Pyruvate dehydrogenase kinase from *Arabidopsis thaliana*: a protein histidine kinase that phosphorylates serine residues

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Pyruvate dehydrogenase kinase (PDK) is the primary regulator of flux through the mitochondrial pyruvate dehydrogenase complex (PDC). Although PDKs inactivate mitochondrial PDC by phosphorylating specific Ser residues, the primary amino acid sequence indicates that they are more closely related to prokaryotic His kinases than to eukaryotic Ser/Thr kinases. Unlike Ser/Thr kinases, His kinases use a conserved His residue for phosphotransfer to Asp residues. To understand these unique kinases better, a presumptive PDK from *Arabidopsis thaliana* was heterologously expressed and purified for this investigation. Purified, recombinant *A. thaliana* PDK could inactivate kinase-depleted maize mitochondrial PDC by phosphorylating Ser residues. Additionally, *A. thaliana* PDK was capable of autophosphorylating Ser residues near its N-terminus, although this reaction is not part of the phosphotransfer pathway. To elucidate the mechanism involved, we performed site-directed mutagenesis of the canonical His residue likely to be involved in phosphotransfer. When His-121 was mutated to Ala or Gln, Ser-autophosphorylation was decreased by 50% and transphosphorylation of PDC was decreased concomitantly. We postulate that either (1) His-121 is not the sole phosphotransfer His residue or (2) mutagenesis of His-121 exposes an additional otherwise cryptic phosphotransfer His residue. Thus His-121 is one residue involved in kinase function.

Key words: autophosphorylation, mechanism, mitochondria, mutagenesis, transphosphorylation.

INTRODUCTION

The pyruvate dehydrogenase complex (PDC) is a multienzyme structure that catalyses the oxidative decarboxylation of pyruvate, yielding CO_2, acetyl-CoA and NADH [1]. This complex is localized to the cytoplasm of bacteria and the mitochondrial matrix of eukaryotic cells. Additionally, plant cells contain a second isoform localized to the plastidial stroma [2–4]. The plastidial and mitochondrial PDCs have distinct regulatory and catalytic properties complementing their unique roles in metabolism [4]. The most striking difference is regulation by reversible phosphorylation. The mitochondrial PDC, unlike the plastid counterpart, has an associated pyruvate dehydrogenase kinase (PDK, EC 2.7.1.99) and phosphopyruvate dehydrogenase phosphatase [5]. These two enzymes catalyse the reversible phosphorylation of mitochondrial pyruvate dehydrogenase. PDK curtails mitochondrial PDC activity by catalysing the transfer of the γ-phosphate from ATP to the α subunit of the pyruvate dehydrogenase (E1) component enzyme [4,5]. Conversely, phospho-pyruvate dehydrogenase phosphatase reactivates this component by catalysing dephosphorylation in a Mg^{2+}-dependent manner [6]. The phosphorylation state, and hence the activity status, of PDC is determined by the net sum of these opposing enzyme activities.

Molecular cloning of the first PDK from mammals revealed a deduced amino acid sequence lacking typical eukaryotic Ser/Thr kinase domains but having the five domains characteristic of prokaryotic two-component histidine kinases [7]. Prokaryotic histidine kinases are multidomain polypeptides that catalyse the first step of two-component signal transduction pathways (reviewed in [8]). The sensor domain responds to a cellular signal by autophosphorylating a His residue within the transmitter domain, followed by phosphotransfer to an Asp residue within the receiver domain of a cognate response regulator protein. The activated response regulator typically functions as a specific transcription factor. Although bacterial two-component systems usually follow this reaction scheme, variations exist. Some histidine kinases contain both the histidine phosphotransfer donor domain and the aspartate receiver domain on a single polypeptide, i.e. a hybrid histidine kinase [9]. Multiple donor and receiver domains can reside on a single polypeptide with His-to-Asp phospho-relay perhaps occurring in *cis* on these kinases [10]. Regardless of mechanistic specificities, all histidine kinases characterized until now transfer phosphate from an autophosphorylated His to an Asp residue, transducing cellular signals [11,12].

On the basis of similarities to these histidine kinases [7], PDKs might also use a His for phosphotransfer. However, unlike histidine kinases, mammalian PDKs phosphorylate on Ser rather than Asp residues [13,14]. This novel class of eukaryotic protein kinases also includes the branched-chain α-keto acid dehydrogenase kinase (BCDK, EC 2.7.1.115), which regulates a related mitochondrial α-keto acid dehydrogenase complex involved in branched-chain amino acid catabolism [15]. Although not yet well understood, rat PDK and BCDK both undergo autophosphorylation [16,17]. The BCDK autophosphorylation site was mapped to a Ser residue near the N-terminus [17].

We recently reported the molecular cloning and functional expression of two PDK isoforms from *Zea mays* [18]. The deduced amino acid sequences for the plant enzymes are approx.

Abbreviations used: AtPDK, *Arabidopsis thaliana* pyruvate dehydrogenase kinase; BCDK, branched-chain α-keto acid dehydrogenase kinase; E1, pyruvate dehydrogenase; E2, dihydrolipoamide acetyltransferase; MBP, maltose-binding protein; PDC, pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase.

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30% identical with those from mammals. Amino acid identity is concentrated within 11 discrete domains, of which 5 are related to the prokaryotic histidine kinases [18]. A presumptive PDK recently identified from *Arabidopsis thaliana* is approx. 70% identical with the maize enzymes and contains the five histidine kinase and six signature PDK domains [19]. At least one of these domains was proposed to be a site for phosphotransfer owing to the presence of an invariant His residue. Here we present evidence that this *A. thaliana* cDNA encodes a genuine PDK that involves one of the conserved His residues for maximal activity.

MATERIALS AND METHODS

Bacterial expression and purification of *A. thaliana* PDK (AtPDK)

Primers DDR 229 (5′-ctcgagctgcagctattaTCAGGGTAAAGG-CTCTTGCGA-3′) and DDR 230 (5′-cccgggtctagaATGGCAG-TGAAGAAAGCCTGC-3′) were used to amplify the open reading frame of the AtPDK corresponding to the region between 130 and 1227 bp [19]. Restriction sites were engineered into each primer at the 5′ end (lower-case letters) to facilitate subcloning. The *Pst*I (DDR 229) and *Xba*I (DDR 230) sites were used to subclone the PCR product into the pMAL-cRI expression vector (New England Biolabs, Beverly, MA, U.S.A.). To ensure translation termination, two nonsense codons were introduced into DDR 229. Thermal cycling reactions (50 μl total volume) contained 10 mM Tris/HCl, pH 7.9, 0.5 mM $MgCl_2$, 200 μM dNTPs, 5 units of *Taq* polymerase (Promega, Madison, WI, U.S.A.), 2 ng of plasmid DNA template and 20 pmol of each primer. Cycling conditions were 94 °C for 5 min, initial denaturation, followed by 30 cycles of 30 s at 94 °C, 30 s at 50 °C, 2 min at 72 °C with 6 s extensions for the last step of each cycle.

The expression and purification of maltose-binding protein (MBP)–PDK polypeptides and the preparation of kinase-depleted PDC from maize mitochondria were performed as described elsewhere [18,20]. After purification, MBP–AtPDK was dialysed against 2 litres of 20 mM Tes/KOH (pH 7.3)/2 mM dithiothreitol for 16 h at 4 °C and was subsequently concentrated on an ultrafiltration membrane (10 kDa cut-off; Amicon, Beverly, MA, U.S.A.). Cleavage of fusion protein was performed with factor Xa protease (New England BioLabs, Beverly, MA, U.S.A.) at a 1:25 molar ratio of protease to fusion protein in 20 mM Tris/HCl (pH 8.0)/0.1 M NaCl/2 mM $CaCl_2$ for 18 h at 25 °C. Size-exclusion chromatography and native molecular mass determination were performed with a Superose 12 column (Pharmacia, Uppsala, Sweden) with thyroglobulin, ferritin, catalase, aldolase and BSA as standards. SDS/PAGE and immunoblotting were performed with standard procedures [21]. Polyclonal antibodies to PDK were characterized previously [18]. Polyclonal antibodies against MBP were affinity-purified from sera raised against a branched-chain α-keto acid dehydrogenase subunit fusion with MBP.

Mitochondria isolation and assays

Mitochondria were isolated from etiolated maize (B73; Illinois Seed Foundation, Champaign, IL, U.S.A.) shoots as described previously [21,22]. For PDC, a four-step purification procedure starting with isolated maize mitochondria yielded an 80–90% pure preparation of PDC that was depleted of PDK activity [18,21]. This preparation was termed 'kinase-depleted PDC' and was used to assay recombinant MBP–AtPDK activity. PDC activity was monitored by the reduction of NAD^+ (ΔA_{340}) as described previously [21]. For kinase assays, MBP or MBP–AtPDK were incubated with kinase-depleted maize mitochondrial PDC for 5 min at 25 °C before the addition of $MgATP^{2-}$ to a final concentration of 0.2 mM. Phosphorylation assays proceeded for 5–60 min before PDC activity was assayed. All reported values are the means of at least three independent reactions. Protein quantification was performed by the method of Bradford [23], with BSA as standard.

Phosphorylation assays were performed by adding a known amount of Mg[γ-^{32}P]ATP^{2-} (10 mCi/mmol) to kinase and/or PDC preparations. Reactions proceeded for 1 h at 25 °C and were stopped by the addition of an equal volume of 2×SDS/PAGE sample buffer [8 M urea/4% (w/v) SDS/4% (v/v) 2-mercaptoethanol] followed by heating at 70 °C for 15 min. Denatured proteins were resolved by SDS/PAGE, electroblotted to nitrocellulose, dried and wrapped in Cellophane before exposure to X-ray film for 5–30 min. The incorporation of radiolabel into MBP–AtPDK was quantified by liquid-scintillation spectrometry of the excised MBP–AtPDK band. Relative phosphorylation efficiency was quantified with QS30 quantification software (PDI, Huntington Station, NY, U.S.A.), which accounted for band area and intensity.

Limited proteolysis of MBP–AtPDK was performed with trypsin (Sigma, St Louis, MO, U.S.A.) at a molar ratio of 3:1 fusion protein to trypsin. Protease digestion was performed in 20 mM Tes (pH 7.5)/1 mM dithiothreitol for 16 h at 25 °C and was terminated by the addition of an equal volume of 2×SDS/PAGE sample buffer followed by heating at 70 °C for 15 min.

Site-directed mutagenesis

Mutagenesis was performed with the GeneEditor site-directed mutagenesis kit (Promega). The pMAL-cRI expression vector containing AtPDK was used as template for mutagenesis performed in accordance with the manufacturer's instructions. The oligonucleotides used for mutagenesis, synthesized at the University of Missouri DNA core facility (Columbia, MO, U.S.A.), were DDR 238 5′-GGGAACCACATTGTTA**GC**CCTTACTT-TGACAGCC-3′ (His-121A oligo) and DDR 270 5′-GGGAA-CCACATTGTT**T**TGCCTTACTTTGACA-3′ (His-121Q oligo). The mutated base(s) are underlined and in bold. The entire reading frames of both mutant constructs were sequenced to verify that no additional changes had been introduced.

Phosphoamino acid analyses

Protein preparations were phosphorylated with [γ-^{32}P]ATP, resolved by SDS/PAGE and transferred to PVDF membranes. Immobilized proteins were excised and phosphoamino acids released by hydrolysis in 6 M HCl for 1 h at 120 °C. The supernatants containing the phosphoamino acids were freeze-dried, then resuspended in 15 μl of pH 1.9 buffer [2.2% (v/v) formic acid/7.8% (v/v) acetic acid] containing phospho-Ser, phospho-Thr and phospho-Tyr standards (1 μg of each). Samples of 1.5 μl (approx. 1000 c.p.m.) were spotted on to cellulose chromatography plates (J. T. Baker, Phillipsburg, NJ, U.S.A.) in intervals. Plates were placed in a model HTLE-7000 Hunter thin-layer electrophoresis apparatus in accordance with the manufacturer's instructions (CBS Scientific Company, Del Mar, CA, U.S.A.). Electrophoresis in the first dimension with pH 1.9 buffer was performed at 1.5 kV for 20 min. After the first dimension the plates were dried and rotated through 90 ° for the second dimension. Electrophoresis in the second dimension, with pH 3.5 buffer [5% (v/v) acetic acid/0.5% (v/v) pyridine], was performed at 1.3 kV for 16 min. Plates were then dried and phosphoamino acid standards were detected by being stained with 0.25% (w/v) ninhydrin in acetone, then being baked at

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65 °C for 15 min. Radiolabelled amino acids were detected by autoradiography.

RESULTS

Characterization of AtPDK

When expressed as a fusion with the *Escherichia coli* MBP, AtPDK migrated as an 87 ± 3 kDa ($n = 3$) polypeptide during SDS/PAGE (Figure 1A). This protein was absent from untransformed bacteria, determined by immunoblotting with anti-PDK antibodies (Figure 1C). Approximately 10–15 % of MBP–AtPDK was soluble under native conditions and this protein was purified to near-homogeneity by amylose affinity chromatography. Protein yields ranged from 0.1 to 0.2 mg/l of culture broth. Removal of the MBP fusion partner by cleavage with factor Xa protease was 40 % efficient after digestion for 18 h. Digestion efficiency was improved to nearly 100 % when equimolar amounts of protease and fusion protein were incubated (Figure 2C). To estimate the native size it was necessary to remove MBP because it is capable of forming dimers [24]. The factor Xa-cleaved PDK migrated at 44 ± 2 kDa ($n = 3$) during SDS/PAGE, with a native molecular mass, as determined by size-exclusion chromatography, of approx. 86 kDa, suggesting a homodimeric native conformation. The His-121A and His-121Q mutants were identical in size with the wild-type protein (Figure 1B).

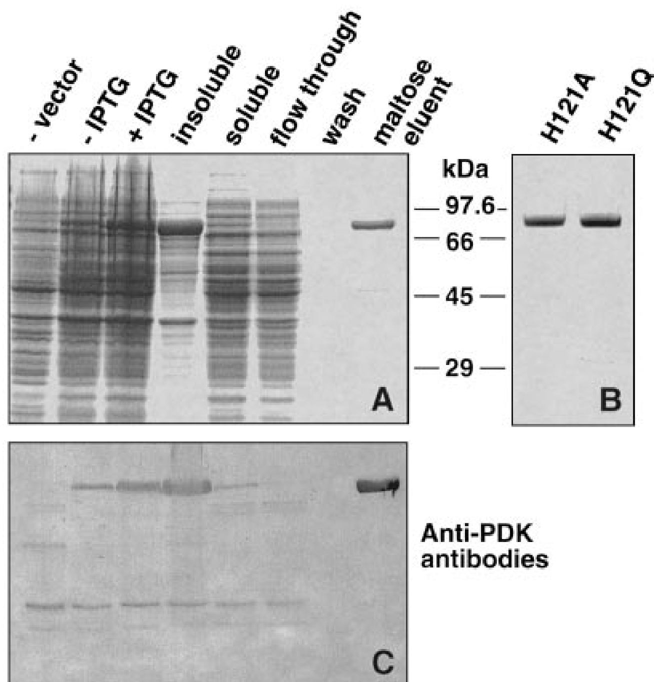

Figure 1 Expression and purification of MBP–AtPDK chimaera

(**A**) Coomassie Blue-stained SDS/PAGE of a typical expression and purification profile. Total protein fractions are shown from untransformed cells (−vector), transformed cells without (−IPTG) or with (+IPTG) the addition of the inducer isopropyl β-D-thiogalactoside at 0.5 mM. After cell disruption the 'insoluble' fraction (lane 4) was removed by centrifugation (9000 ***g***) and the supernatant was termed 'soluble' protein (lane 5). The MBP fusion bound to an amylose-affinity matrix and the unbound fraction was termed 'flow through' (lane 6). The matrix was washed with 50 column vol. of wash buffer (lane 7), after which the bound protein was eluted with maltose-containing buffer (lane 8). (**B**) The His-121A and His-121Q mutants were purified by using the same procedure; they migrated at the same position as wild-type PDK. (**C**) Immunoblot of protein from (**A**) probed with anti-PDK polyclonal antibodies. The positions of molecular mass markers are indicated for (**A**) and (**B**).

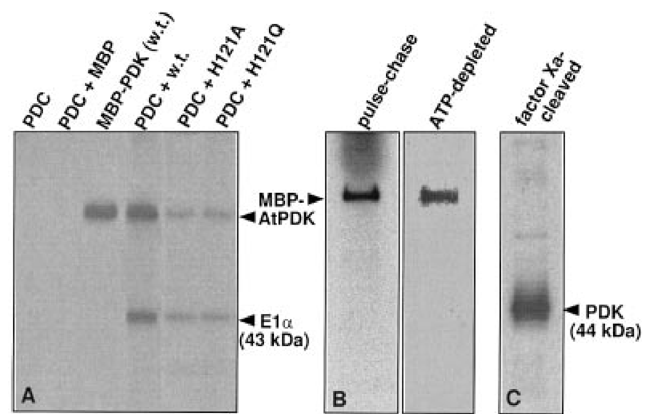

Figure 2 Autophosphorylation of MBP–AtPDK and transphosphorylation of PDC E1α

(**A**) Phosphorylation of kinase-depleted maize mitochondrial PDC by MBP–AtPDK. Kinase-depleted maize PDC (3 μg) was incubated with 2 μg of MBP or MBP–AtPDK for 30 min at 25 °C in the presence of Mg[γ-^{32}P]ATP^{2-} (5 μCi,10 mCi/mmol). Reactions were stopped by the addition of SDS/PAGE sample buffer followed by boiling. Controls lacking MBP–AtPDK (lane 1), containing maize PDC plus MBP (lane 2) and lacking maize PDC (lane 3) are also shown. Abbreviation: w.t., wild-type. (**B**) Lane 1, ^{32}P-phosphorylated MBP–AtPDK was chased with a 10-fold excess of MgATP^{2-} for 5 min before the addition of kinase-depleted maize PDC [in the same ratio as in (**A**)]; the reaction proceeded for 1 h at 25 °C before termination with SDS/PAGE sample buffer. Lane 2, ^{32}P-phosphorylated MBP–AtPDK was desalted by G-50 Sephadex chromatography and subsequently incubated with kinase-depleted maize PDC for 1 h at 25 °C. (**C**) Phosphorylated MBP–AtPDK (0.2 μg) was digested with a molar equivalent of factor Xa protease (New England Biolabs) for 18 h at 25 °C before resolution by SDS/PAGE. The position of the radiolabelled polypeptide coincided with that of AtPDK, which was detected by immunoanalysis.

AtPDK inactivates kinase-depleted maize mitochondrial PDC and phosphorylates maize pyruvate dehydrogenase on Ser residues

When incubated in the presence of MgATP^{2-}, purified MBP–AtPDK inactivated kinase-depleted maize mitochondrial PDC, whereas purified MBP had no effect on PDC activity (Table 1). Although the kinetics of inactivation were slower than observed in previous investigations of plant mitochondrial PDCs [5,6,21], recombinant AtPDK was capable of inactivating 90 % of kinase-depleted maize mitochondrial PDC. The specific activity of MBP–AtPDK was lower (10 PDC units inactivated/min per mg of PDK) than that of maize MBP–PDK2 (40 PDC units inactivated/min per mg of PDK [18]) with maize mitochondrial PDC as substrate. Inactivation of kinase-depleted PDC was coincident with the incorporation of ^{32}P from γ-labelled ATP into a 43 kDa polypeptide (Figure 2A). The radiolabelled 43 kDa polypeptide was not observed in purified MBP or MBP–PDK preparations incubated with [γ-^{32}P]ATP (Figure 2A). Addition of purified MBP to kinase-depleted PDC resulted in no phosphorylation of the 43 kDa protein (Figure 2A), demonstrating that these PDC preparations were kinase-depleted and that recombinant AtPDK was responsible for phosphorylation. The size of the radiolabelled polypeptide is identical with that of maize mitochondrial E1α [21,25]. These results clearly demonstrate that recombinant AtPDK phosphorylates the 43 kDa maize E1α subunit, concomitantly inactivating PDC. Phospho-E1α amino acid(s) were resistant to treatment with acid, indicative

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Table 1 Effect of MBP–AtPDK on kinase-depleted maize PDC activity in the presence of $MgATP^{2-}$

Approximately 40 μg of kinase-depleted maize PDC was incubated with 0.2 mM $MgATP^{2-}$ plus recombinant protein at 25 °C for 5 min, then immediately assayed for activity. All rates were normalized to the rate (0.72 μmol of NADH/min per mg) in the absence of $MgATP^{2-}$ and recombinant protein activity. The final rates were obtained by incubating the sample with $MgATP^{2-}$ for 1 h before assay. All rates are means ± S.D. for three measurements.

Control contents	Relative activity (%)
$-MgATP^{2-}$, −PDK	100 ± 5
$+MgATP^{2-}$, +MBP (15 μg)	95 ± 8
With $MgATP^{2-}$; PDK added (μg)	**Relative activity (%)**
1.5	98 ± 5
3.7	84 ± 11
7.5	65 ± 4
15.0	45 ± 4
Incubation for 60 min	**Relative activity (%)**
PDK (3.7 μg)	10 ± 5
MBP (5.0 μg)	100 ± 3

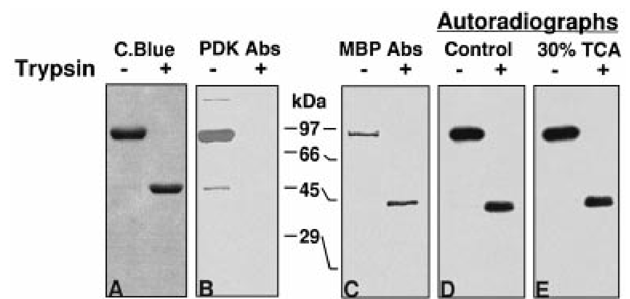

Figure 4 Autophosphorylation and tryptic digestion of MBP–AtPDK

(**A**) Coomassie Blue-stained SDS/PAGE of intact MBP–AtPDK (3 μg) and MBP–AtPDK digested with trypsin (0.5 μg) for 16 h at 25 °C. (**B**) Immunoblot of (**A**) probed with anti-PDK antibodies (PDK Abs). (**C–E**) MBP–AtPDK (0.5 μg) was incubated with [γ-^{32}P]ATP (1 μCi, 10 mCi/mmol) before tryptic digestion (lane 2). (**C**) Immunoblot was probed with anti-MBP antibodies (MBP Abs). (**D**, **E**) Autoradiographs of replicate-blotted ^{32}P-autophosphorylated MBP–AtPDK. The blot in (**E**) was washed with 30% (w/v) trichloroacetic acid (TCA) for 1 h at 25 °C to cleave acid-labile phosphoamino acid bonds. Both autoradiographs were exposed for 4 h. Although radiolabel migrated with the undigested N-terminal half of the fusion protein, which was predominantly composed of MBP, AtPDK was incapable of phosphorylating purified MBP. Furthermore, cleavage of the radiolabelled fusion protein at the fusion junction (with factor Xa) indicated that it was AtPDK that was phosphorylated. On the basis of these results, the phosphorylation site resides near the undigested N-terminus of AtPDK. The positions of molecular mass markers are indicated between (**B**) and (**C**).

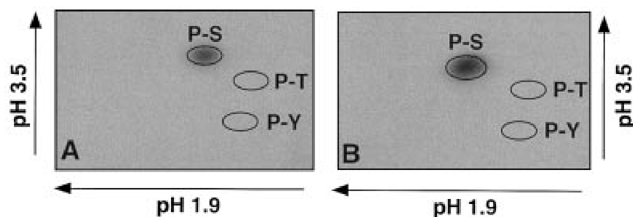

Figure 3 Phosphoamino acid analyses of radiolabelled maize mitochondrial E1α (A) and MBP–AtPDK (B)

(**A**) Kinase-depleted maize PDC was phosphorylated with MBP–AtPDK, resolved by SDS/PAGE and transferred to PVDF membrane. Radiolabelled E1α band was excised and subjected to acid hydrolysis followed by two-dimensional thin-layer electrophoresis. Phosphoamino acid standards were revealed with ninhydrin and ^{32}P-labelled amino acids were detected by autoradiography (16 h exposure). (**B**) Autoradiograph of autophosphorylated MBP–AtPDK subjected to phosphoamino acid analysis as described for (**A**). Arrows indicate the directions of electrophoretic migration; pH buffers are indicated on the axes.

of phosphoester bonds. Acid hydrolysis of the radiolabelled 43 kDa maize E1α, followed by two-dimensional thin-layer electrophoresis, indicated phosphorylation took place exclusively on Ser residues (Figure 3A).

AtPDK is autophosphorylated on Ser residues

When incubated with [γ-^{32}P]ATP, MBP–AtPDK was autophosphorylated in both the presence and the absence of PDC with similar efficiencies (Figure 2A). Autophosphorylation exhibited Michaelis–Menten kinetics, reaching saturation near 40 min. The autophosphorylation reaction was pH-dependent, with an optimum at 7.3, and was abolished when a heat-denatured sample was used. These results indicated that autophosphorylation was enzyme-catalysed and could not be attributed to non-specific binding of ATP. The stoichiometry of AtPDK autophosphorylation was approx. 0.10 mol of phosphate/mol of protein. AtPDK phosphorylation was completely stable to cleavage with 30% (w/v) trichloroacetic acid indicating that a hydroxyamino acid such as Ser, Thr or Tyr was phosphorylated (Figures 4D and 4E). Phosphoamino acid analyses indicated only Ser residues were phosphorylated (Figure 3B). Autophosphorylated AtPDK was incapable of transferring covalently bound phosphate to PDC when label was chased or when incubated with PDC after the chromatographic removal of endogenous ATP by Sephadex G-50 (Figure 2B).

To help to identify the site of autophosphorylation, limited proteolysis of phosphorylated MBP–AtPDK under non-denaturing conditions was employed. Digestion of MBP–AtPDK with trypsin yielded one predominant polypeptide of approx. 45 kDa (Figure 4A). Through immunoblot analyses, with anti-MBP and anti-PDK antibodies, it was determined that the 45 kDa polypeptide corresponded primarily to the MBP half of the fusion protein (Figures 4A–4C). This finding enabled us to map the autophosphorylation site coarsely to the N-terminal half of the fusion protein (Figure 4D). Because the 45 kDa N-terminal half was primarily MBP, it was necessary to determine whether AtPDK was capable of transphosphorylating purified MBP. When purified MBP was incubated with MBP–AtPDK and $MgATP^{2-}$ at a 5:1 molar ratio, no transphosphorylation of MBP was observed, though autophosphorylation of MBP–AtPDK occurred during this period (results not shown). It was therefore concluded that the autophosphorylation site was localized to the undigested N-terminus of AtPDK. This was supported by the observation that the radiolabel from factor-Xa-cleaved MBP–AtPDK co-migrated with the AtPDK polypeptide (Figure 2C).

Mechanism of phosphorylation

Because PDKs contain domains diagnostic of protein histidine kinases yet phosphorylate Ser residues in the target sequence, the mode of phosphotransfer warranted further investigation. We hypothesized that if phosphotransfer occurred through a phosphohistidine intermediate, mutating that residue should

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A. thaliana	VKVR**H**[121]NNVVP.....IRMLIGQ**H**[168]VELHNP
Z. mays I	VKVR**H**[117]NNVVP.....IRMLIGQ**H**[169]VALHDP
Z. mays II	IRVR**H**[117]TNVVP.....IRMLIGQ**H**[169]VALHDP
D. melanogaster	IRNR**H**[123]NDVVQ.....IRMLINQ**H**[174]TLLFGG
A. suum	ILKR**H**[120]SRVVE.....IRMLQNQ**H**[169]LVVFGV
C. elegans	VLKR**H**[120]AHVVE.....IRMLQNQ**H**[169]LVVFGN
R. rattus	IRNR**H**[149]NDVIP.....IRMLLNQ**H**[198]SILFGG
H. sapiens	IRNR**H**[149]NDVIP.....IRMLLNQ**H**[198]SILFGG
R. rattus BCDK	LLDD**H**[162]KDVVT.....IRMLATH**H**[207]LALHED
Consensus	+++R**H** + VV .....IRML++Q**H** ++LHG

Figure 5 Comparison of the amino acids surrounding the two invariant His residues in PDKs and BCDKs

All sequences are PDKs except for *Rattus rattus* BCDK. The invariant His residues proposed to be involved in phosphotransfer are shown in **bold**. If five or more residues are identical in position, this residue is presented in the consensus sequence. If five or more residues are not identical, but are of the same amino acid group, this is indicated by + in the concensus sequence. Genbank accession numbers: *A. thaliana*, AF039406; *Z. mays* I, II, AF038585, AF038586; *Drosophila melanogaster*, D88814; *Ascaris suum*, U94519; *Caenorhabdhitis elegans*, M98552; *R. rattus*, L22294; *Homo sapiens*, L42450; *R. rattus* BCDK, M93271.

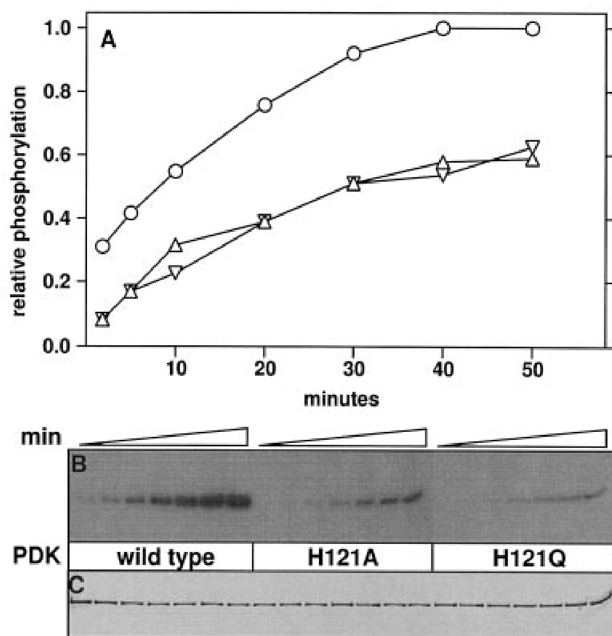

Figure 6 Kinetics of MBP–AtPDK autophosphorylation

Equal amounts (5.4 μg) of purified MBP–AtPDK were incubated with [γ-^{32}P]ATP (10 μCi, 10 mCi/mmol) at 25 °C. Samples were removed at indicated time points and mixed with an equal volume of sample buffer before analysis by SDS/PAGE. (**A**) The relative phosphorylations of MBP–PDK polypeptides [shown in (**B**): ▽, H121A; △, H121Q] were quantified with QS30 software (PDI, Huntington Station, NY, U.S.A.) and plotted relative to that of the wild-type enzyme (○). (**B**) Autoradiograph of MBP–AtPDK autophosphorylation. (**C**) Ponceau S-stained [0.1% (w/v) Ponceau S in 30% (w/v) trichloroacetic acid] loading control for (**B**).

abolish phosphorylation. Although at least three His residues are semi-conserved in all PDKs (and BCDK), only two are invariant, His-121 and His-168 (Figure 5). Because His-121 was previously proposed to be the phosphotransfer His, this residue was mutated to Ala or Gln. On an equivalent protein basis, purified His-121A and His-121Q AtPDKs were autophosphorylated at approx. 40–50% of the level of the wild-type protein during time-course studies (Figure 6). Autophosphorylation was consistent between PDK preparations, indicating that this difference was not due to recombinant expression. The lower degree to which these mutants phosphorylated maize E1α (in comparison with wild-type AtPDK) confirmed the lower V_{max} of these mutants (Figure 2A).

DISCUSSION

Reversible protein phosphorylation is a ubiquitous mechanism for signal transduction. Prokaryotic organsims use His-to-Asp phosphotransfer, whereas eukaryotic signal transduction cascades are predominantly mediated by the reversible phosphorylation of Ser, Thr and Tyr [26,27]. Examples of eukaryotic protein histidine kinases also exist, and His-to-Asp phospho-relays have been proposed [28]. So far, however, there are no conclusive examples of histidine kinases that phosphorylate Ser rather than Asp residues in Nature. Phytochrome from higher plants contains some of the hallmark histidine kinase domains yet phosphorylates Ser residues [29]. Additionally, the *E. coli* histidine kinase CheY phosphorylates hydroxyamino acids such as Ser when no Asp residue is available on its cognate response regulator, although the reaction mechanism is unknown [30]. It was later postulated that the high transfer potential of a phospho-His, the stability of a phospho-Ser and the proper positioning of the accepting amino acid collectively facilitate this unique His-to-Ser phospho-relay [28]. Although the CheY example was created artificially, the mitochondrial PDK might have evolved a similar phospho-relay system naturally.

Aside from PDK, other protein histidine-like kinases have been discovered in plants. One of these histidine-like kinases, 'ethylene triple response' (encoded by *etr1*), is involved in ethylene signal transduction and was discovered as a loss-of-function mutant responsible for a constitutive ethylene response phenotype [31]. Autophosphorylation of ETR1 is sensitive to treatment with acid, which is suggestive of His phosphorylation. Site-directed mutagenesis confirmed that this occurs on a His residue within the putative phosphotransfer domain [32]. The green algal phytochrome undergoes autophosphorylation on a His residue, determined via site-directed mutagenesis [33].

In the present study, a presumptive PDK homologue recently identified from *A. thaliana* was functionally expressed in *E. coli*. Fusing PDK with MBP obviated the difficulty in obtaining soluble AtPDK, observed previously with maize PDKs [18], and enabled the E1α subunit to be resolved from PDK during SDS/PAGE. For these two reasons intact MBP–PDK protein was used in this investigation. Autophosphorylation was not observed with MBP–AtPDK from nematodes [34]; in contrast, mammalian PDKs [16] and BCDKs [17] are capable of Ser-autophosphorylation. Here we demonstrate that the *A. thaliana* PDK is also capable of Ser-autophosphorylation. The phospho-Ser residue for AtPDK is located near the N-terminus as determined by mapping after limited proteolysis. Candidate residues include Ser-13, Ser-30 and Ser-39 because these are closest to the N-terminus on the basis of the deduced amino acid sequence. Recombinant maize PDK2 also undergoes Ser-auto-phosphorylation (results not shown), suggesting that this phenomenon might occur with all plant PDKs. Although the significance of Ser-autophosphorylation is not known, it is not an artifact induced by the absence of natural substrate because autophosphorylation was equivalent in the presence and in the absence of PDC (Figure 2A). Also, this phospho-Ser is not a catalytic intermediate, as evidenced by the inability to transfer this particular phosphate to maize E1α (Figure 2B). Because the stoichiometry of autophosphorylation is less than 1 mol of phosphate per mol of MBP–AtPDK, it is likely that only one

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phosphorylation site is present. The low stoichiometry of phosphate incorporation into purified MBP–AtPDK might indicate that autophosphorylation had taken place during bacterial expression before isolation of the protein.

Recombinant AtPDK inactivated kinase-depleted maize mitochondrial PDC by phosphorylating Ser residues on the 43 kDa E1α subunit. The specific activity of recombinant MBP–AtPDK was approx. one-quarter of that for recombinant maize MBP–PDK2 [18]. This difference was surprising because maize E1α shares 80% overall identity with *A. thaliana* E1α and 100% identity within the predicted phosphorylation motifs [25]. The difference in specific activities is therefore attributed either to PDK properties or docking site(s) to the dihydrolipoamide acetyltransferase (E2) core complex. Reconstitutions of mammalian PDCs have shown a 3–5-fold decrease in PDK activity (inactivation of PDC) in the absence of the E2 core [35–37]. This observation was explained by a proximity model in which the independent binding of E1 and PDK by the E2 core couples E1α phosphorylation. Recently, we demonstrated that dicotyledonous plants, such as *A. thaliana*, contain two mitochondrial E2 isoforms that share 33% amino acid identity [38], the primary difference being the presence of one compared with two lipoyl domains. In contrast, monocots such as maize possess a single mitochondrial E2 that contains one lipoyl domain [21,38]. Because mammalian PDKs have been shown to bind to the E2 core via the lipoyl domains [16,36,37], perhaps the divergence between monocot and dicot E2 cores hinders AtPDK binding to maize PDC, resulting in a lower specific activity *in vitro*. Steric hindrance by the bulky fusion partner (MBP) might also contribute to the decreased activity.

The ability of a histidine-like kinase such as PDK to autophosphorylate and transphosphorylate Ser residues is enigmatic. In an attempt to explain this we employed a site-directed mutagenesis approach. Histidine kinases require a His residue for phosphotransfer. Typically the His residue lies upstream of the Gly-rich ATP-binding domains and lacks conserved flanking sequences [39]. This organization is best described as an island domain. Seven His residues in the AtPDK sequence fit this description. Of those, only His-121 and His-168 are present in all PDKs (Figure 5). Because His-121 was previously postulated as the cardinal phosphotransfer His [15,18], this site was further investigated.

Mutating His-121 to either Ala or Gln did not noticeably alter the stability or yield of recombinant protein in comparison with the wild-type control. However, on an equal protein basis both mutant PDKs had decreased activity compared with wild-type as observed by Ser-autophosphorylation and transphosphorylation of E1α. A lower V_{max} could not be attributed to the substitution of a neutral amino acid because replacement with Gln gave results similar to those with Ala. The simplest interpretation is that His-121 is necessary for maximum activity but is not essential for catalysis. If His-121 is indeed a site for phosphotransfer, site-directed mutagenesis of this residue indicates either that PDKs have multiple phosphotransfer sites or that mutagenesis of His-121 exposes an otherwise cryptic site. In favour of this explanation, the covalent modification of MBP–AtPDK with two different His-modifying agents, diethyl pyrocarbonate and (2,2′:6′,2″-terpyridine)-platinum (II) dihydrate, completely abolished activity [40]. It is therefore evident that multiple His residues are involved in kinase function. The results presented here demonstrate that His-121 is one of these functionally important His residues, although at least one other must exist because activity was not completely abolished. Resolving this issue will require the scanning mutagenesis of other His residues.

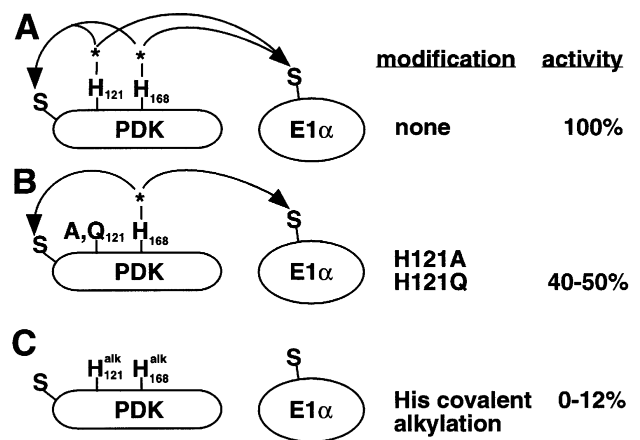

Figure 7 Model of PDK phosphorylation mechanism

The proposed model is based on the evidence that PDKs are descendants of prokaryotic protein histidine kinases, and also on the involvement of His residues in phosphotransfer and analyses revealing that PDKs autophosphorylate and transphosphorylate on Ser residues. Because mutagenesis of His-121 (this paper) did not completely abolish activity, a second phosphotransfer His residue (His-168) is proposed. The two conserved His residues (His-121 and His-168) are numbered in accordance with the AtPDK primary sequence. Mutagenesis or covalent modification of conserved His residues and the effect on relative activity are noted. Covalent modification of His was performed as described previously [40].

In summary, we have functionally expressed the *A. thaliana* PDK to improve understanding of the properties of phosphorylation. Recombinant AtPDK inactivated kinase-depleted maize PDC by phosphorylating Ser residues on the E1α subunit, although the specific activity of AtPDK was one-quarter of that of maize PDK2. During phosphorylation assays it was noticed that the MBP–AtPDK fusion also underwent autophosphorylation. Analyses of phosphoamino acids after limited proteolysis revealed that this occurred on a Ser residue, most probably near the AtPDK N-terminus. The autophosphorylated Ser was stable in pulse–chase reactions and was not an intermediate for E1α phosphorylation. Site-directed mutagenesis of His-121 curtailed autophosphorylation and transphosphorylation efficiencies by approx. 50%. The results presented here support the conclusion that PDKs are protein kinases that use at least one phosphohistidine intermediate for the transfer of phosphoryl groups to Ser residues (Figure 7). In support of this model are the following data. (1) The primary amino acid sequence resembles that of prokaryotic His kinases more closely than that of eukaryotic Ser/Thr protein kinases. (2) Two invariant His residues are present within the primary amino acid sequence of all PDKs and BCDKs. (3) Covalent modification of His residues on AtPDK abolishes activity [40]. (4) Mutagenesis of His-121 to Ala or Gln significantly decreases kinase activity. (5) PDKs autophosphorylate and transphosphorylate Ser residues. Although a phospho-His intermediate has not yet been directly defined, this might be attributed to the instability of this intermediate or to rapid phosphotransfer.

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