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Conformational changes in Akt1 activation probed by amide hydrogen/deuterium exchange and nano-electrospray ionization mass spectrometry[†]

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Abstract

Amide hydrogen exchange coupled to nano-electrospray ionization mass spectrometry (nano-ESI-MS) has been used to identify and characterize localized conformational changes of Akt upon activation. Active or inactive Akt was incubated in D₂O buffer, digested with pepsin, and analyzed by nano-ESI-MS to determine the deuterium incorporation. The hydrogen/deuterium (H/D) exchange profiles revealed that Akt undergoes considerable conformational changes in the core structures of all three individual domains after activation. In the PH domain, four β -strand (β 1, β 2 β 5 and β 6) regions containing membrane-binding residues displayed higher solvent accessibility in the inactive state, suggesting that the PH domain is readily available for the binding to the plasma membrane for activation. In contrast, these β -strands became less exposed or more folded in the active form, which is favored for the dissociation of Akt from the membrane. The beginning α -helix J region and the C-terminal locus (T450-470P) of the regulatory domain showed less folded structures that probably enable substrate entry. Our data also revealed detailed conformational changes of Akt in the kinase domain due to activation, some of which may be attributed to the interaction of the basic residues with phosphorylation sites. Our H/D exchange results indicating the conformational status of Akt at different activation states provided new insight for the regulation of this critical protein involved in cell survival.

Akt is a key serine/threonine kinase that controls many cellular processes such as cell survival, differentiation, proliferation and metabolism.^{1,2} In mammals, Akt comprises three highly homologous isoforms known as Akt1, Akt2 and Akt3. Each isoform contains three distinct functional regions, including an N-terminal pleckstrin homology (PH) domain (residues 1-108), a central kinase domain (KD, residues 150-408), and a C-terminal regulatory domain (RD, residues 409-480).³ It is well established that Akt activation is mediated by membrane phospholipids particularly phosphatidylinositol 3,4,5-trisphosphate (PIP3) which is produced by phosphoinositide 3-kinase (PI3K) in response to the growth factor receptor stimulation. The interaction of the PIP3 with the PH domain of Akt triggers the translocation of cytosolic Akt to the plasma membrane. The membrane interaction results in Akt conformational changes, exposing T308 of the kinase domain and S473 of the regulatory domain. The phosphorylation of both T308 and S473 by phosphoinositide-dependent protein kinase 1 and 2 (PDK1 and PDK2), respectively, leads to full activation of Akt.^{4–8}

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As Akt activation is tightly associated with its conformational changes, identifying and characterizing conformation statuses of Akt have been aimed to provide insight into the molecular mechanism of the activation which is not completely understood. Although the crystal structure of full-length Akt is not available, high-resolution structures of the isolated PH and kinase domains of Akt, with or without ligand binding, have been reported by X-ray crystallography.^{9–14} In addition, NMR and circular dichroism have provided in-solution structural information for Akt at PIP3-bound state.^{12,15} Recently, we have monitored the conformation of full-length Akt during its activation processes in a physiologically relevant condition using chemical cross-linking and mass spectrometry.¹⁶ We demonstrated that Akt undergoes dramatic interdomain conformational changes at each state of the activation processes and substrate binding. Specifically, inactive Akt exists as a folded structure with the PH and RD domains covering parts of the kinase domain. The PH and RD domains unfold upon Akt-membrane interaction and T308 and S473 are exposed for phosphorylation. After activation by phosphorylation, the PH domain folds toward the kinase domain, presumably assisting the dissociation of Akt from the membrane with an open RD domain that enables substrate entrance.¹⁶ Nevertheless, detailed conformational statuses within individual domains still remain unclear.

In the past decade, solution-phase hydrogen/deuterium (H/D) exchange in combination with mass spectrometry (MS) has become a sensitive tool to study protein structures.^{17–21} This approach is based on the fact that the H/D exchange rate at protein amide linkages is highly dependent on the spatial arrangements of proteins. In general, a fast exchange rate is expected when amide hydrogens are solvent-exposed, while a slow exchange rate is observed when amide protons are less accessible to solvent or participating in intramolecular hydrogen bonding. By using amide H/D exchange coupled with proteolytic digestion and nano-electrospray ionization (nano-ESI)-MS, we were able to characterize the structural changes of Akt associated with its activation in this study. Our results provide details of localized conformation of Akt at different activation states.

Experimental

Materials

Inactive and active Akt with 90% purity were purchased from Upstate Cell Signaling Solutions (Lake Placid, NY, USA). As provided by Upstate, the active Akt was phosphorylated at both Thr-308 and Ser-473 while these sites were not phosphorylated in the inactive Akt. Deuterium oxide (99.9%), HEPES, sodium chloride, and trifluoroacetic acid (TFA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Pepsin was obtained from Pierce (Rockford, IL, USA). Solvents used for high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) were purchased from Burdick & Jackson (Muskegon, MI, USA). The K-LISA Akt activity kit was purchased from Calbiochem (La Jolla, CA, USA). C18 Ziptip was obtained from Millipore Corp. (Bedford, MA, USA).

H/D exchange experiments

Both active and inactive forms of Akt were dialyzed overnight against 50 mM HEPES containing 50 mM NaCl at 4°C to remove detergents and keep both samples in an identical buffer condition. Subsequently, 5 µL of the Akt solution (1 µg/µL) were deuterated by the addition of 100 µL D₂O buffer (50 mM HEPES containing 50 mM NaCl). After various incubation time (1 min, 5 min, 10 min, 30 min, 2 h and 24 h), a 10-µL aliquot was taken, added with 1% TFA to lower the pH below 2.5, and quickly mixed with pepsin at a pepsin/protein (mole/mole) ratio of 1:5 allowing for 6 min digestion on ice. Alternatively, the mixture was immediately placed on dry ice to quench the exchange reaction then kept at –80°C for later analysis. The digested sample was desalted with C18 Ziptip using cold solvents and

immediately analyzed using a Q-Star ESI-Qq-ToF mass spectrometer within 5 min to minimize the back-exchange.

Mass spectrometric identification of pepsin-generated Akt fragments

Prior to the H/D exchange studies, fragments generated from the pepsin digestion of both active and inactive forms of Akt were identified either by LC/MS/MS using an Agilent ion trap mass spectrometer (XCT) equipped with an Agilent 1100 nanoflow HPLC system or by off-line static nano-ESI-MS/MS analysis using a Q-Star Pulsar Qq-TOF mass spectrometer equipped with a nano-ESI source (ABI, Applied Biosystems Instruments). For the nano-LC/ESI-MS/MS analysis, peptides were trapped in a C₁₈ enrichment column (Zorbax 300 SB C₁₈ column, 0.3 × 5 mm) and separated on a Zorbax 300 SB C₁₈ column (75 μm × 150 mm, 3.5 μm) using a mobile phase that contained solvent A (0.1% formic acid in H₂O) and solvent B (0.1% formic acid in acetonitrile) at a flow rate of 300 nL/min. The mobile phase composition was held initially at 3% B for 5 min and gradually changed to 15, 45 and 85% B at 8, 50 and 55 min, respectively. The data-dependent automatic scan mode was used. The capillary voltage was set at 1750 V and the mass spectrum was scanned from *m/z* 300 to 2000 Da. The data set generated was analyzed by Mascot software. In the case of the off-line static nano-ESI-MS/MS analysis, the ion source voltage was set at 1100 V in the positive-ion mode. A full mass spectrum was acquired over an *m/z* range of 500–2000 Da, and the ions of interest were selected for collision-induced dissociation (CID) using high-purity nitrogen for MS/MS analysis. MS/MS data for the peptic peptides were interpreted with the assistance of ABI Analyst QS software and the protein analysis work sheet (PAWS).

Akt kinase assay

The Akt kinase activity was measured by an ELISA-based activity assay using streptavidin-coated 96-well plates and a biotinylated peptide substrate (GRPRTSSFAEG) which can be phosphorylated by Akt (Calbiochem).

Data analysis

The extent of deuterium incorporation was determined from the shift in mass of labeled peptides relative to unlabeled peptides. Peptide masses were computed from the centroid of the isotopic envelope using Magtran software (Magtran software, kindly provided by Dr. Zhongqi Zhang²²). The deuterium loss during the analysis was monitored in reference to the fully deuterated samples of both active and inactive Akt which were prepared by incubating samples in a deuterated buffer at pH 7 at room temperature for 24 h. The fully deuterated states of both active and inactive Akt showed the same extent of deuterium incorporation after 24 h. Under our condition, the back exchange was within 5%. The extent of deuterium incorporation (D%) at any given time was calculated according to Eqn. (1):

$$D\% = D/N = (m - m_{0\%}) / (m_{100\%} - m_{0\%}) \quad (1)$$

where *m* is the observed mass of the peptide being analyzed at a given time of deuterium exchange, *m*_{100%} is the mass of the fully deuterated peptide, and *m*_{0%} is the mass of the non-deuterated peptide.

Results and Discussion

Identification of peptic fragments of Akt1

Figure 1(A) shows the mass spectrum of the peptic peptide peaks reconstructed from multiply charged ions detected by off-line static nano-ESI-MS/MS. The identity of these peptides was

determined by tandem mass spectrometry as exemplified by a peptic peptide (I36-55F) in Fig. 1(B). The MS/MS data indicated that the peak with mass of 2388 Da reconstructed from the triply charged ion of m/z 797 originated from the peptic peptide of I36-55F. In addition, minor peptide peaks which were difficult to identify by static nano-ESI-MS/MS were detected by nano-LC/ESI-MS/MS, as demonstrated in Fig. 2 for the peptic peak with mass of 1785 Da reconstructed from the doubly, triply or quadruply charged ions of m/z 892.9, 595.9 or 447.3, respectively. The MS/MS analysis of the triply charged ion at m/z 595.9 revealed the peak originated from V4-18Y (Fig. 2(B)). Using these approaches, a total of 24 peptide peaks were identified in both inactive and active Akt (Table 1). These peptic peptides identified covered 70% of the amino acid sequence of Akt (Fig. 3).

H/D exchange

Typical ESI-MS spectra showing different H/D exchange profiles are shown for a representative peptide I36-55F in Fig. 4. The spectra are expanded to show the isotopic envelope of the peptide derived from undeuterated Akt (Fig. 4(A)), inactive Akt (Fig. 4(B)) after 30-min deuteration, active Akt (Fig. 4(C)) after 30-min deuteration, and fully deuterium exchanged inactive or active Akt after 24 h deuteration (both forms showed identical deuterium incorporation profiles under our condition) (Fig. 4(D)). The number of deuterons incorporated into the peptide fragment were determined by calculating the centroid of each mass envelope and then subtracting the centroid of the corresponding undeuterated peptide mass envelope (Fig. 4). The peptide I36-55F clearly showed more deuterium incorporation in the active form, suggesting that this peptide segment displayed a more exposed conformation in active Akt compared to inactive Akt. The activation states of the Akt samples were confirmed using the K-LISA™ Akt activity assay. As expected, the active Akt sample showed kinase activity, whereas inactive Akt exhibited no kinase activity (Fig. 4, inset).

The deuterium incorporation into Akt was monitored for 30 min as shown for two representative peptides, V4-26Y and T450-470P, in Fig. 5. While the deuterium incorporation level increased gradually during the 30-min exchange period, significant differences in deuterium incorporation between inactive and active states were apparent. Residues V4-26Y in the PH domain of Akt exhibited more deuterium incorporation, while T450-470P in the regulatory domain had less deuterium incorporated in the inactive form. The difference sustained throughout the deuterium exchange period. Most of the peptides identified showed the largest differences in deuterium incorporation between these two forms after exchange for 30 min. Therefore, deuteration levels of inactive and active Akt were compared after 30 min of incubation with D₂O buffer and the results are summarized in Table 1.

Deuterium incorporation into the PH domain

It has been reported that the core structure of the PH domain of Akt consists of seven β -strands and two α -helices¹¹ (Fig. 3). In this domain, changes in deuterium incorporation between the inactive and active forms of Akt were observed for peptide segments V4-26Y, F27-35F, I36-55F, M63-73F and Q79-103I (Fig. 6(A)), suggesting that Akt conformational changes occurred upon activation in these regions. Among these, peptide V4-26Y which contains β 1 (I6-15R) and most of β 2 (W22-29L), as well as the segment of M63-73F enclosing β 5 (Q61-65T) through the beginning of β 6 (T72-79Q), showed significantly higher deuteration levels in the inactive form in comparison to the active form. The data suggested that these β -strand regions were, in general, more exposed to solvent when Akt was inactive. According to the crystal structure of the PH domain of Akt interacting with PIP₃,¹¹ which is a prerequisite step for Akt activation, the locus of β 1 and β 2 contains amino acid residues such as K14, E17, Y18, R23 and R25, which are crucial components of the PIP₃ binding pocket. In addition, R15 and K20 located outside the PIP₃ binding pocket, along with R67 and R69 located at the linkage between β 5 and β 6, form a positively charged flat surface. It has been suggested that these

residues interact with the negatively charged lipid membrane components during activation.¹¹ These models of PH-membrane interaction derived from X-ray diffraction are supported by our H/D exchange data revealing that the membrane-binding residues in inactive Akt are more exposed to solvent therefore readily available for the interaction with the membrane. Moreover, our data suggested that these membrane-binding residues became less exposed (or less available for membrane interaction) in the active form, presumably assisting Akt dissociation from the membrane upon activation. In addition, our data indicated that the peptides F27-35F (containing the end of $\beta 2$ through the beginning of $\beta 3$ (G33-38Y)), F36-55F (enclosing part of $\beta 3$, $\alpha 1$ (V45-49E) and most of $\beta 4$ (N53-56S)), and Q79-103I (spanning $\beta 7$ (T82-89H) through the first half of $\alpha 2$ (P93-113Q)), incorporated deuterium at a slightly higher level in the active form, suggesting that these regions became more solvent-exposed when activated. The increased solvent accessibility was caused possibly by the disruption of hydrogen bondings and ionic interactions between residues such as R86 and N53 during activation processes.¹² No significant changes in deuterium incorporation were observed in the linker region between the PH domain and the kinase domain (residues 108-149).

Deuterium incorporation into the kinase domain

The deuterium incorporation profiles of the kinase domain in both inactive and active Akt are shown in Fig. 6(B). According to the crystal structure, the core structure of the kinase domain of Akt is composed primarily of eight β -strands ($\beta 1$ - $\beta 8$), and eight α -helices (αC - αI)¹³ (Fig. 3). Our data showed that in general six of the β -strands and five of the α -helices exhibited difference in the deuterium incorporation profile, suggesting activation-dependent Akt conformational changes in these regions. The peptide segments of F150-166L containing $\beta 1$ (E149-156L) and the majority of $\beta 2$ (F161-169E), R174-201V and A193-224C spanning $\beta 3$ (Y175-181L), αC (R200-204N), $\beta 4$ (L213-217F), and the beginning of $\beta 5$ (R222-229Y), F245-259S containing most of αE (E247-265H), and I402-410G containing part of αI (D398-403M), showed higher deuterium levels in the inactive form, suggesting more solvent-exposed arrangements of the regions in the resting state. The interaction of R200 and Q203 located at the α -helix C with phospho-S473 in the regulatory domain¹³ and the interaction of H194 and R251 with the phosphorylation site at T308¹⁴ could have contributed in part to the reduced solvent accessibility observed for these segments when Akt is activated by phosphorylation. In addition, the peptide K289-300I (containing $\beta 8$ (H287-290I)) connected to F309-315Y through a disulfide bond between C296 and C310 which spans the activation loop showed less deuterium incorporation in the active state. Previous crystallographic studies of protein kinases have defined auto-inhibition mechanisms, which are mediated by the activation loop.¹³ In the inactive state, the substrate binding site is blocked by the relatively well-structured activation loop residues. It is possible that the residues F309, P313, and L316 in the activation loop may form a hydrophobic groove following activation in a similar manner shown for Akt2 structure in the active form,¹⁴ while this hydrophobic groove may not exist in the inactive form. In addition, once T308 is phosphorylated, the phosphate group may interact with the side chains of R273 and K297, stabilizing the activation loop. These molecular mechanisms may be reflected by the decreased deuterium exchange observed in the peptide containing the disulfide bond. In contrast, V167-175Y enclosing the linkage between $\beta 2$ and $\beta 3$, F236-252F containing αD (L235-242E), the linkage between αD and αE , and the beginning portion of αE , and E322-336G containing the linkage of αE (A317-323D)- αF (A329-345G) and the first half of αF , exhibited more deuterium incorporation in the active form. The H/D rates for peptides of G345-359E containing the linkage of αF - αG (H356-363M) and the beginning parts of αG , and L379-397E containing the last half of αH (P374-386K) and the linkage between αH and αI (A399-404Q), remained unchanged before and after activation.

Deuterium incorporation into the regulatory domain

Dramatic differences in the deuterium incorporation into the regulatory domain were observed between inactive and active Akt, as shown in Fig. 6(C). In general, the regulatory domain adopted a more exposed conformation after activation, evidenced by the peptide segment of I411-432E containing the α -helix J region (W413-418E) and the C-terminal segment (T450-470P) of the regulatory domain. This finding is consistent with our previous report for an open conformation of the regulatory domain in the active form, based on the data that the proximity between K284 of the kinase domain and K426 of the regulatory domain evaluated by cross-linking was apparent only in the inactive Akt but not in the active Akt.¹⁶ The open conformation of the regulatory domain in the active form, suggested by both cross-linking and current H/D exchange data, may enable the substrate entry to the activation loop in the kinase domain. In contrast, the peptide F433-444A in the regulatory domain showed less deuterium incorporation in the active form, suggesting a more folded structure in this region in the activated form. It is conceivable that the peptide segment of F433-444A, which was more exposed in the inactive form, may play a role in blocking the substrate entry into the kinase domain. These findings may provide important new insight for the structure of the regulatory domain, since the crystal structure for the most part (C-terminal) of the regulatory domain is still not available for both inactive and active Akt.

Conclusions

Amide hydrogen/deuterium exchange combined with nano-ESI/MS/MS provided a powerful technique for characterizing the different conformational statuses of full-length Akt during activation. Our H/D exchange results revealed significant conformational changes of Akt within the core structures of all three individual domains upon activation. The membrane-interacting residues in the PH domain displayed more exposed conformation, which may allow these residues to be readily available for the binding to the plasma membrane. These membrane-interacting residues were more folded in the active state, the structure of which is likely in favor of Akt dissociation from membrane upon activation. The structural changes in the kinase domain, β 1, β 2, β 3, β 4, β 8, α C, and α E were consistent with more folded structure of active Akt. Moreover, our data indicated that the regulatory domain generally adopts a more open conformation, possibly allowing substrate entry. Our data provided new in-solution structural information for full-length Akt that is complementary to the existing crystallographic data.

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Figure 1. Peptic peptides identified by static nano-ESI-MS/MS. (A) Reconstructed MS spectrum of the peptic digest of Akt. (B) Identification of the peptic peptide detected at m/z 797 as the triply charged ion by MS/MS.

Figure 2.

A peptic peptide identified by nano-LC/ESI-MS/MS. (A) Extracted ion chromatogram of a peptic peptide eluted from an LC column at 34.6 min. This peptide was detected at m/z 892.9, 595.9 and 447.3 as doubly, triply and quadruply charged ions, respectively (inset). (B) MS/MS spectrum of the triply charged ion at m/z 595.9. MS/MS data revealed the peptide originated from the peptic segment of V4-18Y.

Figure 3. The structure of Akt1. Mass spectrometric detection of the peptic peptides (underlined) covered 70% amino acid sequence under our experimental conditions. The α -helices and β -strands based on crystal structures of Akt^{11,13} are illustrated schematically above the primary sequence.

Figure 4.

ESI-MS spectra of the peptic peptide I36-55F detected as a triply charged ion. (A) Unlabeled Akt. (B) Inactive Akt after 30-min incubation with D_2O buffer. (C) Active Akt after 30-min incubation with D_2O buffer. (D) Fully deuterated Akt after 24-h incubation with D_2O buffer. The centroid values were determined from the mass envelopes of each peptide. Inset shows Akt activity assay using a K-LISA activity assay kit.

Figure 5. Time course of the deuterium incorporation plotted for representative peptides. (A) V4-26Y of the PH domain. (B) T450-470P of the regulatory domain.

Figure 6. Deuterium incorporation levels in 24 peptic peptides in the PH (A), kinase (B), and regulatory (C) domains obtained from inactive and active Akt after 30-min incubation with D₂O buffer.

Table 1
Summary of peptic peptides identified and H/D exchange data for Akt1 *

Residues of Akt1	2 nd structure	No. of D (100% H/ D)	D inactive (30 min)	D active (30 min)	Δm^{**}	D inactive (%)	D active (%)	ΔD (%)
A. PH domain								
V4-18Y	$\beta 1$	15	10	6	-3	66.7	40.0	-26.7
V4-26Y	$\beta 1, \beta 2$	21	13	8	-5	61.9	38.1	-23.8
F27-35F	$\beta 2, \beta 3$	8	4	5	1	50.0	62.5	12.5
I36-55F	$\beta 3, \alpha 1, \beta 4$	17	9	11	2	52.9	64.7	11.8
M63-73F	$\beta 5, \beta 6$	8	8	4	-4	100.0	50.0	-50.0
Q79-103I	$\beta 7, \alpha 2$	23	8	9	1	34.8	39.1	4.3
I103-120F	$\alpha 2$	17	7	7	0	41.2	41.2	0.0
M134-149E		14	8	8	0	57.1	57.1	0.0
E135-149E		13	8	8	0	61.5	61.5	0.0
B. Kinase domain								
F150-166L	$\beta 1, \beta 2$	16	9	7	-2	56.3	43.8	-12.5
V167-175Y	$\beta 2$	8	6	7	1	75.0	87.5	12.5
R174-201V	$\beta 3$	27	13	11	-2	48.1	40.7	-7.4
A193-224C	$\alpha C, \beta 4, \beta 5$	30	19	13	-6	63.3	43.3	-20.0
T211-225F	$\beta 4, \beta 5$	14	7	7	0	50.0	50.0	0.0
F236-252F	$\alpha D, \alpha E$	16	7	9	-2	43.8	56.3	12.5
F245-259S	αE	14	10	6	-4	71.4	42.9	-28.6
D262-279N	$\alpha E, \beta 6, \beta 7$	17	13	13	0	76.5	76.5	0.0
K289-300I-F309-315Y	$\beta 8$	17	9	7	-2	52.9	41.2	-11.8
E322-336G	ef, αF	14	7	9	2	50.0	64.3	14.3
G345-359E	αG	13	7	7	0	53.8	53.8	0.0
L379-397E	αH	17	6	6	2	35.3	35.3	0.0
I402-410G	αI	8	5	4	-1	62.5	50.0	-12.5
C. Regulatory domain								
I411-432E	αJ	18	7	11	4	38.9	61.1	22.2
F425-444A		18	11	6	-5	61.1	33.3	-27.8
T450-470P		19	7	12	5	36.8	63.2	26.3

* The data are representative of two independent experiments.

** The %difference in H/D exchange where Δm values were greater than 1 may be considered significant since Δm of even ± 1 was reproducible for most of the peptides indicated.