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Pro^{42} and Val^{45} of staphylokinase modulate intermolecular interactions of His^{43}–Tyr^{44} pair and specificity of staphylokinase–plasmin activator complex

Satish Singh, Ashish, Kanak L. Dikshit *

Institute of Microbial Technology, CSIR, Sector-39 A, Chandigarh 160036, India

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ABSTRACT

Staphylokinase (SAK) forms a 1:1 stoichiometric complex with plasmin (Pm) and changes its substrate specificity to create a plasminogen (Pg) activator complex. The His^{43}–Tyr^{44} pair of SAK resides within the active site cleft of the partner Pm and generates intermolecular contacts to confer Pg activator ability to the SAK–Pm bimolecular complex. Site-directed mutagenesis and molecular modeling studies unravelled that mutation at 42nd or 45th positions of SAK specifically disrupts cation-pi interaction of His^{43} with Trp^{215} of partner Pm within the active site, whereas pi–pi interaction of Tyr^{44} with Trp^{215} remain energetically favoured.

Structured summary of protein interactions:
Pg binds to **SAK** by surface plasmon resonance (View Interaction: 1, 2, 3)
SAK enzymaticly reacts **Pg** by enzymatic study (View Interaction: 1, 2, 3, 4, 5)

1. Introduction

Staphylokinase (SAK), a 15.5 kDa fibrinolytic protein secreted from the bacterium, *Staphylococcus aureus* [1,2], is a plasminogen (Pg) activator that induces fibrin specific thrombolysis in human plasma [3,4]. Unlike direct human Pg activators such as tissue-plasminogen activator (t-PA) [5] and urokinase (UK), SAK does not have any proteolytic activity of its own but it acts by forming a 1:1 stoichiometric complex with plasmin (Pm) and changing its substrate specificity to activate Pg as its substrate [6,7]. Unlike SAK, Streptokinase (SK) can form a functional activator complex with Pg by modifying its active site without any proteolytic cleavage in Pg molecule [8] while SAK requires the conversion of SAK–Pg complex to SAK–Pm [9] and the removal of ten amino acid residues from the N-terminus to form a functional Pg activator complex [10,11]. Despite these differences in their mechanism of Pg activation, a close structural homology exists between SAK and α domain of SK [12] and their intermolecular contacts with the partner [13–15] near the active site suggesting that the critical contacts at the two respective interfaces have been well conserved in the evolutionary process and may be crucial in Pg binding and activation [12].

The mechanisms by which SAK reprograms the broad trypsin like substrate specificity of Pm in SAK–Pm complex towards sessile peptide bond of Arg^{561}–Val^{562} within the activation loop of substrate Pg is a fascinating aspect of SAK functionality that has not been understood at the molecular level. The crystal structure of ternary complex of SAK, complexed with the partner and substrate μPm [14], has revealed several intermolecular contacts between SAK and the partner μPm molecule in the vicinity of its active site. More or less similar intermolecular interactions occur when SK forms a bimolecular Pg activator complex with Pg/Pm [15,16]. Recent site-directed mutagenesis and molecular modeling studies, conducted in our laboratory, have demonstrated that His^{43}–Tyr^{44} pair of SAK generates crucial contacts at the interface with the partner Pm molecule and may be vital for the specificity switch and functionality of binary activator complex of SAK with Pm [17]. Structural comparison of unbound SAK with the bound one in the SAK–μPm complex revealed distinct movement of Ser^{41}–His^{43} region after complex formation with Pm indicating the involvement of Pro^{42} at this junction. Moreover, Val^{45} another flanking residue of this pair is present at the crucial junction of intermolecular contacts. Biochemical and molecular modeling studies, presented in this work, demonstrated key role of SAKPro^{42} and SAKVal^{45} in modulating the intermolecular interactions of His^{43} and Tyr^{44} residues of SAK with the partner and specificity of the SAK–Pm complex.

2. Materials and methods

2.1. Bacterial strains, plasmids and reagents

The expression vector pET9b and *Escherichia coli* host strains, JM109 and BL21DE3, purchased from Promega (WI, USA), were

* Corresponding author. Fax: +91 172 2690585.
E-mail address: kanak@imtech.res.in (K.L. Dikshit).

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routinely utilized for cloning and expression of recombinant genes using *sak* gene [11,18] as a template. All restriction and modifying enzymes were procured from New England Biolabs or Stratagene (USA). Chromozyme PL (Tosyl-glycyl-polyl-lysine-4-nitranilide-acetate) and human Glu-Pg was obtained from Roche Diagnostics (Germany). Human Pg was also purified from human plasma using Lysine-Sepharose chromatography [19].

2.2. Site-directed mutagenesis of SAK: expression and purification SAK mutants

Site directed mutagenesis of Pro^{42} and Val^{45} residues of SAK was done by using pRM1 plasmid as template through QuickChange site directed mutagenesis kit from Stratagene which uses a set of two primers having the desired mutation in the center. List of oligonucleotide primers used for this purpose is given in Supplementary Table S1. Site directed mutants of SAK were cloned, expressed in *E. coli* and purified as described previously [17].

2.3. Pg activation property of SAK/SAK mutants

To check the Pg activation properties of SAK mutants, 5 nM SAK or SAK mutants were mixed with 1.5 μM Pg in assay buffer (50 mM Tris·HCl, pH 7.5, containing 0.1% BSA, 100 mM NaCl and 0.01% Tween 80) containing 1 mM chromozyme in 96 well microtitre plate and generation of Pm was measured as a function of time at 405 nm at 25 °C in BioTEK Powerwave X 96 wells microtitre plate reader [11,20]. To see the Pg activation by preformed complexes of SAK or SAK mutants with Pm, equimolar mixtures of SAK or SAK mutants (0.5 μM) and Pm (0.5 μM) were preincubated in assay buffer at 37 °C for 5 min to generate the SAK–Pm bimolecular complex. These preformed activator complexes (5 nM) were then mixed with substrate Pg (1.5 μM) and generation of Pm was measured spectrophotometrically at 25 °C using Chromozyme PL (1 μM) as described previously [11,18]. To calculate the steady-state kinetic constants, these preformed complex of SAK–Pm (5 nM) were mixed with different concentrations of Pg (0.5–5 μM) and 1.0 mM Chromozyme PL. The change in absorbance at 405 nm was measured as a function of time at 25 °C as described above. The kinetic constants were determined through Michaelis Menten plot [18,21].

2.4. Amidolysis profile of SAK–Pm bimolecular complex

To see the specificity of the active site generated by SAK/SAK mutants with Pm, an equimolar mixture of Pm (0.5 μM) with SAK/SAK mutant (0.5 μM) in the reaction buffer (50 mM Tris·HCl, pH 7.5, containing 0.1% BSA, 100 mM NaCl) was incubated for 5 min and then 50 nM of the SAK/SAK mutant–Pm complex was added to the reaction buffer containing 1.5 μM Chromozyme in microtitre plate. The amidolysis profile of SAK or SAK mutants was measured spectrophotometrically at 405 nm in microtitre plate as described above [22].

2.5. Determination of the binding affinity of SAK/SAK mutants for Pg by surface plasmon resonance (SPR)

The kinetic constants for binding of SAK/SAK mutants with Pg were determined by SPR [23] using Sensor Chip SA in a BIACORE 3000 biosensor [24]. Pg was purified from human plasma and biotinylated with Sulpho-NHS-LC-Biotin [25]. Approximately 1500 Resonance units of Pg were immobilized on Sensor Chip SA. To calculate the rate and equilibrium binding constants, the different concentrations of SAK/SAK mutants (1–20 μM or 10–100 μM) were passed over the immobilized Pg. All the experiments were performed in HBS running buffer (20 mM HEPES, 1 mM EDTA, 0.15 M NaCl, and 0.005% surfactant P20, pH 7.2) supplemented with NPGB to avoid any Pg/Pm mediated proteolysis [22]. The k_{on} (assosciation rate constant, k_a), k_{off} (dissociation rate constant, k_d) and K_D (equilibrium binding constant) values were calculated by non-linear fitting of the association and dissociation curves using 1:1 Langmuir binding model in BIACORE 3000 evaluation software.

2.6. Molecular modeling studies of SAK/SAK mutants –μPm complex

Energy minimization of the coordinates of SAK (and its mutants) in complex with μPm was carried out using Newton software in the Tinker suite of programs [26,27]. Using the crystal structure 1BUI as starting coordinates, the substrate μPm was excluded from calculations to mimic the binary activator complex. Mutants were generated using SPDB viewer program and all rotameric conformations of each mutant for the side-chain with least disallowed contacts [28] were selected. The calculations were carried out employing implicit dielectric of 80, vdw-cutoff of 9 Å and force-field parameters of optimized parameters for liquid simulation united-atom (OPLSUA). Coordinates were minimized to an RMSD cut-off of 0.01 kcal/mol/Å. Total and interaction energies were estimated using Analyze program.

3. Results

3.1. Intermolecular contacts at the interface of active site of SAK–μPm bimolecular complex

$His^{43}$and Tyr^{44} residues of SAK are uniquely placed inside the active site of μPm in SAK–Pm complex and interact with Trp^{215} (Fig. 1A) through cation–pi and pi–pi interactions respectively. Presence of His^{43}–Tyr^{44} pair in SAK and Lys^{36}–Phe^{37} in SK and their contacts at the two respective interfaces (Fig. 1B) signifies similarity between these interactions that may be required for changing the specificity of active site towards Pg activation. Positioning of Pro^{42} and Val^{45} of SAK at the junction of these crucial interactions led to the speculation that these two residues may be involved in the spatial arrangement of their neighbors for establishing their intermolecular contacts with the partner Pm.

3.2. Site-directed mutagenesis of $SAKPro^{42}$ and $SAKVal^{45}$ and functional properties of SAK mutants

To gain experimental insights for the role of Val^{45} and Pro^{42} in Pg binding and activation, the specific mutants of these residues were generated. Val^{45} residue of SAK was replaced either with Ala in $SAKVal^{45}Ala$ mutant, or Tyr in $Val^{45}Tyr$ mutant. Pro^{42} was mutated to leucine in $SAKPro^{42}Leu$ mutant and a double mutant namely $Tyr^{44}Phe$, $Val^{45}Phe$ was constructed in which both SAK-Tyr^{44} and $SAKVal^{45}$ were mutated to phenylalanine respectively to mimic the residues as present in SK. Surprisingly, all the mutants showed much diminished Pg activation ability as compared to native SAK as evident from their activation profile (Fig. 2A). When preformed complexes of $SAKPro^{42}$ and Val^{45} were used with Pm, these complexes exhibited extremely slow progression and a significant lag (10 min as compared to 2 min in native SAK) in Pg activation suggesting attenuation in catalytic activity of activator complexes formed by these SAK mutants (Fig. 2B).

3.3. Steady state kinetics of Pg activation by equimolar complexes of SAK or $SAKPro^{42}$/$SAKVal^{45}$ mutants with Pm

The k_m and k_{cat} values of Pg activation by $SAKPro^{42}$ and SAK-Val^{45} mutants (Table 1) clearly suggested the crucial role of these residues in complex formation and activation of Pg. These observa-