Intermolecular interactions in staphylokinase–plasmin(ogen) bimolecular complex: Function of His43 and Tyr44

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ABSTRACT

Staphylokinase (SAK) forms a 1:1 stoichiometric complex with human plasmin (Pm) and switches its substrate specificity to generate a plasminogen (Pg) activator complex. Site-directed mutagenesis of SAKHis43 and SAKTyr44 demonstrated the crucial requirement of a positively charged and an aromatic residue, respectively, at these positions for optimal functioning of SAK–Pm activator complex. Molecular modeling studies further revealed the role of these residues in making cation–π and π–π interactions with Trp215 of Pm and thus establishing the crucial intermolecular contacts within the active site cleft of the activator complex for the cofactor activity of SAK.

1. Introduction

Activation of plasminogen (Pg) to plasmin (Pm) is a key event in fibrinolysis. Two bacterial Pg activators, staphylokinase (SAK) and streptokinase (SK), initiate fibrinolysis in humans by forming a cofactor-enzyme complex with Pm or Pg and results in conformational expression of new catalytic active site that specifically cleaves the Arg561–Val562 activation bond in the catalytic domain of Pg to form Pm [1–3]. Thus, both SAK and SK serve as cofactors that alter the substrate specificity of Pm from the cleavage of fibrin to the cleavage of Pm. In contrast with SK–Pg complex, the SAK–Pg complex remains inactive and requires conversion into SAK–Pm form to exhibit high specificity for the Pg activation [4]. Understanding of structural and molecular basis of this “specificity switch” in Pm after complex formation with the SAK is crucial to unravel the molecular mechanism of Pg activation by SAK. SAK is a 15.5 kDa single domain molecule and three times smaller than SK that carries three independently folded domains (α, β and γ). Although α and β domains of SK exhibit close structural similarity with SAK [5–9], Pg activation ability of individual SK domain is highly attenuated unlike single domain SAK which is fully functional as a Pg activator. Therefore, being the smallest and single domain Pg activator, SAK provides an ideal molecule to delineate the mechanism of cofactor mediated specificity switch in Pm that may be helpful in design and development of new thrombolytic molecules.

During Pg activation by SAK–Pm bimolecular complex, the heterotrimer formed between one SAK and two Pg/Pm molecules, generates several intermolecular interactions that are crucial for altering the geometry of the active site for changing the specificity of Pm towards the activation loop of substrate Pg. Thus, SAK residues, interacting and lining the active site cleft of Pm, may be functionally important for contributing to its cofactor function. The crucial region of SAK that remains in close vicinity of active site of Pm spans from Gly36 to Glu46 and participates in generating a network of salt-bridges and hydrogen bonding (e.g. SAKGlu38–PmLys101; SAKSer41–PmGln177; SAKGlu46–PmArg175) at the interface. The importance of Arg175 of Pm in modulating the Pg activator activity of SAK [6,7,10], suggests that a part of this activator interacts with the partner Pm in a manner similar to SK. The interface of the SAK–Pm enzyme complex displays existence of a pair of basic and aromatic residues (His43, Tyr44) in SAK that protrude into the active site cleft of the partner Pm [7] but do not show direct interaction with the partner Pm, thus, a clear picture of their role in modulating the Pg activator activity of SAK–Pm complex is lacking.
The present study has been undertaken to understand the role of His43 and Tyr44 of SAK that constitute an interesting part of the SAK–Pm activator complex and may be playing a crucial role in modulating the change in the specificity of partner Pm and/or Pg activator activity of the activator complex.

2. Materials and methods

2.1. Bacterial strains, plasmids and reagents

Plasmid vectors pBS KS+ (Strategene, Germany), pET9b (Promega, WI, USA) and Escherichia coli host strains, JM109 and BL21 (DE3) (Promega) were utilized for cloning and expression of recombinant genes. SAK expression plasmid, pRM1, has been described earlier [11]. Restriction and DNA modifying enzymes were obtained from New England Biolabs (Beverly, USA). Chromozyme PL (tosyl-glycyl-polyl-lysin-4-nitranilide-acetate) and human Pg were obtained from Roche Diagnostics (Germany). Pg was purified from lysine-Sepharose affinity purification [12].

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

Site-directed mutants of SAK were generated by overlap PCR and were cloned under T7 promoter of plasmid, pET9b, as described previously [13,14]. List of oligonucleotide primers is given in Supplementary Table S1. SAK and its mutants were purified by a two-step purification protocol as mentioned earlier [11,13].

2.3. Pg activation property of SAK mutants

Pg-activation properties of various SAK mutants were checked using published procedures [13,15]. Briefly, 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) in 96 well microtitre plate containing 1 mM chromozyme and generation of Pm was measured at 405 nm at 25 °C as a function of time in Biotech Power Wave X microplate reader as described previously [11,13]. Pg activation by preformed complexes of SAK or SAK mutants with Pg was checked by taking equimolar mixtures of SAK or SAK mutants (0.5 μM) and Pg (0.5 μM) and incubating it in assay buffer at 37 °C for 5 min to generate the SAK–Pg bimolecular complex. These preformed activator complexes (5 nM) were then mixed with substrate Pg (1.5 μM) and generation of Pm was measured using Chromozyme PL (1 mM) as described above [11]. To calculate the steady state kinetic constants for the Pg activation [13], the preformed activator complexes (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 at 25 °C and kinetic constants were determined through Michaelis–Menten plot. Cleavage of substrate Pg to Pm by SAK was monitored on SDS–PAGE by incubating equimolar complex of SAK mutant and Pg (1.5 μM each) at 25 °C in assay buffer and analyzing the conversion of Pg into Pm on 12% SDS–PAGE.

2.4. Time-course acylation in the bimolecular complex of SAK–Pm

Time-course acylation in the SAK–Pm bimolecular complex was monitored after titration with active site inhibitor of serine proteases, i.e., p-nitrophenol-p-guanidinobenzoate (NPG) (100 μM) in 10 mM sodium phosphate buffer, pH 7.5 at room temperature [13,16]. Precipitated mixture of Pg (4 μM) and SAK/SAKmutant (4.5 μM) (for 5 min) was added to an assay cuvette containing 100 μM NPG in 10 mM sodium phosphate buffer and p-nitrophenol burst was monitored spectrophotometrically at 410 nm as a function of time.

2.5. Structure analysis of SAK–Pm complex and molecular modeling of SAK mutants

Energy minimization of the coordinates of SAK (and its mutants) in complex with two molecules of microplasmin (μPm) (PDB ID: 1BUI) were carried out using Newton software in the Tinker suite of programs [17,18]. The calculations were carried out employing implicit dielectric of 80, vdW-cutoff of 15 Å and force-field parameters of optimized parameters for liquid simulation united-atom (OPLSUA) to simulate continuum solvation model to affect the electrostatic interactions. Coordinates were minimized to an RMSD cut-off of 0.01 kcal/mol/Å. Mutants for different rotamer conformations were generated using SPDB viewer program [19]. Total and interaction energies were estimated using Analyze program. Normal mode analysis (NMA) of the ternary complexes was carried out using ElneMO server [20].

3. Results

3.1. Structural features of the active site cleft of the SAK–Pm bimolecular complex

In the bimolecular complex of SAK–μPm, SAK residues spanning from Gly36 to Glu46 lie at the interface and remain in direct contact with the partner μPm via salt bridges and hydrogen bonding (Fig. 1). Unique positioning of His43 and Tyr44 within the active site cleft of SAK–Pm complex led to the speculation that the specific orientation of these residues at the interface might play a role in specificity switch of Pm by altering its active site geometry. Structural analysis of SAK–μPm ternary complex (PDB ID: 1BUI) using PyMOL software [21] indicated that the Trp215, lying within the S3–S4 site of the μPm enzyme, is located within the distance of ~4 Å from His43 and Tyr44 of SAK and may favor a cation–π and π–π interactions with His43 and Tyr44 of SAK, respectively, via its aromatic side chain (Fig. 1) that may be vital for the Pg-activation function of the activator complex.

3.2. Site directed mutagenesis of SAK: functional properties of His43 and Tyr44 mutants

To gain experimental validation for the function of His43 and Tyr44, site-specific mutations were created at these positions of SAK. Two SAK mutants, namely SAKHis43Lys and SAKHis43Glu were constructed to find out the involvement of any charged interaction. Additionally, His43 residue was replaced with phenylalanine in SAKHis43Phe mutant to test the possibility of any aromatic ring interaction with Trp215 at this position. The Pg activation profile of SAKHis43Lys displayed Pg-activator activity similar to native SAK, whereas, SAKHis43Glu, and SAKHis43Phe exhibited drastic reduction in their Pg activation ability (Fig. 2A). When preformed complexes of SAKHis43Lys with Pg were used, Pg activation occurred immediately and progressively without any distinct lag, similar to native SAK but preformed complexes of SAKHis43Phe and SAKHis43Glu exhibited slow progression and a significant lag (10 min as compared to 2 min in native SAK) in Pg activation suggesting that the catalytic activity of these bimolecular complexes is highly attenuated (Fig. 2B). These observations validated the requirement of a positively charged residue at this topological (43rd) position of SAK. These results were further substantiated when conversion of Pg to Pm by the pre-complexes formed by these SAKHis43 mutants were monitored on SDS–PAGE (Fig. 3). Densitometric analysis of the protein profile indicated that almost 50% Pg was converted to Pm in less than 10 min by SAKHis43Lys, similar to native SAK, as compared to SAKHis43Phe and SAKHis43Glu (20 min). Catalytic efficiency of SAKHis43/SAK-
Tyr44 mutants was determined by a two step assay to calculate the steady state kinetic constants. The replacement of His43 with another positively charged residue did not alter its affinity towards substrate and its catalytic processing as indicated by their $K_m$ and $K_{cat}$ values (Table 1). In contrast, replacement of His43 with any other residue reduced the affinity of SAK–Pm enzyme complex.

Fig. 1. Structural features of interface of SAK–Pm biomolecular complex. Ribbon diagram of SAK–Pm interface displaying SAK in red and Pm in blue. The active site residues of catalytic triad (His57, Asp102, Ser195) of Pm are shown as sticks in green. The figure was prepared by PyMOL software.

Fig. 2. (A) Pg activation profile of SAK mutants. The graph shows SAK (−−−−−), SAKHis43Lys (−−−−), SAKHis43Glu (−−−−), SAKHis43Phe (−−−−−), SAKTyr44Phe (−−−−), and SAKTyr44Ala (−−−−). (B) Pg activation profile of preformed complexes of SAK mutants. The graph shows SAK (−−−−), SAKHis43Lys (−−−−), SAKHis43Glu (−−−−), SAKHis43Phe (−−−−−), SAKTyr44Phe (−−−−), and SAKTyr44Ala (−−−−).

Fig. 3. Cleavage of Pg by equimolar complex of Pm with SAKHis43 mutants. Lane 1: Molecular-mass markers; lane 2: Pg; lane 3: purified SAK; lane 4–6: SAK–Pg reaction mixtures at 0, 5 and 10 min; lane 7–10: SAKHis43-Phe-Pg reaction mixture at 0, 5, 10 and 20 min; lane 11–14: SAKHis43Glu-Pg reaction mixture at 0, 5, 10 and 20 min; lane 15–17: SAKHis43Lys-Pg reaction mixture at 0, 5 and 10 min. The arrows indicate the Pg cleavage products.

Table 1

<table>
<thead>
<tr>
<th>Bimolecular complex</th>
<th>$K_m$ (μM)</th>
<th>$K_{cat}$ (s$^{-1}$)</th>
<th>$K_{cat}/K_m$ (s$^{-1}$ μM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAK–Pm</td>
<td>0.80 ± 0.09</td>
<td>0.43 ± 0.06</td>
<td>0.53</td>
</tr>
<tr>
<td>SAKHis43Lys–Pm</td>
<td>0.80 ± 0.1</td>
<td>0.40 ± 0.07</td>
<td>0.50</td>
</tr>
<tr>
<td>SAKHis43Glu–Pm</td>
<td>1.7 ± 0.08</td>
<td>0.1 ± 0.086</td>
<td>0.058</td>
</tr>
<tr>
<td>SAKHis43Phe–Pm</td>
<td>1.9 ± 0.1</td>
<td>0.095 ± 0.006</td>
<td>0.050</td>
</tr>
<tr>
<td>SAKTyr44Phe–Pm</td>
<td>0.85 ± 0.1</td>
<td>0.40 ± 0.08</td>
<td>0.47</td>
</tr>
<tr>
<td>SAKTyr44Ala–Pm</td>
<td>2.0 ± 0.09</td>
<td>0.096 ± 0.004</td>
<td>0.048</td>
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</table>

* The results are means ± S.E.M. of three determinations.
plex towards substrate Pg thereby resulting drastic reduction in the catalytic processing of Pg into Pm. These observations indicated the requirement of a basic residue at this topological position of SAK for the optimal Pg activation.

To probe the role of Tyr44 of SAK, two mutants namely SAK-Tyr44Phe and SAKTyr44Ala were constructed and their Pg activation properties were studied. SAKTyr44Phe exhibited Pg activation properties similar to that of wild type SAK while SAK-Tyr44Ala displayed extremely slow Pg-activator profile (Fig. 2A and B). The high $K_{\text{m}}$ and low $K_{\text{cat}}$ values of SAKTyr44Ala as compared to SAK or SAKTyr44Phe also indicated its highly compromised catalytic activity (Table 1).

3.3. Time-course acylation by SAKHis43 and SAKTyr44 mutants

Time-course acylation in the preincubated mixture of Pg and SAKHis43Lys or SAKTyr44Phe occurred exponentially similar to native SAK as evident by the profile of NPCG burst, whereas, in the case of SAKHis43Glu, SAKHis43Phe and SAKTyr44Ala, there was a long lag and very slow progression in the exposure of active site (Fig. 4) implying that the exposure in the active is hindered in these SAK mutants.

3.4. Molecular modeling and structure analysis

To gain further insight into the function of His43 and Tyr44 residues of SAK, we performed energy minimization of the ternary complex involving native SAK and its various in silico mutants. In energy minimized structure of native SAK, His43 and Tyr44 residues were in a proximal orientation for both cation–pi and pi–pi interactions, respectively, with Trp215 residue of Pm (Fig. 5A) at a distance of $\sim$4 Å (Table 2). The substitution of His43 with another positively charged residue, i.e., Arg or Lys resulted in a change in pattern of local interactions where the relatively longer positively charged side-chains of Arg/Lys established a more stable electrostatic interaction with the COO$^-$ of Glu180 of Pm (Fig. 5B and C) with Trp215 moving more closer to Tyr44 in energy minimized structures. In the virtual mutant, SAKHis43Glu there was a loss of local profile in the energy minimized structure, the negatively charged side-chain of Glu43 was in direct conflict with the negatively charged y-carboxylate group of Glu180 and electron rich cloud of Trp215 of Pm (Fig. 5D). The normal mode analysis of SAKHis43Glu clearly showed that Glu43 caused movement of Glu180 away towards Trp215 which in turn moved away with Tyr44 in tow (Supplementary Fig. S1). In SAKHis43Phe mutant of SAK, the side-chain of Phe residue occupied a very different spatial disposition which ruled out the possibility of pi–pi interaction with Trp215 (Fig. 5E). Energy minimized coordinates of SAKTyr44Phe retained the characteristics of native like architecture (Fig. 5F), but the missing aromatic nature totally handicapped Ala44 (Supplementary Fig. S2) for making intermolecular contacts. Overall, in corroboration to our experimental data, molecular modeling of SAK–Pm predicted that His43 may be complemented by a charged residue like Lys/Arg and 44th position of SAK requires an aromatic residue to retain a native like interaction topology, thus, highlighting the crucial role of His43–Tyr44 pair in modulating the SAK function as a Pg activator.

4. Discussion

During Pg activation, the formation of SAK–Pm biomolecular complex and proper presentation of substrate Pg on the activator complex is crucial and may require a number of intermolecular interactions and topological complementarities between the energetically critical regions of SAK and Pg/Pm. The present experimental and modeling studies demonstrated that the His43 and Tyr44 residues of SAK play a pivotal role in modulating the Pg-activator ability of SAK–Pm enzyme complex by establishing crucial intermolecular interactions with the partner Pm at the interface.

The His43 and Tyr44 pair of SAK occupies a unique position at the interface of SAK–Pm complex and is spaced within the active site cleft of Pm in a way that favors cation–pi and pi–pi interactions, respectively, with Trp215 of Pm at the interface. These interactions may stabilize the SAK–Pm complex simultaneously creating a change in the geometry of the active site of the partner Pm. Experimental studies on His43 and Tyr44 mutants of SAK validated these observations. The steady-state kinetic parameters of SAKHis43 mutants demonstrated the crucial requirement of a positively charged residue at this position. This is also substantiated by the observation that the His43 has been replaced by an arginine residue in a natural variant of SAK having similar Pg activator activity [22]. Since the length and geometric features of the Arg, Lys and His side chains differ significantly from each other, it can be envisaged that the presence of these residues at the interface may affect geometry of active site differently, thus, the requirement of a basic residue at this topological position of SAK may be for establishing crucial contact with the partner. It is known that the protonated form of His residue when positioned appropriately can interact with aromatic residues to form a cation–pi interaction similar to lysine to arginine [23,24].

Experimental studies also validated the requirement of a phenylalanine ring at 44th position of SAK and a clear pi–pi interaction between Tyr44 and Trp215 of Pm was established after modeling and energy minimization, which was maintained even when Tyr44 was replaced with another aromatic residue, i.e., phenylalanine. Importantly, this contact was not disrupted even after local movement of side chains in SAKHis43, SAKHis43Arg and SAKHis43Lys mutants. It has been proposed that Tyr44 makes a hydrophobic shield with Tyr24 and Met26, and may stabilize the internal salt bridges of interface of SAK–Pm complex [6]. Thus, the placement of a phenylalanine residue would serve the similar function. However, our studies suggest that Tyr44 generates an intermolecular contact at the interface apart from stabilizing other intermolecular contacts at the interface for the optimal function of the SAK–Pm activator complex. Intermolecular cation–pi or pi–pi pairs have been found frequently at the interface of protein complexes [23] and tryptophan is the most favorable aromatic residue that can establish energetically significant cation–pi interaction with cationic side chains. The phenylalanine and tyrosine residues prefer to interact with tryptophan in an edge-to-face (T-shaped) or parallel-displaced configuration [25] over parallel pi-stacking. Skewed
T-shaped interaction geometry of tyrosine or phenylalanine observed at 44th position of SAK may confer better stability to the SAK–Pm complex [26]. The sequence comparison of SAK with streptococcal Pg activator, PadA [27] also shows the presence of a phenylalanine (Phe44) residue at the same position. Both SAK and SK activate Pg/Pm in a non-proteolytic manner suggesting a basic similarity in their molecular mechanism of function. The contacts of Pm with SAK are more or less similar to that made by the α domain of SK [6,7]. Molecular modeling studies indicated that

Table 2
Interatomic distances between key residues of SAK and Pm complex after energy minimization.

<table>
<thead>
<tr>
<th>Amino acid (SAK)</th>
<th>Atoms</th>
<th>Distance (Å) from Trp215</th>
<th>Distance (Å) from Glu180</th>
</tr>
</thead>
<tbody>
<tr>
<td>His43</td>
<td>SAK(ND)–Pm(NE)</td>
<td>3.28</td>
<td>–</td>
</tr>
<tr>
<td>Lys43</td>
<td>SAK(HZ)–Pm(OE)</td>
<td>–</td>
<td>2.39</td>
</tr>
<tr>
<td>Arg43</td>
<td>SAK(HH)–Pm(OE)</td>
<td>–</td>
<td>3.10</td>
</tr>
<tr>
<td>Glu43</td>
<td>SAK(OE)–Pm(OE)</td>
<td>–</td>
<td>3.15</td>
</tr>
<tr>
<td>Tyr44</td>
<td>SAK(CE)–Pm(CZ)</td>
<td>3.58</td>
<td>–</td>
</tr>
<tr>
<td>Phe44</td>
<td>SAK(CE)–Pm(CZ)</td>
<td>3.18</td>
<td>–</td>
</tr>
</tbody>
</table>

Fig. 5. Molecular modeling of interaction of His43 and Tyr44 mutants at the interface after energy minimization. The interacting residues are represented as sticks (A) SAKHis43–µPmTrp215 and SAKTyr44–µPmTrp215 (B) SAKLys43–µPmGlu180 (C) SAKArg43–µPmGlu180 (D) SAKGlu43–µPmGlu180 (E) SAKHis43Phe (F) SAKTyr44Phe–µPmTrp215. The figure was prepared by Web Lab Viewer Lite software.
the structural composition of SAK and SK-α, surrounding the active site cleft of μPm, are remarkably similar and the positions equivalent to His43 and Tyr44 of SAK are occupied by a lysine (Lys36) and a phenylalanine residue (Phe37) [7] in SK (Fig. 6) that may also establish a cation–π and a π–π interactions, respectively, at the interface of SK–Pg/Pm complex.

Taken together, the combined experimental and modeling studies presented herein, contain three major findings: (1) His43 and Tyr44 residues of SAK establish a direct contact with the partner via electrostatic cation–π and π–π interactions respectively at the interface of SAK–μPm bimolecular complex (2) these intermolecular interactions are crucial for the function of the SAK–Pm activator complex as topological positions of His43–Tyr44 pair of SAK can be complemented by residues capable of generating similar contacts at the interface without disturbing the local environment of the active site of the bimolecular complex, (3) although intermolecular contact among SAK and μPm was altered when His43 was replaced with another basic residue but local π–π interaction between Tyr44 and Trp215 was maintained in all functional mutants of SAK and may be critical for the specificity switch and/or Pg activation. Overall results, thus, provide a new insight into the molecular mechanism by which His43 and Tyr44 residues of SAK, residing at the interface of SAK–Pm bimolecular complex may generate crucial interactions with the partner Pm and modulate the functionality and/or specificity of SAK–Pm activator complex.

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Appendix A. Supplementary data


References


Fig. 6. Interface of SAK–μPm and SK–μPm complex. Interface of SK and μPm displaying interactions of Lys36 (B) and Phe37 (D) of SK with Glu606 (58) and Trp761 (215) of μPm, respectively, analogous to SAK His43 (A) and Tyr44 of SAK (C). The figure was prepared by Web Lab Viewer Lite software.


