An Essential Role of Glu-243 and His-239 in the Phosphotransfer Reaction Catalyzed by Pyruvate Dehydrogenase Kinase*

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This study was undertaken to examine the mechanistic significance of two highly conserved residues positioned in the active site of pyruvate dehydrogenase kinase, Glu-243 and His-239. We used site-directed mutagenesis to convert Glu-243 to Ala, Asp, or Gln and His-239 to Ala. The resulting mutant kinases demonstrated a greatly reduced capacity for phosphorylation of pyruvate dehydrogenase. The Glu-243 to Asp mutant had \( \sim 2\% \) residual activity, whereas the Glu-243 to Ala or Gln mutants exhibited less than 0.5 and 0.1% residual activity, respectively. Activity of the His-239 to Ala mutant was decreased by \( \sim 90\% \). Active-site titration with \([\alpha-32P]ATP\) revealed that neither Glu-243 nor His-239 mutations affected nucleotide binding. All mutant kinases showed similar or even somewhat greater affinity than the wild-type kinase toward the protein substrate, pyruvate dehydrogenase complex. Furthermore, neither of the mutations affected the inter-subunit interactions. Finally, pyruvate dehydrogenase kinase was found to possess a weak ATP hydrolytic activity, which required Glu-243 and His-239 similar to the kinase activity. Based on these observations, we propose a mechanism according to which the invariant glutamate residue (His-239) acts as a general base catalyst, which polarizes the hydroxyl group on a serine residue of the protein substrate for direct attack on the \( \gamma \) phosphate. The glutamate residue in turn might be further polarized through interaction with the neighboring histidine residue (His-239).

Mammalian pyruvate dehydrogenase complex (PDC) catalyzes oxidative decarboxylation of pyruvate with concomitant formation of acetyl-CoA and NADH. Under physiological conditions this reaction is irreversible and, therefore, largely defines the metabolic fate of pyruvic acid and carbohydrate fuels in general (1). In well oxygenated tissues such as brain, skeletal muscle, heart, and kidney, PDC commits pyruvate to further oxidation through the Krebs cycle, thus supplying the oxidative fuel for the generation of ATP. In lipogenic tissues such as liver, fat, and mammary gland, PDC provides acetyl-CoA primarily for biosynthesis of fatty acids, allowing the excessive carbohydrates obtained with diet to be spared. To fulfill these opposing functions, the activity of mammalian PDC is tightly regulated by reversible phosphorylation (2). Phosphorylation of the dehydrogenase component of the complex (E1 component) by dedicated pyruvate dehydrogenase kinase (PDK) renders the entire complex inactive (3). Phospho-PDC can be re-activated through the action of another dedicated enzyme, pyruvate dehydrogenase phosphatase (4). In mammals, both kinase and phosphatase components exist in several isozymic forms (four for PDK and two for pyruvate dehydrogenase phosphatase (6)), which are likely to contribute to the tissue-specific regulation of PDC. The latter is evidenced by the tissue-specific distribution of different isozymes (5) as well as by their different responses to the naturally occurring inhibitors and activators of the kinase (7) and phosphatase reactions (6).

Despite the important role that phosphorylation of PDC plays in regulation of carbohydrate metabolism, little is known about molecular mechanisms underlying the phosphorylation reaction. Kinase-driven inactivation of PDC occurs as a result of phosphorylation of three serine residues usually referred to as phosphorylation sites 1, 2, and 3 (8). This makes PDK a strictly Ser-specific protein kinase. Surprisingly, PDK (5) along with the homologous mitochondrial protein kinase that phosphorylates the branched chain \( \alpha \)-ketoadid dehydrogenase complex (BCKDC) and BCKDC kinase, respectively (9), does not show any sequence similarity to the other Ser/Thr-specific protein kinases. Instead, it resembles histidine kinases (10), a diverse group of enzymes involved in regulation of various signal transduction pathways in bacteria. All structural elements characteristic of histidine kinases (so-called boxes H, N, G1, G2, and G3) can be readily identified in the amino acid sequence of PDK (11). Recently, the three-dimensional structures of two histidine kinases involved in the regulation of chemotaxis and osmosensitivity (CheA (12) and EnvZ (13), respectively) have been determined by x-ray crystallography and NMR. These structures reveal a very characteristic nucleotide binding domain that, in contrast to the catalytic domains of Ser/Thr-specific protein kinases, folds as an \( \alpha/\beta \) sandwich consisting of five strands and three helices with unique left-handed connectivity. Furthermore, certain amino acids of boxes N, G1, G2, and G3 are intimately involved in the anchoring of the nucleotide substrate in the active site of the kinase molecule (13). When we probed the corresponding residues of PDK2 by site-directed mutagenesis, the resulting mutant kinases were catalytically defective due to the impaired ability to bind the nucleotide

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1 The abbreviations used are: PDC, pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase; PDK1, PDK2, and PDK3, and PDK4, isozymes 1, 2, 3, and 4 of pyruvate dehydrogenase kinase; E1, pyruvate dehydrogenase component of PDC; E2, dihydrolipoyl acetyltransferase component of PDC; E3, dihydrolipoamide dehydrogenase component of PDC; E3BP, E3-binding protein component of PDC; PAGE, polyacrylamide gel electrophoresis.
substrate, strongly suggesting that the nucleotide binding domain of PDK is folded similarly to the nucleotide binding domain of histidine kinases (14).

On the other hand, the differences between these two groups of enzymes are also quite apparent. Histidine kinases use ATP to phosphorylate their own histidine residue positioned within, external to the nucleotide-binding site histidine-bearing domain (12, 13). It is generally believed that the phospho-accepting histidine residue directly attacks the γ phosphate of ATP (12). PDK, in contrast, catalyzes the transfer of γ phosphate to the side chain of the serine residue of the exogenous substrate, E1. To date, there is no evidence for existence of a phospho-the side chain of the serine residue of the exogenous substrate, the nucleophilicity of Glu-243 might be further increased consistent with the idea that, at least in mammalian kinases, phosphorylation reaction. The results reported here are also consistent with the idea that, at least in mammalian kinases, the nucleophilicity of Glu-243 might be further increased through the interaction with the neighboring histidine residue (His-239 of PDK2).

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—Point mutations within the amino acid sequence of rat PDK2 were introduced using oligonucleotide-directed mutagenesis (17). The sequences of mutagenic oligonucleotides were as follows: 5′-CCA CAT GCT CTT CTA GAA GTC C-3′ for Glu-243 to Asp mutant; 5′-CCA CAT GCT CTT TCG ACT CTT CAA GAA TGC C-3′ for Glu-243 to Glu mutant; 5′-CCA CAT GCT CTT TCG ACT CTT CAA GAA TGC C-3′ for Glu-243 to Ala mutant; 5′-CCA CAT CTC GCG CAT CTT CTA GAA GTC C-3′ for Asp-282 to Gln mutant (the amino acid residues are numbered according to the sequence of mature rat PDK2, the altered bases are underlined). Mutagenesis reactions were carried out on double-stranded DNA of rat PDK2 (5) subcloned into µC19 using the ExSite™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). The reactions were set up essentially as recommended by the manufacturer. The mutations as well as the fidelity of the rest of DNAs were confirmed by direct sequencing (19).

**Expression and Purification of the Mutant Kinases**—PDK2 cDNAs (~1.2 kilobases) carrying the point mutations were cut out of the µUC 19 DNA with ScaI and HindIII restriction enzymes and re-ligated into the pET-28a expression vector (Novagen, Madison, WI) between ScaI/HindIII sites of the vector (pPDK2 vector). Plasmids containing the inserts of the correct size (~1.2 kilobases) were identified by restriction analysis. Positive plasmids were co-transfected into BL21(DE3) cells (Novagen) along with pGroESL plasmid carrying the genes coding for molecular chaperones GroEL and GroES under the control of an isopropyl-1-thio-β-galactosidase-inducible promoter (the respective plasmid was obtained as a generous gift from Dr. Anthony Gatby at DuPont Central Research and Development, Wilmington, DE). Double-transformants were selected on yeast-dropyram on agar containing kanamycin and chloramphenicol (50 µg/ml each) (7). Several individual colonies from every transformation were tested for their ability to produce significant amounts of soluble, recombinant kinase. Clones expressing the greatest amount of the soluble kinase were used for further analysis.

The expression of the mutant kinases was performed essentially as described previously (14). Their purification was carried out using TALONTM (CLONTECH Laboratories, Inc., Palo Alto, CA) affinity resin as described elsewhere (7). The protein composition of each preparation was evaluated by SDS/PAGE analysis. Gels were stained with Coomassie Blue R250. All enzyme preparations used in the present study were more than 90% pure as judged by Coomassie-stained SDS/PAGE.

**Other Enzyme Preparations**—Human recombinant PDC was expressed in *Escherichia coli* as described in Harris et al. (20). The complex was purified by polyethylene glycol 8000 (Sigma) precipitation, gel filtration on Sepharose 4B (Amersham Pharmacia Biotech), and high speed centrifugation (20). The E1 component of PDC was expressed and purified as described elsewhere (21). All enzyme preparations used in this study were more than 90% pure as judged by Coomassie-stained SDS/PAGE.

**Kinase Pull-down Assays**—To construct “bait” vectors for pull-down experiments, unique NdeI and XhoI restriction sites flanking the coding region of the kinase cDNA were introduced by site-directed mutagenesis. Respectively cDNAs were subcloned between NdeI and XhoI sites of pET-28a vector (Novagen), producing in-frame fusion with the vector sequence coding for a His10-Tag. “Catch” plasmids were constructed by subcloning kinase cDNAs flanked by SstI and XhoI restriction sites into pET-28a (Novagen) cut with SstI and XhoI, producing in-frame fusion with a T7-tag sequence coded by the vector. An expression cassette carrying the T7 promoter, the cDNA of interest, and a T7 terminator was cut out of catch vectors using BsnI and DraIII restriction enzymes. After blunt-end, it was subcloned into an appropriate bait vector cut with DraIII, blunt-ended with T4 polymerase, and dephosphorylated with calf intestinal phosphatase. Thus, the resulting catch/bait plasmids were carrying two expression cassettes: one directing synthesis of His10-
tagged kinase and the other directing synthesis of T₇-tagged kinase.
Expression of the respective proteins was performed in BL21(DE3) cells
co-transformed with appropriate catch/bait plasmid and pGroESL vec-
tor, essentially as described above. Respective recombinant kinases
were isolated using metal affinity chromatography on TALON™ resin
(CLONTECH) (7). Isolated proteins were analyzed using SDS/PAGE
and Western blotting with anti-His₆-tag antibodies (CLONTECH) or
anti-T₇-tag antibodies (Novagen). Immunoreactive bands were visual-
ized with [125I]protein A (ICN Biomedicals, Inc., Costa Mesa, CA) fol-
lowed by autoradiography (22).

**Standard PDK Activity Assay**—Kinase activity was determined by
following [32P]phosphate incorporation from [γ-32P]ATP into the E1α
subunit of PDC, essentially as described previously (14). Phosphoryla-
tion reactions were incubated at 37 °C in a final volume of 100 µl
containing 20 mM Tris·HCl, pH 7.8, 5 mM MgCl₂, 50 mM KCl, 5 mM
dithiothreitol, 1.0 mg/ml PDC, and nucleotide substrate (the specific
activity of [γ-32P]ATP was ~200–500 cpm/pmol). For assaying recom-
binant kinases, the respective proteins were reconstituted with recom-
binant human PDC before the assay. Reconstituted preparations were
kept on ice for 30 min. The final protein concentrations of the recombin-
ant proteins in the assay mixture were as follows: the E1-E2-E3BP
subcomplex at 1.0 mg/ml and the corresponding wild-type kinase at 5
µg/ml. When we tried to apply the above assay for the analysis of kinase
mutants with minute activity, it was difficult to obtain reliable esti-
mates even when reactions were conducted for 30 min. To improve
the sensitivity of the assay, the amount of kinase protein added to the
reaction was increased (see legend to Fig. 1). Under these conditions,
there was a linear incorporation of [32P]phosphate into PDC with time
for at least 30 min of the reaction for all mutants tested. The rates of
phosphorylation reactions were proportional to the amount of added
kinase, indicating that the protein substrate is not limiting under the
conditions used. Protein-bound radioactivity was determined as de-
scribed previously (14). The activity of wild-type kinase was determined
in the standard assay and was calculated based on incorporation of
[32P]phosphate during the first 30 s of the reaction. The activities of
mutant kinases were calculated based on incorporation of [32P]phos-
phate during 30 min of the reaction. The concentrations of nucleo-	ide substrate (ATP) and the concentrations of inhibitors used in particular
experiments are given in the legends to figures. Radicicol was added
from a solution made with dimethyl sulfoxide. The final concentration
of dimethyl sulfoxide in the reaction mixture was 1% (v/v) at all concen-
trations of inhibitor tested. Under these conditions, dimethyl sul-
oxide had no effect on the kinase activity as established in preliminary
experiments. Phosphorylation reactions in this case were initiated by
the addition of wild-type PDC or reconstituted PDC kinase after equili-
bration at 37 °C for 30 s. The volume of PDC added was 1/10 of the total
reaction volume. All assays were conducted in duplicates.

**ATP Binding Assay**—Nucleotide binding studies were conducted us-
ing a modified vacuum filtration assay developed by Pratt and Roche
(23). Briefly, the recombinant kinases were used in a final concentra-
tion of 0.1 mg/ml in binding buffer (20 mM Tris·HCl, pH 7.4, 5 mM
MgCl₂, 50 mM KCl, 5 mM dithiothreitol). Binding reactions (total vol-
ume of 100 µl) were initiated by the addition of [α-32P]ATP (with a
specific radioactivity of 200–500 cpm/pmol). The final concentration of
ATP in the binding buffer was varied from 2.5 to 50 µM. The reactions
were incubated at room temperature for 2 min. Protein-bound radioac-
tivity was determined essentially as described previously (14). All bind-
ing experiments were conducted in triplicate.

**ATPase Activity Assay**—ATPase activity was assayed essentially as
described by Singh and Cerione (24). Briefly, reactions were set up in a
final volume of 100 µl containing 50 mM Tris·HCl, pH 7.5, 10 mM MgCl₂,
1 mM dithiothreitol, 5% (v/v) glycerol, and 10 µM [γ-32P]ATP (specific
radioactivity of 200–400 cpm/pmol). Reactions were initiated by adding
the respective preparation of recombinant kinase to a final protein
concentration of 0.1 mg/ml. Assays were conducted at room tempera-
ture. At the indicated times, reactions were terminated by the addition
of 1 ml of ice-chilled 5% (w/v) Norit A (Sigma) in 50 mM Na₂HPO₄. The

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**Fig. 2.** [α-32P]ATP binding by the wild-type PDK2 and mutant
enzymes. Recombinant PDK2, wild-type (○), His-239 → Ala (●), Glu-
243 → Ala (△), Glu-243 → Gln (□), or Asp-282 → Glu (Δ) mutants were
incubated with the indicated concentrations of [α-32P]ATP (specific
radioactivity ~200 cpm/pmol) for 2 min. Free and protein-bound nucle-	idease activity was determined using a vacuum-filtration assay as described
under "Experimental Procedures." The specific binding was determined
after subtraction of nonspecific binding from the total binding. The
nonspecific binding was determined in the presence of 1,000-fold excess
of cold ATP.

**Fig. 3.** Binding of wild-type PDK2 and respective mutant ki-
nases to recombinant PDC. Panel A, SDS/PAGE analysis of wild-
type PDK2, recombinant PDC, and PDC-kinase complex centrifuged
through Sephacel S300 columns. Panel B, Western blot analysis of
corresponding preparations with monoclonal antibodies against His₆-
tag. Immunoreactive bands were visualized with [125I]protein A staining
followed by autoradiography. Panel C, concentration-dependence
curves for binding of wild-type PDK2 (○), His-239 → Ala (□), Glu-
239 → Asp (●), Glu-243 → Gln (△), and Asp-282 → Glu (Δ) mutants to
recombinant PDC. Binding curves were constructed based on the results
of scanning densitometry of the respective Western blots stained with
[125I]protein A. Data was analyzed using UN-SCAN-IT gel™ soft-
ware (Silk Scientific, Inc., Orem, UT). Results are expressed as percent
of wild-type PDK2 bound to PDC.
mixture was centrifuged, and 100 µl of supernatant was mixed with 5 ml of scintillation fluid. The release of [32P]phosphate as the outcome was measured in a scintillation counter. Radicicol (final concentration 200 µM) was added from the solution made with dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the reaction mixture was less than 1% (v/v). Reactions made without radicicol received an appropriate amount of dimethyl sulfoxide for control. When E1 component was used as phosphate acceptor, its final concentration was 1.0 mg/ml.

PDC Binding Assay—PDC binding reactions were set up in 100-µl volumes containing 20 mM Tris-HCl, pH 7.8, 5 mM MgCl2, 50 mM KCl, 1 mM dithiothreitol, 1.0 mg/ml recombinant PDC, and various amounts of PDK2. Kinase was allowed to bind to the complex for 15 min at room temperature. By the end of the incubation, reactions were loaded on the columns containing 1 ml of Sephalac S300 (Amersham Pharmacia Bio-tech) equilibrated with 20 mM Tris-HCl, pH 7.8, 5 mM MgCl2, 50 mM KCl, 1 mM dithiothreitol, and 0.1% (w/v) Tween 20. Before the experiment, columns were centrifuged at 2,000 rpm for 1 min in a IEC Centra CL2 centrifuge (International Equipment Co., Needham Heights, MA) equipped with a bucket rotor. Immediately after loading, columns were centrifuged again at 2,000 rpm for 1 min. The flow-through containing the PDC-kinase complex was collected in Eppendorf tubes. The resulting preparations of PDC-bound kinase were analyzed further by Western blotting with anti-PDK2 antibodies essentially as described (22).

Analysis of Kinetic and Binding Data—Raw kinetic and binding data were analyzed using GraFit version 3 software (Erithacus Software Ltd, Middlesex, UK). The apparent inhibition constants were determined by measuring the initial rates of the phosphorylation reaction at various concentrations of nucleotide substrate and inhibitor (matrix of 24 different conditions). The resulting matrices were analyzed as a set to determine the respective kinetic parameters. Shown are representative results obtained with one out of three preparations of each enzyme analyzed for this study. Other Procedures—SDS/PAGE was carried out according to Laemmli (25). Protein concentrations were determined according to Lowry et al. (26) with bovine serum albumin as standard.

RESULTS AND DISCUSSION

Kinase Activity of Wild-type and Mutant Proteins—In this study, we used site-directed mutagenesis to test a hypothesis that the invariant glutamic acid residue of mammalian PDK (Glu-243 in rat PDK2) serves as a catalyst in the phosphotransfer reaction. We also analyzed the functional significance of two additional residues: His-239, which may be involved in polarization of Glu-243, and Asp-282, which contributes to the binding of ATP in the active site (14). The latter residue has been chosen as a control because a substituent produces a kinase with impaired catalytic ability. However, this effect, as previously established (14), is achieved by different means, through the knockout of nucleotide binding. The invariant Glu-243 of rat PDK2 was altered to Ala, Asp, and Gln and His-239 was altered to Ala. The invariant Asp-282 was changed to Glu. Wild-type PDK2 and respective mutant kinases were expressed in E. coli cells and purified to near homogeneity as described under “Experimental Procedures.” When the activi-
tioning of ATP within the active site.

**PDC Binding**—It is generally believed that the kinase component is an integral part of PDC, and every complex contains 2–3 tightly bound molecules of kinase (27). Association with the complex alone accounts for more than a 10-fold increase in the kinase activity (28). Therefore, an essential decrease in activity would be expected if the mutations affected the interaction between kinase and PDC directly or indirectly. To explore this possibility, we measured the PDC-kinase interaction using gel filtration through Sephacel S-300 columns (see “Experimental Procedures”). This procedure allows for fast separation of free and complex-bound kinase, thus decreasing the possibility of kinase dissociation during the procedure. As shown in Fig. 3A and B, under these conditions binding of wild-type PDK2 to the complex was readily detectable. To evaluate the ability of PDK2 active-site mutants to interact with the protein substrate, we conducted a series of experiments to determine their relative affinities for PDC (Fig. 3C). It appeared that all mutant kinases tested bind PDC at levels similar to the wild-type kinase. This suggests that the ability to catalyze the phosphotransfer reaction or the ability to bind the nucleotide substrate has no profound effect on the protein-protein interactions involved in kinase binding. On the other hand, we have noticed that all mutant proteins had a somewhat higher affinity for the protein substrate. The latter was especially apparent for the Asp-282 to Glu mutant, which consistently showed 2–3-fold higher affinity for PDC. The rationale for this phenomenon is currently unknown.

**Inter-subunit Interaction**—In solution, PDK exists as a dimer (3). Dimerization was suggested to be very important for kinase function, allowing the kinase to move around PDC, phosphorylating multiple copies of the E1 component without dissociating from the complex (30). Thus, if the mutations somehow compromise the inter-subunit interactions within the dimer, this might have a deleterious effect on kinase activity. To explore this possibility, we employed a genetic approach, co-expressing His6-tagged and T7-tagged kinase molecules in E. coli. This allowed the use of the His6-tagged kinase as a bait to pull down T7-tagged kinase on an affinity resin. The resulting preparations can be characterized for the presence of T7-tagged species by Western blot analysis with anti-T7-tag antibodies. This way, it is possible to establish the formation of mixed species and draw conclusions about the strength of inter-subunit interaction for different mutant kinases. When the respective constructs were expressed in E. coli, His6-tagged species were purified on TALON™ resin and probed with anti-T7-tag antibodies using Western blot analysis; it was found that all preparations tested contained T7-tagged species, which cannot directly bind TALON™ resin (Fig. 4, panels A and B). The latter strongly suggests that the wild-type PDK2 as well as kinases carrying amino acid substitutions within the kinase domain exist as dimers. Furthermore, the ratios between T7-tagged and His6-tagged species in each preparation were very similar, indicating that the ability to catalyze phosphotransfer reaction or the ability to bind adenyl nucleotides is not required for dimer formation.

**ATPase Activity in Preparations of PDK**—Many phosphokinases (31), including some protein kinases (32, 33), have been shown to hydrolyze ATP in the absence of substrate (34). The ATPase activity of PDK is also enhanced by the presence of E1 component (35). In the absence of E1, the ATPase activity of PDK is minimal (36). The ATPase activity of PDK is also inhibited by radicicol, a specific inhibitor of PDK (37). The inhibition of ATPase activity by radicicol is concentration-dependent, with a half-maximal inhibitory concentration (IC50) of approximately 5 μM (37). The inhibition of ATPase activity by radicicol is not reversed by the addition of E1 component (37). Therefore, the ATPase activity of PDK is controlled by the presence of substrate and inhibitor, and the inhibition of ATPase activity by radicicol is not reversed by the addition of E1 component.
reported to possess an intrinsic hydrolytic activity toward ATP. If PDK uses general base catalysis to activate the hydroxyl group of the serine residue for the direct attack on the ATP γ phosphate, it also must be able to utilize the hydroxyl of water as a phosphoryl acceptor instead of an amino acid hydroxyl group, although at a slower rate. To investigate this possibility, we determined ATPase activity in various preparations of PDK. When wild-type enzyme was incubated in a reaction mixture containing the standard components of the ATPase assay, and aliquots were removed and assayed for the release of $[^{32}P]P_i$, there was a linear formation of $P_i$ with time. Furthermore, when $Mg^{2+}$ was omitted from the standard ATPase assay, no activity was observed, suggesting that, indeed, preparations of PDK contain some ATP hydrolytic activity (data not shown). However, these results do not exclude the possibility that the source of the observed ATPase activity is a contaminating ATP hydrolase.

In several studies, the specificity of ATP hydrolytic reaction was demonstrated using compounds acting as potent inhibitors of ATP binding (34, 35). Unfortunately, to date, there are no PDK inhibitors available that are specific for the nucleotide binding domain. However, we reasoned that some of ATP-binding site inhibitors designed for enzymes with nucleotide binding domains arranged similarly to the nucleotide binding domain of histidine kinases and, therefore, to the nucleotide binding domain of PDK might inhibit PDK activity (18, 36). Indeed, one of these compounds, radicicol (monorden) (36), potently inhibited phosphorylating activity of PDK when tested in standard kinase assay (Fig. 5, panel A). Inhibition was competitive with respect to ATP, with the apparent $K_i$ value of 23.3 ± 1.8 μM, making radicicol an invaluable tool as an inhibitor of the nucleotide binding domain of PDK.

The addition of radicicol to the standard ATPase assay inhibited ~25–30% of ATP hydrolytic activity in preparations of wild-type PDK. The specific activity of radicicol-sensitive ATPase was ~1.0 nmol of $P_i$ released/min/mg (Fig. 5, panel B), suggesting that PDK2 possesses intrinsic ATPase activity that comprises ~2.5% of kinase activity. Having analyzed the ATPase activity in preparations of wild-type PDK2, experiments were performed to establish whether the mutations within the active site of kinase affect the ATP hydrolytic activity. When kinases carrying the substitutions of Glu-243 and His-239 were assayed similarly to the wild-type PDK2, there was little if any radicicol-sensitive ATP hydrolysis that could be assigned to PDK2, indicating that these residues are essential for ATPase activity. To further explore the possible mechanistic significance of Glu-243 and His-239 in an ATP hydrolytic reaction, we prepared several highly purified preparations of PDK2 carrying substitutions of Glu-243 to Gln and His-239 to Ala. These preparations had a drastically reduced radicicol-insensitive ATPase activity. Subsequent analysis of these preparations revealed that His-239 to Ala enzyme had ~7% of the activity of the wild-type PDK2, whereas the activity of Glu-243 to Gln enzyme was very close to the background values, less than 0.5% of the wild type (Fig. 5, panel B). These results strongly suggest that the same residues of PDK2 are involved in catalysis of both hydrolytic and phosphoryltransferase reactions.

To further analyze the relationship between kinase and ATPase activities, we characterized the effect of the substrate of kinase reaction (E1 component of PDK) on the rate of ATPase reaction. As shown in Fig. 5, panel C, inhibition of the total ATPase activity caused by the addition of E1 component was comparable with that caused by the addition of radicicol. This further confirms that PDK2 possesses an intrinsic ATPase activity and also shows that the kinase preferentially catalyzes the phosphorylation reaction under conditions when the native phosphoacceptor is provided.

Thus, it appears that both Glu-243 and His-239 are crucial for kinase activity. In conjunction with the results showing that Glu-243 and His-239 do not contribute to ATP binding, intersubunit interaction, and binding to PDC, these data strongly suggest that Glu-243 and His-239 are required for catalysis of the phosphotransfer reaction. In accord with this idea is the observation that PDK possesses a weak ATP hydrolytic activity, which can be detected only in the absence of physiological substrate, the E1 component of PDC. Furthermore, the ATPase activity appears to depend on the presence of both Glu-243 and His-239. Substitution of either Glu-243 or His-239 resulted in a decrease in ATPase activity that closely corresponded to the decrease in kinase activity.

In conclusion, it is interesting to note that bacteria contain another protein of the same lineage, the so-called anti-sigma factor SpoIIB (29). Its sequence is very similar to the bacterial histidine kinases but, like PDK, contains a properly spaced glutamic acid in the N box. Accordingly, SpoIIB phosphorylates its substrate SpoIIB on Ser-58 (29). This suggests that the ability of this type of catalytic domain to phosphorylate exogenous protein substrates on serine residues might be largely defined by the presence of glutamic acid in the N box.

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