Kinetic Mechansim of Fully Activated S6K1 Protein Kinase

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S6K1 is a member of the AGC subfamily of serine-threonine protein kinases, whereby catalytic activation requires dual phosphorylation of critical residues in the conserved T-loop (T229) and hydrophobic motif (HM; T389). First, we demonstrate by (i) Western analysis, (ii) ESI-TOF, (iii) MonoQ anion exchange chromatography, and (iv) kinetic assays that the N-terminal His_6 affinity tagged catalytic kinase domain construct of the αII isoform of S6K1 [His_6-S6K1αII(ΔAID)-T389E, residues 1−398] can be generated in its most highly active form ($k_{cat} = 0.18 \pm 0.01$ s^{-1}) by baculovirus-mediated expression and purification from Sf9 insect cells that are coinfected with the upstream T229 kinase, PDK1. Next, we report its kinetic mechanism for catalyzing phosphorylation of a model peptide substrate (Tide, RRRLSSLRA). First, two-substrate steady-state kinetics and product inhibition patterns indicated a Steady-State Ordered Bi Bi mechanism, whereby initial high affinity binding of ATP (K_d^{ATP} = 5−6 μM) was followed by low affinity binding of Tide (K_d^{Tide} = 180 μM); and values of K_m^{ATP} = 5−6 μM and K_m^{Tide} = 4−5 μM were expressed in the active ternary complex. Global curve-fitting analysis of ATP, Tide, and ADP titrations of pre-steady state burst kinetics yielded microscopic rate constants for substrate binding, rapid chemical phosphorylation, and rate-limiting product release. Catalytic trapping experiments confirmed rate-limiting steps involving release of ADP. Pre-steady state kinetic and catalytic trapping experiments showed osmotic pressure to increase the rate of ADP release; and direct binding experiments showed osmotic pressure to correspondingly weaken the enzyme's affinity for both ADP and ATP, indicating a less hydrated conformational form of the free enzyme.