 and PDK1, and PDK3 displayed the lowest level of activity (Table I). For site 2 the highest activity was displayed by PDK3 and less by PDK4, PDK2, and PDK1. Only PDK1 had detectable activity with site 3 (24 ± 4 milliunits/mg), which was similar to its activity for site 2. Three PKDs (1, 2, and 4 isoenzymes) had higher activity toward site 1. Interestingly, PDK3 had higher activity toward site 2 than site 1 in reconstituted PDC.

Table II shows kinetic parameters determined with E1 re-
constituted in PDC in the phosphate buffer system only with oxidized lipoyl moieties in the presence of excess NAD\(^+\) and E3. PDK3 had the lowest apparent \(k_{cat}\) value for ATP and \(S_{0.5}\) values for E1s (with site 1 or site 2 only; \(S_{0.5}\) values were determined for E1 as a substrate because of cooperativity displayed by PDKs for E1). Interestingly, PDK3 had higher \(k_{cat}\) for site 2 than for site 1. Apparent \(S_{0.5}\) for PDK3 for site 2 E1 was about 3.6-fold higher than for site 1 E1. Differences between apparent \(S_{0.5}\) values for sites 1 and 2 E1s were not very different for PDK2 (−1.4-fold higher for site 2) and PDK4 (1.1-fold higher for site 2). However, apparent \(S_{0.5}\) values for PDK1 were higher (−3.2-fold for site 2 and 4.5-fold for site 3 compared with site 1. PDK3 had the highest catalytic efficiency (\(k_{cat}/S_{0.5}\)) for both site 1 and site 2 E1s (and also highest catalytic efficiency for ATP) compared with the other three PDK isoenzymes. Catalytic efficiencies were higher for PDK3 and PDK2 than for PDK1 and PDK4 for site 1 E1, as well as for ATP. Activities of Four Isoenzymes of PDKs with Sites 1, 2, and 3 of E1 in the Absence of E2-E3BP—Previously, it was shown that purified PDK preparations (mixtures of isoenzymes) and purified recombinant PDK2 (5, 16) could phosphorylate site 1 and not sites 2 and 3 in the absence of E2. Also, PDK2 and PDK3 were found to have higher activity for wild-type E1 in the presence than in the absence of E2 (22). We have investigated whether different PDK isoenzymes could phosphorylate the three phosphorylation sites with equal efficiency in the absence of E2. Table I depicts the activities of PDKs toward the three phosphorylation sites of E1 (present individually) in the absence of E2-E3BP in phosphate buffer. All four PDKs had higher activity toward site 1 of free E1 than the other two phosphorylation sites. PDK3 displayed the lowest activity toward both sites 1 and 2. Table III shows the extent of activation of PDKs in the presence of E2-E3BP compared with that in the absence of E2-E3BP. Activities of PDK1 (63.7 ± 3.0 milliunits/mg) and PDK4 (69.7 ± 1.8 milliunits/mg) for site 1 E1 did not significantly differ from their activities in the presence of E2-E3BP (with oxidized lipoyl moieties) (Tables I and III). PDK2 was activated 1.7-fold and PDK3 by 6.6-fold for E1 with site 1 only as a substrate in the presence of E2-E3BP (with oxidized lipoyl moieties).

Surprisingly, in contrast to the previous reports in which wild-type E1 was used as a substrate (5), site 2 was phosphorylated by all four PDK isoenzymes in the absence of E2-E3BP (Table I). For site 2 PDK4 had much higher activity than PDK1, PDK2, and PDK3. The activities decreased in the following order PDK4 > PDK1 > PDK2 > PDK3. Activation by E2-E3BP was from 1.7-fold (PDK4, site 1) to 29-fold (PDK3, site 2) compared with oxidized lipoyl moieties of E2. Only PDK1 had activity for site 3 in the absence E2-E3BP (as was the case in the presence of E2-E3BP), and it was slightly higher (7.5 ± 0.3 milliunits/mg) than for site 2 (5.6 ± 0.4 milliunits/mg).

Stimulation of PDK Activity with the Reduction and Acetylation of Lipoyl Moieties on E2—Activities of PDKs depend upon the proportion of oxidized and reduced plus acetylated lipoyl groups of E2 in reconstituted PDC. We therefore investigated the activities of PDKs under the following different experimental conditions: (i) with completely oxidized lipoyl groups in the presence of excess of NAD\(^+\), (ii) with reduced lipoyl groups of E2 in the presence of NADH and E3, and (iii) with both reduced and acetylated lipoyl groups of E2 in the presence of NADH plus E3 and acetyl-CoA (as described under “Experimental Procedures”). MOPS-K\(^+\) buffer with higher concentration of K\(^+\) than that in phosphate buffer was found to be better for stimulation of bovine kidney PDK activity and was suggested to be more physiological (14). We therefore performed the measurements of the activities of PDKs in three conditions mentioned above in the following buffer systems: phosphate buffer and MOPS-K\(^+\) buffer toward reconstituted PDC using mutant E1, E2-E3BP, and E3 (Table I). Activities of all PDKs were higher in the phosphate buffer compared with the MOPS-K\(^+\) buffer (Table III), from 1.4-fold for site 1 phosphorylation by PDK3 to 40-fold for site 2 phosphorylation by PDK2. Activities were in decreasing order as follows: PDK1 > PDK3 > PDK2 > PDK4 for site 1 and PDK3 > PDK1 > PDK4 > PDK2 for site 2 in MOPS-K\(^+\) buffer with oxidized lipoyl moieties. Site 3 was modified only by PDK1 and was similar to the phosphate buffer system.

Table III compares the extent of stimulation for each PDK isoenzyme by both reduction and acetylation of lipoyl moieties of E2 (reduced/acetylated lipoyl groups versus oxidized lipoyl groups) in the two buffer systems. The maximum stimulation was shown by PDK2 in both buffers. For site 1 phosphorylation with PDK2, the extent of stimulation was higher in MOPS-K\(^+\) buffer compared with phosphate buffer from 2.4- to 5-fold. PDK3 and PDK1 were stimulated to a lesser extent in both buffer systems. The extent of stimulation did not significantly differ on the E1 mutants as a substrate (compare 1.5-fold stimulation in phosphate buffer for PDK1 for site 1 and 1.5-fold for site 2; 2.4-fold for PDK2 for site 1 and 2.3-fold for site 2) (Table III).

In this study we investigated the effect of the following variables on the activity of four PDK isoenzymes: (i) phosphorylation site, (ii) presence of E2-E3BP, (iii) phosphate buffer or MOPS-K\(^+\) buffer, and (iv) condition of the lipoyl moieties of E2 (oxidized, reduced, or reduced/acetylated). Activities of PDKs were different for all the three sites, and site 3 was phosphorylated only by PDK1 (Table I). PDK2 had the highest activity toward site 1 in phosphate buffer compared with other PDKs. PDK3 showed the highest activity toward site 2 in both the buffer systems. In the absence of E2-E3BP, PDK3 showed the lowest activity toward site 1, and PDK4 showed the highest
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PDK activities were measured in phosphate buffer and MOPS-K+ buffer as described under “Experimental Procedures.” Activities of PDK2, PDK3 and PDK4 for site 3 were undetectable under experimental conditions employed.

### Table I

| PDK | E1 site | Phosphate buffer | MOPS-K+ buffer |
|-----|---------|------------------|----------------|
|     |         | +E2-E3BP         | -E2-E3BP       | +E2-E3BP       |
|     |         | Oxidized+        | Reduced+        | Reduced/acylated+ |
| PDK1 | 1 | 62.3 ± 4.3 | 89.8 ± 7.6 | 97.5 ± 1.6 | 63.7 ± 3.0 |
|      | 2 | 20.1 ± 2.2 | 26.6 ± 1.0 | 31.0 ± 0.7 | 5.6 ± 0.4 |
|      | 3 | 23.8 ± 3.8 | 30.0 ± 1.2 | 31.3 ± 1.0 | 7.5 ± 0.3 |
| PDK2 | 1 | 124.7 ± 5.7 | 284.4 ± 9.4 | 297.2 ± 8.0 | 74.8 ± 2.7 |
|      | 2 | 32.0 ± 2.7 | 71.9 ± 4.4 | 72.6 ± 1.6 | 5.1 ± 0.6 |
| PDK3 | 1 | 35.7 ± 0.5 | 46.8 ± 2.9 | 45.3 ± 3.4 | 5.4 ± 0.5 |
|      | 2 | 73.9 ± 2.2 | 76.9 ± 2.3 | 81.5 ± 1.3 | 2.6 ± 0.3 |
| PDK4 | 1 | 70.3 ± 3.4 | 103.8 ± 1.7 | 142.7 ± 2.6 | 69.7 ± 1.7 |
|      | 2 | 41.0 ± 5.3 | 56.2 ± 1.8 | 76.4 ± 3.2 | 24.0 ± 0.8 |

### Table II

#### Kinetic parameters of PDK isoenzymes in the presence of E2-E3BP in phosphate buffer

PDC, reconstituted from E1 (E1-S2A/S3A for site 1, E1-S1A/S3A for site 2, and E1-S1A/S2A for site 3), E2-E3BP, E2, and PDK, was incubated for 5 min with NAD+/NADH = 200/1 (796 μM/4 μM) with 50 μM CoA (oxidized lipoyl groups) prior to starting PDK reaction by the addition of [γ-32P]ATP. Activity of PDK was determined in phosphate buffer as described under “Experimental Procedures.” Apparent S0.5 values for E1 were calculated using the Hill equation: \([V/V_{max}] = \left(\frac{S}{S_0.5}\right)^m\). Apparent K_m values for ATP were calculated from the double reciprocal plots.

### Table III

#### Comparison of PDK activities in two buffer systems and activation by E2-E3BP

PDK were measured in phosphate buffer and MOPS-K+ buffer with lipoyl groups oxidized (PDC reconstituted from E1, E2-E3BP, E3, and PDK was preincubated in the presence of NAD+/NADH = 796 μM/4 μM and 50 μM CoA for 5 min before PDK reaction was started by addition of [γ-32P]ATP) as described under “Experimental Procedures.”

Activity toward site 2. PDK3 was activated in the presence of E2-E3BP to a greater extent than the other PDKs (Table III). Activities of all PDKs were higher in phosphate buffer compared with MOPS-K+ buffer (Table I). PDK2 displayed higher stimulation by the reduction/acytlation of the lipoyl moieties of E2 than the other PDKs (Table III).

Effect of Substitution of Site 1 on Phosphorylation of Site 2 by Four PDK Isoenzymes—Because the three phosphorylation sites of E1 are phosphorylated at different rates (16), the question is whether phosphorylation of site 1 affects phosphorylation of site 2. To address this question, the activities of PDK isoenzymes were determined using E1 mutants with the site 1 substituted by either alanine or glutamate. The glutamate substitution at Ser-264 (site 1) was shown previously to mimic phosphorylation of site 1 by inactivating E1 (17). As seen in Table IV, activities of four PDK isoenzymes were almost the same for E1-S1A and E1-S1E mutants with oxidized, reduced, and reduced/acytlated lipoyl moieties of E2. Table IV shows activities of PDK2, PDK3, and PDK4 toward site 2 only, whereas PDK1 phosphorylated both site 2 and site 3. The activity of PDK1 toward E1-S1A (41.4 ± 2.2 milliunits/mg with oxidized lipoyl groups of E2) is nearly equal to the sum of the activities at site 2 alone (20.1 ± 2.2 milliunits/mg) and site 3 alone (23.3 ± 3.8 milliunits/mg) (Table I). The degree of stim-

### Table IV

#### Comparison of PDK activities in two buffer systems and activation by E2-E3BP

| PDK | E1 site | Ratio of PDK activity with E1-E2-E3BP/E3 over E1 | Stimulation of PDK activities by reduction and acetylation of lipoyl moieties of E2 (reduced/acytlated over oxidized) | Increase (fold) of PDK activities in phosphate buffer compared with MOPS-K+ buffer** |
|-----|---------|-----------------------------------------------|----------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------|
| PDK1 | 1 | 1.0 | 1.5 | 2.1 | 2.2 |
|      | 2 | 3.6 | 1.5 | 1.4 | 4.1 |
|      | 3 | 3.2 | 1.3 | 1.7 | 3.7 |
| PDK2 | 1 | 1.7 | 1.3 | 2.4 | 5.0 | 6.2 |
|      | 2 | 6.3 | 2.3 | 4.4 | 40 |
| PDK3 | 1 | 6.8 | 1.3 | 2.7 | 1.4 |
|      | 2 | 29 | 1.1 | 1.3 | 2.6 |
| PDK4 | 1 | 1.0 | 2.0 | 2.6 | 9.4 |
|      | 2 | 1.7 | 1.9 | 2.5 | 12 |

** PDK activities were measured in phosphate buffer and MOPS-K+ buffer with lipoyl groups oxidized (PDC reconstituted from E1, E2-E3BP, E3, and PDK was preincubated in the presence of NAD+/NADH = 796 μM/4 μM and 50 μM CoA for 5 min before PDK reaction was started by addition of [γ-32P]ATP) as described under “Experimental Procedures.”
ulation with reduced lipoyl groups and reduced/acetylated lipoyl groups compared with oxidized lipoyl groups was higher for PDK2 and PDK4. PDK1 and PDK3 showed no effect or very little stimulatory effect in the three conditions. These experiments were performed at saturating concentrations of mutant E1s. We considered the possibility that the saturating concentrations of E1s might have masked any influence of the modified site 1 on phosphorylation of the other two sites by PDKs. We therefore repeated the experiments with oxidized lipoyl groups of E2 with the concentrations of E1s in the range of S0.5. There was no difference toward E1-S1A and E1-S1E as substrates for the activity of each of the four PDK isoenzymes. Experiments were not performed with the corresponding substitutions of sites 2 and 3, because glutamate mutants of sites 2 and 3 showed different kinetic behavior (with significant levels of residual activity) than E1 mutants with phosphorylated sites 2 and 3 (17).

**DISCUSSION**

The importance of regulation of PDC activity in metabolism of carbohydrates and some amino acids is evident from the complexity of its regulation by phosphorylation-dephosphorylation. The heterotetrameric (α2β2) E1 has three phosphorylation sites per α subunit, and of the six potential sites available, phosphorylation of any one site results in near complete inactivation of E1. Recent studies have shown that the rates of phosphorylation are site-specific and that phosphorylation of each of the three sites affects the active site in a site-specific manner for its ability to bind thiamine pyrophosphate and substrates (16, 17). Tissue-specific expression of multiple isoenzymes of PDK add yet another complexity to regulation of E1 (and hence PDC). PDK1 is expressed predominantly in heart, PDK2 is the most abundant isoenzyme present in several tissues such as liver, heart, skeletal muscle, etc. with lower amounts in spleen and lung. PDK3 is expressed mostly in the testis and lung, and PDK4 is expressed in heart and skeletal muscle (15). The results presented here demonstrate for the first time (i) the specificity of the four PDK isoenzymes toward the three phosphorylation sites of E1, (ii) the presence of significant level of PDK activity toward free E1 (PDK1, PDK2, and PDK4 toward site 1, PDK4 toward site 2, and PDK1 only toward site 3), and (iii) a lack of influence of site 1 on phosphorylation of the other two sites by PDK isoenzymes. Hence, site specificity for phosphorylation of four PDK isoenzymes with unique tissue distribution and differential regulation provides an added feature for tissue-specific regulation of PDC in normal and pathological conditions.

Activities of four PDK isoenzymes were measured previously using wild-type E1 (with all three sites functional) in Tris buffer (15). Recently, activities of PDK2 and PDK3 were compared using wild-type E1 in three different buffer systems (phosphate buffer, MOPS-K+ buffer, and Tris buffer) (22). In the present study we have determined activities of PDK isoenzymes for phosphorylation sites 1, 2, and 3 individually in human E1. PDK activities were determined in two buffer systems for E1 sites 1, 2, and 3 individually. PDKs displayed different maximal specific activities for E1s with sites 1, 2, and 3 reconstituted in PDC depending on the buffer system used. In Tris buffer, activities were as follows: PDK2 > PDK4 → PDK1 > PDK3 for site 1 and PDK3 > PDK4 > PDK2 > PDK1 for site 2 (Table I). In MOPS-K+ buffer activities were as follows: PDK1 > PDK3 > PDK2 > PDK4 for site 1 and PDK3 > PDK1 > PDK4 > PDK2 for site 2 (Table I). These data indicate the sensitivity of the PDKs to the slight changes in their microenvironment. Site 3 was phosphorylated only by PDK1 in both buffer systems, and the activities of the other three PDKs toward site 3 were undetectable under our experimental conditions (see Fig. 1 and Table I). PDK1 differs from PDK2, PDK3, and PDK4 by the length of its amino acid sequence (and hence possibly in structure). Human precursor PDK amino acid sequences are composed of 436 residues for PDK1, 407 for PDK2, and 411 for PDK4 (7). Matured PDK1 has the largest molecular mass among the four PDKs and corresponds to the 48-kDa subunit identified in the PDK preparations purified from bovine and porcine tissues (25), whereas matured PDK2, PDK3, and PDK4 have similar molecular masses and correspond to the 45-kDa subunit.

The stimulation of PDK activities with the reduction and acetylation of the lipoyl moieties of E2 was isoenzyme-specific but not site-specific and was dependent on the buffer system employed. The maximal stimulation was observed for PDK2 in both phosphate buffer and MOPS-K+ buffer (Tables I and III). However, the extent of stimulation was not much different for different phosphorylation sites as substrates. Reduction or acetylation of the lipoyl moieties was suggested to induce conformational changes that generated a more active PDK state (14). Earlier it was shown that stimulation of PDK purified from mammalian tissues by reduction and acetylation of lipoyl moieties of E2 required physiological concentrations of potassium salts (26). However, increasing K+ concentration inhibited bovine PDK activity (27). The reduction in activities of all four PDKs observed in our study with MOPS-K+ buffer com-

### Table IV

| PDK  | Condition of lipoyl groups | E1-S1A        | E1-S1E        |
|------|----------------------------|---------------|---------------|
| PDK1 | oxidized                   | 41.4 ± 2.2    | 53.6 ± 4.2    |
|      | reduced                   | 55.0 ± 5.9    | 51.1 ± 3.3    |
|      | reduced/acetylated         | 52.4 ± 6.9    | 51.3 ± 6.0    |
| PDK2 | oxidized                   | 28.6 ± 5.3    | 20.6 ± 1.5    |
|      | reduced                   | 63.5 ± 6.5    | 60.1 ± 7.5    |
|      | reduced/acetylated         | 58.4 ± 9.8    | 58.2 ± 7.3    |
| PDK3 | oxidized                   | 49.5 ± 3.0    | 62.6 ± 2.6    |
|      | reduced                   | 53.2 ± 2.0    | 60.0 ± 7.5    |
|      | reduced/acetylated         | 61.9 ± 5.8    | 59.6 ± 3.1    |
| PDK4 | oxidized                   | 33.2 ± 2.5    | 31.7 ± 0.9    |
|      | reduced                   | 56.6 ± 5.3    | 60.0 ± 4.0    |
|      | reduced/acetylated         | 69.6 ± 3.9    | 70.7 ± 1.3    |

Activities of PDKs towards E1 with unmodified sites 2 and 3 and with modified site 1 were determined by 32P incorporation into mutant E1s (E1-S1A and E1-S1E) reconstituted with E2-E3BP and E3 as described under "Experimental Procedures." PDC was incubated for 5 min with NAD+/NADH = 796 μM/4 μM and 50 μM CoA (oxidized lipoyl groups), NADH/NAD+ = 600 μM/200 μM and 50 μM CoA (reduced lipoyl groups), and NADH/NAD+ = 60 μM/200 μM and 50 μM acetyl-CoA (reduced/acetylated lipoyl groups) prior to starting PDK reaction by the addition of [γ-32P]ATP. Results are means ± S.D. of three independent determinations.
pared with phosphate buffer is consistent with an earlier study (22).

Although PDK is found to be present in limiting amounts in PDC (1–2 PDK molecules per complex), it was proposed to phosphorylate several molecules of E1 in PDC by moving on the surface of E2 core through interactions with the lipoyl domains (13). In our experiments PDK isoenzymes displayed cooperative interaction with its substrate E1 (apparent $S_{0.5}$ values were determined instead of apparent $K_m$ values). If we calculate the E1 concentration under our experimental conditions in phosphate buffer at which the amount of PDK is only one molecule per PDC, the calculated value for E1 concentration of 0.33 $\mu M$ would be close to the observed apparent $S_{0.5}$ values for E1 for PDK1, PDK2, and PDK4 toward site 1 and PDK4 for site 2. The presence of the substrates in the range of $K_m$ values under physiological conditions provides a higher degree of sensitivity for regulation. In the mitochondria the ratio of PDK isoenzyme activities will depend upon the following: (i) the relative amount of each PDK isoenzyme expressed, (ii) the activity of each PDK isoenzyme at the concentrations of ATP and E1, (iii) the level of reduced and acetylated lipoyl moieties of E2, and (iv) the concentration of pyruvate that has been shown to be inhibitory to PDKs (28).

PDKs are known to be activated by binding to E2 (13). The activation of PDK by E2 was explained by enhancement of the catalytic efficiency of PDK and colocalization of PDK and E1 bound to the subunit-binding domain of E2 (22). When saturating concentrations of E1 and ATP were used, activities of PDK1 and PDK4 toward site 1 of E1 by itself and E1 reconstituted in PDC were almost identical (Tables I and III). In contrast PDK3 was maximally activated by E2. Previously highly purified PDK preparations (mixtures of isoenzymes) purified from bovine tissues and recombinant PDK2 were found to phosphorylate only site 1 of wild-type E1 in the absence of E2 (5, 16). The results presented here show clearly that site 2 is also phosphorylated by all four PDK isoenzymes, and site 3 is phosphorylated by PDK1 only in the absence of phosphorylation of site 2 by all PDK isoenzymes and for phosphorylation of site 3 by PDK1 only. Interestingly, activity of PDK4 toward site 2 of E1 in the absence of E2-E3BP was much higher (from 4.3 to 9.2-fold) than activities of other three PDK isoenzymes (Table I). Recent evidence showed the presence of a large amount of free branched-chain $\alpha$-keto acid-dehydrogenase kinase in rat liver mitochondria (29). Earlier it was found that the protein found in rat heart and liver mitochondrial extracts was able to increase phosphorylation of PDC. This protein was identified later as free PDK (30). PDK4 has higher expression in heart and skeletal muscle compared with other tissues, whereas PDK2 is present in large amounts in different tissues including heart, liver, and kidney (15). The amounts of PDK4 in rat heart are increased during starvation and diabetes (4-fold), hyperthyroidism (3.5-fold), and high fat feeding (3.2-fold) (31, 32). Also, starvation resulted in the increased expression of PDK2 and PDK4 in liver, kidney, and lactating mammary gland but not in brain or adipose tissue (33). This increase in PDK4 (and PDK2) may not correlate with the increases, if any, in the amounts of PDC molecules present in the mitochondria, resulting in the presence of more PDK4 (and PDK2) in the free form. The increase in hyperphosphorylation (multiple sites phosphorylation) of E1 on sites 1 and 2 (and hence PDC) not only by PDKs bound to PDC but also by free PDK4 (and possibly by PDK2) is supported by an increased expression of PDK4 in pathological studies.

E1 was shown to be phosphorylated only on half of its potentially available phosphorylation sites. This seems to be true for all four PDKs. PDK1 phosphorylated about three sites (of six potential sites) and PDK2, PDK3, and PDK4 phosphorylated no more than two sites (of four potential sites) (Fig. 1A). The three phosphorylation sites of E1 are located not far from each other, especially site 1 (Ser-264) and site 2 (Ser-271), and they were suggested to affect E1 activity by different mechanisms (17). It was of interest to find out whether phosphorylation of one site affected phosphorylation of the other sites. The results presented here indicate that all four PDK isoenzymes had similar activities both in basal and stimulated conditions toward mutant E1s with serine at site 1 replaced with alanine or glutamate (Table IV). The lack of inhibition of site 1 modification on phosphorylation of the other two sites would allow hyperphosphorylation of E1 to proceed, which is important under some pathophysiological conditions, such as diabetes and starvation (18).

Concluding Remarks—The present study demonstrates another aspect involved in the regulation of PDC activity in the mitochondria. Not only do PDK isoenzymes have different specific activities toward E1, but also their activities depend upon the individual phosphorylation site of E1 being modified. With the changes of the ratio of different isoenzymes, depending on the tissue and the metabolic state of the cell, PDC activity would be modulated by the extent of site(s) being phosphorylated. The number of sites phosphorylated could be increased by the presence of free PDKs capable of phosphorylation of three sites of E1 as indicated by this study. We have reported previously that each phosphorylation site is dephosphorylated with the similar rate, but dephosphorylation (and hence activation, as phosphorylation of each site causes inactivation) of all the three sites will require a longer time (16), resulting in PDC inhibition for an extended period in the cell.

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