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Regulation of PDK1 Protein Kinase Activation by Its C-Terminal Pleckstrin Homology Domain

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UNIVERSITY OF MIAMI

REGULATION OF PDK1 PROTEIN KINASE ACTIVATION BY ITS C-TERMINAL PLECKSTRIN HOMOLOGY DOMAIN

By

Hassan Al-Ali

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Coral Gables, Florida

May 2010
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the requirements for the degree of
Doctor of Philosophy

REGULATION OF PDK1 PROTEIN KINASE ACTIVATION BY ITS C-TERMINAL
PLECKSTRIN HOMOLOGY DOMAIN

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Phosphoinositide-dependent protein kinase-1 (PDK1) plays an integral role in signaling cellular growth and proliferation, one that’s dependent on its ability to autophosphorylate Ser-241 in its T-loop. This process appears to have a strict requirement for its C-terminal pleckstrin homology (PH) domain. Thus, the overall objective of this work was to determine the mechanism by which the PH domain induces an active kinase conformation in unphosphorylated PDK1, capable of Ser-241 autophosphorylation. First, computational modeling and protein cross linking studies were combined with site-directed mutagenesis and kinetic assays in order to provide initial assessment of how the PH domain scaffolds Ser-241 autophosphorylation. A significant number of contacts were identified between the enigmatic “N-bud” region of the PH domain and the kinase domain. Specifically, these studies implicated Glu-432 and Glu-453 of the N-bud region of the PH domain that bind and serve as mimics of the phosphorylated Ser-241 in the T-loop and the phosphorylated C-terminal tail of PDK1 substrates, respectively. Next, a novel method for protein trans-splicing of the regulatory and catalytic kinase domains of PDK1 was developed. The method utilizes the N- and C-terminal split inteins of the gene dnaE from Nostoc punctiforme [(N)NpuDnaE]
and *Synechocystis* sp. strain PCC6803 [(C)SspDnaE], respectively. The cross-reacting KINASE(AEY)-(N)NpuDnaE-His$_6$ and GST-His$_6$-(C)SspDnaE-(CMN)PH fusion constructs generated full length spliced-PDK1 with $k_{obs} = (2.8 \pm 0.3) \times 10^{-5}$ s$^{-1}$. Finally, NMR was used to further characterize the structural and dynamical properties of the PH domain in both its isolated form and in full length PDK1. Whereas, it was not possible to obtain chemical shift assignments of any backbone or side chain nuclear resonances, methods were optimized for $^2$H,$^{13}$C,$^{15}$N-isotopic labeling of the recombinant PH domain. Furthermore, the protein trans-splicing method was significantly improved and utilized for segmental isotopic labeling of the PH domain in full length PDK1. These new findings and developments may provide specific insight and technological improvements towards future studies aimed to better understand and target autoinhibited conformations of PDK1 for translational purposes.
ACKNOWLEDGEMENTS

I am indebted to my supervisor, Dr. Thomas K. Harris, for his guidance and support, which enabled me to carry out this work and develop a keen understanding of the subject. I owe my deepest gratitude to my family and friends for their unconditional love and support. They were, and continue to be, my safety net. Lastly, I offer my regards to everyone who has assisted me during the course of my research, whether by allowing me access to lab equipment or by sharing their minds and experience so that I may proceed in my work with haste and confidence.
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## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>AMPK</td>
<td>AMP-stimulated protein kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine triphosphate</td>
</tr>
<tr>
<td>GSK</td>
<td>Glycogen synthase kinase</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>MSK</td>
<td>Mitogen- and stress-induced protein kinase</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>Npu</td>
<td>Nostoc punctiforme</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phospho-inositide dependent protein kinase-1</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PI(3)P</td>
<td>Phosphatidylinositol 3-phosphate</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIF</td>
<td>PDK1 interacting fragment</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKG</td>
<td>Protein kinase G</td>
</tr>
<tr>
<td>RSK</td>
<td>Ribosomal protein S6K kinase</td>
</tr>
<tr>
<td>S6K</td>
<td>Ribosomal protein S6 kinase</td>
</tr>
<tr>
<td>SGK</td>
<td>Serum- and glucocorticoid-induced protein kinase</td>
</tr>
<tr>
<td>Ssp</td>
<td>Synechocystis sp.</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1. Phosphoinositide-Dependent Protein Kinase-1 (PDK1)

1.1.1. DISCOVERY OF PDK1 AS ACTIVATOR OF PKB/AKT

The seminal identification and characterization of protein kinase Bα (PKB/Akt; 481 total residues) as a serine-threonine protein kinase related to the cAMP-dependent (PKA) and Ca^{2+}-activated protein kinases (PKC) (Coffer and Woodgett, 1991; Jones et al., 1991) gained strong interest on discovering: (i) it is a cellular homolog of the viral oncogene v-Akt (Belacossa et al., 1991) and overexpressed in a significant percentage of ovarian, pancreatic, and breast cancer cells (Jones et al., 1991; Cheng et al., 1992; Cheng et al., 1996); (ii) it is rapidly activated in response to insulin and a variety of growth factors (Kohn et al., 1995; Franke et al., 1995); and (iii) its activation is attenuated by inhibitors of phosphoinositol 3-kinase (PI3K) (Franke et al., 1995; Burgering and Coffer, 1995).

PI3K is a membrane bound phospholipid kinase that is rapidly activated in response to binding of insulin and other growth factors to a number of receptor tyrosine kinases. Upon activation, PI3K specifically phosphorylates the 3’-hydroxyl of the polar inositol head group of phosphatidylinositol 4,5-bisphosphate [P(4,5)P₂] (Cantley, 2002). Generation of the membrane-bound second messenger P(3,4,5)P₃ leads to membrane translocation of PKB/Akt. At the membrane, the C-terminal kinase domain of PKB/Akt (residues 146–481) becomes phosphorylated at Thr-308 (T-loop) and Ser-473 (C-terminus), which
stabilizes the fully active kinase conformation of PKB/Akt. In addition to its catalytic kinase domain, PKB/Akt has an N-terminal pleckstrin homology (PH) domain. It is this domain that specifically binds the PI(3,4,5)P$_3$ second messenger, which causes membrane translocation and leads to the subsequent activating phosphorylations of PKB/Akt (Franke et al., 1997; Frech et al., 1997).

Diligent efforts in the search for the kinase activator(s) of PKB/Akt by Alessi et al. (1997a) produced a 500,000-fold purification of the elusive “phosphoinositide-dependent protein kinase-1” (PDK1) from rabbit skeletal muscle extracts. Due to a high degree of protein sequence conservation, Alessi et al. (1997b) used rabbit PDK1 to successfully clone and express full length human PDK1 (Figure 1.1, 556 total residues). In addition to a near N-terminal catalytic kinase domain (residues 71–359), a putative C-terminal PH regulatory domain (residues 459–550) of PDK1 was noted by Alessi et al. (1997b). Indeed, growth factor stimulation of serum-starved HEK 293 cells, transiently infected with full length human PDK1 in addition to PKB/Akt, dramatically increased phosphorylation of Thr-308 of PKB/Akt. Furthermore, the reaction was directly dependent on PI3K activity, since Thr-308 phosphorylation was attenuated when the cells were treated with small molecule inhibitors of PI3K. Thereafter, it became established that the common pleckstrin homology (PH) domains shared by both PKB/Akt and PDK1 were responsible for their co-localization upon their mutual binding of PI(3,4,5)P$_3$ (Anderson et al., 1998; Stephens et al., 1998; Currie et al., 1999; Filippa et al., 2000; Vanhaesebroeck and Alessi, 2000).
Figure 1.1: Nucleotide coding and amino acid residue sequences of human phosphoinositide-dependent protein kinase-1 (PDK1; Accession No. NM002613). The nucleotide and amino acid sequences of the catalytic kinase domain (red), N-bud region of the PH domain (blue), and the conserved core region of the PH domain (green) are indicated. The sites of autophosphorylation (circles), tyrosine phosphorylation by v-Src (squares), and phosphorylation by unknown upstream kinases (triangles) are indicated.
1.1.2. Role of PDK1 as Activator of Other AGC Kinases

PDK1 is a member of the AGC subfamily of serine-threonine protein kinases, which includes different isoforms of cAMP-dependent protein kinase (PKA), protein kinase B (PKB), Ca\(^{2+}\)-activated protein kinase (PKC), protein kinase G (PKG), 70 kDa 40S ribosomal protein S6 kinase (S6K), 90 kDa 40S ribosomal protein S6 kinase (RSK), serum- and glucocorticoid-induced protein kinase (SGK), and mitogen- and stress-activated protein kinase (MSK) (Peterson and Schreiber, 1999). Among these kinases, amino acid sequences are conserved in a segment of the kinase domain known as the activation loop or T-loop, as well as in a segment C-terminal to the kinase domain known as the hydrophobic motif; and phosphorylation sites or acidic residues in these regions play important roles in their catalytic regulation and/or stability.

PDK1 has been termed the "master kinase" (Biondi et al., 2004) in that it has been shown to phosphorylate the critical residue in the T-loops of AGC kinase family members (Figure 1.2A) including PKB\(\alpha\) (Alessi et al., 1997a; Alessi et al., 1997b; Stokoe et al., 1997; Stephens et al., 1998), PKB\(\beta\) (Walker et al., 1998), PKB\(\gamma\) (Walker et al., 1998), SGK1 (Kobayashi and Cohen, 1999), SGK2 (Kobayashi et al., 1999), SGK3 (Kobayashi et al., 1999), S6K1 (Alessi et al., 1997c; Pullen et al., 1998), PKA (Cheng et al., 1998), PKC\(\alpha\) (Dutil et al., 1998), PKC\(\beta\)II (Dutil et al., 1998), PKC\(\delta\) (Le Good et al., 1998), PKC\(\zeta\) (Le Good et al., 1998; Chou et al., 1998), RSK (Jensen et al., 1999; Richards et al., 1999), and protein kinase N (PKN) (Dong et al., 2000; Torbett et al., 2003). With combined knowledge from available amino acid sequence alignments and X-ray
### A

<table>
<thead>
<tr>
<th>kinase</th>
<th>PDK1 VII</th>
<th>PDK1 VIII</th>
<th>PI3K-dependent HM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDK1</td>
<td>SPESKQARAN SFV GTAQYVSPE</td>
<td>231</td>
<td>252</td>
</tr>
<tr>
<td>PKBα</td>
<td>EGIKDGATMK TFCGTPYEYLAPE</td>
<td>298</td>
<td>319</td>
</tr>
<tr>
<td>PKBβ</td>
<td>EGISDGATMK TFCGTPYEYLAPE</td>
<td>299</td>
<td>320</td>
</tr>
<tr>
<td>PKBγ</td>
<td>EGISDGATMK TFCGTPYEYLAPE</td>
<td>295</td>
<td>316</td>
</tr>
<tr>
<td>SGK1</td>
<td>ENIEHNTSTS TFCGTPYEYLAPE</td>
<td>246</td>
<td>267</td>
</tr>
<tr>
<td>SGK2</td>
<td>EGVEPEDTTT TFCGTPYEYLAPE</td>
<td>183</td>
<td>204</td>
</tr>
<tr>
<td>S6K1</td>
<td>ESITHGTYHT TFCGTEYMAPE</td>
<td>242</td>
<td>263</td>
</tr>
<tr>
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<td>EHMMDGVTRR TFCGTPYIAPE</td>
<td>487</td>
<td>508</td>
</tr>
<tr>
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<td>ENIWDGVTRK TFCGTPYIAPE</td>
<td>490</td>
<td>511</td>
</tr>
<tr>
<td>PKCδ</td>
<td>ENIFGENRAS TFCGTPYIAPE</td>
<td>495</td>
<td>516</td>
</tr>
<tr>
<td>PKCζ</td>
<td>EGLGPGDTTS TFCGTPYIAPE</td>
<td>400</td>
<td>421</td>
</tr>
<tr>
<td>PKA</td>
<td>AKRVKGRTW  TFCGTPYEYLAPE</td>
<td>189</td>
<td>209</td>
</tr>
</tbody>
</table>

| SGK1     | 246       | 267       |
| PDK1     | 231       | 252       |
| PKBα     | 298       | 319       |
| PKBβ     | 299       | 320       |
| PKBγ     | 295       | 316       |
| SGK2     | 183       | 204       |
| S6K1     | 242       | 263       |
| PKCα     | 487       | 508       |
| PKCβII   | 490       | 511       |
| PKCδ     | 495       | 516       |
| PKCζ     | 400       | 421       |
| PKA      | 189       | 209       |

<table>
<thead>
<tr>
<th>PIF-Tide</th>
<th>EPRILSEEEQEM FRDFDYIADWC</th>
</tr>
</thead>
</table>

**Figure 1.2:** Sequence alignment of peptide regions that interact with PDK1. (A) Human AGC kinase family members that have been shown to be substrates for PDK1 show conserved sequences in the segment comprising the VII and VIII subdomains of the catalytic kinase domain, as well as the hydrophobic motif (HM) near the C-terminus. Subdomain VIII is known as the activation loop, and the critical threonine residue that undergoes phosphorylation by PDK1 is indicated by the arrowhead. PI3K-dependent phosphorylation of the critical residue in the HM (arrowhead) enhances PDK1 phosphorylation of the activation loop residue. Identical residues are in bold; and they cluster in the region C-terminal to the threonine phosphorylation site, suggesting a potential consensus motif for PDK1 phosphorylation. (B) T308-Tide represents the consensus motif found in subdomain VIII of PKBα, whereby PDK1 phosphorylates Thr-308 (underlined); PIF-Tide represents the consensus HM, which binds the PIF pocket of PDK1 and activates catalysis; PDK1-Tide is generated by joining the T308-Tide and PIF-Tide sequences.
structures, molecular modeling and biochemical testing now provide strong evidence for a common AGC kinase activation mechanism in which the C-terminal phosphorylated hydrophobic motif interacts with a phosphate binding pocket located in the small N-lobe of the kinase (Frödin et al., 2002). This intramolecular interaction acts synergistically with PDK1-catalyzed T-loop phosphorylation to stabilize the active AGC kinase conformation.

1.2. Regulation of PDK1 Activity

1.2.1. Sites of PDK1 Phosphorylation

Like other members of the AGC kinase family, PDK1 requires phosphorylation of Ser-241 of its T-loop for catalytic activity (Figure 1.2A), and both in vivo and in vitro evidence indicate that PDK1 catalyzes autophosphorylation of this site (Casamayor et al., 1999; Park et al., 2001; Wick et al., 2002; Wick et al., 2003; Scheid et al., 2005). In addition, it has been noted that PDK1 catalyzes in vitro autophosphorylation of Thr-33 (Park et al., 2001), Ser-410 (Casamayor et al., 1999; Park et al., 2001), and Thr-513 (Park et al., 2001; Wick et al., 2002; Wick et al., 2003; Scheid et al., 2005). Although upstream kinases have yet to be identified, cellular metabolic labeling studies indicated PDK1 to be additionally phosphorylated at Ser-25 (Casamayor et al., 1999; Park et al., 2001), Ser-393 (Casamayor et al., 1999), and Ser-396 (Casamayor et al., 1999; Wick et al., 2002; Wick et al., 2003). Finally, it has been shown that co-expression of PDK1 with v-Src tyrosine kinase in cell cultures leads to phosphorylation of Tyr-9, Tyr-373, and Tyr-376 (Park et al., 2001).
**Figure 1.1** shows (i) Tyr-9, Ser-25, and Thr-33 to be in the N-terminal peptide leader sequence preceding the catalytic kinase domain and (ii) Tyr-373, Tyr-376, Ser-393, Ser-396, and Ser-410 to be in the peptide linker region bridging the kinase domain to the C-terminal PH domain. Whereas phosphorylation of Tyr-9, Tyr-373, and Tyr-376 slightly enhanced PDK1 kinase activity (Park et al., 2001), the potential roles of Ser-25, Thr-33, Ser-393, Ser-396, and Ser-410 phosphorylation remain undetermined. Since no significant effect on cellular levels of PDK1 Ser-241 phosphorylation or activity occurred on mutation of these residues to Ala, it has been postulated that their phosphorylation may regulate cellular stability and/or localization. Most interestingly, mutating Thr-516 of mouse PDK1 to Glu (homologous to Thr-513 in human) caused constitutive activation of mouse PDK1 towards phosphorylation of PKB/Akt in cells (Wick et al., 2002). This is particularly noteworthy since Thr-513 is located in the C-terminal PH domain (**Figure 1.1**).

**1.2.2. ROLE OF PH DOMAIN IN REGULATING PDK1 CATALYTIC ACTIVITY**

PH domains are protein structural domains consisting of some 100 to 120 amino acids that share a conserved core fold consisting of a seven-stranded $\beta$-barrel capped on one end by a C-terminal $\alpha$-helix (Rebecchi and Scarlata, 1998). X-ray structural studies indicated that the C-terminal PH domain of PDK1 (residues 408–556) possesses unique features not previously observed in other PH domains (Komander et al., 2004). In addition to a conserved core fold (residues 458–547), the PH domain of PDK1 contains an N-terminal hydrophobic
'bud' that joins at the C-terminus of the kinase domain (residues 408–457) (Figure 1.1). This 'bud' consists of two additional β strands followed by a long α helix, and it has not been observed in any other crystallized PH domain. It remains undetermined as to whether the hydrophobic 'bud' region may play any critical role in regulating either the stability or reactivity of PDK1.

While the functional role of the 'bud' region remains enigmatic, the additional observation that the PDK1 PH domain phosphoinositide binding pocket is significantly more spacious than other phosphoinositide binding PH domains explained the ability of PDK1 to efficiently bind numerous phosphoinositides [e.g., PI(3,4,5)P3, PI(3,4)P2, PI(4,5)P2, PI(3,5)P2, and PI(3)P] and inositol polyphosphates [e.g., I(1,3,4,5)P4, I(1,3,4,6)P4, I(1,3,4,5,6)P5, I(3,4,5,6)P4, IP6, and 5PP-IP5] (Komander et al., 2004; Currie et al., 1999; Stephens et al., 1998). In addition, cellular localization studies of green fluorescent protein (GFP)-tagged full length PDK1 (Komander et al., 2004) confirmed previous studies (Currie et al., 1999) that PDK1 was mainly cytosolic, with a small fraction at the membrane, and was excluded from the nucleus in unstimulated HEK 293 cells; and localization of PDK1 was not affected by either agonists or inhibitors of PI3K. Taken together, these findings are consistent with an overall model in which promiscuous binding of the PH domain anchors PDK1 to both phosphoinositides at the membrane and inositol phosphates in the cytosol (Komander et al., 2004).

The common regulatory pleckstrin homology (PH) domains shared by PDK1 and PKB/Akt provides for mutual binding to PI(3,4,5)P3 and co-localization at the plasma membrane, which is necessary for PDK1-catalyzed
phosphorylation and activation of PKB/Akt (Vanhaesebroeck et al., 2000). More recently, a combination of computational modeling, biochemical assays, and cellular fluorescence lifetime imaging studies indicated that binding of PKB/Akt to PI(3,4,5)P₃ induces a change in the relative orientation of the contiguous PH and kinase domains of PKB/Akt, which allows PDK1 to phosphorylate Thr-308 in the T-loop of PKB/Akt (Calleja et al., 2003; Calleja et al., 2007; Calleja et al., 2009a; Calleja et al., 2009b). Although potential roles of the PH domain of PDK1 binding to other phosphoinositides at the membrane and inositol phosphates in the cytosol remain undetermined, it has been surmised that such binding events likely direct localization.

1.2.3. TRANSACTIVATION OF PDK1 BY AGC KINASE TARGETS

The common ability of T-loop Ser-241 phosphorylated PDK1 to catalyze T-loop phosphorylation of AGC protein kinases other than PKB/Akt has been explained from the intriguing perspective that PDK1 has the ability to 'sense the conformation' of these kinases (Biondi, 2004). Similar to its other AGC kinase family members, X-ray structural and biochemical studies showed PDK1 to contain the phosphate binding pocket in the small N-lobe of its catalytic kinase domain (Biondi et al., 2000; Biondi et al., 2002). Yet in contrast to other AGC kinase family members, PDK1 does not possess a phosphorylated hydrophobic motif C-terminal to its catalytic domain (Figure 1.2A). It has been proposed that the small N-lobe of PDK1 is accessible for intermolecular interaction with the phosphorylated hydrophobic motifs of its AGC kinase targets. Such docking-
based recognition of the AGC kinase target would serve to transactivate PDK1-catalyzed T-loop phosphorylation of that target. Subsequently, the phosphorylated C-terminal hydrophobic motif of the target kinase would be released from PDK1 to form an *intramolecular* interaction with its N-lobe pocket, stabilizing its own active conformation (Biondi et al., 2004). Both in vivo and in vitro studies indicated the important role of intermolecular interactions in activating PDK1-catalyzed T-loop phosphorylation of S6K1, RSK, and SGK (Frödin et al., 2002; Biondi et al., 2001; Frödin et al., 2000). In stark contrast, intermolecular interaction of the C-terminal hydrophobic motif of PKB with the phosphate binding pocket of PDK1 could not be detected (Biondi et al., 2001).

The C-terminal phosphorylated hydrophobic motif of the AGC kinases that interacts with PDK1 has been termed the PDK1-interacting fragment or “PIF”. To further investigate the role of PIF interactions, a model ‘PIF-Tide’ was synthesized to contain the C-terminal hydrophobic motif region of protein kinase C-related kinase-2 (PRK2). The PIF region in PRK2 contains high sequence homology to the PIF regions of PDK1 protein substrates, except that the Ser/Thr phosphorylation site is replaced by a negatively charged Asp residue (*Figure 1.2B*) (Biondi et al., 2000; Balendran et al., 1999). Surface plasmon resonance and competition measurements yielded $K_{d}^{\text{PIF}}$ values of ~0.8–1.5 $\mu$M, indicating a binary complex between PIF-Tide and PDK1 (Balendran et al., 1999). In addition, PIF-Tide was found to activate PDK1-catalyzed phosphorylation of the small T308-Tide, which represents the consensus motif near the site of phosphorylation in PKBα (*Figure 1.2B*) (Biondi et al., 2000). By conjugating the
PIF-Tide to the C-terminus of T308-Tide, the PDK1-Tide was generated (Figure 1.2B). The degree that the proposed PIF interaction activates the catalytic kinase activity of PDK1 is illustrated by the observation that PDK1-Tide undergoes phosphorylation at a rate >100-fold faster than T308-Tide and its $K_m$ of 80 $\mu$M is significantly lower than the $K_m$ of >10 mM estimated for T308-Tide (Biondi et al., 2000).

1.3. Targeting PDK1 for Anticancer Drug Discovery

1.3.1. Identification of PDK1 as Drug Discovery Target

Remarkably, for a drug developed over 30 years ago by ICI (now AstraZeneca), tamoxifen is still the world’s most deployed antibrust-cancer drug. Despite its benefits, almost all breast cancer patients with metastatic disease, and many with non-metastatic disease, relapse and ultimately die due to tumors becoming resistant to tamoxifen. To identify genes and compounds that could affect the sensitivity of cells to tamoxifen, Iorns et al. (2009) reported an innovative dual-screening approach that combined parallel RNAi genetic and chemical compound screens in oestrogen-dependent MCF7 breast cancer cells. Remarkably, seven of the 20 most sensitizing hits identified from the genetic screen were involved in signaling pathways controlled by PDK1; and nine of the top ten tamoxifen-sensitizing hits in the chemical screen were also judged to inhibit PDK1 signaling. The study by Iorns et al. (2009) points out that targeting PDK1 itself would be most effective at sensitizing cells to tamoxifen.
In addition to the above mentioned study, high-throughput protein phosphorylation screening assays have long cited the importance of PDK1 in activating an intertwining network of tumor growth-promoting signaling pathways (Lin et al., 2005; Knuefermann et al., 2003). For example, Figure 1.3 shows how mitogen, growth factor, nutrient, and energy status signaling pathways integrate to initiate translation of a select subgroup of mRNA transcripts required for maintenance of the cancerous phenotype (Tee and Blenis, 2005; Fingar and Blenis, 2004; Richardson et al., 2004; Martin and Blenis, 2002).

Figure 1.3: Mitogen, nutrient, and energy status regulation of S6K1-dependent activation of the translation preinitiation complex. (A) A number of well-characterized mitogens and growth factors bind and activate receptor tyrosine kinases (RTK) (Schlessinger, 2000). Activated RTKs nucleate intracellular membrane-bound complexes that initiate both mitogenic and growth signaling cascades. The guanine nucleotide exchange factor SOS activates the RAS G-protein, which signals through the mitogen-activated protein (MAP) kinase pathway (RAS→RAF→MEK→ERK) to control phosphorylation of the C-terminal AID of S6K1αII and facilitate T389 phosphorylation by mTOR (Ferrari et al., 1992; Cheatham et al., 1995; Weng et al., 1995; Mukopadhayay et al., 1992; Weng et al., 1998; Dufner and Thomas, 1999). The Rheb G-protein mediates nutrient-dependent (e.g., amino acids, nitrogen, and glucose) activation of mTOR (Saucedo et al., 2003; Stocker et al., 2003). Rheb-dependent activation of mTOR is also modulated by the antagonizing effects of low energy status versus mitogens, which act on the tumor suppressor protein tuberous sclerosis complex (TSC). Within the heterodimeric TSC is TSC2-GAP, which functions as the GTPase-activating protein of Rheb thereby causing down regulation of mTOR-mediated S6K1αII phosphorylation and protein translation (Gao et al., 2002; Zhang et al., 2003; Tee et al., 2003; Inoki et al., 2003a; Castro et al., 2003; Garami et al., 2003). In response to low energy status, the AMP-stimulated protein kinase (AMPK) directly phosphorylates TSC2-GAP on T1227 and S1345 and influences additional phosphorylation on S1337 and 1341, which together enhance the GAP activity of TSC2 (Inoki et al., 2003b). A low energy status is further linked to a second tumor suppressor protein kinase, LKB1, which has been shown to activate AMPK by phosphorylating the catalytically critical T172 (Woods et al., 2003; Hawley et al., 2003; Hong et al., 2003). Synergistic with nutrient stimulation, Rheb and mTOR activation is sustained by mitogenic activation of the phosphatidylinositol 3-kinase (PI3K) signaling cascade (RTK→PI3K→PDK1→PKB), whereby PKB phosphorylation of TSC2-GAP at S939 and T1462 leads to its targeted degradation (Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002; Dan et al., 2002). (B) GTP-bound Rheb activates mTOR in the eukaryotic initiation factor 3-preinitiating complex (eIF3-PIC). mTOR phosphorylation of the 4E-BP1 suppressor leads to its dissociation and formation of the capped translation preinitiation complex; and mTOR phosphorylation of S6K1αII at T389 leads to its dissociation (Holz et al., 2006). (C) To become fully activated, S6K1αII must also be phosphorylated at T229 of its T-loop by PDK1 (Alessi et al., 1997c; Pullen et al., 1998). In order for PDK1 to bind and phosphorylate S6K1αII, T513 in the PH domain of PDK1 is autophosphorylated (Gao et al., 2006). S6K1αII phosphorylation of the 40S ribosomal subunit S6 and eIF4B leads to activation of eIF4A, which is the mRNA helicase required to initiation translation of mRNAs with long, structured 5'-UTRs (Holz et al., 2006).
First, it has been reasoned that the mitogen-activated protein (MAP) kinase pathway (RAS→RAF→MEK→ERK) controls the initial or priming S6K1αII phosphorylations at S411, S418, T421, and S424 in the C-terminal autoinhibitory domain (AID; residues 399–502) (**Figure 1.3A**) (Cheatham et al., 1995; Weng et al., 1995; Ferrari et al., 1992; Mukhopadhayay et al., 1992). AID phosphorylation is thought to destabilize an autoinhibited conformation, whereby T229 (T-loop) and T389 (HM motif) in the catalytic kinase domain of S6K1αII become accessible to their upstream kinases, PDK1 and mTOR, respectively (Weng et al., 1998; Dufner and Thomas, 1999; Alessi et al., 1997c; Pullen et al., 1998). Active PDK1 is generated in a phosphatidylinositol 3-kinase (PI3K)-dependent manner, whereby membrane-bound phosphatidylinositol (3,4,5)-triphosphate [PI(3,4,5)P₃] binding to the C-terminal PH domain of PDK1 results in both S241 (T-loop) and T513 (PH domain) autophosphorylation (Gao and Harris, 2006).

In addition, a complex interplay between *growth factor*, *nutrient*, and *energy* levels is required to coordinate Rheb-mediated mTOR activation at the eukaryotic initiation factor 3-preinitiating complex (eIF3-PIC) (**Figure 1.3A,B**) (Saucedo et al., 2003; Stocker et al., 2003; Gao et al., 2002; Zhang et al., 2003; Tee et al., 2003; Inoki et al., 2003a; Castro et al., 2003; Garami et al., 2003; Inoki et al., 2003b; Woods et al., 2003; Hawley et al., 2003; Hong et al., 2003; Inoki et al., 2002; Manning et al., 2002a; Potter et al., 2002; Dan et al., 2002). As a matter of simple depiction, T389 phosphorylation by mTOR is shown to precede T229 phosphorylation by PDK1 (**Figure 1.3C**); however, it remains unclear as to whether any preferred order is actually required. In any case, fully activated
S6K1αII phosphorylates eIF4B, which is a protein cofactor that recruits and activates the eIF4A mRNA helicase that processively unwinds long, structured 5'-untranslated regions leading to the initiator codon in the eIF3:40S ribosomal initiation complex (Figure 1.3C) (Holz et al., 2006). The long, structured 5'-untranslated regions are unique to mRNA transcripts that code for a variety of potent proteins that initiate and sustain cell cycle progression leading to DNA replication and mitosis events. Hence, large pharmaceutical efforts have been directed towards the discovery and development of potent and selective inhibitors of signaling enzymes, which can effectively shut down the mitogenic response in cancer cells.

1.3.2. A NOVEL STRATEGY FOR PDK1 DRUG DISCOVERY

Discovering potent and selective inhibitors of individual members of the protein kinase superfamily has long proved to be an arduous task. Foremost, the protein kinase superfamily is the largest enzyme family with an estimated 518 members in the human genome (Manning et al., 2002b). X-ray structures available for the catalytic domains of different kinases in their phosphorylated and active forms reveal highly homologous structures, especially in the ATP-binding cleft (Huse and Kuriyan, 2002). Whereas a number of high affinity kinase inhibitors of PDK1 have been identified (e.g., staurosporine and UCN01), these compounds have been shown to be highly toxic. Their toxicity results from the common ability to bind in the ATP-binding cleft that is shared among the large number of protein kinases (Noble et al., 2004; Davies et al., 2000; Bain et al.,
Alternatively, new drug targeting strategies may employ methods to identify ligands that bind sites outside the ATP pocket (exosites), which could serve to stabilize autoinhibited structures of multidomain kinases.

The idea of improving inhibitor selectivity by targeting "exosites" is motivated by inspection of primary structure alignments of a number of well established serine-threonine protein kinase drug targets. While the catalytic kinase domains share a high degree of structural homology, the most distinguishing feature among these kinases is the distribution of various types of regulatory domains, many of which are contiguous with the catalytic kinase domain. Better understanding of the mechanisms by which the unusual C-terminal PH domain regulates PDK1 activation and reactivity with downstream protein kinase targets may facilitate design and/or discovery of a new class of highly selective 'exosite' inhibitors.

1.4. Aims of Studies

Thus, the overall objective of this thesis was to determine the mechanisms by which the C-terminal PH domain of PDK1 regulates PDK1 activation. The ability to address this objective was particularly challenging as progress towards molecular understanding of conformational interactions within full length multi-domain kinases remains hampered. The observation that suitable crystals for X-ray diffraction studies of full length multi-domain monomeric protein kinases have not been obtained suggests an important role for intra-domain-domain conformational dynamics.
Chapter 2 begins by describing computational modeling and protein cross-linking combined with site-directed mutagenesis and kinetic assays, which were used to provide initial assessment of how the PH domain activates Ser-241 autophosphorylation. Whereas little or no molecular contacts were identified between the core region of the PH domain with its N-terminal catalytic kinase domain, a significant number of contacts were identified between the enigmatic “N-bud” region of the PH domain and the kinase domain. In specific, these studies implicated two Glu residues of the N-bud region that bind and serve as mimics of the phosphorylated T-loop and phosphorylated PIF. In short, these Glu residues seem to organize and stabilize an active PDK1 kinase conformation capable of catalyzing T-loop Ser-241 autophosphorylation.

In Chapter 3, a novel method for protein trans-splicing of the regulatory and catalytic kinase domains of PDK1, which has been published (Al-Ali et al., 2007). Finally, Chapter 4 describes aggressive attempts to use NMR to study the structural and dynamical properties of the PH domain in both its isolated form and in its native context in full length PDK1. Whereas, it was not possible to obtain chemical shift assignments of any backbone or side chain nuclear resonances, methods were optimized for expression, purification, and $^{2}H, ^{13}C, ^{15}N$-isotopic labeling of the recombinant PH domain. Furthermore, the protein trans-splicing method was significantly improved and utilized for segmental isotopic labeling of the PH domain in full length PDK1. Such new findings and developments may provide specific insight and technological improvements
towards future studies aimed to better understand and target autoinhibited conformations of PDK1 for translational purposes.
CHAPTER 2
REGULATION OF PDK1 AUTOACTIVATION BY ITS C-TERMINAL PLEKSTRIN HOMOLOGY (PH) DOMAIN

2.1. Background and Significance

Phosphoinositide-dependent protein kinase-1 (PDK1) is a member of the AGC subfamily of serine-threonine protein kinases (Peterson and Schrieber, 1999). Among these kinases, amino acid sequences are conserved in a segment of the kinase domain known as the activation loop or T-loop, as well as in a segment C-terminal to the kinase domain known as the hydrophobic motif; and phosphorylation sites or acidic residues in these regions play important roles in their catalytic regulation and/or stability. In contrast to other AGC kinases, PDK1 does not possess a hydrophobic motif C-terminal to its catalytic domain. Rather, a pleckstrin homology (PH) domain resides at the C-terminus. PH domains are protein structural domains consisting of some 100 to 120 amino acids that share a conserved core fold consisting of a seven-stranded \( \beta \)-barrel capped on one end by a C-terminal \( \alpha \)-helix (Rebecchi and Scarlata, 1998). Although more than 100 PH domain-containing proteins are predicted from sequence data, only a few have been characterized, and these have been shown to bind phosphoinositides with a broad range of affinity and specificity (Ferguson et al., 2000).

The common regulatory PH domains shared by PDK1 (Figure 2.1A) and protein kinase B (PKB/Akt) (Figure 2.1B) provides for mutual binding to phosphatidylinositol (3,4,5)-trisphosphate \([\text{PI}(3,4,5)\text{P}_3]\) and co-localization at the
Figure 2.1: Pleckstrin homology (PH) domains of PDK1 and PKBα. X-ray crystal structures are shown for (A) the C-terminal PH domain of PDK1 (residues 409–556) (Komander et al., 2004) and (B) the N-terminal PH domain of PKBα (residues 1–123) (Milburn et al., 2003), each bound to inositol (1,3,4,5)-tetrakisphosphate [top, I(1,3,4,5)P₄]. The PH domain canonical conserved core (green) is comprised of a seven-stranded β-barrel capped by a C-terminal α helix (bottom). The atypical N-terminal bud of the PH domain of PDK1 is shown in blue.

plasma membrane, which is necessary for PDK1-catalyzed T-loop phosphorylation and activation of PKB/Akt (Vanhaesebroeck et al., 2000). In contrast to the high specificity binding of PI(3,4,5)P₃ by the PH domain of PKB/Akt, the PH domain of PDK1 exhibits broad specificity binding to numerous phosphoinositides [e.g., PI(3,4,5)P₃, PI(3,4)P₂, PI(4,5)P₂, PI(3,5)P₂, and PI(3)P] and inositol polyphosphates [e.g., I(1,3,4,5)P₄, I(1,3,4,6)P₄, I(1,3,4,5,6)P₅, I(3,4,5,6)P₄, IP₆, and 5PP-IP₅] (Komander et al., 2004; Currie et al., 1999; Stephens et al., 1998). X-ray structural studies indicated the broad specificity is derived from a significantly more spacious phosphoinositide binding pocket in the PH domain of PDK1 (residues 409–556) (Komander et al., 2004) (Figure 2.1A), as compared to the PH domain of PKBα/Akt1 (residues 1–123) (Thomas et al., 2002) (Figure 2.1B).
Although much progress has been achieved towards characterizing the structure and phosphoinositide binding specificity of the PH domain of PDK1 (Komander et al., 2004), few studies have addressed the potential role that the PH domain may play in regulating PDK1 autoactivation and reactivity with downstream substrates. To address these possibilities, Gao and Harris (2006) (i) generated recombinant full length and catalytic domain constructs of PDK1, (ii) tested their ability to catalyze site-specific autophosphorylation, and (iii) assessed the ability of different phosphorylated forms of each construct to phosphorylate different model peptide substrates (e.g., T308-Tide and PDK1-Tide). The results of these studies provided evidence that the (i) PH domain activates T-loop Ser-241 autophosphorylation, (ii) the PH domain autoinhibits PIF-dependent transactivation of Ser-241 monophosphorylated PDK1, and (iii) autoinhibition of transactivation is relieved upon subsequent autophosphorylation of Thr-513 in the PH domain.

The observation that the PH domain activates PDK1 T-loop autophosphorylation, as well as modulates reactivity with downstream substrates, suggests that the PH domain engages one or more distinct interactions with the kinase domain. On further review, the X-ray structure of the PH domain of PDK1 (Komander et al., 2004) (Figure 2.1A) shows an N-terminal hydrophobic 'bud', which consists of two β strands followed by a long α helix. The N-bud peptide region would join at the C-terminus of the kinase domain (residues 409–457), and it has not been observed in any other crystallized PH domain. Thus, one could hypothesize that the N-bud might play important roles in
regulating PDK1 autoactivation and downstream reactivity by making specific contacts with the kinase domain of PDK1.

In this chapter, a combination of computational macromolecular docking, chemical cross linking, mutagenesis, and kinetic assays were used to provide more detailed understanding of how PDK1 is autoregulated by its C-terminal PH domain. Whereas little or no molecular contacts were identified between the core region of the PH domain with its N-terminal catalytic kinase domain, a significant number of contacts were identified between the enigmatic “N-bud” region of the PH domain and the kinase domain. Specifically, these studies implicated two Glu residues of the N-bud region that bind and serve as mimics of the phosphorylated T-loop and phosphorylated PIF. In short, these Glu residues seem to organize and stabilize an active PDK1 kinase conformation capable of catalyzing T-loop Ser-241 autophosphorylation.

2.2. Experimental Procedures

2.2.1. MATERIALS

‘Full length’ constructs (residues 51–556) of wild type and mutant PDK1s [His6-PDK1, His6-PDK1(K111A), His6-PDK1(S241G), His6-PDK1(E432K), His6-PDK1(E453K), and His6-PDK1(K512E)], as well as the catalytic kinase domain of PDK1 [His6-PDK1(ΔPH); residues 51–359], each containing an N-terminal His6 tag (MHHHHHHH) followed by a PreScission protease recognition sequence (LEVLFQGP) prior to residue 51, were expressed using the Bac-to-Bac® baculovirus expression system (Invitrogen) and His6 affinity purified as described
Fractions containing His\textsubscript{6} affinity tagged kinase were combined and subjected to heparin-Sepharose chromatography, which removed minor contaminants from His\textsubscript{6} affinity chromatography. Mutagenesis was carried out using corrective linear amplification PCR (Appendix A1). Unphosphorylated wild type and mutant forms of His\textsubscript{6}-PDK1 and His\textsubscript{6}-PDK1(ΔPH) were generated by treatment with Lambda protein phosphatase (λPP) as described (Appendix A1; Gao et al., 2005). The C-terminal PH domain of PDK1 (residues 408–556) was generated as described (Chapter 4), except that the natural abundance isotopic forms of water (H\textsubscript{2}O), D-glucose, and ammonium chloride (NH\textsubscript{4}Cl) were used. [γ\textsuperscript{32}P]ATP was from MP-Biomedical (Irvine, CA). All other chemicals, salts, and buffers were from Sigma.

2.2.2. AUTOPHOSPHORYLATION ASSAYS

The ability of the λPP-treated unphosphorylated forms of wild type and mutant PDK1 constructs to catalyze T-loop (S241) autophosphorylation was tested in a reaction mixture containing 5 μM kinase (60 μg or 1 nmol) in 50 mM Tris-HCl buffer, pH 7.5, 1 mM 2-mercaptoethanol, and 10 mM MgCl\textsubscript{2}. The reaction was initiated by addition of 100 μM of either cold ATP or [γ\textsuperscript{32}P]ATP (~500 cpm/pmol) and allowed to proceed with continuous shaking at 30 °C. Control assays were carried out in parallel in which kinase was omitted. Site-specific S241 phosphorylation was confirmed by SDS-PAGE and Western analysis of the PDK1 reaction product taken from assay samples using cold ATP (see below). Reaction rates and stoichiometry of phosphorylation were quantified.
by $^{32}$P-radiometric analysis of the PDK1 reaction product taken from assay samples using [$\gamma^{32}$P]ATP. Here, 10 µL aliquots were removed at different times and quenched by addition to 10 µL of 75 mM phosphoric acid. Each 20 µL quenched sample was applied to P81 phosphocellulose paper (2 × 2 cm). After 30 s, the papers were washed (three times) in 1 L of fresh 75 mM phosphoric acid for 10 min and then rinsed with 50 mL of acetone and placed in the hood to dry. The specific radioactivity of $^{32}$P-labeled kinase (SA$^{PDK1}$, cpm/pmol) was determined from radioactivity detected by scintillation counting of the known amount of the PDK1 construct that was applied to the paper (50 pmol). The concentration of phosphorylated sites in the kinase product was calculated by

$$[E-\text{OPO}_3^{2-}] \, (\mu\text{M}) = (\text{SA}^{PDK1}/\text{SA}^{ATP}) \times [\text{PDK1}]_{\text{tot}} \, (\mu\text{M}).$$

2.2.3. SDS-PAGE AND WESTERN ANALYSIS

Protein samples (1 µg) in SDS sample buffer were heated at 95 °C for 2 min and cooled on ice. Analytical SDS-PAGE was performed on 4–12% gradient polyacrylamide gels (Invitrogen) developed at 150 V (constant) for 1 h or until the tracking dye reached the bottom of the slab. Coomassie staining was used to visualize total protein. For Western analyses, protein was transferred from the gel to Nitrocellulose Membrane Filter Paper Sandwich (Invitrogen) in a semidry blotting apparatus 25 mM Tris, 20 mM glycine, and 20% v/v methanol as transferring solution. According to the manufacturer’s instructions, purified and λPP-treated PDK1 were probed with either PDK1 (general) or phospho-PDK1 (Ser241) polyclonal rabbit antibody (Cell Signaling Technology). Detection of
immuno-protein complexes was carried out using secondary anti-rabbit antibody conjugated to horseradish peroxidase (HRP) and LumiGLO® chemiluminescent reagent and peroxide. Densitometric analysis was performed using the ImageQuant software (Molecular Dynamics).

2.2.4. MACROMOLECULAR DOCKING OF PH AND KINASE DOMAINS OF PDK1

The X-ray structure of the catalytic kinase domain of PDK1 (1H1W) was docked with the X-ray structure of its C-terminal regulatory PH domain (1W1H), using the automated macromolecular docking DOT 2.0 software. In order to do so, the X-ray structure of the catalytic kinase domain required insertion of the \( ^{233}\text{ESKQ}^{236} \) peptide sequence into the T-loop region that was missing in the structure. To better approximate interactions of the PH domain with the unphosphorylated inactive form of the kinase, the X-ray structure of the catalytic kinase domain of PDK1 (1H1W) was modified in that (i) bound ATP was removed and (ii) the phosphate group from S241 in the T-loop was removed. The electrostatic and steric properties of both the PH and kinase domains were mapped to grids, and a systematic rigid-body translational and rotational search was performed. Using correlation functions calculated from the sum of electrostatic and van der Waals terms, a ranked list of lowest energy fits for docked structures was obtained. Using the NAMD molecular dynamics software, lowest energy structures were solvated and energy minimized, which converged to the proposed complex formed between the regulatory PH and catalytic kinase domains of PDK1.
2.2.5. Chemical Cross Linking of PH and Kinase Domains of PDK1

Chemical cross linking experiments were performed in PBS buffer (50 mM sodium phosphate, pH 6.5, 250 mM NaCl, 1 mM 2-mercaptoethanol). Freshly prepared 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide (EDC, Fluka) and the catalyst N-hydroxy-succinimide (NHS, Fluka) were added to the protein sample (10 μM PH domain and 10 μM kinase domain) to yield a final concentration of 50 mM EDC and 200 mM NHS. Control reactions were prepared containing either 10 μM PH domain or 10 μM kinase domain alone. The samples were incubated at room temperature for 30 min before the reaction was terminated by addition of SDS loading buffer and boiling at 95 °C for 5 min. Samples were analyzed by SDS-PAGE by both Coomassie and silverSNAP staining.

2.3. Results

2.3.1. Activation of T-Loop (SER-241) Autophosphorylation by PH Domain

As previously reported (Gao and Harris, 2006), the recombinant catalytic domain construct of PDK1 [His₆-PDK1(ΔPH), residues 51–359] and full length His₆-PDK1 were purified (≥95%) from Sf9 insect cell lysate in their Ser-241 phosphorylated forms, as detected by Western analysis with the phospho-PDK1 (S241) antibody (α-pS241) (Figure 2.2A, lane 1). It is interesting to point out that SDS-PAGE resolution of pS241 His₆-PDK1(ΔPH) showed only a single band, whereas pS241 His₆-PDK1 resolved as two distinct bands. The appearance of multiple bands has often been attributed to multiply phosphorylated states, whereby the presence of additional negatively charged phosphate groups
Figure 2.2: Western analysis of Ser-241 autophosphorylation by PDK1 constructs. (A) SDS-PAGE and Western blotting with phospho-PDK1 (Ser-241) antibody (α-pS241) and general PDK1 antibody (α-PDK1) of the catalytic kinase domain of His₆-PDK1 (ΔPH) and full length His₆-PDK1 (wild type, WT). Lane 1 shows the enzymes after purification from Sf9 insect cells; lane 2 shows the enzymes after removal of phosphates by treatment with λPP; and lane 3 shows the enzymes after incubation with 100 μM ATP and 10 mM MgCl₂ for 5 min. (B) Diagrams showing that the catalytic kinase domain of His₆-PDK1 (ΔPH) does not catalyze Ser-241 autophosphorylation, whereas full length His₆-PDK1 (WT) does catalyze Ser-241 autophosphorylation. (C) SDS-PAGE and Western blotting with α-pS241 and α-PDK1 of the S241G and K111A mutants of full length His₆-PDK1. Western analysis with α-pS241 is also shown for detection of Ser-241 trans-phosphorylation of the K111A mutant by the S241G mutant. Lane 1 shows the enzymes after purification from Sf9 insect cells; lane 2 shows the enzymes after removal of phosphates by treatment with λPP; and lane 3 shows the enzymes after incubation with 100 μM ATP and 10 mM MgCl₂ for 5 min. (D) Diagrams showing that (i) α-pS241 does not nonspecifically detect the S241G mutant, since Gly-241 cannot be phosphorylated; (ii) the K111A mutant does not catalyze any detectable Ser-241 autophosphorylation; and (iii) the unphosphorylated S241G mutant catalyzes Ser-241 phosphorylation of the K111A mutant.
reduces the overall number of bound SDS molecules thereby slightly retarding electrophoretic mobility. In this case, one might expect that multiple bands would collapse into a single band after generating a homogeneous unphosphorylated kinase species. Figure 2.2A (lane 2) shows that after treatment of both purified His₆-PDK1(ΔPH) and His₆-PDK1 with λ.PP for 40 min, no Ser-241 phosphorylation could be detected by Western blotting with α-pS241. However, two distinct bands with slightly faster mobilities (as observed by coomassie staining) continued to be observed for λ.PP-treated unphosphorylated His₆-PDK1, and were detectable by Western analysis with general PDK1 antibody (α-PDK1).

Since both His₆-PDK1(ΔPH) and His₆-PDK1 were purified from the Sf9 insect cells with significant Ser-241 phosphorylation, it seemed reasonable that both constructs should have the ability to catalyze Ser-241 phosphorylation. In order to test this possibility, 5 µM of either λ.PP-treated unphosphorylated His₆-PDK1(ΔPH) or λ.PP-treated unphosphorylated His₆-PDK1 were incubated with 100 µM ATP and 10 mM Mg²⁺ for 5 min. Most surprisingly, Western blotting with α-pS241 showed that His₆-PDK1(ΔPH) catalyzed no detectable amount of Ser-241 autophosphorylation, whereas full length His₆-PDK1 readily catalyzed Ser-241 autophosphorylation (Figure 2.2A, lane 3). Thus, the PH domain appears to be required to catalytically activate the unphosphorylated PDK1 kinase domain (Figure 2.2B). In addition, the detected amounts of Ser-241 phosphorylated His₆-PDK1(ΔPH) purified from Sf9 insect cells likely resulted from trans-phosphorylation by either endogenous active PDK1 or some unidentified Ser-241 kinase. In final consideration, a similar distribution of the slightly faster and
slower migrating species of in vitro Ser-241 autophosphorylated full length His$_6$-PDK1 was detected by Western blotting with both $\alpha$-pS241 and $\alpha$-PDK1 (Figure 2.2A, lane 3). Thus, one is led to speculate that the presence of the double band character of the full length His$_6$-PDK1 is due to covalent modification, possibly other than phosphorylation, of side chain residues within the linker region or the PH domain. Interestingly, this is observed when full length PDK1 is expressed in a eukaryotic system, but not when bacterially expressed.

In general, protein kinases may catalyze autophosphorylation by either of two processes. In one case, an enzyme molecule binds ATP and catalyzes intramolecular (or cis) transfer of the terminal phosphate from ATP to a residue on the same enzyme molecule. Alternatively, one enzyme molecule binds ATP and catalyzes intermolecular (or trans) transfer of the terminal phosphate from ATP to a residue on a different enzyme molecule. In order to distinguish between these two mechanisms, two different mutant His$_6$-PDK1 constructs (S241G and K111A) were (i) purified from Sf9 insect cells, (ii) treated with $\lambda$PP, and (iii) tested for their ability to undergo Ser-241 autophosphorylation, either independently or when mixed together (Figure 2.2C). Mutation of Ser-241 prevents His$_6$-PDK1 from behaving as a protein substrate for phosphorylation; whereas mutation of the strictly conserved catalytic Lys-111 prevents His$_6$-PDK1 from behaving as an active kinase enzyme (Figure 2.2D).

Figure 2.2C (lane 1) shows SDS-PAGE and Western analysis of the S241G and K111A mutants of full length His$_6$-PDK1 after purification from Sf9 insect cells. As expected, no signal was detected for the S241G mutant by
Western blotting with $\alpha$-pS241, since it is incapable of undergoing phosphorylation at Ser-241. The observation that Ser-241 phosphorylation was detected in the purified K111A mutant suggests that it either (i) retained autophosphorylation capability or (ii) was trans-phosphorylated by either endogenous active PDK1 or some unidentified Ser-241 kinase in the Sf9 insect cells. Similar to wild type His$_6$-PDK1, a near equal distribution of the slightly faster and slower migrating species was detected for both the S241G and K111A mutants by Western blotting with $\alpha$-PDK1 (Figure 2.2C, lane 1).

Figure 2.2C (lane 2) shows that after treatment of both the S241G and K111A mutants of full length His$_6$-PDK1 with $\lambda$PP for 40 min, little change was observed in either the relative distribution or mobility of the species detected by Western blotting with $\alpha$-PDK1. Nevertheless, $\lambda$PP treatment effectively removed phosphate from Ser-241 of the K111A mutant, as detected by Western blotting with $\alpha$-pS241. Figure 2.2C (lane 3) shows that upon incubation of the $\lambda$PP-treated K111A mutant with 100 $\mu$M ATP and 10 mM Mg$^{2+}$ for 5 min, no Ser-241 autophosphorylation was detected by Western blotting with $\alpha$-pS241. Here, it can be concluded that the K111A mutant of full length His$_6$-PDK1 (i) does not have in vitro autophosphorylation activity and (ii) was trans-phosphorylated at Ser-241 during expression and purification from Sf9 insect cells. Most revealing was the observation that the $\lambda$PP-treated K111A mutant underwent Ser-241 phosphorylation upon addition of the $\lambda$PP-treated unphosphorylated S241G mutant. Overall, it can be concluded that the PH domain in full length unphosphorylated His$_6$-PDK1 (e.g., S241G) somehow stabilizes an active kinase
domain conformation, which presumably resembles that provided by T-loop Ser-241 phosphorylation and the phosphorylated hydrophobic motif of PDK1 substrates (Figure 2.2D).

2.3.2. MACROMOLECULAR DOCKING OF PH AND KINASE DOMAINS OF PDK1

In order to gain initial insight as to how the PH domain might interact with and stabilize an active PDK1 kinase domain conformation, macromolecular computational docking studies were carried out. Using the automated DOT 2.0 software, the X-ray structure of the catalytic kinase domain of PDK1 (1H1W) was docked with the X-ray structure of its C-terminal regulatory PH domain (1W1H). In order to do so, the X-ray structure of the catalytic kinase domain required insertion of the $^{233}\text{ESKQ}^{236}$ peptide sequence into the T-loop region that was missing in the structure. To better approximate interactions of the PH domain with the unphosphorylated inactive form the kinase, the phosphate group from Ser-241 in the T-loop was removed, as well as bound ATP. Using these two structures, the electrostatic and steric properties of both molecules were mapped to grids, and a systematic rigid-body translational and rotational search was performed. Using correlation functions calculated from the sum of electrostatic and van der Waals terms, a ranked list of lowest energy fits was obtained, yielding the cluster of docked structures shown in Figure 2.3A. Using the NAMD molecular dynamics software, structures were solvated and energy minimized to achieve the proposed complex formed between the regulatory PH and catalytic kinase domains of PDK1 (Figure 2.3B).
Figure 2.3: Computational modeling of the complex between the regulatory PH and catalytic kinase domains of PDK1. (A) Side view of the kinase domain (gray chain) showing the best fitted cluster of PH domains (seven multi-colored chains) that converged on docking the X-ray structure of the PH domain (residues 408–556) onto the X-ray structure of the kinase (residues 51–359), which was obtained using the automated molecular docking DOT 2.0 software. The view is parallel down the N-lobe αC-helix (center), which supports the largely β-sheet structure of the N-lobe (above) and distinguishes these residues from the larger mostly α-helical C-lobe (below). Unphosphorylated Ser-241 in the T-loop is indicated by the yellow stick. (B) The same side view of the kinase domain (gray chain) showing an energy minimized structure of the PH domain in the complex, which was obtained using the NAMD molecular dynamics software. The N-bud region (blue) and the β-sandwich core fold (green) of the PH domain are indicated. Residues important for binding the membrane-bound PI(3,4,5)P₃ second messenger are shown as yellow sticks at the very top of the β-sandwich core fold of the PH domain (K465, K467, R472, R474, Y486, K495, and R521). The area in the black box highlights important catalytic residues that are described in the next two panels. (C) Close view of active site residues in the X-ray structure of the catalytic kinase domain of PDK1 (1H1W). The lower dashed oval indicates salt bridge interactions between the S241 phosphate in the T-loop to Y126, R129, R204, and K228, which partially stabilize the active kinase conformation. The upper dashed oval indicates salt bridge interactions between a sulfate anion to K115, R131, and Q150. These residues are known to bind the phosphorylated residue in the hydrophobic motif of PDK1 protein kinase substrates, which synergizes with pS241 to yield fully activated PDK1 kinase conformation. (D) Close view of active site residues in the docked structure of the catalytic kinase domain of PDK1 with its PH domain. The lower dashed oval indicates salt bridge interactions coming from E432 of the PH domain, which mimic those of the S241 phosphate shown in panel C. The upper dashed oval indicates salt bridge interactions coming from E453 of the PH domain, which mimic those of the sulphate shown in panel C.
Closer inspection of this first approximated complex (Figure 2.3B) already reveals a number of new potential insights regarding the molecular determinants that can account for how the PH domain stabilizes an active kinase conformation in unphosphorylated PDK1. Figures 2.3C and 2.3D compare the active site architectures of the T-loop S241 phosphorylated active PDK1 kinase domain (1W1H) and the unphosphorylated kinase domain docked with the PH domain, respectively. Of foremost interest is the observation that two Glu residues of the PH domain (E432 and E453) make salt bridge interactions with residues of the kinase domain, and seem to mimic the synergistic activating effects given by T-loop Ser-241 phosphorylation (E432) and the phosphorylated hydrophobic motif of PDK1 substrates (E453).

2.3.3. EFFECT OF GLU-432 AND GLU-453 ON SER-241 AUTOPHOSPHORYLATION

If the proposed salt bridge interactions provided by Glu-432 and Glu-453 of the PH domain mimic those provided by the synergistic activating effects of T-loop Ser-241 phosphorylation and the phosphorylated hydrophobic motif of PDK1 substrates, then mutagenesis of these residues should destabilize the active kinase conformation and impair autophosphorylation. Figure 2.4A shows SDS-PAGE and Western blotting with both α-pS241 and α-PDK1 of the E432K and E453K mutants of full length His<sub>6</sub>-PDK1 after (i) purification from Sf9 insect cells (lane 1), (ii) treatment with λ.PP (lane 2), and (iii) 5 min after addition of ATP-Mg<sup>2+</sup> (lane 3). Although α-pS241 detection of Ser-241 phosphorylation of both the E432K and E453K mutants purified from Sf9 insect cells (Figure 2.4A, lane 1)
were nearly comparable to wild type His6-PDK1 (Figure 2.4A, lane 1), Ser-241 autophosphorylation after 5 min was visibly impaired for both mutants (Figure 2.4A, lane 3).

**Figure 2.4: Effect of Glu-432 and Glu-453 on Ser-241 autophosphorylation.** (A) SDS-PAGE and Western blotting with α-pS241 and α-PDK1 of the S241G and K111A mutants of full length His6-PDK1. Lane 1 shows the enzymes after purification from Sf9 insect cells; lane 2 shows the enzymes after removal of phosphates by treatment with λPP; and lane 3 shows the enzymes after incubation with 100 µM ATP and 10 mM MgCl2 for 5 min. (B) Time progress curves for Ser-241 autophosphorylation catalyzed by either the wild type, E432K mutant (●), or E453K mutant (●) construct of His6-PDK1. In each case, 5 µM enzyme was incubated with 100 µM [γ-32P]ATP and 10 mM MgCl2. First-order reaction kinetics were observed, and the bars indicate SE.

In order to better quantify the degree that Glu-432 and Glu-453 contribute towards activating Ser-241 autophosphorylation in PDK1, 32P-radiometric assays were employed. Figure 2.4B shows progress curves for Ser-241 autophosphorylation catalyzed by wild type, E432K, and E453K His6-PDK1. In each case, 5 µM of λPP-treated unphosphorylated kinase was mixed with 100 µM of [γ-32P]ATP in kinase reaction buffer at 30 °C. In all cases, molar 32P-incorporation into the given His6-PDK1 construct increased in a single exponential manner, yielding observed first order rate constants (kobs) of (120 ±
10) \times 10^{-3} \text{ s}^{-1}, (4.3 \pm 0.6) \times 10^{-3} \text{ s}^{-1}, \text{ and } (3.1 \pm 0.5) \times 10^{-3} \text{ s}^{-1} \text{ for wild type, E432K, and E453K, respectively. The molar limits of } ^{32}\text{P-}{}\text{incorporation were } 4.9 \pm 0.1 \mu\text{M, 4.7} \pm 0.2 \mu\text{M, and 4.4} \pm 0.3 \mu\text{M for wild type, E432K, and E453K, respectively. Given that 5 } \mu\text{M total enzyme was used in each case, approximately 0.9 to 1 mole of phosphate was incorporated per mole of each His}_6\text{-PDK1 construct, indicating site-specific Ser-241 autophosphorylation. Thus, it can be concluded that the E432K and E453K mutations decreased the wild type PDK1 Ser-241 autophosphorylation rate by 28-fold and 39-fold, respectively.}

Inspection of the modeled complex (Figure 2.3B) shows solvent exposed Lys-512 to be diametrically opposed to the proposed interface between the PH and kinase domains. A control experiment showed that the K512E mutant of His}_6\text{-PDK1 catalyzed Ser-241 autophosphorylation comparable to wild type His}_6\text{-PDK1 [}\text{ }k_{\text{obs}} = (120 \pm 10) \times 10^{-3} \text{ s}^{-1}, \text{ and 1 mole phosphate per enzyme}], consistent with the proposed model for autoactivation by Glu-432 and Glu-453.

2.3.4. CHEMICAL CROSS LINKING OF PH AND KINASE DOMAINS OF PDK1

The possibility of the critical salt bridge interactions between Glu residues of the PH domain and cationic amines of the kinase domain was further probed by testing whether the individual PH and kinase domains of PDK1 could be chemically cross linked using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC). EDC is a zero-length cross linking agent used to couple carboxyl groups to primary amines. EDC reacts with a carboxyl to form an amine-reactive O-acylisourea intermediate. If this intermediate does not encounter an
amine, it will hydrolyze and regenerate the carboxyl group. In the presence of \(N\)-hydroxysulfosuccinimide (sulfo-NHS), EDC can be used to convert carboxyl groups to amine-reactive sulfo-NHS esters. The sulfo-NHS group can then be substituted by a nearby amine group to yield an amide bond.

**Figure 2.5** shows SDS-PAGE analysis of purified (i) recombinant regulatory PH (residues 408–556) and (ii) purified \(\lambda\)PP-treated unphosphorylated kinase domain of PDK1 (residues 51–356) before and after treatment with EDC and sulfo-NHS. Both Coomassie (**Figure 2.5A**) and silverSNAP staining (**Figure 2.5B**) were used to detect protein species after cross linking reactions. In control

![Figure 2.5: Chemical cross linking of purified recombinant PH and kinase domains of PDK1. (A) Coomassie and (B) silverSNAP SDS-PAGE staining after treatment of 10 \(\mu\)M PH and kinase domain samples with EDC and sulfo-NHS. The PH domain in the absence (lane 1) and presence (lane 2), the kinase domain in the absence (lane 3) and presence (lane 4), and the mixture of PH and kinase domains in the absence (lane 5) and presence (lane 6) of EDC and sulfo-NHS.](image-url)
reactions, treatment of either the isolated PH domain (lanes 1 and 2, $M_r = 17$ kDa) or $\lambda$PP-treated kinase domain (lanes 3 and 4, $M_r = 39$ kDa) alone did not produce any detectable amounts of protein species with relative mobilities ($M_r$) corresponding to cross linked multimers of these domains. After treatment of an equivalent mixture of the two domains (10 $\mu$M), silverSNAP staining detected a small but significant amount of cross linked PH-kinase domains ($M_r = 56$ kDa) (lane 6). Thus, the EDC cross linking experiments confirm the presence of one or more salt bridge interactions between carboxylate and amine groups, which exist between the regulatory PH and catalytic kinase domains of PDK1.

2.4. Discussion

The computationally modeled complex of the PDK1 kinase domain with its PH domain (Figure 2.3D) is well suited to serve as the initial model for understanding how the PH domain activates T-loop Ser-241 autophosphorylation. For instance, the Glu-432 carboxylate of the N-bud $\alpha$-helix of the PH domain occupies the region of the Ser-241 phosphate (T-loop) in active PDK1, making salt bridge contacts with Arg-129 and Tyr-126 of the $\alpha$C-helix and Arg-204 of the RD motif. In addition, Glu-453 carboxylate of the N-bud occupies the region used by the phosphorylated hydrophobic motif of PDK1 substrates, interacting with Lys-115, Arg-131, and Gln-150. The geometrical positioning of Glu-432 and Glu-453 in the N-bud of the PH domain would serve as dual phosphomimics for activating Ser-241 autophosphorylation by unphosphorylated PDK1. In addition, the Ser-241 (T-loop) hydroxyl would be displaced and
accessible for autophosphorylation. Suitability of the modeled complex is again demonstrated, since the PI(3,4,5)P₃ binding pocket of the PH domain is clearly accessible.

**Figure 2.6** shows an amino acid sequence alignment of the kinase domain of PDK1 with those of its AGC kinase family members for which X-ray structures have been determined for the kinase domain in T-loop phosphorylated form (**Figure 2.7**, PKA, PKBβ, PKCθ, and PKCι). Like all protein kinases, the catalytic domains are composed of an N-terminal lobe consisting mainly of β-sheet and a predominantly α-helical C-terminal lobe. The ATP binding site is located between the two lobes. In addition, phosphorylation of a critical residue in the T-loop is required. The AGC protein kinases are unique in that they contain a C-terminal hydrophobic motif peptide region, and phosphorylation of a critical residue or placement of a negatively charged residue in an analogous site is also required for kinase activity.

**Figures 2.6 and 2.7** further illustrate how T-loop phosphorylation synergizes with hydrophobic motif phosphorylation to organize a constellation of catalytic residues important for kinase activity. The phosphorylated residue of the T-loop interacts with (i) a conserved Lys located near the beginning of the T-loop and (ii) a conserved Arg located in the β6-β7 loop. Coordination of the Lys serves to position an Asp in the “DFG motif”, which is critical to Mg²⁺ binding. Coordination of the Arg serves to position the adjacent Asp, and this “RD motif”, is used to orient the incoming residue side chain hydroxyl of protein substrate. In addition, the phosphorylated residue of the T-loop is found to interact with a ARG
Figure 2.6: Structure-based amino acid sequence alignment of PDK1, PKA, PKBβ, PKCθ, and PKCι. Residues comprising common secondary structure elements are indicated as cylindrical rods for helices (orange) and arrows for β-strands (green). Positions of the T-loop (red), turn motif (purple), and hydrophobic motif (HM, blue) are marked. Phosphorylation sites in the T-loop (●), turn motif (●), and HM (●) are indicated. Residues that coordinate the phosphorylated residue in the T-loop (●) and HM (●) are indicated. Conserved hydrophobic residues in the N-lobe that make contacts with HM residues are indicated (●). Black arrows point to catalytic residues and originate from residues that coordinate phosphate in either the T-loop or HM.
Figure 2.7: X-ray structures of T-loop phosphorylated kinase domains of AGC family members. The catalytic kinase domains are shown for (A) PKA with bound ATP and Mn\(^{2+}\) (1ATP), (B) PKB\(\beta\) with bound AMPPNP and Mg\(^{2+}\) (1O6K), (C) PKC\(\theta\) with bound staurosporine inhibitor (1XJD), and (D) PKC\(\iota\) with bound BIM1 inhibitor (1ZRZ). All views are aligned so as to view down the \(\alpha\)C-helix. The T-loop phosphorylated residue and the phosphorylated or negatively charged group in the hydrophobic motif (HM) are indicated. Residues that coordinate these groups serve to stabilize and position the \(\alpha\)C-helix so as to organize catalytic residues in the active site as described in the text.
residue of the αC-helix. Although the identity and/or exact position of the αC-helical residue are not conserved, it serves the common purpose of anchoring the αC-helix so that the strictly conserved αC-helical Glu makes a salt bridge interaction with Lys in the β3-strand. This strictly conserved catalytic Lys orients the phosphate and stabilizes the transition state for phosphoryl transfer. Mutation of this strictly conserved Lys renders any given protein kinase completely inactive, and it is this Lys that is mutated to generate “kinase-dead” mutants. The phosphorylated or negatively charged residue of the C-terminal hydrophobic motif is found to wind from the lower C-lobe to ultimately interact with a cluster of basic residues adjacent to a hydrophobic pocket in the rear of the upper N-lobe. This extensive network of hydrophobic and salt bridge interactions again serves the purpose of orienting and stabilizing the αC-helix, enabling proper positioning of the catalytic Lys. In X-ray structures of either unphosphorylated or T-loop monophosphorylated AGC kinases (e.g., PKBβ, S6K1, and RSK1), large segments of the αC-helix and T-loop were found to be highly disordered, and the N-lobe was significantly displaced from the C-lobe.

From the results of these studies, it was of further interest to devise experimental methods to test more rigorously the proposed structural roles of Glu-432 and Glu-453. Thus, Chapter 3 describes a new method for protein trans-splicing of the regulatory PH and catalytic kinase domains of PDK1; and Chapter 4 describes NMR studies of segmental isotopic labeled PH domain in full length PDK1, as well as the isolated PH domain.
CHAPTER 3
PROTEIN TRANS-SPLICING OF THE REGULATORY PH AND CATALYTIC KINASE DOMAINS OF PDK1

3.1. Background and Significance

Protein kinases comprise the largest enzyme family, with ~518 being encoded by the human genome (Manning et al., 2002b). The large number of serine-threonine protein kinases (~80% of total protein kinases) reflects the large number of intracellular signal transduction pathways required in regulating proper cellular growth, survival, and proliferation. The complexity in elucidating intracellular signaling networks is compounded by analyses indicating that individual protein kinases may target and phosphorylate numerous different protein substrates (Manning and Cantley, 2002; Obenauer and Yaffe, 2004). Further complexities arise in the observation that individual protein kinases can be intricately regulated by multiple phosphorylation events catalyzed by numerous upstream kinases, as well as auto-catalytically. On review of protein kinase sequence alignments, it becomes readily apparent that signaling complexity can be largely accounted for by a distinguishingly diverse arrangement of regulatory domains, which may exist contiguously N- or C-terminal to an otherwise structurally homologous catalytic kinase domain (Neidner et al., 2006). Often such domains may serve in proper localization, but many have been shown to play direct roles in regulating kinase activity either intrinsically or through participation in docking-based substrate recognition.
While the functions of various regulatory domains of different protein kinases are steadily being recognized, progress towards molecular understanding of conformational interactions within full length multi-domain kinases remains hampered. To date, very few three-dimensional structures have been reported for full length, monomeric, multi-domain serine-threonine protein kinases. Rather, for example, many X-ray structures have been reported for the isolated catalytic kinase domains of multi-domain AGC kinases including PDK1 (Biondi et al., 2002), PKBβ (Yang et al., 2002a; Yang et al., 2002b), PKCθ (Xu et al., 2004), and PKCτ (Messerschmidt et al., 2005); multi-domain MAP kinases including B-RAF (Wan et al., 2004), MEK1,2 (Ohren et al., 2004), MK2 (Underwood et al., 2003), and MSK1 (Smith et al., 2004); and multi-domain cell-cycle related kinases including CHK1 (Zhang et al., 2002), Aurora A (Cheetham et al., 2002), and Aurora B (Sessa et al., 2005). Likewise, X-ray or NMR structures have been reported for corresponding isolated regulatory domain constructs including the PH domains of PDK1 (Komander et al., 2004), PKBα (Thomas et al., 2002), and PKBβ (Auguin et al., 2004); the C1 domain of PKCγ (Xu et al., 1997); the C2 domains of PKCα (Ochoa et al., 2002; Ochoa et al., 2003), PKCβ (Sutton and Sprang, 1998), PKCδ (Pappa et al., 1998; Benes et al., 2005), and PKCε (Ochoa et al., 2001); the CR1 domain of RAF1 (Mott et al., 1996); the FHA domain of CHK2 (Li et al., 2002); and the PBD domain of PLK1 (Cheng et al., 2003; Elia et al., 2003). The observation that very few suitable crystals for X-ray diffraction studies of full length multi-domain monomeric protein kinases have been obtained suggests an important role for intra-domain-domain
conformational dynamics. Thus alternative methodologies will be required for advanced understanding of such processes.

One very promising approach towards better understanding of conformational interactions within full length multi-domain kinases involves the use of intein-based technologies to generate a native peptide bond between given pairs of contiguous regulatory and catalytic kinase domains (Muralidharan and Muir, 2006; Xu and Evans, 2005; Muir, 2003; Hofmann and Muir, 2002). Successful peptide conjugation would facilitate domain-specific incorporation of biophysical probes (e.g., fluorophores and paramagnetic spin labels) and stable isotopes (e.g., $^{15}$N and $^{13}$C), enabling spectroscopic characterization of intra-domain-domain conformational dynamics in solution. Whereas a large number of small C-terminal peptides have been successfully ligated to their cognate proteins, few cases have been reported in which two large functional domains have been conjugated to reconstitute regulatory function in a monomeric multi-domain native enzyme (Hofmann and Muir, 2002). To begin addressing the possibility that intein-based technologies may be used to reconstitute a monomeric multi-domain protein kinase for spectroscopic functional studies, we selected the human serine-threonine AGC protein kinase, PDK1, for which X-ray structures are known for both the N-terminal catalytic kinase (Biondi et al., 2002) and the C-terminal PH domains (Komander et al., 2004). The C-terminal PH domain is required for PI(3,4,5)P$_3$-dependent co-localization and phosphorylation of PKB (Vanhaesebroeck and Alessi, 2000).
In this chapter, we report efficient protein trans-splicing of the regulatory PH and catalytic kinase domains of human PDK1 by using naturally split DnaE inteins from two different organisms. In addition, spliced-PDK1 is shown to retain the ability to co-localize and selectively phosphorylate Thr-309 of PKBβ in a PI(3,4,5)P₃-dependent manner. The high-level production and reconstitution of functional spliced-PDK1 establishes the feasibility of generating full length PDK1 with either (i) segmental ¹⁵N-isotopic labeling of the PH domain for NMR studies or (ii) simultaneous site-directed modifications of the PH and kinase domains with small molecule donor and acceptor fluorophores for FRET-based studies of conformational transitions related to docking-based catalytic specificity.

3.2. Experimental Procedures

3.2.1. MATERIALS

PDK1-Tide (acetyl-KTFCTGTPEYLAPEVRPREPLREPRILSEEEQEMFRDYIADWC) and Crosstide (acetyl-GRPRTSSFAEG) were from 21st Century Biochemicals, Inc. (Marlboro, MA). [γ-³²P]ATP was from MP-Biomedical (Irvine, CA). 1,2-dioleoyl-sn-glycerol-3-phosphocholine (PC), 1,2-dioleoyl-sn-glycerol-3-phospho-L-serine (PS), and sn-1-stearoyl-2-arachidonyl D-phosphatidylinositol-(3,4,5)-triphosphate [PI(3,4,5)P₃] were from Echelon Biosciences (Salt Lake City, UT). Full length His₆-PKBβ was expressed using the Bac-to-Bac® Baculovirus Expression System (Invitrogen, Carlsbad, CA) and His₆ affinity purified as described (Gao et al., 2005). Affinity purified His₆-PKBβ was fully
dephosphorylated by treatment with Lambda protein phosphatase (λPP) as described (Gao et al., 2005).

3.2.2. PDK1 TRANS-SPICING STRATEGY

Figure 3.1 shows the protein engineering strategy designed for trans-splicing of the regulatory PH and catalytic kinase domains of human PDK1. This strategy derives from recent reporting of the highly efficient in vivo cross trans-splicing reaction catalyzed between the N-terminal DnaE intein from *Nostoc punctiforme* [(N)NpuDnaE] and the C-terminal DnaE intein from *Synechocystis* sp. strain PCC6803 [(C)SspDnaE] (Iwai et al., 2006). One additional advantage of utilizing (N)NpuDnaE is that it has been C-terminal side of the splice junction (Iwai et al., 2006). Since three-dimensional structures have been reported for the N-terminal catalytic kinase domain (residues 51–359) (Biondi et al., 2002) and the C-terminal regulatory PH domain (residues 409–556) (Komander et al., 2004), C385 was chosen as the optimal site of conjugation. In utilizing the (N)NpuDnaE and (C)SspDnaE cross trans-splicing intein pair, 382QFG384 at the N-terminal side of the splice junction was replaced with 382AEY384. In addition, a conservative Q387N mutation gave 385CMN387 residues at the C-terminal side of the splice junction, which has been shown to be equally effective as the native CFN sequence required for trans-splicing with the native (N/C)SspDnaE pair (Iwai et al., 2006). In order to facilitate purification of spliced-PDK1 from cleaved and uncleaved DnaE intein containing fragments, His₆ affinity tags were joined to the corresponding DnaE inteins. Addition of GST significantly increased the overall
Figure 3.1: Protein trans-splicing of PDK1. (A) Protein trans-splicing reactants. The N-terminal kinase domain of PDK1 (residues 51-381, black oval) is fused to the (N)NpuDnaE trans-splicing intein (gray square) with a C-terminal His6 affinity tag (open square). The N-terminal three residues flanking the splice junction (Q382, F383, and G384) were mutated to the preferred AEY sequence required in trans-splicing. The C-terminal PH domain of PDK1 (residues 388-556, black oval) is fused to the (C)SspDnaE trans-splicing intein (gray square) with an N-terminal GST-His6 tag (open square). Whereas the His6 tag was used for affinity purification, the GST tag significantly improved both expression yield and solution stability. For the native C-terminal three residues flanking the splice junction (C385, M386, and Q387), only Q387 was mutated to yield the preferred CMN sequence optimal for cross trans-splicing of the (C)SspDnaE intein with the (N)NpuDnaE intein. The (N)NpuDnaE and (C)SspDnaE split inteins associate with high affinity and catalyze trans-splicing of the regulatory PH and catalytic kinase domains by forming a native peptide bond between Tyr-384 and Cys-385. (B) Protein trans-splicing products. The primary domain arrangement of PDK1 is regenerated, which contains the Q382A, F383E, G384Y, and Q387N mutations that flank the splice junction. The affinity tagged trans-splicing inteins are indicated. Spliced-PDK1 is separated from the cleaved (N)NpuDnaE-His6 and GST-His6-(C)SspDnaE split inteins, as well as any uncleaved intein fusion constructs by His6 affinity purification. The calculated molecular weights (MW) of each trans-splicing reactant and product are indicated.
protein yield and solubility over that obtained by expression of the His$_6$-(C)SspDnaE-(CMN)PH construct.

3.2.3. Baculovirus-Mediated Expression of KINASE(AEY)-(N)SspDnaE-His$_6$

Experimental procedures describing PCR engineering to generate an EcoRI–XbaI cDNA fragment containing the KINASE(AEY)-(N)NpuDnaE-His$_6$ fusion construct are given as described (Appendix A2). A sequence verified EcoRI–XbaI fragment containing the KINASE(AEY)-(N)NpuDnaE-His$_6$ construct (Figure 3.1) was removed from the pCR$^®$-Blunt II-TOPO$^®$ cloning vector and ligated into pFastBac™1 vector to generate recombinant baculovirus using the Bac-to-Bac$^®$ Baculovirus Expression System (Invitrogen) (Appendix A3). Recombinant P2 viral stocks were used to infect 200 mL spinner flask cultures of Sf9 cells in the mid-logarithmic phase of growth ($2 \times 10^6$ cells/mL) at a multiplicity of infection (MOI) of two viral particles/cell. The infected cells were incubated at 27 °C for 72 h and harvested by centrifugation for 10 min at 4 °C at 3000 rpm in a Beckman tabletop centrifuge. The cells were re-suspended in 50 mL of buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 50 mM imidazole, 1 mM 2-mercaptoethanol, 1 mM benzamidine, and 0.2 mM PMSF), and were immediately stored at −80 °C. The re-suspended frozen cells were allowed to thaw on ice for 1 h, which resulted in efficient cell lysis. Cell debris was pelleted by centrifugation for 30 min at 4 °C at 12,000 rpm. The supernatants containing the soluble components of the cell lysate were collected, and the KINASE(AEY)-(N)NpuDnaE-His$_6$ construct was His$_6$-affinity purified from the soluble lysate as described below.
3.2.4. *E. coli* Expression of GST-His$_6$-(C)SspDnaE-(CMN)PH in Luria Broth

Experimental procedures describing PCR engineering to generate the *Sac*II–*Kpn*I fragment containing the (C)SspDnaE-(CMN)PH fusion construct are given as described (Appendix A1). A sequence verified *Sac*II–*Kpn*I fragment containing the (C)SspDnaE-(CMN)PH construct was removed from the pCR®-Blunt II-TOPO® cloning vector and ligated into the pET-41b protein expression vector (Novagen), which provided N-terminal dual GST-His$_6$ affinity tags to yield the GST-His$_6$-(C)SspDnaE-(CMN)PH construct (Figure 2.1). The protein expression vector was transformed into the Rosetta (DE3) protein expression strain of *E. coli*. Cells were grown in 1 L Luria Broth (LB) containing kanamycin (50 µg/mL) and chloramphenicol (37 µg/mL) at 37 °C until an OD of 0.4. Then the cell culture was transferred to 15 °C and grown until an OD of 0.8 before induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 16 h. The cells were harvested by centrifugation at 3500g for 20 min and then resuspended in 3 mL PBS buffer per gram cells (10 mM Na$_2$HPO$_4$ and 1.8 mM KH$_2$PO$_4$, pH 7.3, 140 mM NaCl, 2.7 mM KCl, 2 mM dithiothreitol, 1 mM benzamidine, and 0.2 mM PMSF). The cells were lysed using the EmulsiFlex®-C3 high pressure homogenizer (Avestin, Inc.), and particulates were removed from the lysate by centrifugation for 20 min at 14,000 rpm in the SS-34 Sorvall rotor. The GST-His$_6$-(C)SspDnaE-(CMN)PH construct was GST-affinity purified from the soluble lysate as described below.
The soluble Sf9 insect cell lysate containing the KINASE(AEY)-(N)NpuDnaE-His$_6$ recombinant fusion construct (Figure 3.1) was directly loaded by FPLC (1 mL/min) onto a 5 mL size Ni$^{2+}$ Sepharose HiTrap HP affinity column (Amersham) equilibrated at 4 °C in 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 50 mM imidazole, 1 mM 2-mercaptoethanol. The soluble E. coli lysate containing the GST-His$_6$-(C)SspDnaE-(CMN)PH recombinant fusion construct (Figure 3.1) was directly loaded by FPLC (1 mL/min) onto a 5 mL size GST HiTrap HP affinity column (Amersham) equilibrated at 4 °C in PBS buffer (10 mM Na$_2$HPO$_4$ and 1.8 mM KH$_2$PO$_4$, pH 7.3, 140 mM NaCl, 2.7 mM KCl, and 2 mM dithiothreitol). The columns were subsequently washed until the absorbances at 260 nm and 280 nm returned to baseline. The KINASE(AEY)-(N)NpuDnaE-His$_6$ construct was eluted by linear increasing of the imidazole concentration from 50 to 500 mM at 1 mL/min for 30 min. The GST-His$_6$-(C)SspDnaE-(CMN)PH construct was eluted with buffer containing 50 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, and 20 mM glutathione. Chromatographic fractions (1 mL) containing the designated fusion protein were detected by absorbance at 280 nm and analyzed by SDS-PAGE. Fractions containing recombinant protein were pooled and subjected to gel exclusion chromatography for exchange to the protein trans-splicing buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.5 mM TCEP, 0.5 mM EGTA, 1 mM EDTA, and 5% (v/v) glycerol). Protein was concentrated using a VivaSpin 20, 6, or 2 mL ultrafiltration concentrator (10 kDa molecular weight cutoff, VivaScience). Protein concentrations were determined by BioRad protein assay. Protease inhibitors
(0.5 mM benzamidine and 0.1 mM PMSF) were added to each purified protein construct. A ~40 µM stock solution of the KINASE(AEY)-(N)NpuDnaE-His₆ construct was prepared using ~2 mg/mL total protein with ≥95% purity. A ~40 µM stock solution of the GST-His₆-(C)SspDna-(CMN)PH construct was prepared using ~2.8 mg/mL total protein with ≥60% purity. These solutions were stored at −80 °C.

3.2.6. PDK1 TRANS-SPlicing

Frozen aliquots containing ~40 µM of either the KINASE(AEY)-(N)NpuDnaE-His₆ or the GST-His₆-(C)SspDna-(CMN)PH trans-splicing constructs were thawed at 21 °C. After 10 min microcentrifugation of each tube, the reaction was initiated by combining the clarified supernatants to yield ~20 µM of each construct. The reaction tube was incubated either at 4 °C or 21 °C, and at varying times (0, 1, 3, 6, 9, 12, and 18 h) 9 µL aliquots containing ~18−20 µg total protein were removed and quenched by addition to 9 µL of 2× SDS sample buffer, heated at 95 °C for 2 min, and placed on ice for SDS-PAGE analysis of trans-splicing progress. At the end of the time course, the reaction mixture was buffer exchanged by gel exclusion chromatography to 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 50 mM imidazole, and 1 mM 2-mercaptoethanol, the equilibration buffer for His₆ affinity chromatography. The cleaved (N)NpuDnaE-His₆ and GST-His₆-(C)SspDna products and any uncleaved KINASE(AEY)-(N)NpuDnaE-His₆ and GST-His₆-(C)SspDna-(CMN)PH reactants were removed by passage over a 5 mL size Ni²⁺ Sepharose HiTrap HP affinity column (Amersham). The column
was subsequently washed until the absorbances at 260 nm and 280 nm returned to baseline. Flow-through fractions containing spliced-PDK1 were desalted and directly loaded by FPLC (0.5 mL/min) onto a Tricorn™ Mono Q™ 5/50 GL anion exchange column (Amersham) equilibrated at 4 °C in Tris-HCl buffer, pH 7.5, containing 2 mM 2-mercaptoethanol. Upon linear increasing of the NaCl concentration from 0 to 1 M at 1 mL/min for 1 h, ≥95% purified spliced-PDK1 eluted between 0.1−0.2 M NaCl. Fractions containing purified spliced-PDK1 (~35 µM or ~2 mg/mL) were combined, and protease inhibitors (0.5 mM benzamidine and 0.1 mM PMSF) were added before storage of either 50 µL or 1 mL aliquots at −80 °C.

3.2.7. SDS-PAGE AND WESTERN ANALYSIS

Protein samples in SDS sample buffer were heated at 95 °C for 2 min and cooled on ice. Analytical SDS-PAGE was performed on 4−20% gradient polyacrylamide gels (GradiPore) developed at 150 V (constant) for 1 h or until the tracking dye reached the bottom of the slab. Coomassie staining was used to visualize total protein. Densitometric analysis was performed using the ImageQuant software (Molecular Dynamics). For Western analyses, protein was transferred from the gel to a Nitrocellulose Membrane Filter Paper Sandwich (Invitrogen) in a semidry blotting apparatus using 0.7% (v/v) acetic acid as transferring solution. According to the manufacturer's instructions, purified KINASE(AEY)-(N)NpuDnaE-His6 was probed with phospho-PDK1 (Ser241) polyclonal rabbit antibody (Cell Signaling Technology); and His6-PKBβ was
probed with either phospho-PKB (Thr-308) or phospho-PKB (Ser473) polyclonal rabbit antibodies (Cell Signaling Technology). Detection of all immuno-protein complexes was carried out using secondary anti-rabbit antibody conjugated to horseradish peroxidase (HRP) and LumiGLO® chemiluminescent reagent and peroxide.

3.2.8. KINETIC ASSAYS AND PHOSPHOPEPTIDE MAPPING

$V_{\text{max}}$ and $K_m$ values at 30 °C and percent inactivation over time of purified 20 μM KINASE(AEY)-(N)NpuDnaE-His$_6$ in protein trans-splicing buffer at 21 °C were measured by quantifying transfer of $^{32}$P-radiolabel from $[^\gamma-^{32}\text{P}]\text{ATP}$ to the model PDK1-Tide substrate as described (Gao et al., 2005). PI(3,4,5)P$_3$-dependent phosphorylation of His$_6$-PKBβ by spliced-PDK1 and measurement of the resulting increase in His$_6$-PKBβ-catalyzed transfer of $^{32}$P-radiolabel from $[^\gamma-^{32}\text{P}]\text{ATP}$ (100 μM) to the model Crosstide substrate (50 μM) was carried out as described (Gao et al., 2005). For both assays, one unit of activity was defined as the amount of enzyme required to catalyze phosphorylation of 1 nmol of peptide substrate in 1 min.

The ability of spliced-PDK1 to selectively phosphorylate Thr-309 in the activation loop of His$_6$-PKBβ was tested in a 1 mL reaction mixture containing 2 μM of each enzyme (60 μg or 1 nmol) in 50 mM Tris-HCl buffer, pH 7.5, 1 mM 2-mercaptoethanol, 10 mM MgCl$_2$, 0.2 mM sodium vanadate, and PC/PS/PI(3,4,5)P$_3$ vesicles. The reaction was initiated by addition of 100 μM $[^\gamma-^{32}\text{P}]\text{ATP}$ (~500 cpm/pmol) and allowed to proceed with continuous shaking at 30
°C for 40 min. The reaction was quenched by addition of urea to 6 M. Control assays were carried out in parallel in which either spliced-PDK1, PI(3,4,5)P₃ in the phospholipid vesicles, or both were omitted.

³²P-radiolabeled His₆-PKBβ was affinity-purified from the quenched reaction mixture by addition of 200 µL of Ni-NTA agarose resin and shaking overnight at room temperature. After low speed centrifugation, the supernatant was removed and the resin was washed twice with 1 mL of 50 mM Tris-HCl, pH 7.5, containing 6 M urea, 50 mM imidazole, 300 mM NaCl, and 1 mM 2-mercaptoethanol. ³²P-radiolabeled His₆-PKBβ was eluted from the Ni-NTA agarose resin pellet with 100 µL of 50 mM Tris-HCl, pH 7.5, containing 6 M urea, 500 mM imidazole, 300 mM NaCl, and 1 mM 2-mercaptoethanol; and approximately 40–50% of enzyme (400–500 pmol) was recovered in the 100 µL eluent. First, protein concentration was determined with the Bio-Rad protein assay kit, using 10 µL of the eluted enzyme. Second, the specific radioactivity of the ³²P-radiolabeled His₆-PKBβ construct (SAPKBβ, cpm/pmol) was determined from radioactivity detected by scintillation counting of the known amount of enzyme (~40–50 pmol) in 10 µL of the eluted enzyme. The mole fraction of total phosphorylated sites (fₘol.movtot) was calculated according to fₘol.movtot = SAPKBβ/SAATP.

To determine site-specific phosphorylation, the remaining amount (~80 µL with ~320–400 pmol) of affinity-purified His₆-PKBβ was used for trypsin digestion and HPLC resolution of ³²P-radiolabeled peptides. First, cysteine residues were reduced by addition of 0.5 mL of 50 mM Tris-HCl, pH 7.5, containing 20 mM 2-mercaptopethanol. Free cysteines were protected from re-oxidation by subsequent
addition of 50 µL of 500 mM iodoacetamide incubation in the dark at room temperature for 20 min. Excess unreacted iodoacetamide was depleted from the reaction mixture by further addition of 30 µL of 500 mM 2-mercaptoethanol and incubation at room temperature for 5 min. Proteolytic digestion was carried out by addition of 1 µg trypsin and incubation at 37 °C for 5 h, followed by subsequent addition of 1 µg trypsin and incubation at 37 °C overnight. The individual reaction mixtures (~200 µL) containing digested ^32^P-radiolabeled His<sub>6</sub>-PKBβ constructs were diluted to 1 mL with solvent A [0.1% (v/v) trifluoroacetic acid in water]. The 1 mL samples were directly loaded by HPLC (0.5 mL/min) onto a µRPC C2/C18 column (Amersham) equilibrated in 100% solvent A. The column was subsequently washed in this buffer for 5 min, and the peptides were eluted by linear increasing solvent B [80% acetonitrile and 0.07% (v/v) trifluoroacetic acid in water] from 0% to 50% in 1 h at a flow rate of 0.4 mL/min, while 300 µL were collected for each fraction. ^32^P-radiolabeled peptides were detected by scintillation counting of 30 µL of each chromatographic fraction.

The identity of the ^32^P-radiolabeled peptide was confirmed by MALDI-TOF mass spectrometric analysis. The 300 µL fraction containing the ^32^P-radiolabeled peptide was evaporated to dryness and resuspended in a small volume (10–30 µL) of 50% acetonitrile to yield a ^32^P-peptide concentration of 10 pmol/µL, as indicated by the amount of radioactivity; 1 µL of saturated 2,5-dihydroxybenzoic acid (2,5-DHB) was added to 1 µL of the concentrated ^32^P-peptide, and 1 µL of this mixture containing ~5 pmol of the ^32^P-peptide was placed on a stainless steel MALDI-TOF target plate and allowed to dry. MALDI-TOF mass spectra were
acquired on a Biflex IV MALDI-TOF mass spectrometer (Bruker Daltronics) in either linear (larger peptides) or reflectron (smaller peptides) mode. A N₂ laser was used to desorb/ionize the matrix/analyte material. Calibration was performed using angiotensin II (monoisotopic mass [MH+] 1046.5423 Da), angiotensin I (monoisotopic mass [MH+] 1296.6900 Da), bombesin (monoisotopic mass [MH+] 1619.8229 Da), and adrenocorticotrophic hormone clip 18–39 (monoisotopic mass [MH+] 2465.2027 Da) (Sigma-Aldrich, St. Louis, MO).

3.3. Results and Discussion

3.3.1. Development of PDK1 Cross Trans-Splicing Strategy

It has been underreported but overestablished that a majority of human protein kinases are poorly expressed and purified as inclusion bodies from both bacterial and yeast protein expression strains under a wide variety of growth and induction conditions. In addition, such kinase preparations have proven difficult to re-solubilize into stable and active conformations. To date, baculovirus-mediated protein expression in either Sf9 or Sf21 insect cells has been the most effective method for generating high levels of soluble and active human protein kinases (Gao et al., 2005). Although the reason is not entirely clear, we observed that high production of the catalytic kinase domain constructs of PDK1, S6K1, and PKBβ, typically achieved by baculovirus-mediated expression in Sf9 insect cells, was severely attenuated when these constructs were fused with a C-terminal active intein (e.g., Mxe GyrA or VMA inteins available from New England Biolabs, Inc.) (unpublished results). In addition, the small amounts of expressed fusion
protein (≤0.5 mg/200 mL Sf9 insect cell culture) were observed to be ≥80% hydrolyzed. Therefore, only very small quantities of N-kinase-intein fusion protein (≤0.1 mg/200 mL Sf9 insect cell culture) were available for thiolysis of the intein to generate the C-terminal thioester required for chemical ligation to the N-terminal cysteine residue of a C-terminal peptide fragment. Thus, we reasoned that high-level baculovirus-mediated expression in Sf9 insect cells of human catalytic kinase domains for engineered ligation could be better achieved if fused to an inactive intein fragment of a protein trans-splicing intein pair.

To date, the naturally occurring split Ssp DnaE intein (Evans et al., 2000; Martin et al., 2001; Nichols and Evans, 2004) and the artificially split Ssp DnaB and Sce VMA inteins (Brenzel et al., 2006; Ludwig et al., 2006) have been best characterized with regard to achieving protein trans-splicing activity in the absence of protein refolding routines typically required of artificially split inteins. However in all these cases, protein trans-splicing under native conditions has been compromised by competing cleavage of the protein trans-splicing reactants upon mixing, resulting in typical yields of only 40–50% spliced protein product. Encouragingly, highly efficient protein trans-splicing (≥98%) of two B1 domains of the IgG binding protein (GB1) was recently demonstrated when the His<sub>6</sub>-GB1-(N)NpuDnaE and (C)SspDnaE-GB1 cross reacting trans-splicing constructs were co-expressed in *E. coli* (Iwai et al., 2006). Since it is difficult to access contributions of endogenous chaperones and the reducing environment to the observed high splicing efficiency in *E. coli*, it became of immediate interest
whether this highly efficient in vivo 'cross' trans-splicing strategy would translate to in vitro conditions.

3.3.2. PREPARATION OF CROSS TRANS-SPlicing CONSTRUCTS

The KINASE(AEY)-(N)NpuDnaE-His6 fusion construct of PDK1 (Figure 3.1) was generated by PCR and subcloned into the pFastBac™1 vector for generation of recombinant baculovirus using the Bac-to-Bac® Baculovirus Expression System (Invitrogen). Using MOI = 2 and a time of harvest 72 h after post infection, KINASE(AEY)-(N)NpuDnaE-His6 was overexpressed and His6 affinity purified, typically yielding 10 ± 2 mg from initial infection of 4 × 10^8 total Sf9 insect cells (Table 3.1). Figure 3.2A shows that KINASE(AEY)-(N)NpuDnaE-His6 was efficiently purified to ≥95% homogeneity (lane 3), as judged by Coomassie blue staining of 4–20% SDS-PAGE. Purified KINASE(AEY)-(N)NpuDnaE-His6 migrates with an apparent molecular weight of 51 kDa, which agrees with its calculated molecular mass of 50,802 Da. Similar to the recombinant catalytic domain construct of PDK1 [His6-PDK1(ΔPH), residues 51–359] (Biondi et al., 2002), Western analysis showed that the KINASE(AEY)-(N)NpuDnaE-His6 construct was purified in its Ser-241 phosphorylated and catalytically active form (Figure 3.2A, lower panel). The values of V_{max} = 200 ± 15 U/mg and \text{Tide}K_m = 70 ± 10 \mu M measured KINASE(AEY)-(N)NpuDnaE-His6-catalyzed phosphorylation of the model PDK1-Tide substrate were in the same range as those measured for native His6-PDK1 (Gao et al., 2005).
Table 3.1: Purification of KINASE(AEY)-(N)$\text{NpuDnaE-His}_6$ from Sf9 insect cells$^a$

<table>
<thead>
<tr>
<th>Purification (Step)</th>
<th>Volume (mL)</th>
<th>Concentration (mg/mL)</th>
<th>Yield (mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude lysate</td>
<td>50</td>
<td>2.2 ± 0.2</td>
<td>110 ± 10</td>
<td>N/A</td>
</tr>
<tr>
<td>Ni Sepharose</td>
<td>10</td>
<td>1.0 ± 0.2</td>
<td>10 ± 2</td>
<td>11</td>
</tr>
</tbody>
</table>

$^a$All values are reported for purification from initial infection of 200 mL of insect Sf9 cells ($2 \times 10^6$ cells/L) with recombinant baculovirus.

Table 3.2: Purification of GST-$\text{His}_6$-(C)$\text{SspDnaE-(CMN)PH}$ from $E.\text{coli}$

<table>
<thead>
<tr>
<th>Purification (Step)</th>
<th>Volume (mL)</th>
<th>Concentration (mg/mL)</th>
<th>Yield (mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude lysate</td>
<td>25</td>
<td>14 ± 2</td>
<td>350 ± 50</td>
<td>N/A</td>
</tr>
<tr>
<td>Ni Sepharose</td>
<td>10</td>
<td>1.7 ± 0.3</td>
<td>17 ± 3</td>
<td>21</td>
</tr>
</tbody>
</table>

$^a$All values are reported for purification from expression in 1 L of $E.\text{coli}$.

Figure 3.2: SDS-PAGE analysis of purification of protein trans-splicing reactants. (A) KINASE(AEY)-(N)$\text{NpuDnaE-His}_6$ was His$_6$ affinity purified from Sf9 insect cell lysate. (B) GST-$\text{His}_6$-(C)$\text{SspDnaE-(CMN)PH}$ was GST affinity purified from $E.\text{coli}$ lysate. Lane 1 shows the total lysate; lane 2 shows protein species that were not retained by the affinity column; and lane 3 shows the affinity purified recombinant protein trans-splicing construct. Molecular weight markers are indicated. Proteins were visualized by Coomassie staining.
The (C)SspDnaE-(CMN)PH fusion construct was cloned into pET-41b vector downstream of a GST-His$_6$ tag, yielding the coding sequence for the GST-His$_6$-(C)SspDnaE-(CMN)PH fusion protein (Figure 3.1). Recombinant protein expression of cell cultures (OD of 0.8) were induced with 0.5 mM IPTG for 16 h at 15 °C. Cell lysate from 1 L culture was collected by homogenization, and GST affinity purification typically yielded 17 ± 3 mg total protein (Table 3.2). Figure 3.2B shows that the GST-His$_6$-(C)SspDnaE-(CMN)PH construct was purified to ~60% homogeneity (lane 3), as judged by Coomassie blue staining of 4–20% SDS-PAGE. Affinity purified GST-His$_6$-(C)SspDnaE-(CMN)PH showed anomalous slightly faster migration corresponding to an apparent molecular weight of 49 kDa compared with its calculated molecular mass of 51,214 Da. The ~40% impurities retained after GST affinity purification of GST-His$_6$-(C)SspDnaE-(CMN)PH from the *E. coli* are primarily composed of a mixture of four protein species that migrate with apparent molecular weights of approximately 24, 25, 26 and 37 kDa, which were efficiently removed in subsequent chromatographic steps used to purify spliced-PDK1 from the trans-splicing reaction (see below).

### 3.3.3. PDK1 CROSS TRANS-SPlicing KINETICS AND YIELD

Figure 3.3A shows the progress of protein trans-splicing that occurs over 12 h. The 10 mL protein trans-splicing reaction (21 °C) was initiated by combining 0.2 µmol of each of the protein trans-splicing reactants, KINASE(AEY)-(N)NpuDnaE-His$_6$ (species I) and GST-His$_6$-(C)SspDna-(CMN)PH (species II) (~20 µM each). SDS-PAGE with Coomassie staining shows
Figure 3.3: PDK1 trans-splicing. (A) Progress of PDK1 trans-splicing. Affinity purified KINASE(AEY)-(N)NpuDnaE-His$_6$ and GST-His$_6$-(C)SspDnaE-(CMN)PH protein trans-splicing reactants were mixed and allowed to react at 21 °C. Aliquots of the reaction mixture were removed and analyzed by SDS-PAGE, which indicates the distribution of reactants and products at varying times of 0 h (lane 1), 1 h (lane 2), 3 h (lane 3), 6 h (lane 4), 9 h (lane 5), and 12 h (lane 6). (B) Time courses for KINASE(AEY)-(N)NpuDnaE-His$_6$ inactivation (□) and yield of spliced-PDK1 (●) at 21 °C. (C) Preparative amounts of trans-splicing reactants were mixed (lane 1) and allowed to react at 21 °C for 18 h (lane 2). Lane 3 shows the protein species in the reaction mixture that were retained by His$_6$ affinity chromatography. Lane 4 shows purified spliced-PDK1 (≥95%), which was resolved by MonoQ ion exchange chromatography from trace amounts of protein species that were not retained by His$_6$ affinity chromatography.
decreasing band intensities corresponding to consumption of the trans-splicing reactants (species I and II) with a concomitant increasing band intensity corresponding to formation of full length spliced-PDK1 (species III). In addition, increasing band intensities were observed for formation of the cleaved affinity tagged intein constructs, GST-His<sub>6</sub>-(C)SspDna (species IV) and (N)<sup>Npu</sup>DnaE-His<sub>6</sub> (species V). Densitometric analysis of multiple reaction gels indicated that PDK1 trans-splicing follows apparent first-order kinetics with \( k_{\text{obs}} = (2.8 \pm 0.3) \times 10^{-5} \text{ s}^{-1} \) (Figure 3.3B). This rate is approximately 10-fold slower than the \( k_{\text{obs}} = (3.0 \pm 0.4) \times 10^{-5} \text{ s}^{-1} \) reported for trans-splicing of the maltose binding protein and thioredoxin utilizing the native (N/C)SspDnaE intein pair, which was also performed at room temperature (Nichols and Evans, 2004). However, the fit of the kinetic data (Figure 3.3B) also indicated that the cross trans-splicing strategy facilitates a limiting reaction extent approaching 90 ± 10%. This is corroborated by observation of the faint band intensity indicated for KINASE(AEY) (species VI ≤ 5%), which results from trans-cleavage or hydrolysis of KINASE(AEY)-(N)<sup>Npu</sup>DnaE-His<sub>6</sub>. The extent of the competing trans-cleavage reaction is significantly lower than those typically reported for the native (N/C)SspDna intein pair (Evans et al., 2000; Martin et al., 2001; Nichols and Evans, 2004). Disappointingly, the trans-splicing reaction was much less efficient when performed at 4 °C.

Although the cross trans-splicing reaction at 21 °C should approach 90% yield, it was not reasonable to allow the reaction to proceed to such limits, as a \( k_{\text{obs}} = (4.4 \pm 0.4) \times 10^{-6} \text{ s}^{-1} \) was determined for KINASE(AEY)-(N)<sup>Npu</sup>DnaE-His<sub>6</sub>
inactivation at 21 °C (Figure 3.3B). Thus, a reaction time of 18 h was selected as an optimal time for maximizing the yield of spliced-PDK1, while retaining significant enzyme stability and function (Figure 3.3C, lane 2). The reaction mixture was then buffer exchanged and subjected to His$_6$ affinity chromatography. Figure 3.3C (lane 3) shows that the cleaved affinity tagged intein constructs, GST-His$_6$-(C)SspDna (species IV) and (N)NpuDnaE-His$_6$ (species V), as well as the remaining uncleaved KINASE(AEY)-(N)NpuDnaE-His$_6$ and GST-His$_6$-(C)SspDna-(CMN)PH reactants were effectively retained by the column. In addition, three of the four contaminating protein impurities (24, 25, and 26 kDa) that co-purified with GST-His$_6$-(C)SspDna-(CMN)PH (Figure 3.3C, lane 3) were retained, suggesting that these may likely result from proteolytic degradation prior to cell lysis. Spliced-PDK1, the contaminating species (37 kDa), and the small amount of trans-cleaved KINASE(AEY), which were not retained by the His$_6$ affinity column were readily resolved by MonoQ ion exchange chromatography. Figure 3.3C (lane 4) shows that spliced-PDK1 is purified to ≥95% homogeneity. For this optimized preparative procedure, ~0.1 µmol of purified spliced-PDK1 could be readily obtained from 0.2 µmol of each of the starting reactants.

3.3.4. PI(3,4,5)P$_3$-DEPENDENT PHOSPHORYLATION OF PKBβ BY SPLICED-PDK1

The best characterized function of the C-terminal PH domain of PDK1 is its ability to bind the PI(3,4,5)P$_3$ second messenger, causing its membrane colocalization with PKB, which also contains a PH domain that selectively binds
PI(3,4,5)P₃ (Alessi et al., 1996; Alessi et al., 1997a, Alessi et al., 1997b; Stokoe et al., 1997; Meier et al., 1997; Andjelkovic et al., 1997; Stephens et al., 1998; Walker et al., 1998; Filippa et al., 2000; McManus et al., 2004; Hanada et al., 2004). At the membrane, PDK1 catalyzes phosphorylation of the critical residue in the activation loop of PKB, which is required for downstream PKB signaling activity. For the three known isoforms of PKB, the phosphorylation sites correspond to Thr-308, Thr-309, and Thr-305 in PKBα, PKBβ, and PKBγ, respectively. In vitro characterization of PDK1-catalyzed site-specific phosphorylation of PKB isoforms, which utilize phosphoinositide-containing vesicles, demonstrated an absolute requirement for PI(3,4,5)P₃-containing vesicles, as well as functional PH domains in both PDK1 and PKB (Alessi et al., 1997b; Stokoe et al., 1997; Walker et al., 1998). Since protein trans-splicing of the regulatory PH and catalytic kinase domains of PDK1 required mutation of 382QFG384 to 382AEY384 at the N-terminal side of the splice junction, as well as the Q387N mutation to give 385CMN387 at the C-terminal side of the splice junction (Figure 2.1), it was very important to assess whether spliced-PDK1 retained the characteristic ability to specifically phosphorylate and activate PKB in a PI(3,4,5)P₃-dependent manner.

First, full length His₆-PKBβ was obtained by baculovirus-mediated expression and purification from Sf9 insect cells (Gao et al., 2005). As we have previously demonstrated, His₆-PKBβ purifies with minimal catalytic activity to the Crosstide substrate (~0.3 U/mg) and displays very small but detectable amounts of both Thr-309 and Ser-474 phosphorylation, as detected by Western analysis.
(Figure 3.4A, lane 1). Previous isoelectric focusing studies, as well as ion exchange chromatography could not resolve the phosphorylated isoforms, suggesting that the small detectable amount of phosphorylated His6-PKBβ likely consists of a mixture of enzyme mono-phosphorylated either at Thr-309 or Ser-474 (Gao et al., 2005). Nevertheless, homogeneous unphosphorylated His6-PKBβ with slightly lower Crosstide activity (~0.2 U/mg) was readily generated by treatment with λPP (Figure 3.4A, lane 2). Western analysis further showed that λPP-treated unphosphorylated His6-PKBβ did not catalyze autophosphorylation at either Thr-309 or Ser-474 when incubated in autophosphorylation reaction mixtures either in the absence (Figure 3.4A, lane 3) or presence of PI(3,4,5)P3-containing vesicles (Figure 3.4A, lane 4). Most importantly, Western analysis detected significant Thr-309 phosphorylation of His6-PKBβ only when incubated with spliced-PDK1 in the presence of PI(3,4,5)P3-containing vesicles; and His6-PKBβ catalytic activity to Crosstide increased ~10-fold (Figure 3.4A, lanes 5 and 6). Affinity purification and phosphopeptide mapping of His6-PKBβ from the phosphorylation reaction mixture containing both spliced-PDK1 and PI(3,4,5)P3-containing vesicles (Figure 3.4A, lane 6) confirmed that spliced-PDK1 catalyzed site-specific Thr-309 phosphorylation of His6-PKBβ (Figure 3.4B), as no other 32P-radiolabeled peptides were detected. The mole fraction of total phosphorylated sites ($f_{\text{mol}}^{\text{tot}}$) was calculated to be 0.65 ± 0.15, in agreement with previous reports (Walker et al., 1998).
**Figure 3.4: Site-specific Thr-309 phosphorylation by spliced-PDK1.** (A) Preparation of His$_{6}$-PKBβ (lane 1); preparation of λ,PP-treated unphosphorylated His$_{6}$-PKBβ (lane 2); and affinity purified λ,PP-treated His$_{6}$-PKBβ after 40 min incubation at 30 °C in phosphorylation reaction mixtures containing [γ-$^{32}$P]ATP-Mg$^{2+}$ (lane 3), [γ-$^{32}$P]ATP-Mg$^{2+}$ in the presence of PC/PS/PI(3,4,5)P$_{3}$ vesicles (lane 4), [γ-$^{32}$P]ATP-Mg$^{2+}$ in the presence of spliced-PDK1 (lane 5), and [γ-$^{32}$P]ATP-Mg$^{2+}$ in the presence of both spliced-PDK1 and PC/PS/PI(3,4,5)P$_{3}$ vesicles (lane 6). SDS-PAGE and Western analysis were used to visualize Thr-309 and Ser-474 phosphorylated His$_{6}$-PKBβ. Activities were determined for His$_{6}$-PKBβ-catalyzed phosphorylation of 100 µM of Crosstide (Ψ). (B) After 40 min incubation at 30 °C in the phosphorylation reaction mixture containing [γ-$^{32}$P]ATP-Mg$^{2+}$, affinity purified λ,PP-treated His$_{6}$-PKBβ was digested with trypsin and subjected to reversed-phase HPLC. Scintillation counting of the individual fractions detected the $^{32}$P-radiolabeled peptide that eluted near 28% acetonitrile, which was identified by MALDI-TOF (m/z = 2469.6) to be the mono-phosphorylated tryptic peptide containing Thr-309 (pTFCGTPEYLAPEVLEDNDYGR$^{325}$).
3.3.5. PROSPECTIVE

Large efforts are being directed towards design of potent and selective inhibitors of well established protein kinase drug targets (Vieth et al., 2005; Cohen, 2002). Since the overwhelming majority of protein kinase inhibitors bind in or near the ATP binding pocket shared by the structurally homologous catalytic domain shared by all kinases, very few serine-threonine protein kinase inhibitors have been clinically approved due to their broad specificity and overall high toxicity (Noble et al., 2004; Davies et al., 2000; Bain et al., 2003). Therefore, we are developing the hypothesis that serine-threonine protein kinase inhibitor selectivity may be better achieved by designing compounds that target interfacial clefts and crevices formed between contiguous regulatory and catalytic kinase domains, thereby 'allosterically' stabilizing inactive or autoinhibited conformations of multi-domain protein kinases. In fact, validation of this hypothesis is exemplified by the recent discovery of the small molecule inhibitors, Akt-I-1 and Akt-I-1,2, which showed remarkable isozyme specificity towards PKB/Akt isozymes and did not significantly inhibit other closely related kinases (Barnett et al., 2005). Most importantly, only the full length PKB/Akt isozymes containing the regulatory PH domain were sensitive to inhibition, suggesting an 'allosteric' mechanism in which high selectivity results from inhibitor binding at a site that stabilizes a PH domain mediated autoinhibited form of the kinase.

The inability to obtain diffraction quality crystals for full length constructs of any of the large number of multi-domain serine-threonine protein kinase drug targets suggests a large degree of conformational heterogeneity, which likely
results from changes in relative orientations of contiguous regulatory and catalytic kinase domains. In order to better facilitate structure-based discovery or design of highly selective inhibitors (e.g., Akt-I-1 and Akt-I,2), development of alternative methodologies will be required to better understand autoinhibitory contiguous domain-domain interactions; and domain-specific incorporation of biophysical probes would greatly facilitate spectroscopic studies of these interactions in solution. Disappointingly, the use of intein-mediated chemical ligation for such purposes is largely precluded, as we found it difficult to purify from Sf9 insect cells adequate quantities of a number of protein kinases when fused with active inteins (e.g., Mxe GyrA and VMA). While the use of naturally [e.g., (N/C)SspDnaE] or artificially split intein pairs [e.g., (N/C)SspDnaB] circumvents decreased protein stability and intein self cleavage issues during baculovirus-mediated expression in Sf9 insect cells, only 40–50% protein trans-splicing yields are typically achieved. On the basis of the recently reported highly efficient in vivo protein trans-splicing (≥98%) catalyzed between split DnaE inteins from two different organisms (Iwai et al., 2006), we implemented and demonstrated that the 'cross' trans-splicing strategy was optimal for in vitro ligation of the regulatory PH and catalytic kinase domains of PDK1; and spliced-PDK1 was shown to retain the ability to specifically phosphorylate Thr-309 and activate PKBβ in a PI(3,4,5)P3-dependent manner. This bioconjugation strategy may prove useful for design of spectroscopic experiments to study autoregulatory mechanisms of such flexible multi-domain monomeric protein kinases.
4.1. Background and Significance

In this chapter, a new procedure for high level \textit{E. coli} expression and purification of $^{13}$C,$^{15}$N-isotopic labeled PH domain of PDK1 (residues 408–556) is first described. Disappointingly, a significant degree of spectral line width broadening was observed in heteronuclear, multidimensional NMR experiments, which precluded chemical shift assignment of any backbone and side chain nuclear resonances. A procedure for side chain deuterium isotopic labeling of $^{13}$C,$^{15}$N-PH domain was developed, which significantly reduced spectral line widths and enabled connectivity to be established between a large number of backbone amide resonances. However, it was not possible to identify residue types due to the loss of side chain hydrogen resonances. The relatively high degree of NMR spectral line width broadening likely results from protein dynamics in which the PH domain exchanges between multiple conformations. From this observation, it was hypothesized that a more rigid PH domain structure might be observed in its native full length PDK1. Using the protein trans-splicing strategy developed in \textbf{Chapter 3}, $^{15}$N-isotopic labeled PH domain was spliced to the kinase domain. Whereas improved preparations of $^{15}$N-isotopic labeled PH greatly enhanced the protein splicing reaction, an even higher degree of NMR spectral line width broadening was observed compared to PH domain alone.
4.2. Experimental Procedures

4.2.1. MATERIALS

GST-PreScission™ protease, ÄKTAbasic 100 (FPLC), prepacked GSTrap FF affinity columns (5 mL), and the Superdex™ 75 16/60 size-exclusion column were from GE Healthcare Biosciences (Piscataway, NJ). The pET-41b protein expression vector and BL21 (DE3) protein expression strain of E. coli was from Novagen (Madison, WI). The cOmplete™ Protease Inhibitor Cocktail Tablets were from Roche Applied Science (Indianapolis, IN). DNA restriction enzymes were from New England Biolabs (Ipswich, MA). (U-13C6, 99%) D-glucose, (15N, 99%) ammonium chloride, and (D, 99%) deuterium oxide were from Cambridge Isotope Laboratories (Andover, MA). D-myo-inositol-1,4,5-triphosphate (sodium salt) was from Cayman Chemicals (Ann Arbor, MI). All other chemicals, salts, and buffers were from Sigma, Inc. (St. Louis, MO). For preparation of isotopic labeled PH domain, chromatographic and sample buffers were treated with Chelex, which was from Bio-Rad Laboratories (Hercules, CA).

4.2.2. PCR ENGINEERING OF GST-HIS6-(PRESCISSION)-PH

The PH domain of PDK1 (residues 408–556) was subcloned into the pET-41b protein expression vector, which contains an upstream dual GST-His6 affinity tag. A PreScission™ protease recognition sequence was inserted prior to residue 408 of the PH domain to facilitate effective removal of the GST-His6 tag. Full length PDK1 in the pCR®-Blunt II-TOPO® plasmid vector was used as the template. The forward or upstream 5’-primer was complementary to the coding
region (residues 408–515, italic letters) and extended to include to include a PreScission™ protease cleavage site (LEVLFQGP, bold) and a SacII recognition sequence (lower case letters) (SacII-PreScission-PH-F, 5′-ccgcccGTT CTG TTC CAG GGG CCC TCA GGC AGC AAC ATA GAG CAG TAC-3′). The additional GT nucleotides were inserted adjacent to the SacII recognition sequence to maintain the coding frame. The reverse or downstream 3′-primer was complementary to the coding region (residues 551–stop, italic letters) and extended to include the KpnI recognition sequence (lower case letters) (PH-KpnI-R, 5′-ggtagc TCA CTG CAC AGC GGC GTC CGG-3′). The T_m values of the overlapping regions for each primer were between 70 and 72 °C.

The 50-µL PCR reaction contained 5 µL of 10× PCR Buffer (1.2 mM Tris-HCl, pH 8 at 25 °C, 100 mM KCl, 60 mM (NH₄)₂SO₄, 1% Triton X-100, and 0.01% bovine serum albumin), 2 µL of 25 mM MgSO₄, 2 µL of 50× dNTP mix (2 mM each of dATP, dCTP, dGTP, and dTTP), 1 µL of 50× High-Fidelity KOD Hot Start Polymerase Mix (Novagen), 1 µL of 10 µM of the SacII-PreScission-PH-F primer, 1 µL of 10 µM of the reverse PH-KpnI-R primer, and 100 ng of template. The reaction was carried out in a 0.2 mL sterile thin-walled PCR tube in a MWG Primus 25 “hot-lid” thermal cycler using the following protocol: incubation at 95 °C for 2 min; 20 cycles of 94 °C for 15 s, 60 °C for 30 s, and 68 °C for 2 min; and incubation at 72 °C for 10 min. The resulting blunt-ended cDNA product was gel-purified (1% agarose), isolated using the QIAquick® Gel Extraction Kit (Qiagen), cloned into the pCR®-Blunt II-TOPO® plasmid vector (Invitrogen), and transformed into One Shot® TOP10 Chemically Competent E. coli. Selected
colonies were grown in 10 mL LB/Amp (100 µg/mL); plasmids were isolated using the QIAprep® Spin Miniprep Kit (Qiagen) and digested with ScaI and KpnI to verify the presence of the correct-sized insert. A sequence verified ScaI-(PreScission)-PH-KpnI fragment was doubly digested from the pCR®-Blunt II-TOPO® cloning vector and directionally ligated into the pET-41b protein expression vector (Novagen), downstream of a GST-His6 tag, yielding the coding sequence for the GST-His6-(PreScission)-PH fusion construct.

4.2.3. EXPRESSION AND UNIFORM DOUBLE $^{13}$C, $^{15}$N-ISOTOPIC LABELING

GST-His6-(PreScission)-PH was transformed into the BL21 Star™ E. coli strain (Invitrogen) for expression and uniform $^{13}$C, $^{15}$N-isotopic labeling in PG minimal medium. PG minimal medium (Studier, 2005) was prepared with the following modifications. First, 1.2 L of 50 mM Na$_2$HPO$_4$, 50 mM KH$_2$PO$_4$, and 5 mM Na$_2$SO$_4$ was mixed. One 50-mL aliquot was placed in a 250-mL flask, two 500-mL aliquots were each placed in 2-L baffled-bottom flasks, and the growth flasks were subjected to autoclave sterilization. Immediately before inoculation of sterilized growth flasks with starter culture, final concentrations of 2 mM MgSO$_4$, 56 mM $^{15}$NH$_4$Cl, 0.6% $^{13}$C-glucose, kanamycin (50 µg/mL), and 0.2× of a trace metal mixture were added to each growth flask. A 1000× stock trace metal mixture in 60 mM HCl was prepared as described (Studier, 2005) and contained 50 mM FeCl$_3$, 20 mM CaCl$_2$, 10 mM MnCl$_2$-4H$_2$O, 10 mM ZnSO$_4$-7H$_2$O, and 2 mM each of CoCl$_2$-6H$_2$O, CuCl$_2$-2H$_2$O, NiCl$_2$-6H$_2$O, Na$_2$MoO$_4$-2H$_2$O, Na$_2$SeO$_3$, and H$_3$BO$_3$. 
A single colony of a given transformed BL21 Star™ *E. coli* strain, grown from an LB/kanamycin (50 µg/mL) agar plate, was used to inoculate a starter culture of 5 mL LB/kanamycin (50 µg/mL) medium, which was incubated overnight with shaking at 37 °C. Then, the starter culture was used to inoculate a 50 mL starter culture of PG minimal medium. After conditioning the cells to the PG minimal medium overnight at 30 °C, 20 mL of starter culture were used to inoculate each 2-L baffled-bottom growth flask containing 500 mL of PG minimal medium. The cells were allowed to grow at 37 °C to an optical cell density of 0.8 OD₆₀₀. At this time, cultures were supplemented with an equivalent amount of kanamycin (50 µg/mL), and protein expression was induced with 0.5 mM IPTG for 4 hours. Cells were harvested by centrifugation for 15 min at 5000g in an SLA-3000 rotor (Sorvall). Approximately 4 mL/(g cells) of cell wash buffer (50 mM phosphate buffer, pH 7.3, and 500 mM NaCl) was used to re-suspend, wash, and collect by centrifugation a single cell pellet (~6 g per 1 L of culture), which was stored at −80 °C.

### 4.2.4. Expression and Uniform Triple ²H, ¹³C, ¹⁵N-Isotopic Labeling

A single colony of the transformed BL21 Star™ *E. coli* strain was used to inoculate a starter culture of 5 mL LB/kanamycin (50 µg/mL) medium, which was incubated overnight with shaking at 37 °C. Then, the starter culture was used to inoculate 5 mL culture of PG minimal medium (80% D₂O), which was grown at 37 °C with shaking until the end of the growth phase. This culture was then used to inoculate a new 50 ml culture of PG minimal medium (80% D₂O) and grown
again overnight at 37 °C. A volume of 20 mL of the D$_2$O-adapted culture was used to inoculate each 2-L baffled-bottom growth flask containing 500 mL of PG minimal medium (80% D$_2$O). The cells were allowed to grow at 37 °C to an optical cell density of 0.8 OD$_{600}$. At this time, cultures were supplemented with an equivalent amount of kanamycin (50 µg/mL), and protein expression was induced with 0.5 mM IPTG for 4 hours. Cells were harvested by centrifugation for 15 min at 5000 g in an SLA-3000 rotor (Sorvall). Approximately 4 mL/(g cells) of cell wash buffer (50 mM phosphate buffer, pH 7.3, and 500 mM NaCl) was used to re-suspend, wash, and collect by centrifugation a single cell pellet (~6 g per 1 L of culture), which was stored at −80 °C.

4.2.5. PURIFICATION AND GENERATION OF ISOTOPIC-LABELED PH DOMAIN

The frozen cell pellet was thawed and re-suspended in GST-binding buffer (10 mL per g cell pellet; 50 mM phosphate buffer, pH 7.3, 500 mM NaCl, and 1 mM dithiothreitol). The cells were lysed using the EmulsiFlex-C3 high pressure homogenizer (Avestin, Inc.), and particulates were removed from the lysate by centrifugation for 30 min at 35,000 g in an SS-34 rotor (Sorvall). The soluble lysate containing GST-His$_6$-(PreScission)-PH was directly loaded by FPLC (1 mL/min) onto a pair of tandem connected 5 mL GSTrap FF affinity columns equilibrated at 4 °C in GST-binding buffer (see above). The column was first washed with 50 mL of GST-binding buffer containing 0.01% Triton X-100 (1 mL/min). Then detergent was removed by subsequent washing with detergent free GST-binding buffer until the absorbance at 280 nm returned to baseline.
GST-His\textsubscript{6}-(PreScission)-PH was eluted with 50 mM Tris-HCl, pH 8, containing 500 mM NaCl, 2 mM dithiothreitol, and 10 mM glutathione.

Fractions containing GST-His\textsubscript{6}-(PreScission)-PH were combined and exchanged back into GST-binding buffer using a HiPrep 26/10 desalting column. The N-terminal GST-His\textsubscript{6} tag was removed by direct addition of PreScission\textsuperscript{TM} protease (10 units of protease per mg of fusion construct) and incubating at 4 °C for 16 h. The cleavage reaction products were directly loaded by FPLC (1 mL/min) onto the tandem GSTrap FF affinity columns, which retained cleaved tag and any uncleaved GST affinity-tagged PH domain. The cleaved PH domain that was not retained was collected, concentrated to ~1 mL using Amicon ultrafiltration concentrators (3 kDa molecular weight cutoff), and directly loaded by FPLC (1 mL/min) onto a Superdex\textsuperscript{TM} 75 16/60 size-exclusion column equilibrated at 4 °C in 50 mM phosphate buffer, pH 6.5, and 250 mM NaCl. The PH domain was resolved from all other protein components by isocratic elution with this buffer at 1 mL/min. Fractions containing purified PH domain were combined, concentrated (~1.5 mM), and stored in 10% glycerol at −80 °C. The concentration of the cleaved and purified PH domain was measured using an extinction coefficient of 30,940 M\(^{-1}\) cm\(^{-1}\) (Gill and von Hippel, 1989).

4.2.6. PRODUCTION OF SEGMENTAL \(^{15}\)N-ISOTOPIc LABELED PDK1\((^{15}\)N-PH)  

Production of S241 Unphosphorylated KINASE(AEY)-(N)NpuDnaE-His\textsubscript{6}− 

Experimental procedures describing generation of recombinant baculovirus for expression of KINASE(AEY)-(N)NpuDnaE-His\textsubscript{6} is given as described (Al-Ali et
al., 2007; **Appendices A1 and A2**). The KINASE(AEY)-(N)NpuDnaE-His$_6$ construct was His$_6$ affinity purified to $\geq 95\%$ homogeneity after baculovirus-mediated expression in Sf9 insect cells as described (Al-Ali et al., 2007; **Chapter 3**). For removal of phosphate from S241 and any other potential serine, threonine, and tyrosine residues, $\sim 40$ $\mu$M of PDK1 kinase was diluted to $\sim 5$ $\mu$M in buffer containing 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 5 mM dithiothreitol, 0.01% Brij 35, and 2 mM MnCl$_2$. Then, 5 $\mu$g/mL of lambda protein phosphatase ($\lambda$PP) was added, and the reaction was allowed to proceed for 1 h at 30 $^\circ$C. The reaction mixture was adjusted to contain 50 mM imidazole and 300 mM NaCl, and dephosphorylated kinase was His$_6$ affinity purified. Chromatographic fractions containing His$_6$ affinity tagged dephosphorylated kinase were combined and exchanged to storage buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% 2-mercaptoethanol, and 0.1 mM EGTA), using a HiPrep 26/10 desalting column. Protein was concentrated, adjusted to $\sim 40$ $\mu$M with addition of protease inhibitors (0.5 mM benzamidine and 0.1 mM PMSF), and stored at $-80$ $^\circ$C. KINASE(AEY)-(N)NpuDnaE-His$_6$ concentration was determined by BioRad protein assay.

Production of $^{15}$N-Labeled GST-His$_6$-(C)SspDnaE-(CMN)–Experimental procedures describing generation of the pET-41b protein expression vector (Novagen) containing the coding sequence for the GST-His$_6$-(C)SspDnaE-(CMN)PH domain protein trans-splicing construct is given as described (Al-Ali et al., 2007; **Appendix A2 and Chapter 3**). GST-His$_6$-(C)SspDnaE-(CMN)PH was transformed into the BL21 Star™ *E. coli* strain (Invitrogen), expressed and uniform $^{15}$N-isotopic labeled in PG minimal medium, and tandem His$_6$ and GST
affinity purified. Fractions containing GST-His$_6$-(C)SspDnaE-(CMN)PH were combined and exchanged into protein trans-splicing buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.5 mM TCEP, 0.5 mM EGTA, 1 mM EDTA, and 5% (v/v) glycerol). Protein was concentrated, adjusted to ~40 µM with addition of protease inhibitors (0.5 mM benzamidine and 0.1 mM PMSF), and stored at −80 °C. GST-His$_6$-(C)SspDnaE-(CMN)PH concentration was determined by BioRad protein assay.

**Protein Cross Trans-Splicing**—The protein cross trans-splicing reaction between unlabeled dephosphorylated λPP-treated KINASE(AEY)-(N)NpuDnaE-His$_6$ (~40 µM) and $^{15}$N-isotopic labeled GST-His$_6$-(C)SspDnaE-(CMN)PH (~40 µM) was carried out exactly as described (Al-Ali et al., 2007; Chapter 3). His$_6$ affinity chromatography flow-through fractions containing segmental $^{15}$N-isotopic labeled PDK1($^{15}$N-PH) were pooled and exchanged to storage buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% 2-mercaptoethanol, and 0.1 mM EGTA). Protein was concentrated, adjusted to ~40 µM with addition of protease inhibitors (0.5 mM benzamidine and 0.1 mM PMSF), and stored at −80 °C. PDK1($^{15}$N-PH) concentration was determined by BioRad protein assay.

4.2.7. SDS-PAGE Analysis

Protein samples in SDS sample buffer were heated at 95 °C for 2 min and cooled on ice. Analytical SDS-PAGE was performed on 4–12% gradient polyacrylamide gels (Invitrogen) developed at 150 V (constant) for 1 h or until the
tracking dye reached the bottom of the slab. Coomassie staining was used to visualize total protein.

4.2.8. NMR SPECTROSCOPY

A 1 mM sample of either doubly $^{13}$C,$^{15}$N-labeled or triply $^2$H,$^{13}$C,$^{15}$N-labeled PH domain was prepared in 50 mM sodium phosphate buffer, pH 6.5, 250 mM NaCl, 0.5 mM dithiothreitol, and 5% D$_2$O. A 0.5 mM sample of segmental $^{15}$N-isotopic labeled PDK1($^{15}$N-PH) was prepared in 50 mM sodium phosphate buffer, pH 6.5, 150 mM NaCl, 0.5 mM dithiothreitol, and 5% D$_2$O. In addition, samples were prepared in identical buffers containing 4 mM inositol (1,3,4,5)-tetrakisphosphate [I(1,3,4,5)P$_4$]. NMR data were collected at 25 °C with a Bruker DMX500 NMR spectrometer (500 MHz for protons) equipped with pulsed-field gradients, four frequency channels, and a triple resonance cryoprobe with shielded z-gradient.

Two dimensional (2D) $^1$H–$^{15}$N HSQC data were recorded using a pulse sequence in which the detection scheme was optimized to avoid water saturation (Mori et al, 1995) and by using the states-TTPI method (Marion et al, 1989) in the indirect dimension, with a relaxation delay of 0.8 s. The data were obtained with spectral widths of 1800 and 8000 Hz in $F1(^{15}$N) and $F2(^1$H) dimensions, respectively. A total of 256 complex $t1(^{15}$N) and 1024 real $t2(^1$H) data points were collected. Sixteen transients were acquired for each hypercomplex $t1$ point with $^1$H and $^{15}$N carriers placed at 4.7 and 118 ppm, respectively.
The three-dimensional (3D) HNCA, HN(CO)CA, HNCO, and HN(CA)CO experiments were performed using the hncagp3d, hncocagp3d, hncogp3d, and hncacogp3d pulse sequences, respectively (Grzesiek et al., 1992; Schleucher et al., 1993; Kay et al., 1994). Data for each of these experiments were recorded using the Echo-Antiecho and states-TTPI methods in the $F_2^{(15N)}$ and $F_1^{(13C)}$ dimensions, respectively, each with a relaxation delay of 1 s; and spectral widths were 1800 and 8000 Hz in the $F_2^{(15N)}$, and $F_3^{(1H)}$ dimensions, respectively. A spectral width of 5000 Hz was used in the $F_1^{(13C)}$ dimension of the HNCA and HN(CO)CA experiments, whereas a spectral width of 2700 Hz was used in the $F_1^{(13C)}$ dimension of the HNCO and HN(CA)CO experiments. A total of 128 complex $t_1^{(13C)}$, 40 real $t_2^{(15N)}$, and 1024 real $t_3^{(1H)}$ data points were collected for the HNCA and HN(CO)CA experiments. A total of 128 complex $t_1^{(13C)}$, 32 real $t_2^{(15N)}$, and 1024 real $t_3^{(1H)}$ data points were collected for the HNCO and HN(CA)CO experiments. Sixteen transients were acquired for each hypercomplex $t_1$ point with $^{1}H$, $^{15}N$, and $^{13}C$ carriers placed at 4.7, 118 ppm, and 57 ppm, respectively, in the HNCA and HN(CO)CA experiments. Sixteen transients were acquired for each hypercomplex $t_1$ point with $^{1}H$, $^{15}N$, and $^{13}C$ carriers placed at 4.7, 118 ppm, and 176 ppm, respectively, in the HNCO and HN(CA)CO experiments. The $^{13}C$ and $^{15}N$ decouplings were achieved with the GARP decoupling scheme. Suppression of water resonance was done using water-flip-back with PEP.

The 3D HCCH-TOCSY experiment was carried out using the hcchdigp3d pulse sequence (Kay et al., 1993). Data for the HCCH-TOCSY experiment was
recorded using states-TPPI in the $F2$ and $F1$ dimensions with a relaxation delay of 1 s. Spectral widths were 20000, 4500, and 5000 Hz in the $F1(^1H)$, $F2(^{13}C)$, and $F3(^1H)$ dimensions, respectively. A total of 128 complex $t1(^1H)$, 80 complex $t2(^{13}C)$, and 1024 real $t3(^1H)$ data points were collected. Sixteen transients were acquired for each hypercomplex $t1$ point with $^1H$ and $^{13}C$ carriers placed at 4.7 and 39 ppm, respectively. Water suppression was achieved by two $z$-filters and a pair of proton spin lock pulses.

The program NMRPipes (Delaglio et al., 1995) was used to process all NMR data. Proton chemical shifts were given with respect to the HDO signal to be 4.71 ppm relative to external TSP (0.0 ppm) at 25 °C. The $^{15}N$ and $^{13}C$ chemical shifts were indirectly referenced. Processed spectra were analyzed using the CcpNmr© software suite.

### 4.3. Results and Discussion

#### 4.3.1. Production of Isotopic-Labeled PH Domain of PDK1

Nucleotides coding for the C-terminal PH domain (Figure 1.1, residues 408–556) with an N-terminal PreScission protease cleavage site (LEVLFQGP) were cloned into the pET-41b protein expression vector, which includes a further N-terminal GST-His$_6$ tag (Figure 4.1A). Figure 4.1B shows Coomassie blue staining of 4–12% SDS-PAGE for purification of uniform $^{13}C$, $^{15}N$-isotopic doubly labeled PH domain after expression in PG minimal medium prepared in 100% H$_2$O. An essentially identical SDS-PAGE gel was observed for purification of uniform $^2H$, $^{15}N$, $^{13}C$-isotopic triply labeled PH domain from PG minimal medium.
Figure 4.1: *E. coli* expression and purification of isotopic-labeled PH domain of PDK1. (A) The PH domain of PDK1 (residues 408–556) was inserted into the pET-41b protein expression vector, which contains an N-terminal GST-His$_6$ dual affinity tag. In addition, the PreScission$^\text{TM}$ protease recognition sequence and site of cleavage was inserted for proteolytic removal of the N-terminal GST-His$_6$ dual affinity tag. (B) SDS-PAGE analysis with Coomassie staining and indicated molecular weight markers. Lane 1 shows the total soluble cell lysate after IPTG-induced expression of GST-His$_6$(PreScission)-PH domain in *E. coli* grown in PG minimal media. Lane 2 shows proteins from the soluble lysate that were not retained after passage over the GST Sepharose High Trap (GSTrap) FF affinity column. Lane 3 shows the proteins that were retained and subsequently eluted from the GSTrap FF column. Lane 4 shows the proteins obtained after overnight digestion with GST-PreScission$^\text{TM}$ protease. Lane 5 shows the proteins in the digestion mixture that were retained after re-passage over the GSTrap FF column. Lane 6 shows the proteins in the digestion mixture that were not retained on the GSTrap FF column. Lane 7 shows purified PH domain that was ultimately resolved on a Superdex$^\text{TM}$ 75 size-exclusion column.

| Table 4.1: Purification of $^{13}$C,$^{15}$N-labeled GST-His$_6$(PreScission)-PH from *E. coli*  
<table>
<thead>
<tr>
<th>Purification (Step)</th>
<th>Volume (mL)</th>
<th>Concentration (mg/mL)</th>
<th>Yield (mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude lysate</td>
<td>25</td>
<td>16 ± 2</td>
<td>400 ± 50</td>
<td>N/A</td>
</tr>
<tr>
<td>GSTrap FF</td>
<td>15</td>
<td>5.0 ± 0.6</td>
<td>75 ± 9</td>
<td>5.3</td>
</tr>
<tr>
<td>Superdex 75</td>
<td>10</td>
<td>1.5 ± 0.2</td>
<td>15 ± 2</td>
<td>27</td>
</tr>
</tbody>
</table>

$^a$All values are reported for purification from expression in 1 L of *E. coli*.

| Table 4.2: Purification of $^2$H,$^{13}$C,$^{15}$N-labeled GST-His$_6$(PreScission)-PH from *E. coli*  
<table>
<thead>
<tr>
<th>Purification (Step)</th>
<th>Volume (mL)</th>
<th>Concentration (mg/mL)</th>
<th>Yield (mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude lysate</td>
<td>25</td>
<td>14 ± 2</td>
<td>350 ± 50</td>
<td>N/A</td>
</tr>
<tr>
<td>GSTrap FF</td>
<td>15</td>
<td>3.1 ± 0.5</td>
<td>58 ± 8</td>
<td>6.0</td>
</tr>
<tr>
<td>Superdex 75</td>
<td>10</td>
<td>1.0 ± 0.2</td>
<td>10 ± 2</td>
<td>35</td>
</tr>
</tbody>
</table>

$^a$All values are reported for purification from expression in 1 L of *E. coli*. 
containing 70% D$_2$O and 30% H$_2$O (data not shown). The protein yields after each purification step are reported for doubly $^{13}$C,$^{15}$N-isotopic labeled (Table 4.1) and triply $^2$H,$^{15}$N,$^{13}$C-isotopic labeled PH domain (Table 4.2).

GST affinity chromatography typically retained (i) 75 ± 9 mg of uniform $^{13}$C,$^{15}$N-isotopic doubly labeled and (ii) 58 ± 8 mg of uniform $^2$H,$^{15}$N,$^{13}$C-isotopic triply labeled PH domain from the total soluble lysate. Figure 4.1B (lane 3) shows a number of protein species that co-eluted with GST-His$_6$-(PreScission)-PH ($M_r = 45$ kDa), namely one high molecular weight contaminant ($M_r = 70$ kDa; likely Hsp70 chaperone) and a series of protein species with relative mobilities ranging between those of full length GST-(PreScission)-PH and GST alone ($M_r = 23$ kDa). Western analysis with GST antibody confirmed the presence of GST in all of the latter species. Control experiments in which protein was GST affinity purified from E. coli that was not induced with IPTG indicated the GST species with $M_r = 27$ kDa to be endogenous.

Figure 4.1B (lane 4) shows that GST-PreScission™ protease efficiently cleaved the fusion construct, yielding the GST affinity tag and full length PH domain with the additional N-terminal Gly-Pro (Figure 4.1A). Lane 5 shows that cleaved recombinant and endogenous GST were effectively retained by the affinity column, and lane 6 shows that the cleaved PH domain ($M_r = 17$ kDa) to be fully intact. Lane 7 shows that the low molecular weight PH domain could be effectively resolved from the dispersed higher molecular weight contaminants by passage over a Superdex™ 75 16/60 size-exclusion column. Thus, a final yield of (i) 15 ± 2 mg of uniform $^{15}$N,$^{13}$C-isotopic doubly labeled (Table 4.1) and (ii) 10
± 2 mg of uniform $^2$H, $^{15}$N, $^{13}$C-isotopic triply labeled PH domain was obtained (Table 4.2), which were each judged by Coomassie blue staining of 4–12% SDS-PAGE to be of $\geq$95% homogeneity.

4.3.2. EFFECT OF SIDE CHAIN DEUTERATION ON NMR STUDIES OF $^{13}$C, $^{15}$N-PH

In order to determine the optimal conditions for NMR data acquisition, an array of two-dimensional (2D) $^1$H–$^{15}$N HSQC spectra were obtained for the purified recombinant doubly $^{13}$C, $^{15}$N-isotopic labeled PH domain of PDK1 at different temperatures (5–35 °C) in buffers (pH 6–9) with varying amounts of cosolutes (NaCl, Triton X-100, and PEG) and inositol polyphosphate ligands. The two-dimensional $^1$H–$^{15}$N HSQC NMR experiment correlates the $^1$H and $^{15}$N chemical shifts ($\delta$) of directly bonded $^1$H–$^{15}$N pairs (i.e., backbone and side chain amide groups). Figure 4.2A shows the highest quality $^1$H–$^{15}$N HSQC spectrum, which was obtained at 25 °C, using 1 mM $^{13}$C, $^{15}$N-PH domain in 50 mM sodium phosphate buffer, pH 6.5, 375 mM NaCl, 4 mM inositol (1,3,4,5)-tetrakisphosphate [I(1,3,4,5)P$_4$], 0.5 mM dithiothreitol, and 5% D$_2$O. Resonance cross peaks showed good dispersion in both $^1$H (6.5–10.5 ppm) and $^{15}$N chemical shifts (105–135 ppm), indicating a well folded structure. However, a recognizable degree of line broadening was observed.

Line broadening primarily results from orientation changes between the bonded proton and protons nearby in space, which increase the transverse magnetization relaxation rate ($R_2$) by changing the electronic environment surrounding a given nucleus. In such cases, line broadening can be significantly
Figure 4.2: Effect of side chain deuteration on NMR spectra of the isolated $^{13}$C,$^{15}$N-labeled PH domain of PDK1. (A,B) Two-dimensional (2D) $^1$H–$^{15}$N HSQC spectra of the isolated $^{13}$C,$^{15}$N-labeled PH domain of PDK1 (residues 408–556) with side chain carbon-bound hydrogens substituted with either (A) 0% deuterium or (B) 70% deuterium. Each contour represents a volumetric cross peak that correlates the $^1$H and $^{15}$N chemical shifts of directly bonded $^1$H–$^{15}$N pairs. The majority of peaks derive from backbone amide ($^1$H–$^{15}$N) resonances, whereas those in boxed insets derive from side chain amide resonances of Asn and Gln residues (solid) and Trp residues (dashed). (C,D) 2D projections of $^1$H–$^{13}$C planes from the 3D $^{15}$N and $^{13}$C-edited HNCA spectra of the isolated $^{13}$C,$^{15}$N-labeled PH domain of PDK1 with side chain carbon-bound hydrogens substituted with either (C) 0% deuterium or (D) 70% deuterium. Each contour represents a volumetric cross peak that correlates the $^1$H and $^{13}$C chemical shifts of directly bonded $^1$H–$^{13}$C pairs.
decreased if NMR active carbon-bound hydrogens are replaced with NMR inactive deuterium. In this way, higher resolution 3D heteronuclear $^1$H, $^{15}$N, and $^{13}$C NMR experiments can be obtained for resonance assignments.

**Figure 4.2B** shows the $^1$H−$^{15}$N HSQC spectrum obtained under identical conditions, using $^2$H,$^{13}$C,$^{15}$N-isotopic triply labeled PH domain. It can be seen that deuterium labeling significantly increased the spectral resolution by greatly narrowing the line widths of each backbone and side chain amide group. In addition, deuterium labeling significantly increased the spectral resolution of $^{13}$C resonances as can be seen in the 2D $^1$H−$^{13}$C projections from the 3D $^{13}$C- and $^{15}$N-edited HNCA acquired for side chain protonated (**Figure 4.2C**) versus side chain deuterated PH domain (**Figure 4.2D**).

### 4.3.3. PROTEIN DYNAMICS PRECLUDE CHEMICAL SHIFT ASSIGNMENTS

Some degree of backbone connectivity could be established from the HNCA and HN(CO)CA 3D NMR data acquired for the $^2$H,$^{15}$N,$^{13}$C-isotopic triply labeled PH domain (**Figure 4.3A**). However, very little backbone connectivity could be obtained from the corresponding HNCO and HN(CA)CO 3D NMR data (**Figure 4.3B**). In order to identify specific residue types for sequential assignment, it was necessary to collect a 3D $^{13}$C- and $^{15}$N-edited HSQC-TOCSY and 3D $^{13}$C-edited HCCH-TOCSY, using the fully protonated $^{13}$C,$^{15}$N-isotopic doubly labeled PH domain. Similar to the other 3D NMR spectra obtained for the fully protonated form, a considerable degree of line broadening was observed. Thus, it was not possible to obtain chemical shift
assignment for any backbone or side chain resonance of any residue in the isolated PH domain construct.

Figure 4.3: Protein dynamics preclude sequential chemical shift assignments. (A) Selected strips from the 3D $^{15}$N and $^{13}$C-edited HNCA spectrum (red contours) overlaid with HN(CO)CA spectrum (blue contours), which show sequential inter-residue backbone Cα correlations. The strips were taken at the $^{15}$N chemical shifts (indicated on top of every strip) and are centered about the corresponding $^1$H$^N$ chemical shifts. (B) Selected strips from the 3D $^{15}$N and $^{13}$C-edited HNCO spectrum (red contours) overlaid with HN(CA)CO spectrum (blue contours; none present). In this case, no sequential inter-residue backbone carbonyl (C=O) correlations were detected. The strips were taken at the $^{15}$N chemical shifts (indicated on top of every strip) and are centered about the corresponding $^1$H$^N$ chemical shifts.

4.3.4. SEGMENTAL ISOTOPIC LABELING OF PH DOMAIN IN FULL LENGTH PDK1

The relatively high degree of NMR spectral line width broadening likely resulted from protein dynamics in which the isolated PH domain exchanges between multiple conformations. From this observation, it was hypothesized that a more rigid PH domain structure might be observed in its native full length PDK1. Thus, Chapter 3 described a newly developed method for protein transsplicing of the regulatory PH and catalytic kinase domains of PDK1, which would
enable “segmental isotopic labeling” of the PH domain in full length PDK1. If indeed the PH domain acquired a more ordered structure, then NMR chemical shifts could be assigned and used to identify PH domain residues that potentially interact with the kinase domain.

In order to generate full length PDK1 in which only the C-terminal PH domain is uniformly $^{15}$N-isotopically labeled [PDK1($^{15}$N-PH)], the previously developed in vitro PDK1 protein trans-splicing strategy was used (Al-Ali et al., 2007; Chapter 3). Briefly, the N-terminal catalytic kinase domain of PDK1 (residues 51–384) was fused at the C-terminus with the N-terminal split intein of the gene dnaE from *Nostoc punctiforme* [(N)NpuDnaE] containing a His$_6$ affinity tag [KINASE(AEY)-(N)NpuDnaE-His$_6$] (Figure 4.4, trans-splicing reactants). The KINASE(AEY)-(N)NpuDnaE-His$_6$ construct was generated by baculovirus-mediated expression in Sf9 insect cells and purified to $\geq$95% homogeneity by His$_6$ affinity and heparin Sepharose chromatography.

The C-terminal PH domain of PDK1 (residues 385–556) was fused at the N-terminus with the C-terminal split intein of the gene dnaE from *Synechocystis* sp. strain PCC6803 [(C)SspDnaE] containing dual GST-His$_6$ affinity tags [GST-His$_6$-(C)SspDnaE-(CMN)PH] (Figure 4.4, trans-splicing reactants). In the previous account (Al-Ali et al., 2007; Chapter 3), the GST-His$_6$-(C)SspDnaE-(CMN)PH construct was generated by *E. coli* expression in Luria Broth (LB) and purified to $\sim$60% homogeneity by GST affinity purification. In contrast, $^{15}$N-isotopic labeling of the GST-His$_6$-(C)SspDnaE-(CMN)PH construct required *E. coli* expression in PG minimal medium supplemented with $^{15}$NH$_4$Cl as the sole
source of nitrogen. In this case, GST affinity chromatography yielded $^{15}$N-labeled GST-His$_6$-(C)SspDnaE-(CMN)PH with ≥95% homogeneity.

In the previous account (Al-Ali et al., 2007; Chapter 3), (i) purified Ser-241 phosphorylated KINASE(AEY)-(N)NpuDnaE-His$_6$ and (ii) partially purified GST-His$_6$-(C)SspDnaE-(CMN) were mixed for 18 h at 21 °C. The cross reacting (N)NpuDnaE and (C)SspDnaE inteins generated ~75% yield of full length spliced-PDK1, which was purified to ≥95% homogeneity from the reaction mixture by subsequent His$_6$ affinity and ion exchange chromatography steps. For segmental isotopic labeling, mixing of (i) purified Ser-241 phosphorylated KINASE(AEY)-(N)NpuDnaE-His$_6$ and (ii) purified $^{15}$N-labeled GST-His$_6$-(C)SspDnaE-(CMN)-PH at 21 °C (Figure 4.4, lane 1) required only 12 h to generate ≥95% yield of full length spliced-PDK1($^{15}$N-PH) (Figure 4.4, lane 6). Furthermore, segmental isotopic labeled PDK1($^{15}$N-PH) could be purified to ≥95% homogeneity from the reaction mixture by a single His$_6$ affinity chromatography step (Figure 4.4, lane 8).

It is most interesting to point out that trans-splicing of homogeneous preparations of both the regulatory PH and catalytic kinase domains generated a single band corresponding to full length PDK1 in SDS-PAGE (Figure 4.4, lanes 1 and 8). This suggests that the dual migrating character observed in SDS-PAGE for wild type and mutant full length His$_6$-PDK1 (Figures 2.2 and 2.4) derives from one or more covalent modifications under the action of eukaryotic enzymes that specifically recognize the full length PDK1. Alternatively, it is possible that the interaction between the PH and kinase domains renders certain phosphorylation
sites on the eukaryotically expressed full length PDK1 inaccessible for removal by \( \lambda \)PP. Importantly, \( \lambda \)PP-treated unphosphorylated spliced-PDK1\((^{15}\text{N-PH})\) is comparable to \( \lambda \)PP-treated unphosphorylated wild type PDK1 (Figure 2.2A) in its ability to catalyze in vitro Ser-241 autophosphorylation.

4.3.5. NMR STUDIES OF PDK1\((^{15}\text{N-PH})\)

Figure 4.5A shows the \(^1\text{H}--^{15}\text{N} \) HSQC spectrum that was obtained at 25 \({\degree}\text{C}\), using 0.5 mM segmental \(^{15}\text{N}-\)isotopic labeled PDK1\((^{15}\text{N-PH})\) in 50 mM sodium phosphate buffer, pH 6.5, 150 mM NaCl, 0.5 mM dithiothreitol, and 5% \( \text{D}_2\text{O}\). The \(^1\text{H}--^{15}\text{N} \) HSQC spectrum of the isolated PH domain that was obtained under similar conditions is shown in Figure 4.5B. By comparison, it can be seen
that incorporation of the isolated PH domain into the context of full length PDK1 caused nearly all of the PH domain backbone and side chain amide resonances ($^{15}$N–H) to undergo broadening beyond detection. Variations in sample temperature, pH, and buffer composition (e.g., NaCl, Triton X-100, and PEG) did not at all improve the $^1$H–$^{15}$N HSQC spectrum of segmental $^{15}$N-isotopic labeled PDK1($^{15}$N-PH). In addition to the contribution from spin-lattice relaxation caused by the NMR active protons on the kinase domain, the observed line broadening effects could perhaps result from chemical exchange phenomena on the NMR chemical shift time scale (milliseconds) occurring either (i) due to structural
disorder in the PH domain itself or (ii) due to association-dissociation events between a structurally ordered PH domain and the kinase domain.
5.1. Summary and Conclusions

Since its discovery by Alessi et al (1997a), PDK1 has been the subject of intense study. This is owed, at least in part, to its ability to phosphorylate the critical conserved residue in the T-loop of over a third of all AGC kinases (Pearce et al, 2010). Moreover, screening studies have shown PDK1 to be intimately linked to survival and drug resistance in cancer cells (Iorns et al, 2009), making it a highly desirable target for drug discovery. Unfortunately, developing selective kinase inhibitors continues to be a challenge, perhaps due to the remarkable similarity shared between the kinase domains of many human kinases, and consequently their common ability to bind ATP and ATP-analogue inhibitors (Noble et al., 2004; Davies et al., 2000; Bain et al., 2003). Thus, new approaches are being devised to target sites distinct from the ATP pocket that derive from unique domain-domain architectures within the individual kinases. The purpose of such binding would be to selectively stabilize auto-inhibited conformations. This, however, relies heavily on a priori knowledge of the spatial multi-domain arrangement and intramolecular contacts. To date, efforts to obtain an Xray structure for PDK1 remain unsuccessful due to the inability to form diffraction quality crystals. In this document, alternative methods that have been devised for approaching the problem are reported.

As a first approach, computational macromolecular docking was utilized in discovering low energy complexes between the individual kinase and PH
domains of the human PDK1 protein. The model implicated the involvement of two glutamate residues in stabilizing an active kinase conformation. Kinetic analysis demonstrated a strict requirement for the PH domain for PDK1’s ability to autophosphorylate Ser-241 within its own T-loop. Further enzymatic analysis of PDK1 mutants revealed a real involvement of Glu-432 and Glu-453 in robust autophosphorylation. A charge switch mutation at Lys-512, diametrically opposite to the domain-domain interface in the model, produced no discernible effect on autophosphorylation, further supporting the hypothesis that the PH domain interacts with, and activates, the kinase domain at the interface spanning the two glutamate residues.

A novel method for protein trans-splicing was also developed and optimized for the production of isotopically segmentally labeled PDK1. The method made use of the N- and C-terminal split inteins of the gene dnaE from *Nostoc punctiforme* [(N)NpuDnaE] and *Synechocystis* sp. strain PCC6803 [(C)SspDnaE], respectively. Individual PDK1 subdomains were fused with split intein constructs in order to produce the KINASE(AEY)-(N)NpuDnaE-His₆ and GST-His₆-(C)SspDnaE-(CMN)PH cross trans-splicing fusion protein constructs, which upon encounter generated full length spliced-PDK1 in high yield. The goal of this method was to generate segmental isotopic labeled PDK1 in order to obtain NMR distance restraints between the kinase and PH domains. Such information would have been used in refining the structure of the modeled complex. Unfortunately, and despite aggressive efforts, chemical shifts could not be assigned for the backbone amide resonances of the PH domain. This is likely
due to peculiar conformational dynamics the PH domain undergoes in solution. Also, splicing the PH domain onto the kinase domain caused further broadening of spectral peaks, rendering any analysis unfeasible. Whereas this technique might prove robust for other multidomain kinases, the characteristic behavior of the PH domain, in both the free and trans-spliced state, precluded its application to PDK1.

5.2. Significance

The findings in this work provide detailed insight on the interplay between the regulatory PH and the catalytic kinase domains of PDK1. A critical role for the unique N-bud of PDK1’s PH domain has been proposed in the process of autoactivation by trans-phosphorylation at the T-loop’s Ser-241 residue. This information might prove pivotal for future studies aimed at furthering our understanding of the mechanisms of regulation of PDK1 activity. Specifically, mapping out the interface and modes of interaction will scaffold the design of small molecule inhibitors that target and stabilize autoinhibited conformations of the enzyme. No less important, the techniques developed for the reconstitution of active PDK1 from independently produced individual domains constitutes a solid technology for segmental labeling of proteins, whether by paramagnetic, fluorescent, or NMR-active probes.
5.3. Prospective

In order to better understand the mechanisms of autoactivation and autoinhibition of PDK1 by its regulatory PH domain, high resolution structures must be determined which depict the full length enzyme in distinct functional states. Eukaryotically expressed PDK1 exhibits a peculiar profile in PAGE analysis, one that is absent in trans-spliced PDK1 produced from eukaryotic expressed kinase domain and prokaryotic expressed PH domain. Namely, subjecting full length PDK1 to SDS PAGE partitions it between slightly faster and slightly slower migrating subspecies. Interestingly, the faster and slower migrating subspecies of purified PDK1 did not collapse into a single species upon treatment with lambda protein phosphates ($\lambda$PP). In addition, stoichiometric autophosphorylation was obtained from the total of the two species observed after treatment of purified PDK1 by $\lambda$PP. Thus, it appears that the two subspecies must be distinguished by a covalent post-translational modification other than phosphorylation. Given exact knowledge of the nature of this modification, purification protocols can be devised to separate the two forms using affinity chromatography. With truly homogenous preparations of full length PDK1, good quality crystals might finally be attainable for structural determination using x-ray crystallography.

In related consideration, the extent of modification of native PDK1 in eukaryotic cells suggests it to be relevant to the in vivo functions of PDK1. Following the confirmation of the identity of the modifier(s) using methods such as mass spectrometry and Western analysis, experiments must be designed to
investigate potential roles in subcellular localization as well as enzymatic activity regulation. This information might prove invaluable for research aimed at modulating in vivo PDK1 activity for therapeutic purposes.
REFERENCES


APPENDIX A1

GENERATION OF RECOMBINANT BACULOVIRUS OF PDK1 CONSTRUCTS

A1.1. PCR Engineering of Recombinant pFastBac PDK1 Constructs

PCR was used to generate the cDNA encoding for either ‘full length’ PDK1 (His6-PDK1, residues 51–556) or the catalytic kinase domain of PDK1 (His6-PDK1(ΔPH), residues 51–359). A stop codon was inserted at position 557 of His6-PDK1 and position 360 of His6-PDK1(ΔPH). An N-terminal His6 tag followed by a PreScission protease recognition sequence was inserted prior to residue 51 of both His6-PDK1 and His6-PDK1(ΔPH). In order to facilitate restriction digestion and directional ligation into the pFastbac™1 vector, an EcoRI recognition sequence was inserted upstream of the ATG start codon of His6 tag and an XbaI recognition sequence was inserted downstream of the stop codon. Full length PDK1 in the pCR®-Blunt II-TOPO® plasmid vector (Gao et al., 2005) was used as the template to generate the PDK1 coding region, which does not contain any EcoRI or XbaI recognition sequences.

For His6-PDK1, the reverse or downstream 3′-primer was complementary to the coding region (residues 551–stop, italic) and extended to include the XbaI recognition sequence (lower case letters) (PDK1-R, 5′-ATATtctaga TCA CTG CAC AGC GGC GTC CGG CGT-3′). For His6-PDK1(ΔPH), the reverse or downstream 3′-primer was complementary to the coding region (residues 353–359, italic) and extended to include a stop codon and XbaI recognition sequence (lower case letters) [PDK1(ΔPH)-R, 5′-ATATtctaga TCA GGT GAG CTT CGG AGG CGT CTG-3′]. For both His6-PDK1 and His6-PDK1(ΔPH), two forward or upstream 5′-
primers were used to extend the cDNA to include a His$_6$ tag with a PreScission protease cleavage site and a flanking EcoRI recognition sequence. The PreScission-PDK1-F primer was complementary to the coding region (residues 51–58, italic) and extended to include the protease peptide recognition sequence (LEVLFQGP, bold) (PreScission-PDK1-F, 5′-CTG GAA GTT CTG TTC CAG GGG CCC ATG GAC GGC ACT GCA GCC GAG CCT-3′). The EcoRI-His$_6$-PreScission primer was complementary to the protease peptide recognition sequence (LEVLFQGP, bold) and extended to include the N-terminal His$_6$ tag coding (italic) and the EcoRI recognition sequences (lower case letters) (EcoRI-His$_6$-PreScission, 5′-gaattcTATAAAT ATG GCA CAT CAT CAT CAT CAT CAT CTG GAA GTT CTG TTC CAG GGG CCC-3′). The $T_m$ values for all of the overlapping regions were between 70–72 °C.

Each 50-µL PCR reaction contained 5 µL of 10× PCR Buffer (1.2 mM Tris-HCl, pH 8 at 25 °C, 100 mM KCl, 60 mM (NH$_4$)$_2$SO$_4$, 1% Triton X-100, and 0.01% bovine serum albumin), 2 µL of 25 mM MgSO$_4$, 2 µL of 50× dNTP mix (2 mM each of dATP, dCTP, dGTP, and dTTP), 1 µL of 50× High-Fidelity KOD Hot Start Polymerase Mix (Novagen), 1 µL of 10 µM of the PreScission-PDK1-F primer, 1.5 µL of 10 µM of the EcoRI-His$_6$-PreScission primer, 2.5 µL of 10 µM of the reverse DNA primer (either PDK1-R or PDK1($\Delta$PH)-R), and 100 ng of template. The reactions were carried out in 0.2 mL sterile thin-walled PCR tubes in a MWG Primus 25 “hot-lid” thermal cycler using the following protocol: incubation at 95 °C for 2 min; 20 cycles of 94 °C for 15 s, 60 °C for 30 s, and 68 °C for 2 min; and incubation at 72 °C for 10 min. The resulting blunt-ended cDNA products were
gel-purified (1% agarose), isolated using the QIAquick® Gel Extraction Kit (Qiagen), cloned into the pCR®-Blunt II-TOPO® plasmid vector (Invitrogen), and transformed into One Shot® TOP10 Chemically Competent E. coli. Selected colonies were grown in 10 mL LB/Amp (100 µg/mL); plasmids were isolated using the QIAprep® Spin Miniprep Kit (Qiagen) and digested with EcoRI and XbaI to verify the presence of the correct-sized insert. A sequence verified EcoRI-XbaI fragment of either the His₆-PDK1 or His₆-PDK1(ΔPH) construct was ligated into pFastBac™1 vector (Invitrogen) for baculovirus-mediated protein expression in Sf9 insect cells.

### A1.2. Site-Directed Mutagenesis

The pFastbac™1 vector containing His₆-PDK1 was used as the template for PCR generation of the S241G, K111A, E432K, E453K, and K512K mutants of His₆-PDK1. The pFastbac™1 vector containing His₆-PDK1(ΔPH) was used as the template for PCR generation of the S241G mutant of His₆-PDK1(ΔPH).

**Table A1.1: Forward (-F) and reverse (-R) oligonucleotide primers for His₆-PDK1 mutagenesis***

<table>
<thead>
<tr>
<th>primer</th>
<th>sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>K111A-F</td>
<td>5’-ggcaaacctcagagaatagcgatt(gcg)attctggagaagcagcataatc-3’</td>
</tr>
<tr>
<td>K111A-R</td>
<td>3’-ccggtgaggtctctcttatataggctaa(cgt)taagaccccttgctgtataag-5’</td>
</tr>
<tr>
<td>S241G-F</td>
<td>5’-gagagcaacaacgccaggcgaac(gga)ttcgtggaacagcagcgtacgttt-3’</td>
</tr>
<tr>
<td>S241G-R</td>
<td>3’-ctctccttttgtccggtcgcgtcatgcaa-5’</td>
</tr>
<tr>
<td>E432K-F</td>
<td>5’-ctccttggaggtctctcttatataggctaa(aaa)gatgagaagaggtgtgtgtttgagaag-3’</td>
</tr>
<tr>
<td>E432K-R</td>
<td>3’-ggagaccccttgcacgacccttcccaaanaggtcttaaatc-5’</td>
</tr>
<tr>
<td>E453K-F</td>
<td>5’-gcgggaaaccctttgccacgagttta(aaa)aatataatataactaaagatggc-3’</td>
</tr>
<tr>
<td>E453K-R</td>
<td>3’-cgcccttggaggtctctcttatataggctaa(atct)ttatattaattatgattttctacccgg-5’</td>
</tr>
<tr>
<td>K512E-F</td>
<td>5’-ctctcgacccagcgccgctaatctttttgtcccacagc-3’</td>
</tr>
<tr>
<td>K512E-R</td>
<td>3’-ggacagtgtctccggtcctttaaaa(ctc)tgaagacaggtgtgca-5’</td>
</tr>
</tbody>
</table>

*The nucleotides coding for the mutated residue are indicated in parentheses, and the nucleotide(s) that were altered are indicated in bold.*
Table A1.1 lists the oligonucleotide forward and reverse primers used for each site-directed mutagenesis reaction. Briefly, the complementary primers containing the centrally located mutated bases generate full length corrected copies of the entire template plasmid. Then, the methylated template plasmid is selectively digested away using DpnI endonuclease.

Each 50-µL PCR reaction contained 5 µL of 10× PCR Buffer (1.2 mM Tris-HCl, pH 8 at 25 °C, 100 mM KCl, 60 mM (NH₄)₂SO₄, 1% Triton X-100, and 0.01% bovine serum albumin), 2 µL of 25 mM MgSO₄, 2 µL of 50× dNTP mix (2 mM each of dATP, dCTP, dGTP, and dTTP), 1 µL of 50× High-Fidelity KOD Hot Start Polymerase Mix (Novagen), 1 µL of 10 µM of both the forward and reverse primers, and 50 ng of template. The reactions were carried out in 0.2 mL sterile thin-walled PCR tubes in a MWG Primus 25 “hot-lid” thermal cycler using the following protocol: incubation at 95 °C for 2 min; 15 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 7 min. Each reaction was then incubated with DpnI restriction enzyme for 1 hr at 37 °C to digest template plasmid DNA. The resulting mutant plasmid was transformed into One Shot® TOP10 Chemically Competent E. coli. Selected colonies were grown in 10 mL LB/Amp (100 µg/mL); plasmids were isolated using the QIAprep® Spin Miniprep Kit (Qiagen) and verified by sequencing.

A1.3. Generating Recombinant Bacmid

The recombinant pFastBac™1 vector containing the designated PDK1 construct was used to generate recombinant baculovirus using the Bac-to-Bac®
Baculovirus Expression System (Invitrogen). Briefly, the recombinant FastBac<sup>TM</sup> plasmid was transformed into DH10Bac<sup>TM</sup> competent cells of *E. coli*. The DH10Bac<sup>TM</sup> cells contain a baculovirus shuttle vector (bacmid) with a mini-*att*<sup>Tn7</sup> target site and a helper plasmid. Upon transformation, transposition occurs between the mini-Tn7 element on the recombinant pFastBac<sup>TM</sup>1 vector and the mini-*att*<sup>Tn7</sup> target site on the bacmid to generate a recombinant bacmid. The transposition reaction is catalyzed by transposition proteins supplied by the helper plasmid. Insertion of the mini-Tn7 into the mini-*att*<sup>Tn7</sup> attachment site on the bacmid disrupts expression of the LacZ<sub>a</sub> peptide. When transformed DH10Bac<sup>TM</sup> cells were grown on LB agar plates containing kanamycin (50 µg/mL), gentamicin (7 µg/mL), tetracycline (10 µg/mL), Bluo-gal (100 µg/mL), and IPTG (40 µg/mL), colonies containing recombinant bacmid were white, while colonies containing unaltered bacmid were blue. After selected white colonies were re-streaked, a single isolated large white colony was used to inoculate LB media containing kanamycin (50 µg/mL), gentamicin (7 µg/mL), tetracycline (10 µg/mL), and the high molecular weight recombinant bacmid DNA was isolated using the S.N.A.P.<sup>TM</sup> MidiPrep Kit (Invitrogen). Since the recombinant bacmid DNA is >135 kb in size, PCR analysis was used to verify the presence of the His<sub>6</sub>-PDK1 construct using High-Fidelity KOD Hot Start Polymerase Mix (Novagen), the M13 Forward (-20) (5'-GTAAAACGACGGCCAG-3') and M13 Reverse (5'-CAGGAAACAGCTATGAC-3') primers, and the protocol provided by Invitrogen.
**A1.4. Producing Recombinant Baculovirus in Sf9 Insect Cells**

Recombinant bacmids containing the designated PDK1 construct were purified using the S.N.A.P."TM MidiPrep Kit (Invitrogen). As suggested in the manufacturer's protocol, 1 µg of recombinant bacmid and 6 µL of Cellfectin® Reagent diluted in 200 µL of unsupplemented Grace's Medium were incubated for 45 min at room temperature and then further diluted with 0.8 mL of unsupplemented Grace's Medium (i.e., no antibiotics). This mixture was then added to individual wells of a 35 mm tissue culture plate that contained 9 × 10^5 attached Sf9 cells per well (>97% viability), and the cells were incubated at 27 °C for 5 h. Then, the bacmid solution was replaced with 2 mL of complete growth media (i.e., Sf-900 II serum-free media with antibiotics), and the cells were incubated at 27 °C for 72 h. The recombinant P1 viral stock was collected as the clarified supernatant after centrifugation of the media containing the cells from which viral budding had been inferred from a granular or vesicular appearance of the cells when viewed with an inverted phase microscope at 250–400×. The recombinant P1 viral stock was amplified twice by infection of a 50 mL suspension culture at 2 × 10^6 cells/mL. After centrifugation, the recombinant P2 and P3 stock was collected as the clarified supernatant, and a titer of 1 × 10^7 and 3 × 10^9 plaque forming units (pfu) / mL was obtained for P2 and P3 respectively. Aliquots of the recombinant P3 viral stock were stored either at −80 °C (long term storage) or 4 °C (immediate use). Viral titers were determined according to the manufacturers.
A1.5. Expression and Purification of His₆-PDK1 Constructs

Recombinant P3 viral stocks of PDK1 constructs were used to infect 200 mL spinner flask cultures of Sf9 cells in the mid-logarithmic phase of growth (2 × 10⁶ cells/mL) so that a multiplicity of infection (MOI) of 0.1 viral particles/cell was obtained. Infected cells were incubated at 27 °C for 56–60 h and harvested by centrifugation for 10 min at 4 °C at 3000 rpm in a Beckman tabletop centrifuge. The cells were re-suspended in 50 mL of buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 50 mM imidazole, 1 mM 2-mercaptoethanol, 1 mM benzamidine, 0.2 mM PMSF, and cOmplete™ Protease Inhibitor Cocktail), and were immediately stored at −80 °C. The re-suspended frozen cells were allowed to thaw on ice for 15 min before addition of 2 μL of benzonase and 1 mM MgCl₂. Then, the cells were allowed to continue thawing for 1 h, which resulted in efficient cell lysis and oligonucleotide degradation. Cell debris was pelleted by centrifugation for 45 min at 4 °C at 14,000 rpm in a SS34 rotor.

The soluble lysate containing the designated His₆-PDK1 construct was directly loaded by FPLC (1 mL/min) onto a 5 mL size Ni²⁺ Sepharose HiTrap FF affinity column equilibrated at 4 °C in 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 50 mM imidazole, 1 mM 2-mercaptoethanol, and 5% (v/v) glycerol. The column was subsequently washed until the absorbance at 280 nm returned to baseline, and the recombinant His₆ affinity tagged enzyme was eluted by linear increasing of the imidazole concentration from 50 to 500 mM at 1 mL/min for 30 min. Chromatographic fractions (1 mL) containing His₆ affinity tagged enzyme were detected by absorbance at 280 nm and confirmed by SDS-PAGE. Fractions
containing His₆ affinity tagged kinase were pooled and exchanged to storage buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% 2-mercaptoethanol, and 0.1 mM EGTA), using a HiPrep 26/10 desalting column. Protein was concentrated using a VivaSpin ultrafiltration concentrator (10 kDa molecular weight cutoff, VivaScience), adjusted to ~25 µM with addition of protease inhibitors (0.5 mM benzamidine and 0.1 mM PMSF), and stored as 0.1–1 mL aliquots at −80 °C. His₆-PDK1 and His₆-PDK1(ΔPH) concentrations were determined using the Bio-Rad Protein Assay Kit with bovine serum albumin as a standard.

A1.6. Removal of Phosphates

For removal of phosphate from S241 and any other potential serine, threonine, and tyrosine residues, ~25 µM of PDK1 kinase was diluted to ~5 µM in buffer containing 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 5 mM dithiothreitol, 0.01% Brij 35, and 2 mM MnCl₂. Then, 5 µg/mL of lambda protein phosphatase (λPP) was added, and the reaction was allowed to proceed for 1 h at 30 °C. The reaction mixture was adjusted to contain 50 mM imidazole and 300 mM NaCl, and dephosphorylated kinase was His₆ affinity purified. Chromatographic fractions containing His₆ affinity tagged kinase were combined and exchanged to storage buffer as described above.
APPENDIX A2

PCR ENGINEERING OF PDK1 TRANS-SPLICING CONSTRUCTS

PCR engineering of the cDNA coding sequences of the EcoRI-KINASE(AEY)-(N)NpuDnaE-His6-XbaI (Figure A2.1) and SacII-(C)SspDnaE-(CMN)PH-KpnI (Figure A2.2) fusion constructs are described in three steps:

A2.1. PCR Generation of EcoRI-KINASE(AEY) and (CMN)PH-KpnI (STEP 1)

The cDNA encoding for residues 51–381 of the kinase domain of PDK1 (Figure A2.1, red colored sequence) was obtained using the recombinant pCR®-Blunt II-TOPO® vector containing full length PDK1 (Gao et al., 2005) as the template with EcoRI-KINASE-F (5′-gaattc ATG GAC GGC ACT GCA GCC-3′) and KINASE(AEY)-R (5′-GTA TTC CGC GCT CAG GAG ATT GCC-3′) as the forward and reverse primers, respectively. The EcoRI-KINASE-F primer was complementary to the N-terminal kinase coding region (residues 51–56) and was extended in the 5′ direction to include an EcoRI recognition sequence (lower case letters). The KINASE(AEY)-R primer overlapped with the C-terminal coding region (residues 374–381) and was extended in the 5′ direction to encode for the required C-terminal AEY residues (bold), which replace native QFG (residues 382–384).

The cDNA encoding for residues 385–556 of the PH domain of PDK1 with the Q387N mutation (Figure A2.2, red colored sequence) was obtained using the recombinant pCR®-Blunt II-TOPO® vector containing full length PDK1 (Gao et al., 2005) as the template with (CMN)PH-F (5′-TGC ATG AAT GTC GAG ATT GCC-3′) as the forward and reverse primers, respectively.
TCC TCC TCA-3') and KpnI-(CMN)PH-R (5'- ggtacc TCA CTG CAC AGC GGC GTC-3') as the forward and reverse primers, respectively. The (CMN)PH-F primer was complementary to the C-terminal PH domain coding region (residues 385–394), but encoded for the Q387N mutation (bold). The KpnI-(CMN)PH-R primer overlapped with the C-terminal coding region (residues 552–stop) and was extended to contain a KpnI recognition sequence (lower case letters).

Figure A2.1: Coding sequence of EcoRI-KINASE(AYE)-(N)NpuDnaE-His6-Xbal. Colors indicate residues 51–384 of the catalytic domain of PDK1 (red) with the C-terminal triple QFG (blue) to 384 QFG (red) mutation (red block) fused to (N)NpuDnaE (blue) with a C-terminal His6 affinity tag (green). The coding sequence also contains flanking EcoRI and XbaI recognition sequences for unidirectional ligation into pFastBacTM1 (Invitrogen) for baculovirus-mediated protein expression in Sf9 insect cells.
Inc.), which provides an N-terminal dual GST-His6 affinity tag.

Extraction Kit (Qiagen).

Products were gel-purified (1% agarose) and isolated using the QIAquick

Tube in a MWG Primus 25 "hot-lid" thermal cycler using the following protocol:

Each 50-µL PCR reaction contained 5 µL of 10× PCR Buffer (1.2 mM Tris-
HCl, pH 8 at 25 °C, 100 mM KCl, 60 mM (NH₄)₂SO₄, 1% Triton X-100, and 0.01%
bovine serum albumin), 2 µL of 25 mM MgSO₄, 2 µL of 50× dNTP mix (2 mM
each of dATP, dCTP, dGTP, and dTTP), 1 µL of 50× High-Fidelity KOD Hot Start
Polymerase Mix (Novagen), 1 µL of 10 µM of both forward and reverse primers,
and 100 ng of plasmid template. The Tₘ values for all of the overlapping regions
were 64 ± 3 °C. Each reaction was carried out in a 0.2 mL sterile thin-walled PCR
tube in a MWG Primus 25 “hot-lid” thermal cycler using the following protocol:
in incubation at 95 °C for 1 min; 25 cycles of 94 °C for 15 s, 60 °C for 30 s, and 68
°C for 2 min; and incubation at 68°C for 10 min. The resulting blunt-ended cDNA
products were gel-purified (1% agarose) and isolated using the QIAquick® Gel
Extraction Kit (Qiagen).
**A2.2. Custom Gene Synthesis of KINASE(AEY)-(N)NpuDnaE-His$_6$-XbaI and SacII-(C)SspDnaE-(CMN)PH (STEP 2)**

The cDNA fragments encoding the KINASE(AEY)-(N)NpuDnaE-His$_6$-XbaI and SacII-(C)SspDnaE-(CMN)PH were custom synthesized by the TBIO method of gene synthesis (Gao et al., 2003). The (N)NpuDnaE split intein gene sequence (**Figure A2.1**, blue colored sequence) was extended in the (i) 5'-direction to overlap with the 3'-terminal 24 nucleotides of the EcoRI-KINASE(AEY) fragment and (ii) in the 3'-direction to contain a His$_6$ affinity tag and XbaI recognition sequence. The (C)SspDnaE split intein gene sequence (**Figure A2.2**, blue colored sequence) was further extended in the (i) 5'-direction to contain a SacII recognition sequence and (ii) in the 3'-direction to overlap with the 5'-terminal 23 nucleotides of the (CMN)PH-KpnI fragment.

For the TBIO method, the 3'-terminal ends of the first pair of 60-mer sense- and antisense-strand TBIO primers (IO-S01 and IO-A01) were chosen to overlap in the middle of the overall synthetic gene sequence to yield the optimal $T_m$ value ($64 \pm 2 \, ^\circ\text{C}$) for PCR elongation using the High-Fidelity KOD Hot Start Polymerase Mix (Novagen). Then, 60-mer sense-strand primers (**Tables A2.1A and A2.2A**) were chosen to sequentially extend in the 5'-direction to the 'amino'-terminus of the coding sequence. The 3'-end of each outside amino-terminal sense-strand primer (red) was selected by determining the number of base pairs required to generate an overlap with the complementary nucleotides at the 5'-end of the inside sense-strand primer (blue) to yield the optimal $T_m$ value. Likewise, the antisense-strand primers (**Tables A2.1B and A2.2B**) were chosen to obtain
overlapping regions with optimal \( T_m \) values and to sequentially extend in their 5'-direction to the 'carboxy'-terminus of the coding sequence.

Tables A2.1 and A2.2: The coding- or "sense"-strand oligonucleotide primers (5'→3') and the noncoding- or "antisense"-strand oligonucleotide primers (3'→5') designed for the TBIO PCR-based gene synthesis are listed for (A) the amino-terminal half and (B) the carboxy-terminal half of the target gene sequence. For each primer, the overall length (nt), the coding region of the gene sequence (according to either Figure A1.1 or A1.2), the optimized melting temperature, \( T_m \), and the number of base pairs (bp) for each overlapping region are given. For each primer, the 5'-terminal overlapping nucleotides are indicated in blue color, and the 3'-terminal nucleotides are indicated in red color. Green colored regions of primers indicate nucleotides that are common to both the 5' - and 3'-overlapping regions. Underlined nucleotides indicate overlapping regions with corresponding PDK1 domains; capitalized nucleotides indicating coding for His6; and italicized nucleotides indicate restriction enzyme recognition sequences.

### Table A2.1A: TBIO 'sense' strand primers for synthesis of KINASE(AEY)-(N)NpuDnaE-His6-XbaI

<table>
<thead>
<tr>
<th>Name</th>
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<th>Overlap (bp)</th>
<th>Length (nt)</th>
<th>Cod region</th>
<th>Name</th>
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<tr>
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<td>33</td>
<td>53</td>
<td>985-1037</td>
<td>I0-306</td>
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<tr>
<td>S02</td>
<td>63.6</td>
<td>30</td>
<td>60</td>
<td>1005-1064</td>
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<tr>
<td>S03</td>
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</tr>
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<td>S04</td>
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<td>60</td>
<td>1035-1094</td>
<td>I0-304</td>
</tr>
<tr>
<td>S05</td>
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<td>60</td>
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<td>I0-303</td>
</tr>
<tr>
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<td>60</td>
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<tr>
<td>S07</td>
<td>64.8</td>
<td>26</td>
<td>60</td>
<td>1123-1182</td>
<td>I0-301</td>
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### Table A2.1B: TBIO 'antisense' strand primers for synthesis of KINASE(AEY)-(N)NpuDnaE-His6-XbaI

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<tr>
<th>Name</th>
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<th>Length (nt)</th>
<th>Noncoding region (nt coding)</th>
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<tbody>
<tr>
<td>I0-A01</td>
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<td>I0-A05</td>
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### Table A2.2A: TBIO 'sense' strand primers for synthesis of SacII-(C)SspDnaE-(CMN)PH

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### Table A2.2B: TBIO 'antisense' strand primers for synthesis of SacII-(C)SspDnaE-(CMN)PH

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<th>Length (nt)</th>
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<tr>
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<td>63.0</td>
<td>10</td>
<td>80</td>
<td>80-10</td>
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</table>
The TBIO-designed primer sets were used for PCR-based gene synthesis of either the KINASE(AEY)-(N)NpuDnaE-His$_6$-XbaI or the SacII-(C)SspDnaE-(CMN)PH constructs by a one-step sequential "inside-out" bidirectional elongation reaction from the middle to both the amino- and carboxy-termini of the gene sequence. The 50-µL PCR reaction contained 5 µL of 10× PCR buffer (60 mM (NH$_4$)$_2$SO$_4$, 100 mM KCl, 1.2 mM Tris-HCl pH 8, 1% Triton X-100, 0.01% BSA), 2 µL of 25 mM MgSO$_4$, 1 µL of 50× dNTP mix (2 mM each of dATP, dCTP, dGTP, and dTTT), 1 µL of 50× High-Fidelity KOD Hot Start Polymerase Mix, and a concentration gradient of TBIO primer pairs. For synthesis of (C)kinase-(N)NpuDnaE-His$_6$-XbaI (Tables A2.1A and A2.1B), the concentration gradient was generated by addition of 1.0, 1.5, 2.0, 2.5, 3.0, and 5.0 µL of 2 µM stock concentrations of the TBIO primer pairs A,S-01, A,S-02, A,S-03, A,S-04, A,S-05, and A,S-06, respectively, yielding final concentrations of 40, 60, 80, 100, 120, and 200 nM, respectively. For synthesis of SacII-(C)SspDnaE-(N)PH (Tables A1.2A and A1.2B), the concentration gradient was generated by addition of 1.0 and 5.0 µL of 2 µM stock concentrations of the TBIO primer pairs A,S-01 and A,S-02, respectively, yielding final concentrations of 40 and 200 nM, respectively. Each reaction was carried out in a 0.2 mL sterile thin-walled PCR tube using the following optimized protocol: incubation at 95 °C for 2 min ("hot start") and 25 cycles [94 °C for 15 s, 60 °C for 30 s, and 68 °C for 30 s (60 s per 1000 bp of DNA being generated)]. The resulting blunt-ended cDNA products were gel-purified (1% agarose) and isolated using the QIAquick® Gel Extraction Kit (Qiagen).
A2.3. PCR Generation of EcoRI-KINASE(AEY)-(N)NpuDnaE-His\textsubscript{6}-Xbal and SacII-(C)SspDnaE-(CMN)PH-KpnI (STEP 3)

The overlapping pairs of corresponding PCR products \([\text{EcoRI}-\text{KINASE(AEY)} \text{ with } \text{KINASE(AEY)}-(\text{N})\text{NpuDnaE-His}_6-\text{Xbal}; \text{ SacII}-(\text{C})\text{SspDnaE-(CMN)PH with (CMN)PH-KpnI}]\) were joined by overlapping primer extension. Overlapping cDNA products were joined using the same PCR protocol (STEP 1) with 20 nM of each corresponding purified PCR product pair and 200 nM of the outermost primers. The \text{EcoRI}-\text{KINASE(AEY)}-(\text{N})\text{NpuDnaE-His}_6-\text{Xbal} and \text{SacII}-(\text{C})\text{SspDnaE-(CMN)PH-KpnI} blunt-ended fusion products were gel purified and ligated to the pCR\textsuperscript{®}-Blunt II-TOPO\textsuperscript{®} cloning vector. All ligation products were transformed into One Shot\textsuperscript{®} TOP10 Chemically Competent \textit{E. coli}. Selected colonies were grown in 5 mL LB containing 50 \(\mu\text{g/mL}\) ampicillin, 50 \(\mu\text{g/mL}\) kanamycin, and 170 \(\mu\text{g/mL}\) chloramphenicol. Plasmids were isolated using the QIAgprep\textsuperscript{®} Spin Miniprep Kit (Qiagen) and sequence verified using the T7 and T7 reverse primers.
APPENDIX A3

SINGLE PROTEIN PRODUCTION OF ISOTOPIC-LABELED PH DOMAIN

A3.1. Background and Significance

One important aim of the thesis was to determine whether the C-terminal PH domain interacts with the N-terminal catalytic kinase domain of PDK1. To approach this aim, NMR spectroscopy was used to compare spectral characteristics of the isolated PH domain to those of the PH domain in full length PDK1. Since proteins have such a large number of hydrogen atoms, individual proton signals cannot be resolved in one dimensional $^1$H NMR spectra. By correlating individual proton chemical shifts with the chemical shifts of the heteronucleus to which they are bonded, proton signals can be resolved in heteronuclear, multidimensional NMR experiments. However, proteins are primarily comprised of $^{14}$N and $^{12}$C NMR inactive nuclei, which precludes heteronuclear, multidimensional NMR experiments.

In order to carry out heteronuclear, multidimensional NMR experiments, it is necessary to generate protein samples comprised of the heavier $^{15}$N and $^{13}$C isotopes, which are NMR active nuclei. This is accomplished by inducing protein expression in *E. coli* grown in minimal medium that contains $^{15}$NH$_4$Cl and $^{13}$C-glucose as sole sources of nitrogen and carbon. Whereas such methods are well established, the large volumes of cell cultures (e.g., liters) require substantial weighed amounts of expensive heavy isotope-labeled sources to attain sufficient concentrations that satisfy growth and labeling requirements. Thus, the recently developed single protein production (SPP) system (Takara, Inc.) was tested and
further optimized for overexpression and isotopic labeling of the GST-(PreScission)-PH fusion construct.

The SPP system exploits use of the MazF gene product from *E. coli*. MazF is an unusual mRNA nuclease, which specifically recognizes and cleaves cellular mRNA at ACA sequences. Its induction in *E. coli* induces degradation of mRNA while the cell remains metabolically active and capable of synthesizing protein. By combining MazF expression with expression of an ACA-less recombinant gene, high yield single protein expression can be achieved. In addition, cell cultures can be condensed to only a fraction of the original volume with no loss in protein expression yield. This is particularly useful for isotopic labeling of proteins for NMR studies, whereby equivalent concentrations of $^{15}$NH$_4$Cl, $^{13}$C-glucose, and D$_2$O can be maintained while reducing their overall weighed amounts. In this section, the SPP protocol is described for expression and isotopic labeling of the GST-(PreScission)-PH fusion construct. By using this method, the cell culture volumes could be reduced by 20-fold, which thereby reduced utilization costs for $^{15}$NH$_4$Cl and $^{13}$C-glucose by the same amount. Although no protein products were used in any of the experiments of this thesis, this optimized protocol will be useful for future work in the laboratory.

A3.2. Experimental Procedures

A3.2.1. Design of ACA-Less GST-(PreScission)-PH cDNA Fusion Construct

The single protein production (SPP) system (Takara, Inc.) was developed as an alternative and more cost effective method for overexpression and isotopic
labeling of the GST-(PreScission)-PH fusion construct. As required for this system, the nucleotide sequence coding for the GST-(PreScission)-PH construct was altered to remove all ACA regions, while maintaining the overall protein coding sequence. The PreScission™ protease recognition sequence was inserted to facilitate efficient removal of the GST affinity tag. The GST-(PreScission)-PH cDNA fusion construct lacking ACA was custom synthesized and supplied in a pUC57 plasmid (GenScript, Inc.).

A3.2.2. SPP PRODUCTION OF GST-(PRESCISSION)-PH

The ACA-less GST-(PreScission)-PH cDNA fusion construct was then subcloned into the pColdIII expression plasmid of the SPP system (Takara, Inc.). This protein expression construct was co-transformed with the pMazF protein expression plasmid (Takara, Inc.) into the BL21 Star™ E. coli expression strain. Co-transformed colonies were selected by growth on agar plates containing 50 µg/mL carabenecillin and 25 µg/mL chloramphenicol.

A single colony of the co-transformed BL21 Star™ E. coli strain was used to inoculate a starter culture of 50 mL of PG minimal medium containing 50 µg/mL carabenecillin and 25 µg/mL chloramphenicol, which was incubated overnight with shaking at 37 °C. The next day, 20 mL of the starter culture was used to inoculate growth flasks containing 500 mL of PG minimal medium, which were shaken at 37 °C until an optical cell density of 0.8 OD_{600}. Expression of the MazF ribonuclease in the pMazF plasmid was induced by transfer of the growth
flasks to 15 °C and shaking for 3 h. Cell cultures were pelleted by centrifugation and combined (~6 g cells per liter).

Expression of the recombinant GST-(PreScission)-PH fusion construct was tested in varying degrees of condensed cell cultures. The minimum volume that gave adequate yield of GST-(PreScission)-PH was achieved by resuspending the combined cell pellet (~6 g) in 50 mL of PG minimal medium containing 50 µg/mL carabenecillin, and 25 µg/mL chloramphenicol. Expression of GST-(PreScission)-PH was induced by addition of 1 mM IPTG and incubating overnight with shaking at 15 °C. For uniform 15N- and 13C-isotopic labeling of PH domain fusion constructs, NH₄Cl and glucose in the 50 mL growth flask containing IPTG were replaced with identical amounts of 15NH₄Cl and 13C-glucose, respectively. Cell harvesting and protein purification was carried out exactly as described (Chapter 4).