Activity of the mammalian pyruvate dehydrogenase complex (PDC) is regulated by phosphorylation-dephosphorylation of three serine residues (designated site 1, Ser-264; site 2, Ser-271; site 3, Ser-203) in the α subunit of the pyruvate dehydrogenase (E1) component. Substitutions of the phosphorylation sites were generated by site-directed mutagenesis. Glutamate (S1E) and aspartate (S1D) substitutions at site 1 resulted in the complete protection from phosphorylation by thiamine pyrophosphate and 8-fold increase in the apparent Km values for pyruvate increased for the mutants of site 1 when determined in the 2,6-dichlorophenolindophenol assay. The substitutions at sites 2 and 3 caused only moderate reductions in activity for thiamine pyrophosphate and 8-fold increase in the apparent Km for pyrophosphate. Site 3 was almost completely protected from phosphorylation by thiamine pyrophosphate. The results show that the size rather than negative charge of the substituted amino acid residue affects the active site of E1 and that modification of each of the three serine residues affects the active site in a site-specific manner for its ability to bind the cofactor and substrates.

The mammalian pyruvate dehydrogenase complex (PDC) plays an important role in defining the fate of three-carbon compounds derived largely from carbohydrates and to some extent from amino acids. PDC comprises multiple copies of three catalytic enzymes, namely pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2), and dihydrolipoamide dehydrogenase (E3); in addition, the complex also contains a binding protein referred to as E3-binding protein (E3BP), and two regulatory enzymes, namely pyruvate dehydrogenase kinase (PDK) and phosphopyruvate dehydrogenase phosphatase. E1 carries out the decarboxylation of pyruvric acid and the reductive acetylation of lipoyl moieties covalently linked to E2. E2 then catalyzes the transfer of acetyl groups to CoA, forming acetyl-CoA and fully reduced lipoyl moieties. E3 reoxidizes the reduced lipoyl groups of E2 and transfers the electrons to NAD+, forming NADH. Sixty subunits of mammalian E2 and 12 subunits of E3BP form the inner core structure, whereas 20–30 tetrameric E1 (αβγγ) components bind this core and 6 E3 homodimers bind to the E3BP subunits (1, 2).

Regulation of mammalian PDC activity is accomplished in large part by phosphorylation (resulting in inactivation) of the E1 component by a family of pyruvate dehydrogenase kinases (PDK 1–4 isozymes) and dephosphorylation (leading to activation) of phosphorylated E1 by a set of specific phosphatases (phosphopyruvate dehydrogenase phosphatase 1–2 isozymes) (1, 3–6). The α subunit of the E1 component has three phosphorylation sites, named site 1 (Ser-264), site 2 (Ser-271), and site 3 (Ser-203), and phosphorylation of any one of these three sites results in inactivation (7–9). In vivo inactivation of PDC correlates mostly with phosphorylation of site 1 (10). Phosphorylation of site 1 can occur in E1 alone, whereas further phosphorylation at sites 2 and 3 in E1 requires the presence of E2 component (7, 11). E2 activates PDKs by increasing their catalytic efficiency and mediating colocalization of E1 and PDK (12). The rates of phosphorylation (and hence inactivation) of the three sites are site-specific (9). However, the rates of dephosphorylation are similar for the three sites (9).

E1 has two active sites formed by contribution of amino acid residues of both the α and the β subunits (13–19). E1 exhibits half-of-the-site reactivity, as phosphorylation of only one serine residue of one of the two α subunits is sufficient for inactivation of the enzyme (20). Phosphorylation of E1 causes a reduction in its affinity for thiamine pyrophosphate (TPP) as well as substrate binding, and impedes the formation of the 2-α-hydroxyethylidene-TPP (HE=TPP) intermediate of the E1-catalyzed reaction (13, 21–24). The mechanism of inactivation by phosphorylation of E1 is not understood. In an earlier study, we investigated several human E1 mutants with single, double, and triple mutations by replacing serine with alanine and characterized their site-specific rates of phosphorylation and a random mechanism of dephosphorylation (9). To probe the mechanism of inactivation of human E1 by phosphorylation, we have generated a family of mutant human E1s in which one of the three serine residues is replaced individually by amino acid residues of different size and charge. The results of this study indicate that phosphorylation of site 1 leads to the steric interference in catalysis and phosphorylation of site 3 may affect coenzyme binding.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes were purchased from Promega and Roche Molecular Biochemicals. Sequenase 2.0 was from Amersham Pharmacia Biotech. [1-14C]Pyruvate and [35S]ATP were from PerkinElmer Life Sciences. Nickel-nitrilotriacetate (Ni2+-NTA)-agarose was

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‡ The abbreviations used are: PDC, pyruvate dehydrogenase complex; E1, pyruvate dehydrogenase; E2, dihydrolipoamide acetyltransferase; E3, dihydrolipoamide dehydrogenase; E3BP, E3-binding protein; PDK, pyruvate dehydrogenase kinase; TPP, thiamine pyrophosphate; HE=TPP, 2-α-hydroxyethylidene-TPP; N5'-NTA-agarose, N5'-nitrilotriacetate-agarose; DCPIP, 2,6-dichlorophenolindophenol; BCKDH, branched-chain α-keto acid dehydrogenase; BCKDC, branched-chain α-keto acid dehydrogenase complex; WT, wild-type.
obtained from Qiagen. The protein assay reagent was from Bio-Rad. pPDK1 and pPD2 expression vectors and Echerichia coli strain BL21 with pPDHE2/E3BP were generously provided by Dr. Robert A. Harris (Indiana University School of Medicine).

Site-directed Mutagenesis—Several mutants of the three phosphorylation sites in the E1α that were used were: serine 264 (site 1) was replaced with alanine (S1A), glutamate (S1E), glutamine (S1Q), and aspartate (S1D); serine 271 (site 2) was replaced with alanine (S2A), glutamate (S2E), and glutamine (S2Q); and serine 203 (site 3) was replaced with alanine (S3A) and glutamate (S3E) and recombinant human E3 in the PDC assay (to measure the overall reaction catalyzed by E1). The complete coding sequence of each mutant E1 cDNA was ligated to PQE-9 (containing a polyhistidine extension at the amino terminus). The purified enzyme preparations had purity of more than 90% as judged by densitometry of SDS-polyacrylamide gels (results not shown). Recombinant human E2-3BP was constructed previously (26). Some E1 mutants (S2A,S3A, S1A,S3A, and S1A,S2A) were used in this study to verify the DNA sequencing (26). To overexpress the mutant E1s, coexpression vectors carrying both E1a and E1β were constructed and transformed into E. coli M15 cells containing pMD1.3 plasmid, which encoded the lac repressor as described previously (26). Some E1 mutants (S2A,S3A, S1A,S3A, and S1A,S2A E1) used in this study were overexpressed in E. coli M15 cells and purified using Ni^2+ NTA-agarose chromatography (16). The purified enzyme preparations had purity of more than 90% as judged by densitometry of SDS-polyacrylamide gels (results not shown). Recombinant human E2-3BP was purified (about 92% purity) from BL21 cells harboring pPDHE2/E3BP as described by Yang et al. (27) for E2 with some modifications: (i) the resuspension buffer was 50 mM potassium phosphate buffer, pH 7.5, 0.5 mM EDTA, 1 mM dithiothreitol, and a mixture of protease inhibitors; (ii) cells were disrupted by a French press; (iii) polyethylene glycol precipitation was performed, followed by measuring activity of bacterial PDC and E2-3BP in PDC assay (after E2-3BP reconstitution with E1 and E3), and precipitated E2-3BP was separated from bacterial PDC by centrifugation; and (iv) Sepharose CL-4B was used for gel-filtration. Recombinant rat PDK1 and PDK2 were purified to about 92–95% purity from BL21 cells transformed with pPD1 and pPD2 expression vectors according to Bowker-Kinley et al. (28). Mutations (i) the resuspension buffer was 50 mM potassium phosphate, pH 7.5, 100 mM KCl, 5 mM β-mercaptoethanol, and a mixture of protease inhibitors; (ii) a French press was used to disrupt the cells instead of sonication; and (iii) Ni^2+ NTA-agarose was used instead of Talon resin. Recombinant human E3 was overexpressed and purified to about 96% purity as described by Liu et al. (29).

Determination of E1 Activity and Kinetic Parameters—E1 activity was assayed by three different assay systems: (i) by the formation of NADH after reconstitution of E1 with recombinant human E2-3BP and recombinant human E3 in the PDC assay (to measure the overall complex activity) (21); (ii) by the reduction of 2,6-dichlorophenolindophenol (DCPIP), the artificial electron acceptor in the DCPIP assay (to measure E1-catalyzed reaction only, decarboxylation of pyruvate, and oxidation of HE–TTP to acetate) (30); and (iii) by the production of 14CO2 from [1-14C]pyruvate in the absence of an electron acceptor in the decarboxylation assay (25) using pQE-9–6HE1 mutants (S1D, S1E, S1Q, S2A, S2E, S3A, S3E) and E2-3BP as described by Yang et al. (26) with three modifications: (i) the resuspension buffer was 50 mM potassium phosphate buffer, pH 7.5, 0.1 mM EDTA, 1 mM MgCl2, 2 mM NAD, 4 mM TPP, and 0.2 mg/ml E1, followed by precipitation of the enzyme with 30% polyethylene glycol. The pellet was reconstituted in PDC and free E1 (1–20 μM) and the concentrations of pyruvate from 10 to 500 μM (PDC assay) or from 0.2 to 200 μM (DCPIP assay) and the concentrations of pyruvate from 10 to 800 μM (PDC assay) or from 1 to 1500 μM (DCPIP assay). The linear portions (initial rates) of the time courses were used to calculate the kinetic parameters from double-reciprocal plots. One unit of enzyme activity is defined as 1 μmol of product formed/min at 37 °C. Correlation coefficients in all kinetic experiments were at least 95%. Protein was measured by the Bradford method using bovine serum albumin as the standard (33).

Phosphorylation of E1 and Its Mutants—E1 mutants with site 1, 2, or 3 only (S2A,S3A, S1A,S3A, and S1A,S2A, respectively) were phosphorylated by PDK1 or PDK2 in the presence of E2-3BP and 100–500 μM ATP depending upon the site to be phosphorylated. Phosphorylation was performed at 30 °C in 20 mM potassium phosphate buffer, pH 7.0, 0.1 mM EDTA, 1 mM MgCl2, 2 mM dithiothreitol. To measure decarboxylation and DCPIP activity of the phosphorylated mutant E1s, modified E1s were washed on Millipore Ultrafree (UFDV5Bk25) filters and concentrated (without phosphorylation) as described in the Materials and Methods. Phosphorylation of E1 was blocked either before or after phosphorylation of E1 with unlabeled ATP and different compounds as specified in the figure legends. Aliquots (0.8–1 μg of E1) were withdrawn at the indicated times and added to a 1-ml cuvette containing the components for the PDC assay. PDC was allowed to reconstitute for 1 min, and the reaction was started by the addition of pyruvate.

The results of all experiments were calculated using SigmaPlot software (Jandel Scientific, San Rafael, CA).

RESULTS

Activity Changes in Phosphorylation Site Mutant E1s—Highly purified recombinant human wild-type and phosphorylation site-specific mutant E1s were used to determine activities using three different assays. A comparison of the activities of site 1 E1 mutants (S1A, S1E, S1Q, and S1D) in the PDC assay, DCPIP assay, and decarboxylation assay is shown in Fig. 1A. Mutants S1E and S1D with a single negative charge at that site had undetectable activity in the PDC assay (Fig. 1A), which was expected if these mutations mimicked phosphorylation of site 1 in inhibiting PDC activity (Fig. 1B). The S1Q E1 mutant (without a negative charge at that site) had about 5% of the activity in the PDC assay, whereas S1A mutant E1 had about 58% of the control PDC activity. These results indicate that the size of the substituted residue and possibly its orientation and not only the negative charge at site 1 contributes to inactivation of E1. All the site 1 mutants demonstrated considerable levels of activity (46–62%) in the decarboxylation assay and in the DCPIP assay (63–90% activity). Only the S1D mutant E1 had a very low activity (about 10%) in the DCPIP assay, probably due to high inactivation during the catalytic reaction. The progress curve of the PDC reaction for the S1D mutant E1 was linear during the first 30 s, after which the rate of the reaction decreased significantly (results not shown).

We do not have an explanation for this kinetic behavior, as this effect was not studied further. Wild-type E1 phosphorylated at site 1 only had ~5% activity in the decarboxylation assay (Fig. 1B), similar to that observed for the substitution mutants at site 1 (Fig. 1A). Activity in the DCPIP assay was 45% for the phosphorylated wild-type E1, and PDC activity was undetectable.
E2-E3BP and 0.5 mM ATP for 60 min and activity was measured in the PDC assay, and 135 units/mg of E1 in the PDC assay, 122 ± 11 milliunits/mg in the decarboxylation assay, and 242 ± 10 milliunits/mg in the DCPIP assay. B, the S2A,S3A E1 was phosphorylated on site 1 only by PDK2 in the presence of E2-E3BP and 0.1 mM ATP for 30 min and its activity was measured in the PDC (black bars), decarboxylation (hatched bars), and DCPIP (open bars) assays. *, PDC activity was undetectable. One hundred percent of activity corresponds to 20.0 ± 0.9 units/mg of E1 in the PDC assay, 63 ± 5 milliunits/mg in the decarboxylation assay, and 37 ± 8 milliunits/mg in the decarboxylation assay. The results are means ± S.D. of three to five independent determinations.

As seen from the three different assays, the reduction in the activities for the site 2 or site 3 E1 mutants was less variable for each mutant (about 20%) and ranged from 30% to 70% inhibition (Fig. 1C). It should be noted that the substitution of serine at S1 with glutamate resulted in complete inactivation (Fig. 1A). In contrast to the findings with the site 2 or site 3 substitution E1 mutants, phosphorylation of either site 2 or site 3 alone, drastically reduced (more than 90%) PDC activity (Fig. 1D), as opposed to only about 40% reduction in the decarboxylation assay. Substitution of site 2 or 3 with glutamate did not mimic the phosphorylation effect.

To investigate whether the binding of the mutant E1s in the PDC could affect its activity, we measured the activities of the site 1 mutant E1s bound to E2 (in PDC) or in the free state, using the decarboxylation assay. As expected, wild-type E1-PDC activity as measured by the 14CO2 formation (25.8 ± 3.4 units/mg of E1; Fig. 2A) was similar to that determined by NADH formation in the PDC assay (29.0 ± 0.9 units/mg of E1; Fig. 1A). Site 1 mutants showed variable inhibitory effects on PDC activity (measured by 14CO2 formation), which were similar to the ones reported for PDC activity in the PDC assay (NADH formation) in Fig. 1A. The measurements of E1 activity in the S1 substitution mutants bound to E2 and in the free states showed similar activities except for the S1D E1 mutant, which had about twice as much E1 activity when bound compared with the free state (Fig. 2A). Phosphorylation of site 1 in wild-type E1 had similar levels (about 45%) of E1 activity in the decarboxylation assay when E1 was bound to E2 or was in the free state (Fig. 2B). These results suggest that the interaction of the site 1 E1 mutants (except the S1D mutant) with E2 had no effect on their E1 activity in the decarboxylation assay.

Kinetic Parameters of Phosphorylation Site Mutant E1s—Kinetic parameters of the site 1 mutants were determined in the DCPIP assay (Table I) because three of the four site 1 mutants had no activity or negligible activity in the PDC assay (Fig. 1A). Apparent $K_m$ values for pyruvate increased for E1 mutants with alanine (3.2-fold), glutamate (4.5-fold), and glutamine (2.6-fold) substitutions at site 1. In contrast, apparent $K_m$ values for TPP remained unaffected for the glutamate-
Catalytic parameters of wild-type and site-specific mutant E1s determined in PDC and DCPIP assays

| Assay | E1 | kcat | Km for TPP | kcat/Km for TPP | Km for pyruvate | kcat/Km for pyruvate | Ki for phosphophosphate | Pyruvate-induced lag-phase |
|-------|----|------|------------|----------------|---------------|---------------------|------------------------|---------------------------|
|       |    |      | s⁻¹ µM⁻¹ | s⁻¹ µM⁻¹ | µM           | s⁻¹ µM⁻¹ | µM              | µM           | %                         |
| PDC   | WT | 68   | 0.08      | 850          | 52           | 121              | 0.36               | 133          | Yes                       |
|       | S1A| 44   | 1.00      | 44           | 121          | 0.36              | 133          | No                        |
|       | S2A| 44   | 0.19      | 232          | 41           | 1.07              | 143          | Yes                       |
|       | S2E| 33   | 0.78      | 42           | 78           | 0.42              | 478          | No                        |
|       | S2Q| 46   | 0.26      | 175          | 59           | 0.78              | 195          | Yes                       |
|       | S3A| 54   | 0.09      | 600          | 54           | 1.00              | 65           | Yes                       |
|       | S3E| 39   | 2.16      | 18           | 148          | 0.26              | 499          | No                        |
| DCPIP | WT | 0.66 | 0.98      | 0.67         | 5.1          | 0.13              | 54.6        |                           |
|       | S1A| 0.60 | 2.67      | 0.22         | 16.2         | 0.037             | 25.5        |                           |
|       | S1E| 0.59 | 1.11      | 0.53         | 22.8         | 0.019             | 27.1        |                           |
|       | S1Q| 0.39 | 1.71      | 0.23         | 13.5         | 0.029             | 27.0        |                           |
|       | S2A|      |           |              |              |                   | 47.2        |                           |
|       | S2E|      |           |              |              |                   | 32.8        |                           |
|       | S2Q|      |           |              |              |                   | 38.8        |                           |
|       | S3A|      |           |              |              |                   | 46.5        |                           |
|       | S3E|      |           |              |              |                   | 9.9         |                           |

Substituted mutant but only slightly increased for the glutamine-substituted (1.7-fold), and alanine-substituted (2.7-fold) site 1 mutant E1s. Kinetic parameters were not determined for the S1D E1 due to its strong inactivation under the assay conditions.

For the mutants of sites 2 and 3, kinetic parameters were determined using the PDC assay (Table I). Apparent Kₘ values for pyruvate did not change for the mutants of sites 2 and 3 (except about 2.8-fold increase for S3E). In contrast, apparent Kₘ values for TPP increased for the site 2 mutants (2.4-, 9.8-, and 3.2-fold for S2A, S2E, and S2Q mutant E1s, respectively) and ~27-fold for S3E (with only about 2% catalytic efficiency with TPP; Table I). This latter result indicates a possible effect of glutamate substitution for serine at site 3 on TPP interaction with this mutant E1.

Earlier mammalian E1 was reported to have a lag-phase in the progress curve of the PDC reaction (34). Human E1 displayed a lag-phase only in the presence of low TPP concentrations (below 1 µM); the lag-phase was suggested to reflect E1 activation during catalysis (34). Of several mutant E1s investigated, only S1A, S2E, and S3E did not show the lag-phase in the PDC reaction under the experimental conditions employed (Table I). The absence of the lag-phase could reflect a change in the conformation of the E1 active site caused by these mutations. The information about the presence of the lag-phase may be due to the lack or negligible PDC activity of these mutant proteins. Human E1 was shown previously to be subject to substrate-induced inactivation during incubation with TPP plus pyruvate involving acetylation of the protein groups (35). As shown in Table I, except for the mutant S3E E1 with only about 10% inhibition, all other mutant E1s tested showed inhibition from 25% to 55%. Mutation of site 3 (S3E) could affect TPP binding, which is required to achieve the substrate-induced inactivation. It is noteworthy that inactivation of the site 1 mutant E1s (S1A, S1E, and S1Q) was only about one-half (~26%) as compared with that observed for the wild-type E1 (~55%). This may be due to the different conformation of the active site and/or protection of the protein groups involved in the substrate-induced inactivation (S1A, S1E, S1Q).

To further investigate the changes in TPP binding, pyrophosphate was used as a competitive inhibitor of TPP in wild-type and mutant E1s in the PDC assay (Table I). Glutamate substitution at site 2 or site 3 caused a marked reduction in its affinity for pyrophosphate that correlated with the increased apparent Kₘ values for TPP (Table I). The less efficient binding of pyrophosphate may be due to the presence of the negative charge in the S2E and S3E mutant E1s. However, the mutant S2Q also showed a 3-fold increase in Kᵢ. Unfortunately, the Kᵢ value for the S1E and S1Q mutant E1s could not be determined due to the lack of activity of this enzyme in the PDC assay.

**Substrate Recognition by Phosphorylation Site Mutant E1s**—E1 is able to use α-ketobutyrate and α-ketoisovalerate as substrates in the DCPIP assay (36) and only α-ketobutyrate in the PDC assay (due to specificity of the E2 component) (37). We therefore investigated the possible changes in substrate recognition by the E1 mutants (Table II). The mutants of site 1 (except S1D) had higher activity with α-ketobutyrate than wild-type E1. The most striking observation was a 5-fold higher activity of the S1Q mutant E1 compared with wild-type E1. The DCPIP activity of S1Q with α-ketobutyrate as a substrate (169 milliunits/mg) was as high as the activity with pyruvate as a substrate (153 milliunits/mg), whereas the activity of the wild-type E1 with α-ketobutyrate as a substrate (34 milliunits/mg) was much less than with pyruvate (242 milliunits/mg) (Table II). The E1 mutants of sites 2 and 3 displayed activity nearly similar to the wild-type E1 with α-ketobutyrate as a substrate, whereas their activity with pyruvate was less than that observed for the wild-type E1. When α-ketoisovalerate, the α-keto acid of isoleucine, was used as a substrate, all of the mutants displayed significantly decreased levels of activity; the greatest decrease occurred with the substitution generating a negative charge (residual activity: 7.7% for S1E, 9% for S1D, 33.6% for S2E, and 20.9% for S3E) (Table II). The general direction of the reduction in activities of the mutants of sites 2 and 3 with α-ketoisocaproate was similar to that observed for these mutant E1s with pyruvate as a substrate (Table II).

**Interaction of the Site-specific Mutant E1s with E2**—The subunit-binding domain in mammalian PDC-E2 binds only E1. The possible effect of the mutations on the interaction of E1 with E2 was investigated using competition by mutant E1 with the wild-type E1 for its interaction with E2 (38, 39). Wild-type and mutant E1s were preincubated with E1-E2-3BP in different ratio; aliquots were taken to measure PDC activity after E1-E2-3BP reconstitution with E3. This assay includes interactions of the wild-type and mutant E1s with E2 in the preincubation mixture and under turnover conditions. As shown in Fig. 3, the three E1 mutants of site 1 (S1E, S1Q, and S1D), having almost no activity in the PDC assay, competed for the interaction with E2 with less affinity than the wild-type E1.
curves were displaced from the expected line for the equal binding affinity for E2). Activity was 80% for WT/S1E, 87% for WT/S1Q, and 83% for WT/S1D at 5/5 ratio of WT/mutant E1 (calculated as the best fit of the hyperbolic curves by computer) instead of the theoretical 50%. The same approach was used in the earlier study to demonstrate the higher affinity of the active E1 for E2 compared with the phosphorylated E1 (38). We have confirmed this observation with E1 phosphorylated at site 1 (results not shown). E1 mutants of sites 2 and 3 were not investigated using this experimental approach as they retain nearly 50% of wild-type activity in the PDC assay (Fig. 1).

Effect of Coenzyme and Substrates on Phosphorylation of Different Sites in E1—To probe the possible spatial arrangements of the three phosphorylation sites in E1 in relation to the active site, phosphorylation of each individual site was investigated in the presence of different ligands. Fig. 4 depicts inactivation of E1 harboring mutations at site 1 (E1 S2A,S3A), site 2 (E1 S1A,S3A), and site 3 (E1 S1A,S2A) by PDK1 in the absence and presence of varying concentrations of TPP (up to 200 mM). TPP afforded protection of all three sites, and the level of protection was in the order site 1, site 2, site 3. Site 3 was protected almost completely by 200 mM TPP. The inactivation rates ($k_{app}$) decreased at 200 mM as follows: 3.9-fold for site 1, 7.1-fold for site 2, and 41-fold for site 3, suggesting that site 3 is closer to the TPP-binding site and that phosphorylation of this site may affect the interaction of E1 with the coenzyme.

Because phosphorylation of pigeon breast muscle E1 was shown to prevent pyruvate binding (24), it was of interest to...
investigate if pyruvate could also protect human E1 from inactivation by phosphorylation. Fig. 5A shows phosphorylation of site 1 of the wild-type human E1 by PDK2, which phosphorylates only site 1 in the absence of E2-ESBP and in the presence of different compounds. Aliquots were taken to measure PDC activity. A, no addition; 1, 52 μM ATP; 2, 52 μM ATP and 50 μM pyruvate; 3, 52 μM ATP and 100 μM pyruvate; 4, 52 μM ATP, 1 mM MgCl₂, 0.2 mM TPP, and 0.2 mM pyruvate; 5, 52 μM ATP, 1 mM MgCl₂, 0.2 mM TPP, 0.05 mM pyruvate; 6, 52 μM ATP and 0.2 mM α-ketoisovalerate; 7, 52 μM ATP, 1 mM MgCl₂, 0.2 mM TPP, and 0.2 mM α-ketoisovalerate.

FIG. 5. Effect of different compounds on phosphorylation of site 1. The wild-type E1 was phosphorylated at site 1 only by PDK2 in the absence of E2-ESBP and in the presence of different compounds. Aliquots were taken to measure PDC activity. A, no addition; 1, 52 μM ATP; 2, 52 μM ATP and 50 μM pyruvate; 3, 52 μM ATP and 100 μM pyruvate; 4, 52 μM ATP, 1 mM MgCl₂, 0.2 mM TPP, and 0.2 mM pyruvate; 5, 52 μM ATP, 1 mM MgCl₂, 0.2 mM TPP, 0.05 mM pyruvate; 6, 52 μM ATP and 0.2 mM α-ketoisovalerate; 7, 52 μM ATP, 1 mM MgCl₂, 0.2 mM TPP, and 0.2 mM α-ketoisovalerate.

In the earlier studies when E1 was most probably phosphorylated at one active site due to its half-of-the-site reactivity (7, 20), the residual activity of E1 in the reactions other than the overall PDC reaction (22, 23) could arguably be explained by functioning of the second active site. We excluded this possibility by introducing glutamate or aspartate at the phosphorylation site 1 of both α subunits by site-directed mutagenesis, i.e. modifying both active sites. The moderate reduction of activity in the individual E1-catalyzed reactions may indicate the effect of phosphorylation on the reductive acetylation step. However, we cannot exclude the possibility that the decrease in activity levels could be explained by the differences in the catalytic rates of different reactions: 29 units/mg in the DCPIP assay, 242 milliunits/mg in the DCPIP assay, and 122 milliunits/mg in the decarboxylation assay.

Phosphorylated pigeon breast muscle E1 was shown to form the charge transfer complex accompanying TPP binding. In contrast to WT-E1, the intensity of this band did not change after addition of pyruvate to phospho-E1, indicating possible interaction between the phosphorylated serine and the substrate-binding site (23). Besides the disturbance in the charge transfer complex as a result of pyruvate binding, the other possible explanation could be the conformational transition of the E1 active site after decarboxylation. Apparent \( K_m \) values for pyruvate determined in the DCPIP assay increased not only for the glutamate- and glutamine-substituted E1 mutants of site 1 but also for alanine substitution (Table I). Similarly, replacement of Ser-293 (site 1) with alanine in the BCKDH resulted in an increased apparent \( K_m \) for the substrate without affecting the \( V_{max} \) (43). The substitution of serine at site 1 or 2 in nematode PDC-E1 with alanine did not change the apparent \( K_m \) value for pyruvate but decreased the \( V_{max} \) value (44). Probably even if phosphorylation of site 1 affects the substrate
binding (change in apparent $K_m$ values), it would not account for the complete inactivation. It was suggested that phosphorylation of pigeon breast muscle E1 may prevent pyruvate binding based on pyruvate-dependent protection against phosphorylation (24). In our study, pyruvate decreased inactivation by inhibiting PDK in the absence of TPP (Fig. 5; see also Ref. 40). A substrate analog, $\alpha$-ketoisovalerate (not an inhibitor of PDK activity under our experimental conditions), did not afford protection from phosphorylation (Fig. 5). The recent crystal structure of Pseudomonas putida BCKDH suggested that a serine may be involved in the substrate binding through a water molecule (45). It is possible that Ser-264 (site 1) of human PDC-E1 is involved in stabilization of the substrate binding and any substitution, even with alanine, would affect the substrate binding; however, it would not eliminate E1 activity (Fig. 1A).

An interesting finding is that substitution of serine 264 (site 1) with glutamine forced this mutant E1 to lose its substrate specificity and use pyruvate and $\alpha$-ketobutyrate with nearly the same rates in the DCPIP assay. An increase in the size of the amino acid at position 264 increased E1 activity with $\alpha$-ketobutyrate (Table II). Substitution of site 1 with glutamine may result in a conformational change of the substrate channel, allowing it to better accommodate a larger substrate, $\alpha$-ketobutyrate. The branched-chain keto acid, $\alpha$-ketoisovalerate, seems to be a poor substrate for S1E and S1Q due to the interference of the bulky, substituted substrate.

The earlier studies demonstrated a greater affinity of the active E1 form to the E2 sites compared with the phosphorylated form (38, 46). Our results are in support of these findings and suggest that amino acid substituted mutants of site 1 can not easily replace the wild-type E1 when interacting with E2 (Fig. 3). Inactivation of E1 is not increased because of its binding onto E2, as E1 in the presence or absence of E2 showed the same level of reduction in its activity in the decarboxylation assay (Fig. 2). The recently obtained three-dimensional structure of the human BCKDH provides evidence for location of the E2-binding site close to the C termini of $\beta$ subunits, which is far from the active sites and possibly from the phosphorylation site 1 (47).

Phosphorylation of E1 probably alters its interaction with the lipoyl domains of E2 more than that with pyruvate. The conformational requirements for the reductive acetylation step could be different from that of decarboxylation; thus, E1 may undergo a transition before catalyzing its rate-limiting second step. Berg et al. (48) suggested that the conformational change takes place following pyruvate decarboxylation that opens the binding cleft on E1 for the lipoyl domain of Azotobacter vinelandii PDC-E2. An attractive idea is that phosphorylation may prevent the conformational transition thus eliminating the reductive acetylation step. We have several indications for a change in conformational mobility of E1. The differences in apparent $K_m$ values for pyruvate and TPP and the decrease in the substrate-induced inactivation may reflect a different conformation of the E1 active site. The absence of the lag-phase for the alanine substitution of site 1 and glutamate substitution of sites 2 or 3 (Table I) may indicate the inactivity of the phosphorylated E1 to undergo the activation during catalysis. Gong et al. (49) suggested that it is not the decarboxylation but interaction of lipoyl domain with E1 that facilitates structural changes within E1 and subsequently the efficient reductive acetylation. The negative charge and the size of the phosphoryl group located in the substrate channel could prevent the interaction of E1 with the negatively charged region (Glu-162, Glu-167) of the lipoyl domain (12).

All mammalian PDCs have three phosphorylation sites and phosphorylation of each site leads to inactivation of E1. Nematode PDC-E1 has only two inactivating phosphorylation sites, and their positions in the sequence are conserved with sites 1 and 2 of mammalian PDC-E1 (44). In contrast, mammalian BCKDC-E1 has two phosphorylation sites and phosphorylation of one of them, Ser-293 (site 1), results in complete inactivation (and is conserved with site 1 of PDC-E1) (43). Phosphorylation of Ser-303 (site 2; 3 amino acid residues farther from site 2 of PDC-E1) does not affect the BCKDC activity. Our study showed that the substitution of serine at sites 2 or 3 in E1 with glutamate or glutamine caused only 30–70% reduction of activity in all three assays (Fig. 1C). However, phosphorylation of site 2 or 3 resulted in drastic reductions (>90%) of PDC activity (Fig. 1D). A possible explanation is the larger size of PO$_4^-$ group or compensation in the protein structure occurring during protein folding of the substitution mutant E1s. This situation is different for site 1 phosphorylation, which correlates with serine replacement with glutamate and aspartate (Fig. 1A). This observation lends support to the importance of site 1 and its major contribution to the inactivation during phosphorylation.

Apparent $K_m$ values for TPP determined in the PDC assay were higher for S2E and S3E mutant E1s compared to that for wild-type E1 (Table I). This correlated well with the results of the inhibition study with pyrophosphate that demonstrated less affinity for pyrophosphate of S2E and S3E mutant E1s (Table I). In a separate experiment, TPP protection from phosphorylation-dependent inactivation increased from site 1 to site 2 and was maximal for site 3 (Fig. 4). It is possible that site 2 and especially site 3 are localized in close vicinity to the TPP-binding site and phosphorylation of site 3 affects TPP binding.

Concluding Remarks—Phosphorylation of a serine residue has been shown to alter the activities of several other enzymes by one of these mechanisms: (i) a long range conformational change (50), (ii) an electrostatic repulsion and steric hindrance (51), and (iii) an impairment of protonation by histidine (52). In the case of mammalian PDC-E1, site 1 is most likely exposed at the interface of the $\alpha$ and $\beta$ subunits and may be at the substrate channel directing and positioning the lipoyl moiety in the E1 active site. Hence, the phosphorylation of this site might interfere with the second partial reaction catalyzed by E1, using E2 as a substrate. Serine at site 1 by its juxtaposition in the substrate channel eliminates PDC activity by its replacement with a large group with or without a negative charge. Sites 2 and 3 are exposed only after binding of E1 onto E2, and hence are most likely localized differently and may have different mechanism of inhibition. Site 3 seems to be close to the TPP pyrophosphate moeity-binding site, and its phosphorylation may affect TPP binding.

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