Kinetic Mechanism of AKT/PKB Enzyme Family*

Xiaoling Zhang†, Shiwen Zhang‡, Harvey Yamane§, Robert Wahl¶, Arisha Ali†, Julie A. Lofgren†, and Richard L. Kendall‡

From the Departments of †Oncology Research, §Protein Science, and ¶Molecular Pharmacology, Amgen Inc., Thousand Oaks, California 91320

AKT/PKB is a phosphoinositide-dependent serine/threonine protein kinase that plays a critical role in the signal transduction of receptors. It also serves as an oncogene in the tumorigenesis of cancer cells when aberrantly activated by genetic lesions of the PTEN tumor suppressor, phosphatidylinositol 3-kinase, and receptor tyrosine kinase overexpression. Here we have characterized and suggested that the AKT family of kinases catalyzes reactions via an Ordered Bi Bi sequential mechanism with ATP binding to the enzymes prior to the peptide substrate and ADP being released after the phosphopeptide product. These results suggest that ATP is an initiating factor for the catalysis of AKT enzymes and may play a role in the regulation AKT enzyme activity in cells.

AKT/PKB, the cellular homologue of the murine transforming retrovirus oncogene v-akt (1–5), is a 60-kDa serine/threonine protein kinase composed of three functional domains as follows: a catalytic kinase domain and a short hydrophobic C-terminal tail. Three AKT/PKB isoforms, AKT1 (PKBα), AKT2 (PKBβ), and AKT3 (PKBγ), (GenBank™ accession numbers NM_005163, NM_001626, and NM_005465) have been identified with 73% identity (96% similarity) overall and 84% identity (99% similarity) in the kinase domain (6–11). The kinase domain of AKT shares 45% identity (about 60% similarity) with that of protein kinase A and protein kinase C and thus was classified as a member of AGC protein kinase family. The importance of AKT in growth factor signaling was first demonstrated by showing that it is activated by PI 3-kinase in a PH domain-dependent fashion (12–13). Subsequent studies revealed that AKT is activated by the phosphorylation of two critical residues as follows: Thr-308 (AKT1) in the kinase domain and Ser-473 (AKT1) in the C-terminal hydrophobic motif (14). Activation of Akt is triggered by the interaction between the lipid products of PI 3-kinase and the PH domain of AKT, which induces a conformational change in AKT exposing the activation segment of the kinase domain. This serves to recruit the 3-phosphoinositide-dependent protein kinase PDK1 to the proximity of the activation segment allowing the phosphorylation of Thr-308 (Thr-309 for AKT2 and Thr-305 for AKT3) (15–18, 20). The phosphorylation of Thr-308 only partially activates AKT; the phosphorylation of Ser-473 (Ser-474 for AKT2 and Ser-472 for AKT3) of the C-terminal hydrophobic motif completes the transition of AKT from inactive to a fully active conformation and stabilizes the protein in the active state (16–21). Although the Thr-308 kinase has been clearly shown to be PDK1, the identity of the kinase that phosphorylates Ser-473 has been in dispute. Recent studies suggested several candidate enzymes, including DNA-PKcs, ATM, and the mTOR-Rictor complex (22–25). Interestingly, all three enzymes belong to the PI 3-kinase superfamily, which is consistent with the fact that the phosphorylation of Ser-473 is inhibited by the PI 3-kinase inhibitor wortmannin and LY294002. It is possible that the regulation of Ser-473 involves multiple activating mechanisms under different conditions and therefore offers another level of specificity in signaling.

The physiological functions of AKT are executed through a wide range of downstream effectors involved in cell survival, cell proliferation, and cell growth (for reviews see Refs. 26–29). These substrates all share a common signature motif RXRXX(S/T), where the S/T is the phosphorylation site (27, 30). A peptide library screening approach has further defined the preferred residues flanking the phosphorylation site (31). The studies of individual isoforms of AKT have not shown obvious substrate specificity among the isoforms (19, 32). However, genetic deletion experiments in mice have demonstrated distinct roles of AKT1, AKT2, and AKT3 (33–36). One possible explanation for the isoform-specific functions could simply be the differential tissue expression pattern of the AKT isoforms because tissues types where the major defect resides in the AKT1, AKT2, and AKT3 knock-out mice somewhat correlate with the expression of a particular AKT isoform. However, isoform-specific phenotypes could not be completely explained by the tissue-specific expression pattern. For example, overexpression of AKT1 in adipocytes derived from AKT2−/− mouse embryo fibroblast cells was not able to rescue insulin response (37). In addition, although both AKT1 and AKT3 gene deletion in mice led to a brain size reduction, AKT1 deleted mice only exhibited decreased cell number, whereas AKT3 deleted mice exhibited both decreased cell number and volume (36).

Although AKT has been extensively studied in the last decade, the kinetic mechanism of its intrinsic kinase activity has not been described in detail. Thus there is little understanding of whether the enzyme catalytic properties of AKT contribute to the isoform-specific functions. One of the first detailed kinetic mechanistic studies on a protein kinase was described by Cook et al. (38) and Whitehouse et al. (39). The prototype kinase in these studies was cAMP-dependent protein kinase

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Oncology Research, Amgen Inc., One Amgen Center Dr., Thousand Oaks, CA 91320. Tel.: 805-447-1362; Fax: 805-499-2751; E-mail: xzhang@amgen.com.

‡ The abbreviations used are: PH, pleckstrin homology; AMP-PCP; S′-phosphoadenosine α,β-methylene diphosphonate; DTT, dithiothreitol; PIP2, inositol 1,4,5-trisphosphate; myr, myristoylated; HA, hemagglutinin; PKA, cAMP-dependent protein kinase; AMPK, AMP-activated protein kinase; PI, phosphatidylinositol.

© 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
AKT Kinetic Mechanism

FIGURE 1. Characterization of AKT enzymes used in the study. A, AKT1, AKT2, AKT3 were activated and purified in vitro as described under “Experimental Procedures.” Aliquots of 0.5 and 0.1 μg of proteins were analyzed by SDS-PAGE and stained by SimplyBlue™ SafeStain (catalogue number LC6060; Invitrogen). The positions of molecular weight markers were labeled to the left. B, Western analysis of the level of phospho-Ser-473 and phospho-Thr-308 of the same set of AKT isoforms as in A. Aliquots of 1, 2, and 5 ng from each isoform were loaded to SDS-PAGE, transferred to nitrocellulose, and blotted by phospho-Ser-473, phospho-Thr-308, and pan-AKT antibodies. C, Western blots of immunoprecipitates from Rat-1 cell lines expressing myr-HA-AKT1, myr-HA-AKT2, myr-HA-AKT3, and vector. Aliquots of 150 μg of total protein lysates from each cell line were immunoprecipitated by HA-monoclonal antibody. Each immunoprecipitate was split into 2 aliquots in a ratio of 1:3 and run by SDS-PAGE. The blots were detected by phospho-Ser-473, phospho-Thr-308, and pan-AKT antibodies.

EXPEDIMENTAL PROCEDURES

Materials—ATP, ADP, and AMP-PCP were purchased from Sigma. [γ-32P]ATP (3000 Ci/mmol) was purchased from PerkinElmer Life Sciences. All peptides were synthesized by the Amgen Peptide Technology. Full-length AKT1, AKT2, and AKT3 cDNA was purchased from AG Scientific. phabePuroL plasmids containing myr-HA-AKT1, myr-HA-AKT2, and myr-AKT3 cDNAs were gifts from Dr. William R. Sellers (Dana-Farber Cancer Institute, Harvard Medical School) (42, 43). PKD1 and MAPKAPK-2 were generated by Amgen. The 96-well MultiScreen® phosphocellulose filter plates (catalogue number MAPH N0B) were from Millipore. The 96-well Reacti-Bind™ protein G plates (catalogue number 15131) were from Pierce. Anti-AKT (catalogue number 9272) and antiphospho-AKT (catalogue numbers 9271 and 9275) antibodies were from Cell Signaling Technology. Anti-HA antibody (12CA5) was from Roche Applied Science. Anti-phospho-Thr-308, and pan-AKT antibodies were gifts from Dr. William R. Sellers (Dana-Farber Cancer Institute, Harvard Medical School) (42, 43). PDK1 and MAPKAPK-2 were generated by Amgen. The 96-well MultiScreen® phosphocellulose filter plates (catalogue number MAPH N0B) were from Millipore. The 96-well Reacti-Bind™ protein G plates (catalogue number 15131) were from Pierce. Anti-AKT (catalogue number 9272) and antiphospho-AKT (catalogue numbers 9271 and 9275) antibodies were from Cell Signaling Technology. Anti-HA antibody (12CA5) was from Roche Applied Science (catalogue number 11583816). Cell culture reagents were from Invitrogen. All other reagents were from Sigma.

Enzyme Preparation—Full-length AKT1, AKT2, and AKT3 cDNA were cloned into pFastBacHT baculovirus expression vector (catalogue number10584-027; Invitrogen), which added a His6 tag to the N terminus of the AKT sequence. Recombinant baculoviruses expressing the AKT cDNAs were generated using the Bac-to-Bac baculovirus expression system (Invitrogen) and used to infect S9 cells to produce cell pastes with recombinant AKT proteins. For all three isoforms, insect cell paste (60–100 g) was resuspended in 5 ml of lysis buffer per g (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM imidazole, 10% glycerol, 5 mM DTT. 1× Complete (Roche Applied Science)) and lysed by nitrogen cavitation in a Parr bomb. Lysates were clarified by centrifugation at 20,000 × g followed by 0.45-μm filtration. The filtered lysates were batch bound to Talon (Clontech), washed, and eluted with lysis buffer containing 100 mM imidazole. For Akt3 and Akt1, Talon elution fractions were applied to a HiTrap Q HP column (Amersham Biosciences) and eluted with a linear gradient of 0–0.5 M NaCl. For Akt1, peak fractions were concentrated and further purified on a Superdex 200 XK 26/60 column (Amersham Biosciences). For Akt2, Talon elution fractions were applied to a HiTrap Blue column (Amersham Biosciences) and eluted with a linear gradient of 0–2 M NaCl. Fractions containing Akt2 were pooled, diluted, and applied to a HiTrap Q HP column and eluted with a linear gradient of 0–0.5 M NaCl. Peak fractions were pooled, concentrated, and further purified on a Superdex 200 XK 26/60 column.

Each isoform of Akt was activated by PDK1 and MAPKAPK-2 as follows. Lipid vesicles were prepared by drying 200 μg (0.17 μmol) of PIP2 (AG Scientific), 1.68 μmol of PC, and 1.68 μmol of PS (Avanti Polar Lipids) in a siliconized glass tube in a SpeedVac. Lipids were resuspended in 1.68 ml of activation buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT) and freeze-thawed three times. The resulting suspension was extruded 15 times through a 0.1-μm membrane in a mini-extruder (Avanti). Akt (4 mg), PDK1 (0.4 mg), and p38-activated MAPKAPK-2 (0.4 mg) were added to 1.6 ml of PIP2/PC/PS vesicles. ATP and MgCl2 were added to 1 and 10 mM, respectively, and the final reaction volume was adjusted to 8 ml with activation buffer. The activation reaction was allowed to proceed for 1 h at room temperature. Reactions were quenched by the addition of 1/19 volume of 20% Triton X-100 (1% final) and then centrifuged at 100,000 × g for 1 h. The supernatants were diluted and applied to a HiTrap Q HP column and eluted with a linear gradient of 0–0.5 M NaCl. Peak fractions were pooled, and protein concentration was determined by the method of Bradford using bovine serum albumin as a standard.

AKT Kinase Reaction—The kinase activity of AKT was measured by phosphorylating an optimal AKT peptide substrate identified from peptide library screening by Obata et al. (31) as follows: ARKRERTYSFGHHA, where S9 is the phosphorylation site. The reac-
tion mixture contained 20 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 1 mM DTT, 1 mM EGTA, 1 mM Na3VO4, 0.1% bovine serum albumin (kinase reaction buffer), with the addition of activated AKT. The kinase reactions were performed ranging from 5 to 20 min and enzyme concentrations from 0.4 to 3.3 nM to determine linearity. The optimal concentration of AKT1, AKT2, and AKT3 in the reactions was determined to be 1.7, 3.3, and 1.7 nM, respectively. The reactions were initiated by adding the peptide substrate and [γ-33P]ATP/ATP in a final reaction volume of 100 µL. The reaction mixtures were incubated at room temperature for 10 min and stopped by adding 100 µL of 5% phosphoric acid. Under these conditions, all initial velocity reactions utilized less than 10% of the substrates. For the measurement of the products, the total mixtures were transferred to phosphocellulose filter plates. The peptide substrates were captured by the phosphocellulose filter paper after vacuum

A. AKT1 two-substrate kinetics

B. AKT2 two-substrate kinetics

C. AKT3 two-substrate kinetics

FIGURE 2. Two-substrate kinetic analysis of AKT1, AKT2, and AKT3 enzymes. Double-reciprocal plots of 1/v versus 1/ATP (left) and 1/v versus 1/peptide (right) from initial velocity studies. Conditions were as described under “Experimental Procedures.” A, AKT1 velocities were measured in the presence of 7.8–250 µM ATP and 0.47–30 µM of peptide substrate; B, AKT2 velocities were measured in the presence of 15.6–500 µM ATP and 0.47–30 µM of peptide substrate; C, AKT3 velocities were measured in the presence of 15.6–500 µM ATP and 3.125–100 µM of peptide substrate.
AKT Kinetic Mechanism

TABLE 1
Comparison of the kinetic constants of AKT1, AKT2 and AKT3

AKT represents purified and in vitro activated AKT isoforms, and myr-AKT-IP represents recombinant myristoylated AKT isoforms expressed and immunoprecipitated from Rat-1 cells. Peptide = ARKRERTYSFGHHAA. The experimental details and data analysis are as described under “Experimental Procedures.”

<table>
<thead>
<tr>
<th>Varied substrate</th>
<th>( K_{m} , \mu M )</th>
<th>( K_{m} , \mu M )</th>
<th>( K_{m} , \mu M )</th>
<th>( k_{cat} , \text{min}^{-1} )</th>
<th>( \alpha ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Two-substrate kinetics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AKT1</td>
<td>ATP</td>
<td>55.2 ± 8.1</td>
<td>154.0 ± 58.2</td>
<td>1.6 ± 0.4</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Peptide</td>
<td>0.5 ± 0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AKT2</td>
<td>ATP</td>
<td>358.4 ± 47.2</td>
<td>296.6 ± 99.4</td>
<td>2.5 ± 0.5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Peptide</td>
<td>3.4 ± 0.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AKT3</td>
<td>ATP</td>
<td>87.9 ± 22.7</td>
<td>111.6 ± 62.0</td>
<td>119</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>Peptide</td>
<td>12.4 ± 3.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>B. Single substrate kinetics from cellular complex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( K_{m} ) for ATP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>myr-AKT1-IP</td>
<td>143.3 ± 21.9</td>
<td>2.9 ± 0.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>myr-AKT2-IP</td>
<td>564 ± 69.1</td>
<td>2.3 ± 1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>myr-AKT3-IP</td>
<td>118.7 ± 46.7</td>
<td>2.3 ± 1.1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

and

\[
\nu = \frac{V_{\text{max}}}{(K_{d}/[A] + K_{d}/[B] + 1)} \text{ (ping-pong)} \quad (\text{Eq. 3})
\]

For the determination of enzyme inhibition mechanism, data were fitted into Equations 4–6,

\[
\nu = \frac{V_{\text{max}}}{(K_{m}([1 + [I]/K_{i}] + [S])} \text{ (competitive)} \quad (\text{Eq. 4})
\]

\[
\nu = \frac{V_{\text{max}}}{[S]/(K_{m}([1 + [I]/K_{i}] + [S])} \text{ (noncompetitive)} \quad (\text{Eq. 5})
\]


In these equations, \( \nu \) represents the measured velocity; \( V_{\text{max}} \) is the maximum velocity; \( A, B, \) and \( S \) are substrates; \( K_{d}, K_{i}, \) and \( K_{m} \) are the Michaelis constants for \( A, B, \) and \( S; \) \( K_{d} \) is the dissociation constant for \( A; \) \( K_{i} \) is the dissociation constant for \( B; \) and \( K_{m} \) is the inhibitor constant.

For the determination of interaction between the two substrate sites, data were fitted into Equation 7,

\[
\nu = \frac{V_{\text{max}}}{(\alpha K_{d}/[A] + \alpha K_{d}/[B] + \alpha K_{d}/[A][B] + 1)} \quad (\text{Eq. 7})
\]

where the \( \alpha \) factor indicates whether or not the two sites are independent (48). When \( \alpha = 1 \), there is no interaction, and the two substrates are independent. When \( \alpha < 1 \), the two substrate sites interact with each other with positive cooperativity; the binding of one substrate increases the affinity of the other substrate. When \( \alpha > 1 \), the two substrate sites interact with each other with negative cooperativity; the binding of one substrate decreases the affinity of the other substrate.

RESULTS

Initial Velocity Studies—The three AKT isoforms AKT1, AKT2, and AKT3 were His-tagged and expressed in an Sf9 cell baculovirus system and purified to homogeneity. The AKT proteins were activated by PDK1 in the presence of PIP2 and subsequently by MAPKAPK-2 as described under “Experimental Procedures.” The reactions were driven to saturation by excess ATP to allow maximum activation of AKT. The application and washed three times by 1% phosphoric acid. The filter plates were counted by Wallac MicroBeta® liquid scintillation counter (PerkinElmer Life Sciences).

Kinase Reaction of Immunoprecipitated AKT—Rat-1 fibroblast cells stably expressing myr-HA-AKT1, myr-HA-AKT2, and myr-HA-AKT3 were generated using pbabePuroL-AKT expression plasmids and were expanded in Dulbecco’s modified Eagle’s media containing 10% fetal bovine serum, 2 mM l-glutamine, and 3 μg/ml puromycin (selective agent). To prepare for the cell lysate, cells were washed by PBS and lysed on ice by the addition of a lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na3VO4, 1 μM microcystin-LR, and 1× EDTA-free protease inhibitor mixture (catalogue number 1873580; Roche Applied Science). Clear lysate was isolated from cell debris by a refrigerated bench-top centrifuge at 14,000 × g for 15 min. To specifically pull down the myr-HA-AKT complex, lysates were diluted to a buffer containing 20 mM Tris-HCl, pH 7.5, and 150 mM NaCl (TBS) at a 1:1 ratio, applied to a Reacti-Bind™ protein G plate coated with anti-HA antibody, allowed to bind for 1.5 h at room temperature, and washed three times in TBS with 0.2% Triton X-100 followed by a final wash with kinase reaction buffer. The kinase reactions were initiated by adding 50 μl kinase reaction buffer containing peptide and [γ-32P]ATP/ATP in the indicated amount. The reaction was incubated at room temperature for 10 min and stopped and counted as described above.

Data Analysis—Kinetic data were fitted to equations using the iterative minimum \( \chi^{2} \) nonlinear regression method of Leatherbarrow (44) and converted into double-reciprocal Lineweaver-Burk plots. All kinetic studies were performed in duplicate when using purified enzymes and in quadruplicate when using the immunoprecipitated enzymes. The equations for kinetics analysis were as described by Cleland (45–47) and Segel (48).

For single substrate kinetics measurements (Equation 1),

\[
\nu = \frac{V_{\text{max}}[S]}{(K_{m} + [S])} \quad (\text{Eq. 1})
\]

For the discrimination between sequential and ping-pong mechanisms of two-substrate kinetics, data were fitted into Equations 2 and 3,

\[
\nu = \frac{V_{\text{max}}}{(K_{d}/[A] + K_{d}/[B] + K_{d}/[A][B] + 1)} \quad (\text{sequential}) \quad (\text{Eq. 2})
\]
activated AKT proteins were re-purified, quantified, and examined by Western analysis as shown in Fig. 1. All three isoforms were purified to greater than 90% homogeneity (Fig. 1A). The Thr-308 and Ser-473 sites were phosphorylated to similar levels across the isoforms with a subtle difference as follows: the highest percentage being in AKT2 followed by AKT1 and AKT3 (Fig. 1B).

Two-substrate site kinetics of the forward reactions was performed using these enzymes in the absence of added products. Initial velocities were measured in a matrix of varied ATP concentration and peptide substrate concentrations, and the data were fitted to the equations for sequential and ping-pong mechanism (see Equations 2 and 3 under "Experimental Procedures") to distinguish between the two mechanisms. The double-reciprocal plots of AKT1, AKT2, and AKT3 two-substrate kinetics all best fitted to the pattern of a typical sequential mechanism where the plots of one substrate at varied concentrations of the other one converged to the left of the y axis (Fig. 2), whereas if the reactions followed the ping-pong mechanism, the plots should have been parallel lines with the same slope factors. These results suggest that the catalysis of AKT enzymes requires the formation of a ternary complex of the enzyme with the two substrates before product release. The kinetic constants obtained from the two-substrate kinetic studies were summarized in Table I, part A.

**FIGURE 3. Product inhibition of AKT1, AKT2, and AKT3 enzymes by ADP.** A, AKT1, double-reciprocal plots of 1/v versus 1/ATP with 7.8–250 μM ATP and 20 μM peptide (left); and 1/v versus 1/peptide with 0.47–7.5 μM peptide and 20 μM ATP (right). B, AKT2, double-reciprocal plots of 1/v versus 1/ATP with 15.6–500 μM ATP and 20 μM peptide (left); and 1/v versus 1/peptide with 0.94–15 μM peptide and 200 μM ATP (right). C, AKT3, double-reciprocal plots of 1/v versus 1/ATP with 15.6–500 μM ATP and 20 μM peptide (left); and 1/v versus 1/peptide with 0.94–15 μM peptide and 100 μM ATP (right). ADP concentrations were as indicated. Conditions were as described under "Experimental Procedures."
### AKT Kinetic Mechanism

The double-reciprocal lines did not converge exactly on the x axis, suggesting that binding of one substrate affects the binding of the other substrate, i.e. the two substrate sites are interactive. This finding is similar to what has been observed for PKA in its kinetic study (39) and the crystal structure study with protein kinase inhibitor (PKI) and ATP (49). The two-site interaction factor \( \alpha \) was determined using the rapid equilibrium sequential model (see "Experimental Procedures," Equation 7; Table 1, part A). In the case of AKT1 and AKT3, binding of one substrate increased the binding of the other substrate. However, for AKT2, the binding of one substrate decreased the binding of the other substrate, although the degree of interaction was within 2-fold. It is important to note that the interaction factor was determined from purified recombinant proteins, possibly providing a mechanism for the feedback regulation of AKT activity.

Unexpected from the high degree of homology in the kinase domains, different AKT isoforms showed different kinetic parameters. AKT2 exhibited the greatest dissociation constant and apparent \( K_m \) value for ATP compared with AKT1 and AKT3. However, AKT3 showed the highest catalytic efficiency with a \( k_{cat} \) of 119, whereas the \( k_{cat} \) values of AKT1 and AKT2 were similar (22 for AKT1 and 25 for AKT2). To further confirm that the differences of the AKT isoforms in their kinetic constants are relevant to their activated state *in situ*, immunoprecipitates of constitutively active AKT1, AKT2, and AKT3 expressed in Rat-1 fibroblast cells (myr-HA-AKTS) were used to obtain the \( K_m \) values for ATP and peptide substrate. The three myr-HA-AKT isoforms were all dually phosphorylated at Thr-308 and Ser-473 sites as shown in Fig. 1C. Consistent with the results from purified enzymes, AKT2 also showed the highest \( K_m \) value for ATP (Table 1, part B), suggesting that the three AKT isoforms are different in their intrinsic kinetic properties.

**Dead-end Inhibition**—Dead-end inhibitors often provide definitive answers in discriminating among the different kinetic mechanisms. Two dead-end inhibitors were used in this study to confirm the results from product inhibition as follows: AMP-PCP, a nonhydrolyzable ATP analogue, and Ala peptide, the substrate peptide with the phosphate acceptor residue serine altered to alanine. When the peptide was the varied substrate, the pattern of the double-reciprocal lines at different concentrations of Ala peptide converged to the y axis (Fig. 4), suggesting that Ala peptide is a competitive inhibitor of the peptide substrate and the Ala peptide shares the binding site with the peptide substrate excluding the two substrate sites are interactive. This finding is similar to what has been observed for PKA in its kinetic study (39) and the crystal structure study with protein kinase inhibitor (PKI) and ATP (49). The two-site interaction factor \( \alpha \) was determined using the rapid equilibrium sequential model (see "Experimental Procedures," Equation 7; Table 1, part A). In the case of AKT1 and AKT3, binding of one substrate increased the binding of the other substrate. However, for AKT2, the binding of one substrate decreased the binding of the other substrate, although the degree of interaction was within 2-fold. It is important to note that the interaction factor was determined from purified recombinant proteins, possibly providing a mechanism for the feedback regulation of AKT activity.

Unexpected from the high degree of homology in the kinase domains, different AKT isoforms showed different kinetic parameters. AKT2 exhibited the greatest dissociation constant and apparent \( K_m \) value for ATP compared with AKT1 and AKT3. However, AKT3 showed the highest catalytic efficiency with a \( k_{cat} \) of 119, whereas the \( k_{cat} \) values of AKT1 and AKT2 were similar (22 for AKT1 and 25 for AKT2). To further confirm that the differences of the AKT isoforms in their kinetic constants are relevant to their activated state *in situ*, immunoprecipitates of constitutively active AKT1, AKT2, and AKT3 expressed in Rat-1 fibroblast cells (myr-HA-AKTS) were used to obtain the \( K_m \) values for ATP and peptide substrate. The three myr-HA-AKT isoforms were all dually phosphorylated at Thr-308 and Ser-473 sites as shown in Fig. 1C. Consistent with the results from purified enzymes, AKT2 also showed the highest \( K_m \) value for ATP (Table 1, part B), suggesting that the three AKT isoforms are different in their intrinsic kinetic properties.

**Product Inhibition**—To distinguish between a random or ordered substrate addition mechanism for the AKT two-substrate kinetics, initial velocities of AKT isoforms were measured in the presence of the reaction product ADP (Fig. 3). ADP inhibited the reactions of all three AKT isoforms with a similar pattern in which ADP was a competitive inhibitor *versus* ATP and a noncompetitive inhibitor *versus* peptide. This pattern is the same as in PKA reactions as shown by Whitehouse *et al.* (39) and is consistent with a steady state Ordered Bi Bi mechanism in which the ATP is the first substrate to bind to the enzyme, and ADP is the last product released. However, if the two substrates bind to the enzyme randomly, the inhibition would have been noncompetitive *versus* both ATP and peptide (39, 48). In addition, these data suggest that under the conditions tested the three AKT isoforms share the same kinetic mechanism, and thus AKT1 was used in the subsequent studies as a model to further elucidate the steady state kinetics of the AKT enzymes.

**Dead-end Inhibition**—Dead-end inhibitors often provide definitive answers in discriminating among the different kinetic mechanisms. Two dead-end inhibitors were used in this study to confirm the results from product inhibition as follows: AMP-PCP, a nonhydrolyzable ATP analogue, and Ala peptide, the substrate peptide with the phosphate acceptor residue serine altered to alanine. When the peptide was the varied substrate, the pattern of the double-reciprocal lines at different concentrations of Ala peptide converged to the y axis (Fig. 4), suggesting that Ala peptide is a competitive inhibitor of the peptide substrate and the Ala peptide shares the binding site with the peptide substrate excluding...
sively. When ATP was the varied substrate, the Ala peptide behaves as an uncompetitive inhibitor where the double-reciprocal lines at different concentrations of Ala peptide were parallel to each other (Fig. 4). These data are consistent with a mechanism where the peptide substrate site is only available when ATP is complexed with the enzyme, i.e. the binding of peptide substrate to the enzyme requires the presence of ATP. If the binding of the two substrates were random, the inhibition pattern would have been noncompetitive versus ATP. These results provided strong support for an Ordered Bi Bi kinetic mechanism where ATP binding to the enzyme is a prerequisite for peptide substrate binding. The second dead-end inhibitor ATP analogue AMP-PCP inhibited the reaction as a competitive inhibitor when ATP was the varied substrate and a noncompetitive inhibitor when peptide was the varied substrate (Fig. 5). These data further confirmed that ATP is the first substrate to bind to the AKT enzymes and thus the Ordered Bi Bi kinetic mechanism for AKT. The patterns of inhibition and kinetic constants of the product and dead-end inhibition study of AKT1 are summarized in Table 2. Therefore, the data taken together were consistent with the kinetic scheme as illustrated in Scheme 1.

**DISCUSSION**

Our initial velocity studies, product inhibition, and dead-end inhibition have consistently shown that AKT enzymes follow an ordered sequential kinetic mechanism by which ATP binds to the enzyme first and protein substrate binding is dependent on the ATP binding, which has also been shown for PKA, another member of AGC family (39). This kinetic mechanism suggests that the ATP, rather than protein substrate of AKTs, initiates the AKT reaction and perhaps provides a rate-limiting step in the regulation of its activity. One argument against this hypothesis can be that the cellular ATP concentration is too high (considered in the low millimolar range) and that the magnitude of ATP concentration change in cells can hardly impact the enzyme activity of a kinase. Therefore studies investigating the cellular concentrations of ATP and the product inhibitor ADP under the physiological conditions will allow further testing of this hypothesis. It is possible that cells in tissues under low oxygen tension have a much lower ATP level than what is measured in cell cultures where excess oxygen and glucose are supplemented. Therefore, the ATP level in tissues under hypoxic conditions may set the threshold for AKT activity and thus the survival signal. In fact, there is increasing evidence that AKT activity is regulated by ATP levels in cells. A study by Hresko et al. (50) showed that reduced intracellular ATP by glucosamine treatment impaired insulin response. Although the study did not directly measure AKT kinase activity in response to ATP reduction, it is likely that the effect of ATP depletion on insulin response was through its major downstream target AKT. Recent studies have also shown that the activity of AKT and another kinase in the same pathway mTOR is dependent on the glucose level and the mitochondrial hexokinase activity, and both play key roles in the regulation of cellular ATP levels (51–53). Perhaps more direct evidence is available from a study by Nagata et al. (54) examining the role of AMP-activated protein kinase (AMPK) in the angiogenic activity of endothelial cells under hypoxic conditions. AMPK is an ATP sensor kinase that increases the cellular ATP level in responses to hypoxia and other cellular energy stress. The study showed that AMPK was required for vascular endothelial growth factor-stimulated AKT kinase activity only under hypoxia conditions but not under normoxia conditions, suggesting that AKT activity is dependent on the cellular ATP level, which is maintained by AMPK under hypoxia. Recent studies also showed that AKT can in turn increase cellular ATP levels (53, 55), suggesting that the cellular energy state and ATP level can provide a positive feedback loop to drive the AKT activity.

Although the three AKT isoforms seem to share the same kinetic mechanism, the kinetic constants of these isoforms were different thus suggesting different catalytic properties among the isoforms. The difference was mainly associated with the affinity of the isoforms for ATP and the catalytic efficiency. AKT2 showed the lowest affinity for ATP and thus about a 5-fold higher $K_m$ value than that of AKT1 and AKT3. Interestingly, the physiological role of AKT2 is also distinct from that of AKT1 and AKT3 in that AKT2 has been shown to be the major effector in insulin signaling and glucose metabolism, whereas AKT1 and AKT3 are mainly involved in growth and survival with AKT1 functioning in all organs and AKT3 specifically in the brain (33–36). The high expression of AKT2 in insulin-responsive tissues has not been able to completely explain this functional difference (37). It is possible that AKT2 is adapted to these specific tissues in such way that through a dependence on its catalytic properties, only this isoform is able to sense a change in ATP concentration and thus glucose metabolism. AKT3, the lowest abundant isoform of the three, showed the highest catalytic activity with a 5-fold higher $k_{cat}$ value than AKT1 and AKT2. Genetic deletion of AKT3 in mice led to a specific brain size reduction (36). Protein expression analysis in mouse brain showed that AKT3 represents 50% of total AKT activity, whereas AKT1 and AKT2 make up the other 50% of the activity (36). It will be interesting to test whether simply elevated expression of AKT1 or AKT2 can compensate for the loss of AKT3 activity in brain cells or if there is a specific requirement for AKT3.

### Table 2

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Varied substrate</th>
<th>Inhibition pattern</th>
<th>$K_i$ ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Product inhibition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ATP</td>
<td>C</td>
<td>104.5 ± 7.6</td>
</tr>
<tr>
<td>Phosphopeptide&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Peptide</td>
<td>NC</td>
<td>119.9 ± 7.1</td>
</tr>
<tr>
<td></td>
<td>ATP</td>
<td>NC</td>
<td>1367 ± 237.6</td>
</tr>
<tr>
<td></td>
<td>Peptide</td>
<td>NC</td>
<td>1373.5 ± 187.1</td>
</tr>
<tr>
<td>B. Dead-end inhibition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMP-PCP&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ATP</td>
<td>C</td>
<td>1075.2 ± 149.8</td>
</tr>
<tr>
<td></td>
<td>Peptide</td>
<td>NC</td>
<td>1272.4 ± 125.1</td>
</tr>
<tr>
<td>Ala peptide&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ATP</td>
<td>UC</td>
<td>12.9 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Peptide</td>
<td>C</td>
<td>9.4 ± 0.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> The concentration of peptide and ATP was fixed at 20 $\mu$M when ATP and peptide are varied substrates. Peptide = ARRERTYSFGHHA.

<sup>b</sup> The concentration of peptide was fixed at 10 $\mu$M when ATP was the varied substrate. Peptide = RPRAAT; phosphopeptide = RPRAAT(H<sub>2</sub>PO<sub>4</sub>)F.

<sup>c</sup> The concentration of AMP-PCP was fixed at 4 $\mu$M when ATP was the varied substrate; the concentration of ATP was fixed at 50 $\mu$M when peptide was the varied substrate. Peptide = ARRERTYSFGHHA.

<sup>d</sup> The concentration of peptide was fixed at 2 $\mu$M when ATP was the varied substrate; the concentration of ATP was fixed at 50 $\mu$M when peptide was the varied substrate. Peptide = ARRERTYSFGHHA; Ala peptide = ARRERTYAFGHHA.
AKT Kinetic Mechanism

AKT resides in the central node of receptor signaling and has been demonstrated to be a critical factor in both normal and tumor cell function. The fact that AKT enzyme activity is constitutively activated in a wide range of cancers has positioned AKT as an attractive drug target for cancer therapy. Distinct functions of AKT isozymes shown by mouse genetic studies have suggested that an isozyme-selective AKT inhibitor can be efficacious on selected tumor types. The different intrinsic catalytic properties of these isozymes may provide an opportunity for the development of isoform-selective kinase inhibitors of AKT.

Acknowledgments—We thank the peptide technology group at Amgen for synthesizing the peptides used in this study, Dr. William R. Sellers for providing us the Rat-1-AKT cell lines, and Dr. Murray Robinson for helpful discussions.

REFERENCES

Kinetic Mechanism of AKT/PKB Enzyme Family
Xiaoling Zhang, Shiwen Zhang, Harvey Yamane, Robert Wahl, Arisha Ali, Julie A. Lofgren and Richard L. Kendall

doi: 10.1074/jbc.M601384200 originally published online March 15, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M601384200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 53 references, 30 of which can be accessed free at http://www.jbc.org/content/281/20/13949.full.html#ref-list-1