Nucleotide Release Sequences in the Protein Kinase SRPK1 Accelerate Substrate Phosphorylation[†]

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Abstract

Protein kinases are essential signaling enzymes that transfer phosphates from bound ATP to select amino acids in protein targets. For most kinases, the phosphoryl transfer step is highly efficient while the rate-limiting step for substrate processing involves slow release of the product ADP. It is generally thought that structural factors intrinsic to the kinase domain and the nucleotide-binding pocket control this step and consequently the efficiency of protein phosphorylation for these cases. However, the kinase domains of protein kinases are commonly flanked by sequences that regulate catalytic function. To address whether such sequences could alter nucleotide exchange and, thus, regulate protein phosphorylation, the presence of activating residues external to the kinase domain was probed in the serine protein kinase SRPK1. Deletion analyses indicate that a small segment of a large spacer insert domain and a portion of an N-terminal extension function cooperatively to increase nucleotide exchange. The data point to a new mode of protein kinase regulation in which select sequences outside the kinase domain constitute a nucleotide release factor that likely interacts with the small lobe of the kinase domain and enhances protein substrate phosphorylation through increases in ADP dissociation rate.

Protein phosphorylation represents an essential posttranslational modification that regulates signaling processes in the cell. There are now over 500 known protein kinases in the human genome and it is estimated that up to 30% of the proteins in the cell may be phosphorylated by this large enzyme family at one or more sites (1, 2). The protein kinases consist of a conserved kinase domain comprised of a small, ATP/ binding lobe and a larger substrate-binding lobe. Sequences flanking the kinase domain can serve to either positively or negatively regulate the activity of the kinase domain. Some protein kinases contain autoinhibitor sequences outside the kinase domain that bind in the active site prohibiting substrate association in a classic competitive manner (3). Activation is oftentimes achieved by phosphorylation within the kinase domain at the activation loop and/or by subunit binding that optimally arranges active-site residues (4, 5). These activation modes generally involve large increases in the phosphoryl transfer rate and sometimes a shift in mechanism from slow transfer to rate-limiting ADP release (6). Indeed, many active protein kinases have highly efficient phosphoryl transfer steps and slow ADP release steps that limit substrate turnover (7-14). Given the general role of rate-limiting ADP release for activated protein kinases (15) an exchange factor capable of enhancing or repressing this step would directly affect protein phosphorylation rates. Other nucleoside triphosphate-utilizing enzymes are known to incorporate such mechanisms. For example, Rho GTPases are activated by a guanine exchange factor that accelerates GDP release (16, 17). Based on the

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kinetic data, factors that enhance ADP release in protein kinases have the potential of accelerating protein phosphorylation rates and impacting cell signaling pathways.

The splicing of early mRNA transcripts is catalyzed by a large macromolecular complex known as the spliceosome. In addition to several core RNA molecules, the spliceosome contains numerous auxiliary proteins important for assembly and splice/ site selection. SR proteins are essential splicing factors that establish 5′ and 3′ splice sites on precursor mRNA. They are composed of one or two RNA recognition motifs (RRMs)[1] that bind specific mRNA regions establishing intro-exon boundaries. All SR proteins contain a C-terminal RS domain rich in long Arg-Ser repeats. Phosphorylation of these repeats by the serine protein kinase SRPK1 is essential for nuclear translocation of the SR protein and interaction with the spliceosome (18-20). SRPK1 is considered constitutively active since it does not require prior phosphorylation in its kinase domain for SR protein phosphorylation. SRPK1 and its family members are unique protein kinases whose kinase domains are bifurcated by a spacer insert domain [SID] (Fig.1A). The SID is as large as the kinase domain, interacts with chaperones and maintains a cytoplasmic pool of the kinase for SR protein phosphorylation (21). SRPK1 also contains a long N-terminal extension whose physiological function is poorly understood (Fig.1A). This region outside the kinase domain may be important for physiological control as it has been shown that phosphorylation of the N-terminus enhances activity (22) whereas binding of the nuclear scaffold protein SAFB to the N-terminus represses activity (23).

Much of our knowledge regarding how SRPKs recognize and phosphorylate SR proteins has been informed by the structural and kinetic analyses of SRPK1 and one of its substrate targets, SRSF1 (aka ASF/SF2) (24). Kinetic studies have shown that SRPK1 binds tightly to the RS domain near the C-terminal end of a long Arg-Ser dipeptide repeat known as the RS1 segment and then moves in an N-terminal direction modifying approximately 10-12 serines (25). X-ray structural studies of a truncated form of SRPK1 with SRSF1 reveal that this lengthy polyphosphorylation reaction is facilitated by an electronegative docking groove in the large lobe of the kinase domain (26). The docking groove initially binds the N-terminal portion of RS1 feeding the C-terminal end into the active site for phosphorylation initiation. The N-terminal Arg-Ser repeats then sequentially translate from the docking groove to the active site, a process that leads to unfolding of the final β strand in RRM2 which then occupies the docking groove (27). Rapid quench flow studies indicate that the rate of serine phosphorylation and translocation of the Arg-Ser stretch through the docking groove and active site are fast whereas the release of the product ADP is rate-limiting at each step (11). Although SRSF1 binds with very high affinity at the start of the reaction (K_d =20-50 nM) (28, 29), the addition of the first eight phosphates reduces binding affinity by up to two orders of magnitude (11). These changes along with phosphorylation-dependent decreases in ADP release rate help to terminate the multi-site reaction and release the processed SR protein from SRPK1.

Although the present X-ray model of SRPK1 has provided keen insights into the mechanism of SR protein binding and phosphorylation, the N-terminus and most of the SID (about ½ of the kinase) have been omitted to aid in crystallization. The SID is highly charged and predicted to lack regular secondary structure. Recent hydrogen-deuterium [H-D] exchange data indicate that most of the SID with the exception of two short segments near the SID/ kinase boundaries is unstable and likely to be unfolded (30). Such a large, unstructured region in the SID could provide attachment points for the chaperones, thus, pinning the

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[1]Abbreviations: CATTRAP, catalytic trapping; RRM, RNA recognition motif; RS domain, domain rich in arginine-serine repeats; SID, spacer insert domain; SR protein, splicing factor containing arginine-serine repeats; SRPK1, SR-specific protein kinase 1; SRSF1, SR protein splicing factor 1 (aka ASF/SF2).

kinase in the cytoplasm. A small segment of the N- and C-terminal ends of the SID, included in the expression construct to aid in crystallization, adopts helical conformations possibly explaining the observed stability of these regions in the H-D exchange experiments. While not included in the X-ray structure, the N-terminus appears to possess stable regions based on H-D exchange studies suggesting that it may adopt some secondary structure and/ or pack onto the kinase domain (30).

Prior studies have shown that while the kinase domain of SRPK1 is active and capable of phosphorylating SR proteins, the rate of this process is increased by the presence of the SID and N-terminus (30). Given the enormous size of these noncataytic regions (Fig.1A), we wished to determine how they enhance SR protein phosphorylation and whether a smaller region or regions within the SID and N-terminus could be responsible for the observed catalytic enhancements. Using a combination of rapid quench flow, viscosometric and deletion analyses we showed that a small segment of the SID predicted to adopt a helical conformation ($S_{\alpha 1}$) and a portion of the N-terminus function cooperatively to increase the rate of ADP release and consequently increase the phosphorylation of the SR protein SRSF1. Overall, the data imply that these noncatalytic regions in SRPK1 constitute a nucleotide release factor that is likely to interact with the N-terminal lobe of the kinase domain. This is the first such observation of a structural segment outside the critical kinase domain that up-regulates protein phosphorylation through modulation of the nucleotide pocket and ATP/ADP exchange rates.

Materials and Methods

Materials

Adenosine triphosphate (ATP), 3-(*N*-morpholino)propanesulphonic acid (Mops), 2-[N-morpholino]ethanesulphonic acid (MES), Tris (hydroxymethyl) aminomethane (Tris), $MgCl_2$, NaCl, EDTA, glycerol, sucrose, acetic acid, Phenix imaging film, BSA, and liquid scintillant were obtained from Fisher Scientific. [γ-^{32}P] ATP was obtained from NEN Products, a division of PerkiN-Elmer Life Sciences.

Expression and Purification of Recombinant proteins

SRPK1 was expressed from a pRSETb vector containing a 6xHis Tag at the N terminus (31). All single-site mutations in SRPK1 were generated by polymerase change reactions using the Velocity PCR kit (Bioline) and relevant primers. Deletion mutants were constructed by engineering BamH1 sites in pRSETb at desired locations, digesting the vector and religating. SRPK1(ΔS_{INT}), SRPK1($\Delta S_{\alpha 1}$), SRPK1($\Delta S_{\alpha 2}$) were made by deleting residues 248-483, 224-249 and 484-491, respectively. Deletion constructs SRPK1(ΔN) and SRPK1(ΔS) lack residues 1-73 and 224/492 and were previously described (30). SRSF1 was expressed from a pET19b vector containing a 10xHis Tag at the N terminus (32). The plasmids for SRSF1 and SRPK1 were transformed into the BL21 (DE3) *E. coli* strain and the cells were then grown at 37°C in LB broth supplemented with 100 Δg/ml ampicillin or 50 Δg/ml kanamycin depending on the type of plasmid vector. Protein expression was induced with 0.4 mM IPTG at room temperature for 5 hours for SRSF1 and 12 hours for SRPK constructs. All SRPK1 constructs were purified by Ni-resin affinity chromatography using a published procedure (29). SRSF1 was refolded and purified using a previously published protocol (25).

Phosphorylation Reactions

The phosphorylation of SRSF1 by SRPK1 was carried out in the presence of 100 mM Mops (pH 7.4), 10 mM free Mg^{2+}, 5 mg/mL BSA, and 3-400 PM [γ-^{32}P]ATP (4000-8000 cpm $pmol^{-1}$) at 23 °C according to previously published procedures (25). Reactions were

typically initiated with the addition of SRSF1 (15-1000 nM) or SRPK1 in a total reaction volume of 10 ΔL and then were quenched with 90 μL 30% acetic acid. Phosphorylated SRSF1 was separated from unreacted ATP by a filter binding assay (33). A portion of each quenched reaction (50 ΔL) was spotted onto a phosphocellulose filter disk and was washed 3 times with 0.5% phosphoric acid. The filter disks were rinsed with acetone, dried, and counted on the ^{32}P channel in liquid scintillant. The total amount of phosphate incorporated into the substrate was then determined by considering the specific activity (cpm/min) of the reaction mixture, and the background retention of ^{32}P ATP in the absence of SRPK1. Full retention of the phosphorylated product on the filters was confirmed by running quenched reaction samples on SDS-PAGE and counting the bands.

Viscosity Experiments

The steady-state phosphorylation of SRSF1 was monitored using the filter binding assay as described above in the presence of 0-30% sucrose. The relative solvent viscosity (η^{rel}) of the buffer (100 mM Mops, pH 7.4) containing 0-30% sucrose was measured using an Ostwald viscometer and a previously published protocol (8). Values of 1.44, 1.83, 2.32, and 3.43 for η^{rel} were measured for buffers containing 10, 20, 25, and 30% sucrose at 23 °C, respectively.

Rapid Quench Flow Experiments

Phosphorylation of SRSF1 by SRPK1 was monitored using a KinTek Corp. Model RGF-3 quench flow apparatus. The apparatus consists of three syringes driven by a stepping motor. Typical experiments were performed by mixing equal volumes of the SRPK1-SRSF1 complex in one reaction loop and ^{32}P-ATP (5000-15000 cpm/pmol) in the second reaction loop in 100 mM Mops (pH 7.4), 10 mM free Mg^{2+}, 5 mg/mL BSA. All enzyme and ligand concentrations are those in the mixing chamber unless otherwise noted. The reactions were quenched with 30% acetic acid in the third syringe and were analyzed using the filter binding assay described above. Control experiments lacking SRPK1 were run to define a background correction.

Data Analysis

The CPMs from the rapid quench flow experiments corrected for background were converted to concentrations of phosphoproduct using the specific activity of ATP. Pre-steady-state kinetic transients were either simulated using the program DynaFit (34) or fit to equation (1)

$$\frac{[P]}{[E]} = \alpha \left(1 - \exp\left(-k_b t\right) \right) + k_L t \quad (1)$$

where α, k_b, and k_L are the 'burst' phase amplitude, 'burst' phase rate constant and the linear phase rate constant, respectively. The initial velocity data in manual mixing experiments were fit to the Michaelis-Menten equation to obtain K_m and V_{max}. The V_{max} values were converted to k_{cat} using the total enzyme concentration determined from a Bradford assay ($k_{cat} = V_{max}/E_{tot}$).

Results

N-Terminus & SID Control SR Protein & Nucleotide Entry Into the Active Site

Previous studies showed that removal of regions outside the kinase domain of SRPK1 alters kinetic parameters for SR protein phosphorylation (30). We confirm these results and show that deletion of either the N-terminus [SRPK1(ΔN)] or SID [SRPK1(ΔS)] (Fig.1A) lowers

k_{cat} for SRSF1 phosphorylation by similar amounts (10-13-fold) (Table 1). The larger effect observed on k_{cat}/K_{SR} for SRPK1(ΔN), the apparent association rate constant for SRSF1, is the result of changes in the K_m for the SR protein substrate SRSF1 (K_{SR}). Prior competition studies revealed that the higher K_m for SRPK1(ΔN) is the direct result of reduced affinity for SRSF1 (30). In comparison, deletion of the SID has no effect on K_m or the true affinity of the SR protein. Deletion has no effect on the K_m for ATP (K_{ATP}) or the K_I for ADP ($K_{i,ADP}$) suggesting that removal of the N-terminus or SID does not impact overall nucleotide binding affinity and the folding of the kinase domain (Table 1). To determine whether the N-terminus and SID affect ligand diffusion in the active site we performed viscosometric studies (8, 35). Increasing the relative solvent viscosity of the buffer using sucrose leads to reduced steady-state kinetic parameters. Changes in relative k_{cat}/K_{ATP} and k_{cat}/K_{SR} (ratios in the absence & presence of viscosogen) versus relative solvent viscosity (η^{rel}) are linear with slope values [$(k_{cat}/K_{SR})^\eta$ & $(k_{cat}/K_{ATP})^\eta$] near the theoretical limit of one (Fig. 1B,C). We showed previously that the association rate constant for a substrate can be measured directly from the ratio of k_{cat}/K_m and its viscosity sensitivity as measured by $(k_{cat}/K_{SR})^\eta$ & $(k_{cat}/K_{ATP})^\eta$ (8, 11, 12, 35). This analysis indicates that the steady-state kinetic parameters are limited by diffusive events and that the reductions upon deletion are the result of approximately 20-fold reductions in the association rate constants for both ATP and SRSF1 (Fig. 1D). Overall, these findings demonstrate that the N-terminus and SID regulate the diffusion of ligands into the active site of SRPK1.

N-Terminus & SID Control Rate-limiting ADP Release

We showed previously that the maximum phosphorylation rate of SRSF1 by SRPK1 (k_{cat}) is limited by the release of ADP (11). Since removal of the N-terminus and SID result in large decreases in k_{cat} (Table 1), we wondered whether this could be due to changes in the release of this product. To address this possibility, we initially performed viscosometric experiments to show that k_{cat} is fully sensitive to solvent viscosity for wild-type SRPK1, SRPK1(ΔS) and SRPK1(ΔN) (Fig. 2A). These findings suggest that a product release event controls maximum turnover for these enzymes. To determine whether this step corresponds to ADP release, we performed catalytic trapping ($C_{AT}T_{RAP}$) experiments (7). In this experiment, SRPK1, SRSF1 and ADP are preequilibrated before reaction start with excess ATP in the rapid quench flow instrument (Fig. 2B). In the absence of ADP, a fast, enzyme-stoichiometric 'burst' in phosphoproduct ($t_{1/2} \approx 50$ msec) is observed prior to the linear, steady-state phase (Fig. 2C). In the presence of bound ADP, the 'burst' phase disappears and is replaced by a small lag in phosphoproduct. The linear phases in the absence and presence of ADP are similar implying that sufficient ATP is added to compete with ADP. The data were simulated in the absence of ADP using the kinetic mechanism in Fig.2B where the association rate constant for ATP (k_1) was set to k_{cat}/K_{ATP} (Table 1). Since a maximal viscosity effect is observed, k_{cat}/K_{ATP} (Fig.1B) is assumed to be similar in value to k_1. This assumption was previously validated using ATP-dependent binding experiments in the rapid quench flow instrument (11). Also, in this mechanism the product of the reaction (SR_{n+1}) becomes the substrate for the next phosphorylation event. Optimal fits to the data were achieved with rate constants of 30 and 1.1 sec^{-1} for k_2 and k_3, the rate constants for the phosphoryl transfer step and net product release (Table 2). The data were best fit with a starting enzyme-substrate complex concentration ($[E*SR_n]$) that is near the total enzyme concentration (Table 2), indicating that most of the enzyme is active and bound with the SR protein. The data in the presence of pre-equilibrated ADP were then simulated using these values to obtain a dissociation rate constant for ADP (k_{off}) of 1.1 sec^{-1} (Table 2). Since k_{off} is identical to k_3 and k_{cat}, the release of ADP is rate-limiting for SRPK1 (Fig. 2F).

$C_{AT}T_{RAP}$ experiments were then performed on SRPK1(ΔS) and SRPK1(ΔN) to determine how the noncatalytic regions impact ADP release. Since 'burst' phases are observed for

SRPK1(ΔS) and SRPK1(ΔN) (Fig. 2D,E), the phosphoryl transfer step is not rate-limiting for either deletion mutant. The linear phases are much slower than that for SRPK1, in line with lower k_{cat} values. Also, since the enzyme-normalized amplitudes of the 'burst' phases are near 1 ([E]≈[E*SR$_n$]; Table 2), the reductions in k_{cat} are the result of real changes in the kinetic mechanism and not to inactive enzyme from the protein preparation. The curves in the absence of ADP were simulated using fixed values of k_1 (based on k_{cat}/K_{ATP}) to obtain values for k_2 and k_3 (Table 2). While the best fits to the 'burst' data were obtained using reduced phosphoryl transfer rate constants, the low apparent ATP association rates (k_1[ATP]) made it difficult to obtain accurate measurements of this step for both deletions. Owing to low specific activity, we were not able to use higher ATP concentrations to increase the apparent association rate for the nucleotide. Despite this limitation, the data indicate that removal of the N-terminus or SID is likely to negatively impact the phosphoryl transfer step. More importantly, deletion lowers the rate constant for net product release (k_3) by about 11-13-fold. Using these rate constants we found that deletion lowers the ADP release rate by 11-fold, a value similar to that for the k_{cat} values (Fig. 2F). Thus, while deletion reduces the rate of the phosphoryl transfer step, ADP release is still rate-limiting for SRPK1(ΔN) and SRPK1(ΔS). Together, these data indicate that both the N-terminus and SID enhance the release rate of ADP without affecting overall nucleotide binding affinity.

A Minimal Sequence in the SID Enhances ADP Release

Using H-D exchange methods, we showed previously that most of the SID is unstructured with the exception of two small segments in the extreme N- and C-terminal regions of the SID near the kinase domain (30). Peptide fragments spanning 221-237 and 479-491 exhibit slower backbone amide exchange compared to most of the SID, suggesting that these boundary regions may contain some stable structure. In keeping with this idea, two helical conformations within these segments have been observed in the X-ray structure of a truncated form of SRPK1 (26, 36). To determine whether these limited regions could be responsible for controlling ADP exchange, we expressed three deletion constructs [SRPK1(ΔS$_{INT}$), SRPK1(ΔS$_{\alpha 1}$) & SRPK1(ΔS$_{\alpha 2}$)] that excluded the unstructured regions or the segments containing the putative helical elements (Fig. 3A). Removal of the unstructured portion [SRPK1(ΔS$_{INT}$)] or the second helix [SRPK1(ΔS$_{\alpha 2}$)] had no significant effect on maximum turnover, a parameter limited by ADP release (Table 1). While K_{ATP} is raised by 2-4-fold for the deletions, no significant changes in K_{SR} were observed compared to wild-type SRPK1 (Table 1). Given the large cellular concentrations of ATP (approx. 1 mM), the effects on K_{ATP} are unlikely to have an impact on physiological kinase function. In comparison, removing sequences corresponding to $S_{\alpha 1}$ lowers k_{cat} by 5-fold compared to wild-type SRPK1 (Table 1), suggesting that sequences near the N-terminal end of the SID play the greatest role in controlling SR protein turnover.

To determine whether deletion of the N-terminal region in the SID lowers the rate of ADP release, we first measured the effects of viscosity on the steady-state kinetic parameters. Increasing the viscosity of the buffer had a maximal effect on k_{cat} for SRPK1(ΔS$_{INT}$), SRPK1(ΔS$_{\alpha 1}$) & SRPK1(ΔS$_{\alpha 2}$) (Fig. 3B) suggesting that a product release step controls this parameter. Solvent viscosity also had a maximum effect on k_{cat}/K_{SR} and k_{cat}/K_{ATP} suggesting that the association rate constants for these two ligands limit these parameters (Fig. 3C,D). Consistent with the viscosity data, a rapid 'burst' in phosphoproduct is observed for SRPK1(ΔS$_{INT}$) implying that the phosphoryl transfer step is fast and does not limit k_{cat} (Fig. 3E). Also, $C_{AT}T_{RAP}$ experiments indicate that ADP release limits turnover for this deletion and is the same in value as that for wild-type SRPK1 (Fig. 3E & Table 2). To confirm that the lower k_{cat} for SRPK1(ΔS$_{\alpha 1}$) is the result of a lower k_{off} for ADP, $C_{AT}T_{RAP}$ experiments were performed. The pre-steady-state kinetic data indicate that the phosphoryl transfer step for SRPK1(ΔS$_{\alpha 1}$) is fast compared to k_{cat}, in line with the viscosity

data (Fig. 3F & Table 2). Simulation of the kinetic data in the absence and presence of pre-equilibrated ADP indicates that the lower k_{cat} for this deletion mutant is the result of a reduced k_{off} for ADP (Fig.3F & Table 2). $C_{AT}T_{RAP}$ experiments also showed that phosphoryl transfer is fast and ADP release limits k_{cat} for SRPK1($\Delta S_{\alpha2}$) (data not shown). Since SRPK1($\Delta S_{\alpha1}$) does not fully mimic SRPK1(ΔS) with regard to decreased ADP release rates, it is possible that additional residues in the SID could subtly contribute to the observed nucleotide exchange phenomena. Nonetheless, these studies suggest that a small N-terminal region of the SID, presumed to possess secondary structure, is mostly capable of enhancing ADP release and regulating SR protein phosphorylation.

Probing a Key Residue Bridging the SID & Kinase Domains

Based on the X-ray model of the truncated SRPK1, the N-terminal region of the SID responsible for controlling ADP release contains a helix ($S_{\alpha1}$) positioned near the small lobe of the kinase domain. Within $S_{\alpha1}$, a single residue (Ile-228) makes a direct interaction with Val-167 in the linker between the small and large lobes (Fig. 4A). Assuming that this interaction is present in the full-length kinase we made two mutations (I228G & I228K) in this residue to probe its catalytic function. Neither mutation significantly impacts k_{cat} and has only small effects on K_{SR} (Table 1). While glycine has no significant effect, lysine substitution increases K_{ATP} by 8-fold (Table 1) suggesting that this Ile-228 is near the nucleotide pocket and a large, charged side chain can disrupt ATP binding. To determine whether either mutation affects ADP release, we performed viscosity and $C_{AT}T_{RAP}$ experiments. While k_{cat} for SRPK1(I228G) was maximally affected by solvent viscosity, k_{cat} for SRPK1(I228K) is unaffected (Fig. 4B). In keeping with these observations, a rapid 'burst' in phosphoproduct is observed for SRPK1(I228G) while no such phase is observed for SRPK1(I228K) (Fig. 4C,D). These findings indicate that while glycine substitution has no significant effect on the phosphoryl transfer or ADP release rate, lysine substitution lowers the rate of phosphoryl transfer such that it is now rate-limiting. Although we cannot directly measure k_{off} without a 'burst' phase, we estimate that ADP release is, at least, 5-fold faster for SRPK1(I228K) compared to wild/ type SRPK1. In total, since we did not observe any decreases in k_{off} for ADP upon mutation, the ability of $S_{\alpha1}$ in the SID to accelerate ADP exchange is not due to a specific interaction between Ile-228 and any part of the kinase domain. Overall, these results suggest that either direct interactions between the helix and the kinase core are unnecessary for enhancing nucleotide exchange or this segment of the SID may adopt a different conformation and potentially unique contacts in the full-length SRPK1.

N-Terminal Regions Responsible For ADP Exchange & SR Protein Binding

Having shown that the full N-terminus accelerates ADP release and enhances SR protein binding, we next wished to address whether some or all of the residues in this region are important for this phenomenon. Lacking X-ray structural data for contact mapping, we made two deletion constructs that separate the N-terminus into mostly positively and negatively charged halves (Fig. 5A). Removal of the N-terminal sequences [SRPK1(ΔN_{NT})] has no effect on k_{cat} and only a modest 2-fold increase in K_{ATP} (Table 1). Interestingly, K_{SR} for SRPK1(ΔN_{NT}) is about 3-fold lower than that for the wild-type enzyme suggesting that this portion of the N-terminus may have a destabilizing effect on SR protein association (Table 1). SRPK1(ΔN_{NT}) exhibits full viscosity effects on the steady-state kinetic parameters (Fig. 5B-D) and a 'burst' in product formation consistent with fast phosphoryl transfer and rate-limiting ADP release (Fig. 5E & Table 2). These findings, along with little effect on k_{cat}/K_{ATP} (Table 1), indicate that the positively charged residues in the N-terminus do not control nucleotide exchange rates.

Unlike SRPK1(ΔN_{NT}), SRPK1(ΔN_{CT}) exhibits impaired steady-state kinetic parameters (Table 1). The reduced value for k_{cat} is due to a direct decrease in the rate of the phosphoryl transfer step based on viscosity and pre-steady-state kinetic analyses (Fig.5B-E). The rate of the phosphoryl transfer step is about 100-fold lower for SRPK1(ΔN_{CT}) compared to wild-type SRPK1 (Table 2), implying that replacement of charged segments near the C-terminal end disrupts active-site residues. Although we cannot measure ADP release in the absence of a 'burst' phase, it is likely that deletion of the C-terminal residues in SRPK1(ΔN_{CT}) either has no affect on ADP or increases its release rate. The large increase in K_{ATP} for SRPK1(ΔN_{CT}) suggests that charge changes alter the N-terminal lobe and likely increase ADP release rates. Furthermore, the C-terminal portion appears to be important in stabilizing SRSF1 and may be the cause of the reduced affinity of the substrate upon complete deletion of the N-terminus (Table 1). Overall, the deletion of the C-terminal sequences in the N-terminus did not mimic the ADP effects of complete N-terminal deletion [SRPK1(ΔN)]. This could be due to replacement of the negatively charged residues toward the C-terminus with positively charged residues from the N-terminal end in SRPK1(ΔN_{CT}) that may disrupt the N-terminal lobe. Nonetheless, the kinetic properties of SRPK1(ΔN_{NT}) suggest that only the negatively charged C-terminal residues in the N-terminus of SRPK1 are responsible for stabilizing SR protein binding and it is likely that this segment provides key elements for enhancing ADP release.

Cooperative Interplay of N-Terminal & SID Sequences

Deletion analyses indicate that the SID and N-terminus separately up-regulate ADP release by 10-fold and enhance SR protein association rates by 20-40-fold (Table 2). To determine whether these two noncatalytic segments work cooperatively, we initially made a deletion construct that removed both the N-terminus and the full SID but found that this protein was insolubly expressed in *E.coli*. To avoid these expression problems, we made a more restricted deletion mutant that removes the N-terminus and only the region containing $S_{\alpha 1}$ [SRPK1($\Delta NS_{\alpha 1}$)] (Fig.6A). We elected to remove the entire N-terminus in SRPK1($\Delta NS_{\alpha 1}$) rather than just the negatively charged C-terminal residues given the deleterious effects observed in SRPK1(ΔN_{CT}) for the phosphoryl transfer rate (Fig. 5E) when these residues are replaced with positively charged sequences from the N-terminal region. SRPK1($\Delta NS_{\alpha 1}$) is soluble and displays a k_{cat} that was reduced 10-fold relative to wild-type SRPK1 (Table 1). While K_{ATP} is unaffected, K_{SR} is raised by about 3-fold consistent with results obtained for deletion of the N-terminus in SRPK1(ΔN) (Table 1). The steady-state kinetic parameters for SRPK1($\Delta NS_{\alpha 1}$) are still maximally sensitive to solvent viscosity (data not shown), indicating that a product release step limits maximum turnover and ligand association limits catalysis at low ATP and SRSF1 concentrations. Pre-steady-state kinetic experiments demonstrate that the phosphoryl transfer step is still fast relative to k_{cat} (Fig.6B & Table 2).

To determine whether the lower turnover rate for SRPK1($\Delta NS_{\alpha 1}$) compared to wild-type SRPK1 is the result of slower ADP release, $C_{AT}T_{RAP}$ experiments were performed. As expected from the viscosity experiments, the release rate for ADP is about 10-fold lower compared to wild-type SRPK1 and fully limits k_{cat} (Fig.6B & Table 2). Overall, while deletion of each segment separately lowers ADP release and the association rate of SRSF1, the combined deletions in SRPK1($\Delta NS_{\alpha 1}$) are not additive (Fig.6C). Since separate deletion of the region containing $S_{\alpha 1}$ and the N-terminus lowers ADP release by 5- and 10-fold, a purely additive contribution in SRPK1($\Delta NS_{\alpha 1}$) would have reduced ADP release by 50-fold. Using a similar analysis we anticipate a decrease in k_{on} for SFSF1 of about 140-fold. The more modest effects on ADP k_{off} (10-fold decrease) and SRSF1 k_1 (30-fold decrease) indicate that the N-terminal and SID sequences function cooperatively to both accelerate the release of ADP and the association of the SR protein substrate. To demonstrate the effects of both sequences on multisite SR protein phosphorylation, progress curves for wt-SRPK1 and

SRPK1($\Delta NS_{\alpha 1}$) were performed using saturating ATP and SRSF1 (Fig.6D). These findings show that the effects of deletion extend over numerous catalytic steps and result in a general decrease in SR protein phosphorylation efficiency.

Discussion

Protein kinases are essential signaling enzymes that support a vast number of cellular processes. For most active protein kinases, the phosphoryl transfer step is highly optimized such that the delivery of the γ phosphate of ATP is very fast and does not limit overall substrate turnover. For these kinases, release of one of the reaction products, ADP, mostly limits maximum turnover and defines overall catalytic efficiency (15). The fast-slow phosphoryl transfer and ADP release steps can serve a clamping function for otherwise weakly bound protein substrates as observed in the case of Sky1p and its physiological target Npl3 (12). It is interesting that for many protein kinases that fit this release-dependent mechanism, the binding affinity of ADP is not well correlated with its dissociation rate constant. For example, the K_I values of ADP for PKA and SRPK1, two serine-dependent protein kinases limited by ADP release, are the same (10 ΔM) yet the dissociation rate constants differ by 20-fold (7, 11). Such findings suggest that kinase domains possess common architectural features for stable ligand contacts in the ground state complex but differ substantially with regard to the kinetic barriers associated with attaining this complex. Although transit in and out of the nucleotide pocket (nucleotide exchange) is undoubtedly a critical determinant for catalytic function in protein kinases, an insubstantial amount of attention has been focused on factors that control this pivotal rate-limiting step.

Defining A Nucleotide Release Factor in SRPK1

In earlier studies we showed that removal of the N-terminus or SID lowers maximum SR protein phosphorylation rates (30) raising the possibility that a subset of residues within these noncatalytic regions controls activity through adjustments in nucleotide exchange. We now show that two short sequences in the SID and the N-terminus of SRPK1 enhance the phosphorylation efficiency of the kinase domain by accelerating ADP release. We propose that these sequence elements constitute a nucleotide release factor that is not part of the canonical kinase domain but, nonetheless, is likely to make contacts with the N-terminal lobe of this domain regulating its function (Fig.7A). The region in the SID that promotes ADP release contains a helical conformation ($S_{\alpha 1}$) near the N-terminal lobe of the kinase domain (Fig. 4A). Although the N-terminus is not part of the crystallization construct, it is reasonable to assume that it interacts with the N-terminal lobe as well. Furthermore, there is good evidence that the N-terminus and $S_{\alpha 1}$ interact based on H-D exchange data. Removal of the SID significantly increases backbone exchange rates in the N-terminus whereas removal of the N-terminus increases amide exchange rates in $S_{\alpha 1}$ (30). Taken together with the cooperative effects on ADP release (Fig. 6), we believe that the two sequence stretches flanking the small lobe of the kinase domain interact with this lobe and function as a module to augment nucleotide exchange. The effects of this module on SR protein activation are quite significant. The phosphorylation of approximately 10 serines is necessary to direct SRSF1 from the cytoplasm to the nucleus for splicing functions (37). While this bulk threshold is readily attained within 3-4 minutes in progress curves, about 50 minutes is needed for SRPK1 lacking the nucleotide exchange sequences (Fig.6D).

Although the nucleotide exchange factor in SRPK1 promotes rapid SR protein phosphorylation by enhancing product release, it plays no role in ADP binding affinity. Such findings indicate that this factor does not alter the relative ground state energies of the free and nucleotide-bound enzyme and does not impact general folding. Instead, this structural module acts only in a kinetic manner raising the question of how it facilitates nucleotide entry/exit in the active site. In general, ATP binds in a deep pocket in protein kinases

removed from solvent by hydrophobic contacts and a glycine-rich loop that ensconces the charged triphosphate moiety (38). Surprisingly, although peptides and proteins bind in a shallow groove better exposed to solvent, the kinetic mechanism infrequently involves ordered addition of ATP and substrate (15). To harmonize these structural and kinetic observations it has been suggested that protein kinases are highly dynamic molecules that use rotations in the small and large lobes to accommodate nucleotide access (39). In keeping with this idea, the exchange factor in SRPK1 may promote ADP release by accelerating inter-lobe rotations and attainment of more open form of the nucleotide pocket (Fig 7A). This would also promote more productive collisions between the nucleotide and kinase, a phenomenon observed in the deletion constructs. The ability to accomplish this exchange task may be related to its position near the N-terminal lobe. Protein kinases are believed to possess a conserved catalytic spine that runs from helix F in the large lobe through the nucleotide-binding pocket and into the small lobe (40, 41). It is possible that the nucleotide exchange factor in SRPK1 modifies natural flexion points in this spine, thereby regulating the ADP release and the SR protein phosphorylation rates. Whatever the structural underpinnings, it is highly likely that this mechanism is shared within the SRPK family. The sequences identified in this study are conserved within the family suggesting that they may serve a common function (Fig. 7B).

SR Protein Binding Modulation Through the Nucleotide Release Factor

In addition to supporting productive collisions between SRSF1 and SRPK1, the N-terminal sequences in the nucleotide release factor appear to play a role in stabilizing the bound SR protein. In prior studies we showed that deletion of the N-terminus results in about a 3- and 5-fold increase in K_d and K_m for SRSF1 (30). Removal of the charged residues in SRPK1(ΔN_{CT}) leads to a similar K_m increase suggesting that this segment of the N-terminus could assist SR protein binding. In fact, the N-terminus could make several direct contacts with the RS domain that stabilize the SR protein. Further investigations using different SRPKs and SR proteins may lead to a better understanding of this phenomenon. The N-termini of SRPKs vary significantly in length and appear to have only sequence homology toward the C-terminal end. This region of the N-terminus is negatively charged (Fig. 7B) and could interact with arginines from the RS domain. On the other hand, the N-terminus has a cluster of positively charged residues that may support the generation of phosphoserines during consecutive rounds of RS domain phosphorylation. While SRPKs vary in their N-termini, the RS domains of SR proteins differ enormously in length (50-300 residues) and the positioning of Arg-Ser repeats are not conserved. Overall, the studies presented herein indicate that the nucleotide release sequences in SRPK1 can operate at multiple levels that include reducing kinetic barriers for entry/exit and by stabilizing the substrate in active site.

Conclusions

Since the early observations that ADP release controls PKA catalysis (7, 8) and that other protein kinases operate through a similar mechanism (7-14), regulation through a product release step has remained a compelling supposition. In this study we now demonstrate using fast-mixing techniques that two short sequences outside the kinase domain of the splicing kinase SRPK1 directly up-regulate the ADP release rate and, thus, enhance the phosphorylation rate of an SR protein substrate. The two short sequence elements located in the N-terminus and SID that constitute this nucleotide release factor are likely to interact with the small N-terminal lobe in a cooperative manner. The addition of either the SID sequences or the N-terminal sequences alone is insufficient to accelerate ADP release and enhance SR protein phosphorylation. Interestingly, these sequences do not alter the overall binding affinity of ADP indicating that they behave in only a kinetic manner facilitating entry/exit to the active site. These sequences also increase productive collisions with the SR

protein. Several reports suggest that some factors in the cell can up- and down-regulate SRPK activity through modifications to the N-terminus, a key element of the nucleotide release factor (22, 23, 42). Our observations present a novel mechanism for such activity regulation through the nucleotide-binding pocket that may be conserved within the SRPK family. Furthermore, it is possible that nucleotide-directed release sequences are present in other protein kinase families or within their regulators and substrate targets.

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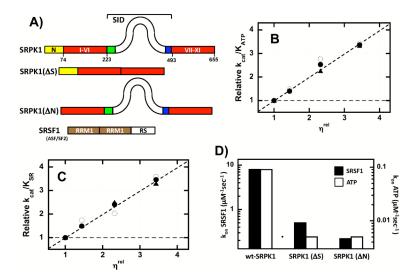
Figure 1. Diffusion Limits of ATP and SRSF1 in the Active Site of Wild-Type & Mutant Forms of SRPK1

A) Enzyme & Substrate Constructs. B,C) Viscosity Effects on Steady-State Kinetic Parameters. Relative values for k_{cat}/K_{ATP} (B), and k_{cat}/K_{SR} (C) in the absence and presence of varying sucrose concentrations are measured as a function of relative solvent viscosity (η^{rel}) for wild-type SRPK1 (○), SRPK1(ΔN) (•) and SRPK1(ΔS) (▲). Dotted lines reflect theoretical slope limits of 0 and 1. D) Bar graph showing changes in the association rate constants for SRSF1 and ATP.
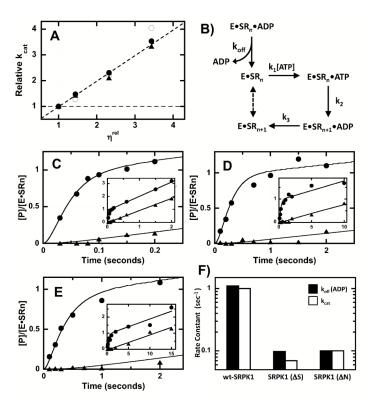
Figure 2. N-Terminus & SID Promote ADP Dissociation from SRPK1

A) Viscosity effects on k_{cat} for wild-type SRPK1 (○), SRPK1(ΔN) (•) and SRPK1(ΔS) (▲). Dotted lines reflect theoretical slope limits of 0 and 1. B) Kinetic Mechanism. Enzyme (E) and SRSF1 (SR_n) are preequilibrated in the absence or presence of ADP and the reaction is started with ATP (k_1). SRSF1 (SR_n) is phosphorylated at the first site (k_2), ADP is released (k_3) and the product (SR_{n+1}) becomes the substrate for the next phosphorylation event (dotted line). C-E) $C_{AT}T_{RAP}$ Experiments. SRSF1 and wild-type SRPK1 (C), SRPK1(ΔN) (D) and SRPK1(ΔS) (E) are prequilibrated in the absence (•) and presence (▲) of 120 ΔM ADP (in syringe) before the reaction is initiated with 600 ΔM ^{32}P-ATP. Insets show extended time frames for the reactions (2-15 sec). The data are simulated to obtain values for k_{off}, k_2, k_3 and [E*SR_n] (Table 2). F) Bar graph showing changes in k_{off} for ADP and k_{cat}.
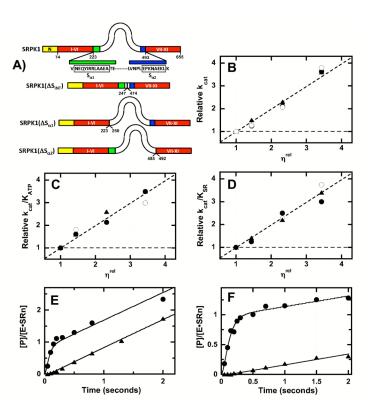
Figure 3. Minimal Sequence Requirements in the SID for Efficient SRSF1 Phosphorylation
A) SID Deletion Constructs. Green and blue regions in the SID represent sequences
spanning 223-249 and 484-493. B-D) Viscosity Effects on Steady-State Kinetic Parameters.
Relative values for k_{cat} (B), k_{cat}/K_{ATP} (C) and k_{cat}/K_{SR} (D) in the absence and presence of
varying sucrose concentrations are measured as a function of relative solvent viscosity (η^{rel})
for SRPK1[ΔS_{INT}] (•), SRPK1[$\Delta S_{\alpha 1}$] (○), and SRPK1[$\Delta S_{\alpha 2}$] (▲). Dotted lines reflect
theoretical slope limits of 0 and 1. E, F) $C_{AT}T_{RAP}$ Experiments for SRPK1[ΔS_{INT}] (E) and
SRPK1[$\Delta S_{\alpha 1}$] (F). SRSF1 and enzyme are preequilibrated in the absence (•) and presence
(▲) of 120 ΔM ADP (in syringe) before the reaction is initiated with 600 ΔM ^{32}P-ATP. The
data are simulated to obtain values for k_{off}, k_2, k_3, and [E*SR$_n$] (Table 2).
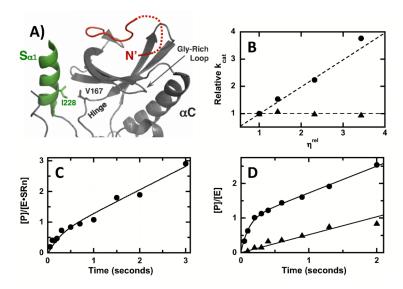
Figure 4. Effects of Mutations in $S_{\alpha 1}$ on Phosphorylation Kinetics
A) Molecular Contacts Between the $S_{\alpha 1}$ and Kinase Domain. $S_{\alpha 1}$ and small lobe of kinase domain are colored green and gray. N-terminal extension from residues 63-73 is shown in red (dotted line for 3 residues with insufficient electron density). B) Viscosity Effects on Turnover. Relative k_{cat} for SRPK1(I228G) (•) and SRPK1(I228K) (▲) in the absence and presence of varying sucrose concentrations are measured as a function of relative solvent viscosity (η^{rel}). Dotted lines reflect theoretical slope limits of 0 and 1. C) Pre-steady-state kinetic phosphorylation of SRSF1 using SRPK1(I228G). Final concentrations of enzyme, SRSF1 and ATP are 0.65, 2 and 50 ΔM, respectively. Data are simulated using the kinetic mechanism in Fig.2A and values are displayed in Table 2. D) Pre-steady-state kinetic phosphorylation of SRSF1 using SRPK1(I228K) (▲) and wt-SRPK1 (•). Final concentrations of wt-SRPK1, SRPK1(I228K), SRSF1 and ATP are 0.16, 0.2, 2 and 100 ΔM, respectively. Data for wt-SRPK1 are fit to equation (1) to obtain values of $0.9 \pm .05$, 9 ± 1 sec^{-1}, and 0.7 ± 0.1 sec^{-1} for α, k_b and k_L, respectively. Data for SRPK1(I228K) are fit to a line function with a slope of $0.5 \pm .05$ sec^{-1}.
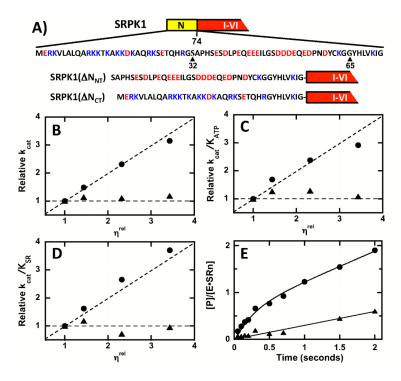
Figure 5. N-Terminal Residues Responsible for Efficient SRSF1 Phosphorylation
A) N-terminal Deletion Constructs. B-D) Viscosity Effects on Steady-State Kinetic Parameters. Relative values for k_{cat} (B), k_{cat}/K_{ATP} (C) and k_{cat}/K_{SR} (D) in the absence and presence of varying sucrose concentrations are measured as a function of relative solvent viscosity (η^{rel}) for SRPK1(ΔN_{NT}) (•) and SRPK1(ΔN_{CT}) (▲). Dotted lines reflect theoretical slope limits of 0 and 1. E) Pre-steady-state kinetic phosphorylation of SRSF1 using SRPK1(ΔN_{NT}) (•) and SRPK1(ΔN_{CT}) (▲). Final concentrations of enzyme, SRSF1 and ATP are 0.5, 2 and 50 ΔM, respectively. Data for SRPK1(ΔN_{NT}) are simulated using the kinetic mechanism in Fig. 2A and values are displayed in Table 2. Data for SRPK1(ΔN_{CT}) are normalized to total enzyme concentration and are fit to a linear function with a slope of 0.3 sec^{-1}.
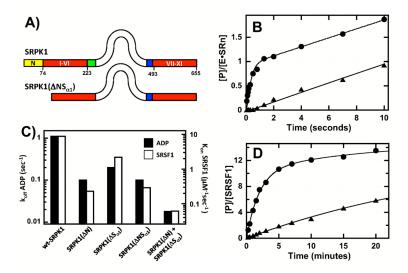
Figure 6. Cooperative Interaction Between the N-Terminus & SID
A) Deletion Construct. B) $C_{AT}T_{RAP}$ Experiment for SRPK1[$\Delta NS_{\alpha 1}$]. SRSF1 (2 ΔM) and enzyme (0.5 ΔM) are prequilibrated in the absence (•) and presence (▲) of 120 ΔM ADP (in syringe) before the reaction is initiated with 600 ΔM ^{32}P-ATP. The data are simulated to obtain values for k_{off}, k_2, k_3, and [E*SR$_n$] (Table 2). C) Bar graph showing the effects on k_{off} for ADP and k_{on} for SRSF1. The bars corresponding to SRPK1[ΔN] + SRPK1[$\Delta S_{\alpha 1}$] reflect theoretical values expected for SRPK1[$\Delta NS_{\alpha 1}$] assuming both individual deletions are energetically additive. D) Steady-State Progress Curve. SRSF1 (1 ΔM) phosphorylation is monitored using 60 nM SRPK1[$\Delta NS_{\alpha 1}$] (▲) or wt-SRPK1 (•) and 100 ΔM ATP. Phosphoproduct is normalized to the total concentration of SRSF1. The data for SRPK1 are fit to a double exponential function with amplitudes of 10 and 5 and rate constants of 0.5 and 0.05 min^{-1}. The data for SRPK1[$\Delta NS_{\alpha 1}$] are fit to a single exponential function with an amplitude and rate constant of 14 and 0.027 min^{-1}.
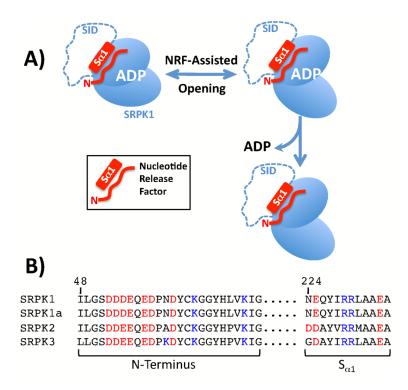
Figure 7. Sequences Constituting the Nucleotide Release Factor in SRPK1
A) Mechanism for the acceleration of ADP release through the N-terminus and $S_{\alpha 1}$. B) Sequence homology within the SRPK family.

Table 1

Steady-State Kinetic Properties of SRPK1 Mutants[a]

Protein	k_{cat} (s^{-1})	K_{ATP} (μM)	k_{cat}/K_{ATP} (M^{-1}s^{-1} ×10^{-3})	K_{SR} (nM)	k_{cat}/K_{SR} (M^{-1}s^{-1}×10^{-5})	$K_{i,ADP}$ (μM)
SRPK1	1.0 (.05)	11 (1.8)	91 (16)	120 (16)	83 (12)	10 (1)
SRPK1(ΔS)	0.07 (0.01)	14 (1)	5.0 (.4)	140 (20)	5.0 (.8)	13 (1)
SRPK1(ΔN)	0.1 (008)	20 (3)	5.0 (.48)	440 (100)	2.3 (.6)	13 (3)
SRPK1(ΔS$_{INT}$)	1.0 (.04)	50 (7)	20 (3)	190 (30)	53 (9)	54 (7)
SRPK1(ΔS$_{αi}$)	0.20 (.01)	30 (7)	6.7 (1.5)	90 (20)	22 (5)	18 (5)
SRPK1(ΔS$_{α2}$)	0.90 (.06)	16 (2)	56 (9)	130 (20)	69 (14)	
SRPK1(I228G)	1.1 (.05)	24 (7)	46 (14)	70 (18)	160 (41)	
SRPK1(I228K)	0.8 (.07)	90 (13)	8.8 (1.5)	240 (90)	33 (13)	
SRPK1(ΔN$_{NT}$)	1.0 (.05)	25 (2)	40 (4)	38 (6)	260 (43)	
SRPK1(ΔN$_{CT}$)	0.4 (.04)	36 (8)	11 (3)	410 (50)	9.1 (1.4)	
SRPK1(ΔNS$_{α1}$)	0.10 (.01)	12 (2)	8.3 (1.4)	340 (78)	2.9 (.72)	

[a]Numbers in parentheses next to data represent standard deviations on each fit.

Table 2

Simulations of Rapid Quench Flow Kinetic Transients for SRPK1 Mutants

Protein	$[E]^a$ (μM)	$[ATP]^a$ (μM)	$[E \cdot SR_n]^b$ (μM)	$k_1[ATP]^c$ (s^{-1})	k_2 (s^{-1})^b	k_3 (S^{-1})^b	k_{off} (s^{-1})^b
SRPK1	0.25	600	0.20	54	30	1.1	1.1
SRPK1(ΔS)	0.60	600	0.60	3	7	0.085	0.10
SRPK1(ΔN)	0.60	600	0.51	6	4	0.10	0.10
SRPK1(ΔS$_{INT}$)	1.0	600	0.77	12	69	0.94	1.0
SRPK1(ΔS$_{\alpha1}$)	0.30	600	0.30	12	19	0.18	0.19
SRPK1(I228G)	1.0	50	0.65	2.5	50	1.1	
SRPK1(ΔN$_{NT}$)	0.50	50	0.45	3	50	0.86	
SRPK1(ΔNS$_{\alpha1}$)	0.50	600	0.46	5	12	0.10	0.10

^{a}Total amounts of enzyme and ATP in kinetic experiments.

^{b}Values from kinetic simulations using DynaFit and the mechanism in Fig. 2B that best fit the kinetic transients in the absence or presence of preequilibrated ADP.

^{c}Values for k_1[ATP] are fixed from the product of total [ATP] and k_{cat}/KATP.