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Mechanistic characterization for c-jun-N-Terminal Kinase 1α1

Brian Ember, Philip LoGrasso *

Department of Molecular Therapeutics and Drug Discovery, The Scripps Research Institute, Scripps Florida, 5353 Parkside Drive, Jupiter, FL 33458, USA

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ABSTRACT

c-jun-N-terminal kinase 1α1 (JNK1α1) is a serine/threonine kinase of the mitogen-activated protein (MAP) kinase family that phosphorylates protein transcription factors after activation by a variety of environmental stressors. In this study, the kinetic mechanism for JNK1α1 phosphorylation of activating transcription factor 2 (ATF2) was determined utilizing steady-state kinetics in the presence and absence of both ATF2 and ATP competitive inhibitors. Data from initial velocity studies were consistent with a sequential mechanism for JNK1α1. AMP-PCP exhibited competitive inhibition versus ATP and pure non-competitive inhibition versus ATF2. JIP-1 peptide (RPKRPTTLNLF) was competitive versus ATF2 and mixed noncompetitive versus ATP. These data suggest that JNK1α1 proceeded via a random sequential kinetic mechanism with non-interacting ATF2 and ATP substrate sites.

The c-jun NH_2-terminal kinases (JNKs)[1] are stress-activated serine/threonine kinases involved in a range of cellular signaling pathways. As a part of the mitogen-activated protein (MAP) kinase family, JNKs are activated by a variety of stimuli including cytokines, UV radiation, osmotic shock, and hypoxia [1,2]. Environmental stressors lead to JNK phosphorylation by MKK4 and MKK7 [3] on the threonine and tyrosine residues in the conserved MAP kinase activation loop (TXY) [4,5]. Upon activation, JNKs phosphorylate c-jun, ATF2, Elk-1, as well as other transcription factors leading to a myriad of cellular responses [4,6,7]. Neurodegeneration, diabetes, inflammatory diseases, and cancer have all been linked to malfunctions in MAP kinase pathways [8,9].

The JNK subfamily is comprised of three distinct genes encoding JNK1, JNK2, and JNK3 which are alternately spliced into at least 10 isozymes [4]. There is high sequence identity within the kinase domains of the isoforms with JNK3 containing 92% amino acid identity with JNK1 and 87% identity with JNK2 [5]. Sequence disparity between the JNK splice variants occurring within subdomains IX–XI and extensions at the C-terminus have proven to be instrumental in differential JNK isozyme-substrate binding and autophosphorylation [10,11]. JNK1/JNK2 chimera studies have indicated that a nine amino acid region within domain IX of JNK2α2 may be responsible for selective c-jun affinity [10] or autophosphorylation activity [11]. Other amino acid domains of interest include a region in subdomain XI of JNK2α2 which may be required for efficient c-jun binding and the C-terminal extension on the 55-kDa JNK isoforms which enhances autophosphorylation intensity [11]. The C-terminal extension domain has also shown functional significance in primary human glial tumors where 86% of tumors show almost exclusive activation of the 55-kDa, as opposed to the 46-kDa, splice variants [12]. In healthy tissue, JNK1 and JNK2 are expressed ubiquitously while JNK3 is preferentially expressed in the brain with lower levels in the heart and testis [13].

KO studies have defined the role of JNK in peripheral diseases such as diabetes mellitus and obesity. In 2002, Hirosumi et al. showed that *Jnk1* −/− mice had decreased adiposity, significantly improved insulin sensitivity, and enhanced insulin receptor signaling capacity in both a diet-induced obesity (DIO) model and a genetic obesity model (using *ob/ob* mice) [14]. In addition, the DIO *Jnk1* −/− mice had decreased body weights compared to DIO wild type mice and also showed decreased blood glucose levels. These data are strong evidence that a JNK1 inhibitor may be efficacious in the treatment of diabetes mellitus as well as in obesity.

A detailed kinetic mechanism of enzyme catalysis is a necessity for the design of specific and potent inhibitors. All kinases catalyze a two-substrate reaction between ATP and a protein phosphate acceptor. Two-substrate mechanisms can demonstrate a sequential pattern involving the association of both substrates to yield a ternary complex followed by dissociation of two products. Sequential catalysis can further be divided into ordered or random models depending on the requirement for an order of association and dissociation between the two substrates. Contrastingly, a ping–pong catalysis model entails binding, transformation, and dissociation of one substrate followed by the same sequence by the second sub-

* Corresponding author. Fax: +1 561 7998959.
E-mail address: lograsso@scripps.edu (P. LoGrasso).

[1] *Abbreviations used:* ATF2, activating transcription factor 2; JNKs, c-jun NH_2-terminal kinases; MAP, mitogen-activated protein; DIO, diet-induced obesity; b-FL-ATF2, biotinylated-FLAG-ATF2; JIP-1, JNK Interacting Protein-1; LB, Miller's Luria Broth; IPTG, isopropylthio-β-galactoside.

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strate [15]. Recently, the mechanisms for both JNK2α2 [16] and JNK3α1 [17] have been reported as random sequential mechanisms with ATP and ATF2.

The current study was designed to determine the kinetic mechanism and steady-state constants for JNK1α1 with ATP and ATF2. Steady-state kinetic analysis of the phosphorylation of biotinylated-FLAG-ATF2 (b-FL-ATF2) was used to determine catalytic efficiency and inhibitor mechanism. AMP-PCP, a non-hydrolyzable ATP analogue, was utilized as an ATP competitive inhibitor and the JIP-1 peptide, derived from the δ-site of JNK Interacting Protein-1 (JIP-1) and an ATF2 competitive inhibitor of JNK2α2 [16] and JNK3α1 [17], was employed as an ATF2 competitive inhibitor. The study establishes that JNK1α1 kinase proceeded via a random sequential kinetic mechanism. Additionally, the JIP-1 peptide inhibits via a competitive mechanism versus b-FL-ATF2. These findings are consistent with the mechanisms for JNK2α2 and JNK3α1 [16,17].

Materials and methods

Expression and purification of proteins

JNK1α1 was purchased from Millipore. Biotinylated-FLAG-ATF2 (b-FL-ATF2) (72 amino acid Bioease [18]—FLAG (DYKDDDDK)—ATF2 1-115 [19]) was expressed in *Escherichia Coli* strain BL21 DE3star (Invitrogen) under the following conditions: 2 mL of a log phase culture grown in Miller's Luria Broth (LB) (Cellgro) supplemented with 100 μg/mL ampicillin (Sigma) and 34 μg/mL chloramphenicol (Sigma) was transferred to 1 L of the same medium with shaking at 210 rpm and 37 °C. At A_{600} = 0.6, 0.05 mM d-biotin (Supelco) was added to biotinylate the bioease tag followed immediately by induction with 0.5 mM (final concentration) isopropylthio-β-galactoside (IPTG) (Gibco) and grown with shaking at 210 rpm and 37 °C for 2 h and harvested at 4 °C by centrifugation at 4000*g*. Cell pellets were frozen at −70 °C for subsequent purification.

Frozen pellets from a 1 L culture were resuspended in 10 mL of B-per lysis buffer (Pierce) with complete Mini EDTA-free protease Inhibitor Cocktail (Roche). Cells were lysed by the addition of Lysozyme chloride Grade VI from chicken egg white and Deoxyribonuclease I from bovine pancreas (Sigma) followed by sonication (20 s at 10 watts with 2 min intervals in ice; 3 times). The lysate was centrifuged at 20,000 rpm for 30 min at 4 °C to provide the supernatant. The supernatant was applied to an Anti-FLAG M2-Agarose column (Sigma), preincubated with a wash buffer (50 mM HEPES, 150 mM NaCl, pH 7.0), followed by 5 column volumes of wash buffer. The protein was eluted with glycine buffer (100 mM glycine, pH 3.5) in 1 mL fractions into eppendorfs containing 100 μL 1 M HEPES pH 8.0. The collected fractions were concentrated using Amicon Ultra-4 (Millipore). SimplyBlue (Invitrogen) staining of 4–12% gradient NuPage Bis–Tris gels (Invitrogen) revealed greater than 95% homogeneity. The concentration of b-FL-ATF2 was determined by BCA protein assay (Pierce).

Steady-state kinetics

Initial velocity studies utilized to determine the steady-state constants for ATP and b-FL-ATF2 were carried out in 50 μL volumes containing the final concentrations of the following: 25 mM HEPES (Sigma), pH 7.4, 10 mM $MgCl_2$ (Sigma); 2 mM DTT (Sigma); 1 mg/mL BSA; 3 μCi [γ-^{33}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) (Perkin Elmer), 0.25–16 μM ATP (Sigma) and 0.125–8 μM b-FL-ATF2. The reactions were initiated with 0.5 nM JNK1α1 (final concentration) and incubated for 1 h at 30 °C. Under these conditions, less than 10% of substrate was converted to product and the reaction was linear over a time course of up to 2 h. Reactions were quenched with 50 μL of a 100 mM EDTA, 15 mM sodium pyrophosphate solution. Twenty microliters of the stopped reaction was spotted in duplicate onto a p81 phosphocellulose circle (Fisher). The samples were allowed to dry before being washed 3 times with 75 mM phosphoric acid for 3 min each to remove unincorporated [γ-^{33}P]-ATP. A fourth wash of Ethanol for 30 s reduced the drying time necessary before addition to 3 mL of ScintiSafe Econo 2 scintillation cocktail (VWR) and counting on a Scintillation counter. Data are the average of two experiments spotted in duplicate. The initial velocities as a function of ATP and b-FL-ATF2 were fitted to equations for ternary complex, ternary complex with non-interacting substrate sites and ping–pong mechanisms [20]. Kinetic constants were determined from a robust nonlinear least-squares analysis [20].

Enzyme inhibition

Enzyme inhibition studies were performed as described for steady-state kinetics. When ATP was the varied substrate, [b-FL-ATF2] was fixed at 0.5 μM, and when b-FL-ATF2 was the varied substrate, [ATP] was fixed at 2 μM for ATF2 competitive inhibitors and 8 μM for ATP competitive inhibitors. For inhibition experiments, the concentration of AMP-PCP (Sigma) ranged from 400 to 25 μM and JIP-1 δ-site peptide (Peptidogenic) ranged from 200 to 12.5 nM. The initial velocities were fitted to equations for competitive, uncompetitive, pure non-competitive, and mixed non-competitive inhibition, and steady-state rate constants were determined from a robust nonlinear squares fit [20]. Reactions were spotted in duplicate and inhibition reactions were performed in duplicate for all inhibitors versus both ATP and b-FL-ATF2.

Kinetic analysis

The kinetic analysis was performed using GraFit version 5 [20] fitted to the following equations:

For two substrate kinetics, ternary mechanism (1), ternary mechanism with non-interacting substrate sites (2), and ping–pong mechanism (3)

$$v = V_{max}[A][B]/(K_{ia}K_{mB} + K_{mB}[A] + K_{mA}[B] + [A][B]) \quad (1)$$

$$v = V_{max}[A][B]/(K_{mA}K_{mB} + K_{mB}[A] + K_{mA}[B] + [A][B]) \quad (2)$$

$$v = V_{max}[A][B]/(K_{mB}[A] + K_{mA}[B] + [A][B]) \quad (3)$$

where V_{max} is the maximum initial velocity, A and B represent the substrates, K_{ia} is the dissociation constant for substrate A from free enzyme, K_{mA} and K_{mB} are the corresponding Michaelis–Menten constants for A and B.

Inhibition experiments were fitted to competitive (4), pure noncompetitive (5), mixed noncompetitive (6), and uncompetitive (7) models

$$v = V_{max}[S]/(K_m(1 + [I]/K_{is}) + [S]) \quad (4)$$

$$v = V_{max}[S]/(K_m(1 + [I]/K_i) + [S](1 + [I]/K_i)) \quad (5)$$

$$v = V_{max}[S]/(K_m(1 + [I]/K_{is}) + [S](1 + [I]/K_{ii})) \quad (6)$$

$$v = V_{max}[S]/(K_m + [S](1 + [I]/K_{ii})) \quad (7)$$

where S is the varied substrate for which K_m refers, I is the inhibitor, K_{is} is the inhibition constant for the EI complex, derived from the slope of the Lineweaver–Burk plots, and K_{ii} is the inhibition constant for the ESI complex, derived from the y-intercept of the Lineweaver–Burk plots. For noncompetitive inhibitors, if K_{is} and K_{ii} are equal, Eq. (6) simplifies to Eq. (5) giving pure noncompetitive inhibition with the inhibitor being recognized by the free enzyme and the enzyme-substrate complex with similar affinity. If K_{is} and K_{ii} are not equal, there is mixed noncompetitive inhibition with the inhibitor being preferentially recognized by either the free enzyme or the enzyme-substrate complex.

Results

Initial velocity studies

The kinetic mechanism for JNK1α1 was determined utilizing a two-dimensional matrix of b-FL-ATF2 and ATP at varying concentrations. Double-reciprocal plots of $1/v$ versus 1/[b-FL-ATF2] and $1/v$ versus 1/[ATP] both revealed patterns of converging lines indicating a sequential or ternary mechanism (Fig. 1) [21]. Equations for ternary mechanisms with and without interacting substrate binding sites were fit to the two-substrate profile data. The corresponding data indicated similar constants for equilibrium dissociation (K_{ia} = 3.6 ± 1.1 μM from Eq. (1) and substrate affinity (K_m = 3.6 ± 0.3 μM from Eq. (1) when varying either b-FL-ATF2 or ATP. Similar K_{ia} and K_m constants illustrate non-interacting substrate sites and thus, the ternary equation simplifies to the ternary equation with non-interacting sites. The steady-state rate constants from two experiments analyzed in duplicate generated utilizing the equation for ternary complexes with non-interacting substrate sites are summarized in Table 1.

Inhibition by dead end inhibitor AMP-PCP

The binding order of the ternary mechanism suggested by the initial velocity studies was determined utilizing dead-end inhibitors. Analysis of the double reciprocal plots of the nonhydrolyzable ATP analogue AMP-PCP (Fig. 2) supported a competitive inhibition mechanism for AMP-PCP versus ATP and noncompetitive inhibition versus b-FL-ATF2. Regression analysis along with standard error comparison for fits for competitive, pure noncompetitive, mixed noncompetitive and uncompetitive inhibition corroborated a competitive inhibition mode for AMP-PCP versus ATP and supported a pure noncompetitive mode of inhibition versus b-FL-