Extended Mechanism and Rate-Limiting Step of the Plasminogen Activator Staphylokinase Revealed by Global Kinetic Analysis

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ABSTRACT: The plasminogen activator staphylokinase is a fibrin-specific thrombolytic biomolecule and an attractive target for the development of effective myocardial infarction and stroke therapy. To engineer the protein rationally, a detailed understanding of the biochemical mechanism and limiting steps is essential. Conventional fitting to equations derived based on simplifying approximations may be inaccurate for complex mechanisms like that of staphylokinase. We employed a modern numerical approach of global kinetic data analysis whereby steady-state kinetics and binding affinity datasets were analyzed in parallel. Our approach provided an extended, revised understanding of the staphylokinase mechanism without simplifying approximations and determined the value of turnover number k_{cat} of 117 s^{-1} that was 10,000-fold higher than that reported in the literature. The model further showed that the rate-limiting step of the catalytic cycle is binding of staphylokinase to plasmin molecules, which occurs via an induced-fit mechanism. The overall staphylokinase effectivity is further influenced by the formation of an inactive staphylokinase.plasminogen complex. Here, we describe a quick and simplified guide for obtaining reliable estimates of key parameters whose determination is critical to fully understand the staphylokinase catalytic functionality and define rational strategies for its engineering. Our study provides an interesting example of how global numerical analysis of kinetic data can be used to better understand the mechanism and limiting factors of complex biochemical processes.

INTRODUCTION

Cardiovascular diseases, mainly myocardial infarction and ischemic stroke, are the leading causes of morbidity and mortality worldwide responsible for nearly 18 million deaths every year.[1] Such alarming statistics are a clear motivation to develop efficient thrombolytic treatment strategies.[2,3] One of the current clinically approved approaches involves using plasminogen activators, i.e., biocatalytic therapeutic molecules with enzymatic activities that help dissolve fibrin clots by generating plasmin (Plm) on the clot surface.[4,5] However, the efficiency of plasminogen activators is still far from ideal and is often accompanied by side effects and bleeding complications. Thus, there is a need to improve current approaches or find new thrombolytic biocatalysts.[2,3] Staphylokinase (SAK) is an attractive potential thrombolytic agent owing to its fibrin specificity and low production costs.[6,7] Even though it is immunogenic, it has the potential to be clinically applied, especially in developing countries where cost-effectiveness is prioritized.[8−11]

SAK belongs to the group of so-called indirect plasminogen activators. This means it does not have any biocatalytic activity and cannot initiate fibrinolysis alone. Instead, it follows a complex molecular mechanism (Figure 1) that has been intensively studied.[2,12−18] SAK forms an equimolar complex with Plm, changing its substrate specificity toward plasminogen (Plg). Consequently, the SAK.Plm complex becomes a typical plasminogen activator capable of Plg-to-Plm catalytic conversion. Finally, generated Plm molecules cleave the fibrin network, resulting in dissolution of fibrin clots to fibrin degradation products. Because two Plm molecules can bind SAK, the terms Plm "partner" and Plm "product" are often used to distinguish between them.[19,20]

Even though there have been several attempts to improve the SAK thrombolytic biocatalytic potential by engineering, none of the generated proteins exhibited a significant increase of effectivity.[15,21−28] This was mainly due to the complex SAK molecular mechanism, which makes it difficult to properly identify the real limiting step, which is critical to be known for rational protein engineering.[29,30] The major challenge is that the generated Plm "product" can also act as a "partner" and form more of the SAK.Plm complex, continuously changing equilibria without reaching a real steady-state phase. Such a phenomenon makes it impossible to precisely characterize the kinetics of the protein using the conventional approach of initial rate analysis without major approximations. In contrast, global kinetic analysis using numerical integration allows fitting of raw kinetic data directly without any simplifying assumptions.[31] Thus, even complex multimolecular systems, such as the herein presented SAK case, can be solved with no approximations.

In this study, we systematically collected kinetic data for each step of the SAK molecular mechanism and analyzed them globally using a modern numerical analysis approach. This allowed us to describe an extended mechanism of SAK. Importantly, we identified that there is a substantial underestimation of kinetic parameters when they are calculated by the conventional approach. Based on the newly acquired mechanistic knowledge, we propose an upgraded guide to derive close-to-real estimates of key parameters of SAK kinetics while still using the simple conventional fitting to equations. This method of data processing is available to a wide community and requires no extensive expertise in advanced numerical methods. We believe that the simple upgraded protocol for more detailed SAK characterization will improve its rational engineering toward an optimized therapeutic biocatalyst with a high level of effectivity. In this example, we also demonstrate how the study of other complex biochemical systems could benefit from rigorous global

kinetic analysis compared to conventional analytical fitting with simplifying approximations.[31–33]

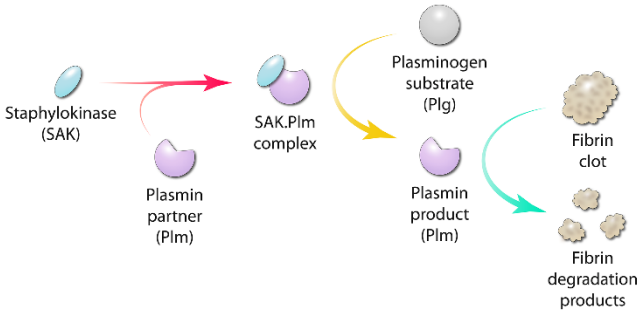

Figure 1. Generally accepted mechanism of staphylokinase. Staphylokinase (SAK) has no enzymatic activity, but it creates an equimolar 1:1 complex with a plasmin (Plm) "partner" to form a catalytically active compound (red arrow). SAK changes the substrate specificity of the Plm "partner" so that the SAK.Plm complex activates the plasminogen (Plg) "substrate" and converts it to additional Plm molecules (yellow arrow). Free generated Plm "products" finally cleave the fibrin network to release degradation products and dissolve fibrin clots (green arrow).

RESULTS

Biophysical characterization confirms protein quality. After heterologous gene overexpression in *E. coli*, SAK protein was successfully purified via its His-tag by metal affinity chromatography followed by size-exclusion chromatography, resulting in a purity higher than 95 % (Supplementary Figure S1a) and average yield of 30 mg per one liter of bacterial culture. Subsequent circular dichroism spectroscopy and thermostability measurements confirmed correct folding of the protein (Supplementary Figure S1b) as well as the corresponding melting temperature of 59.4 °C (Supplementary Figure S1c). Activity measurements using both the standardized fibrin plate assay (Supplementary Figure S1d,e) and indirect chromogenic substrate assay (Supplementary Figure S1f) confirmed that the protein was fully active with a plasminogen activation effectivity comparable to the clinically approved thrombolytic drug alteplase.

Kinetic analysis by conventional approach confirms consistency with previous results. Previous studies have analyzed steady-state kinetics with a premixed solution of SAK and Plm in a 1:1 ratio, assuming that 100 % molecules form the catalytic SAK.Plm complex (Figure 1). The concentration dependence of initial rates then gives a typical Michaelis-Menten relationship from which steady-state kinetic parameters can be determined. However, this approach does not consider that only a fraction of SAK molecules is complexed and, moreover, the equilibrium constantly changes during the reaction due to Plm accumulation.

We applied the same methodology of conventional initial rate analysis to our kinetic data (Figure 2a,b,d,e; Supplementary Table S1) and compared the obtained values with those from the literature.[19,24,25,28,34–39] Values similar to the reported range (Table 1) were obtained for both the turnover number k_{cat} (0.011 ± 0.001 s^{-1}) and Michaelis constant K_m (21 ± 1 μM). Such results confirmed the consistency of our kinetic data with those previously reported.

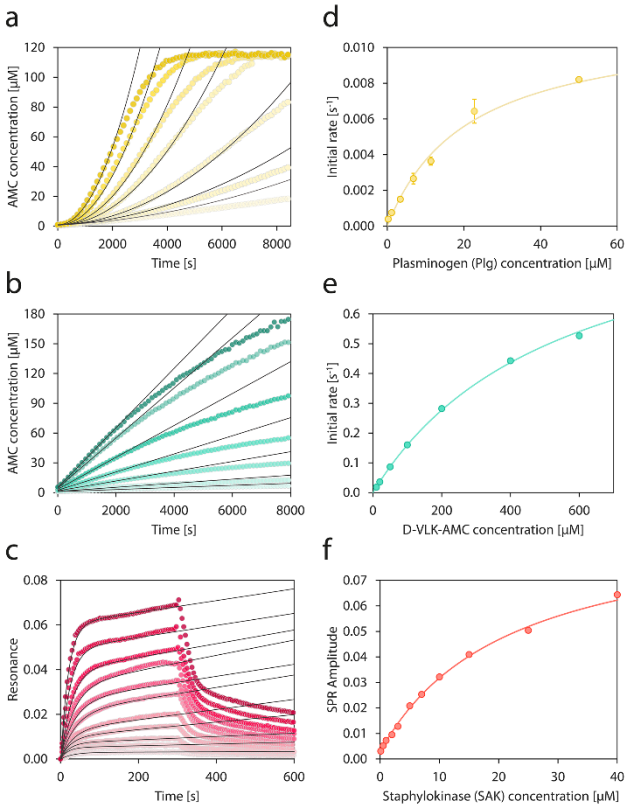

Figure 2. Conventional analytical fitting of staphylokinase kinetic data. The fitting of raw data (a–c) requires the generation of secondary plots (d–f), analyzing concentration dependencies of initial rates and amplitudes separately, losing connections in between them. Analytical fitting was performed for conversion of plasminogen Plg by staphylokinase SAK (yellow curves; a and d), conversion of D-VLK-AMC by plasmin Plm (green curves; b and e), and binding of SAK to immobilized Plm (red curves; c and f). Solid lines represent the best fit. All the experiments were performed in physiological phosphate-buffered saline (PBS) pH 7.4 at 25 °C in biological duplicates, each in technical triplicates.

The affinity-corrected analysis provides close-to-real values of kinetic parameters. The most significant inaccuracy using the conventional approach is caused by underestimation of the turnover number k_{cat}. This is due to the assumption that all SAK molecules form the catalytic SAK.Plm complex. In reality, a dynamic equilibrium between the free and complexed active forms always exists. If the SAK affinity toward the Plm "partner" is low, only a fraction of the molecules forms the active SAK.Plm complex. Therefore, the actual value of k_{cat} is proportionally higher.

Using surface plasmon resonance (SPR) affinity measurements (Figure 2c,f), we determined the equilibrium dissociation constant K_d of the SAK.Plm complex of 22 ± 2 μM. This was in agreement with previously reported SAK affinity experiments applying analogical experimental conditions.[19,40,41] Based on this affinity value, we calculated that only 0.03 % of SAK formed the SAK.Plm complex under the experimental steady-state conditions. Hence, the apparent value of k_{cat} was underestimated by an enormous factor of 3,400. By simply applying the known SAK.Plm dissociation constant K_d, the affinity-corrected value of k_{cat} can be calculated using Equation 1, where c_{SAK} and c_{Plm} correspond to

the concentrations of SAK and Plm applied during the steady-state experiment to pre-form the SAK.Plm complex.

$$k_{cat} = k_{cat,app} \cdot \frac{2 \cdot c_{SAK}}{K_d + c_{SAK} + c_{Plm} - \sqrt{(K_d + c_{SAK} + c_{Plm})^2 - 4 \cdot c_{SAK} \cdot c_{Plm}}} \quad (1)$$

This simple adjustment to the conventional data fitting provides results comparable to the advanced global numerical analysis described below (Table 1).

Table 1. Comparison of staphylokinase steady-state kinetic parameters.[a]

Kinetic parameter	Scientific literature[b]	Conventional analysis	Conventional affinity corrected	Global kinetic analysis
$k_{cat,SAK}$ [s^{-1}]	0.01–0.52	0.011 ± 0.01	39.5 ± 0.4	117 ± 9
$K_{m,SAK}$ [μM]	1–20	21 ± 1	21 ± 1	56 ± 4
$K_{p,SAK}$ [μM]	n.a.[c]	n.a.[c]	n.a.[c]	> 20
$K_{d,Plm}$ [μM]	n.a.[c]	n.a.[c]	22 ± 2	26 ± 2
$K_{d,Plg}$ [μM]	n.a.[c]	n.a.[c]	n.a.[c]	28 ± 13

[a]The values reported in the literature are compared with the parameters determined herein by different approaches of data analysis. The parameters were derived from kinetic experiments performed in physiological phosphate-buffered saline (PBS) pH 7.4 at 25 °C. [b]The range is based on previously published results.[19,24,25,28,34–39] [c]n.a. = not applicable.

Global kinetic analysis reveals an extended mechanism and rate-limiting step of SAK. As illustrated in Figure 2, the conventional analysis is strictly focused on the initial phases of the reaction and does not consider additional mechanistic information that can be inferred from later kinetic phases. This additional understanding can only be obtained by parallel fitting the full progress curves using numerical data analysis (Figure 3). An extended SAK kinetic mechanism was revealed by applying this approach (Figure 4). The extension provided an accurate quantitative description of the known kinetic steps but also revealed previously undescribed phases. In addition to the basic steady-state kinetic parameters k_{cat} and K_m (Table 2), the analysis of the entire time course of the kinetic data allowed estimation of the product inhibition constants K_p for both enzymatic reactions (Figure 4 – $K_{p,SAK}$ and $K_{p,Plm}$). Their values were comparable to their respective Michaelis constants K_m (Table 2), suggesting that the products could act as potent inhibitors after their accumulation during later stages of the conversion. The newly identified phases of the SAK mechanism included (i) a two-step induced-fit mechanism of SAK binding to Plm (Figure 4 – red pathway), and (ii) an inhibitory effect of Plg "partner" binding on Plg "substrate" activation by SAK (Figure 4 – blue pathway).

The proposed two-step mechanism of Plm binding was based on the SPR data analysis (Figure 3c). The SPR signal exhibited two distinct kinetic phases for both association and dissociation, which could not be explained by a simple one-step binding. Detailed analysis (Supplementary Note S1) revealed that interaction between SAK and Plm occurred via a two-step induced-fit mechanism comprising initial binding of SAK to Plm, followed by conformational change of Plm induced by bound SAK. This result is in accordance with previous mechanistic studies suggesting that binding of SAK induces changes in the substrate specificity of Plm.[18,19,42]

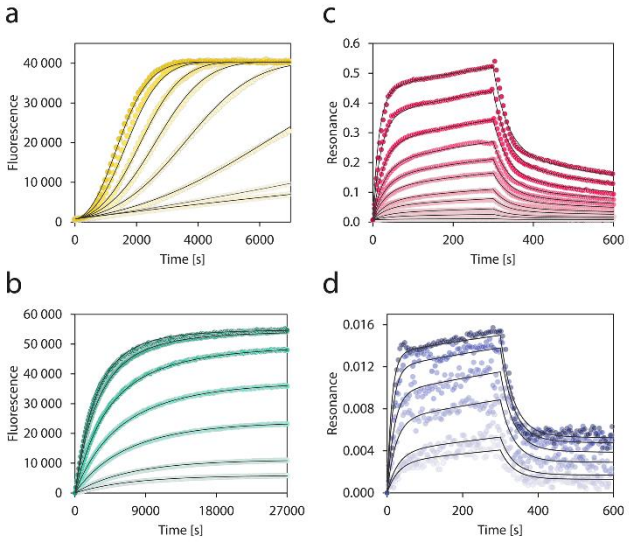

Figure 3. Global numerical analysis of staphylokinase kinetic data. Our approach is based on simultaneous global fitting of the full-progress curves obtained from four different experiments: (a) plasminogen activation and conversion to plasmin by staphylokinase (SAK), (b) D-VLK-AMC conversion to 7-amino-4-methylcoumarin (AMC) by plasmin, (c) binding of SAK to plasmin, and (d) binding of SAK to plasminogen. Solid lines represent the best fit. All the experiments were performed in physiological phosphate-buffered saline (PBS) pH 7.4 at 25 °C in biological duplicates, each in technical triplicates.

The important effect of Plg "partner" binding on the catalysis was derived from the steady-state kinetic curves of Plg conversion by SAK.Plm. When the data were analyzed globally, it was possible to obtain a reasonable fit of the initial phase of Plg activation, but there was an evident slowdown at later stages compared to the expected fit (Supplementary Figure S2a). This deviation could potentially be explained by a product inhibition effect only (Supplementary Figure S3), but the obtained $K_{p,SAK}$ value of 0.07 ± 0.01 μM was unrealistically low (Supplementary Table S2); a value 500-fold lower than $K_{m,SAK}$ was judged to be unlikely. Thus, this mechanism was assumed to be biochemically irrelevant despite providing a relatively good fit. Another possible cause of the fit deviation could be attributed to the inactive SAK.Plg complex formation. SAK has been reported to bind not only Plm but also Plg molecules as "partners" to form an equimolar complex but the SAK.Plg complex is catalytically inactive and cannot serve as a plasminogen activator.[6,14,40] We tested this phenomenon using global kinetic analysis by including an additional dataset obtained from an SPR affinity binding experiment between SAK and Plg (Figure 3d). Plg binding exhibited an induced-fit mechanism similar to that of Plm (Supplementary Note S2). Strikingly, when formation of the SAK.Plg complex was included in the complex kinetic model, the goodness of the global fit was significantly improved and the model fully matched the experimental data (Figure 3 and Supplementary Figure S4). The value of $k_{cat,SAK}$ was further increased when Plg binding was considered, resulting in an overall underestimation factor of 11,700 when compared to the conventional analysis. A rigorous

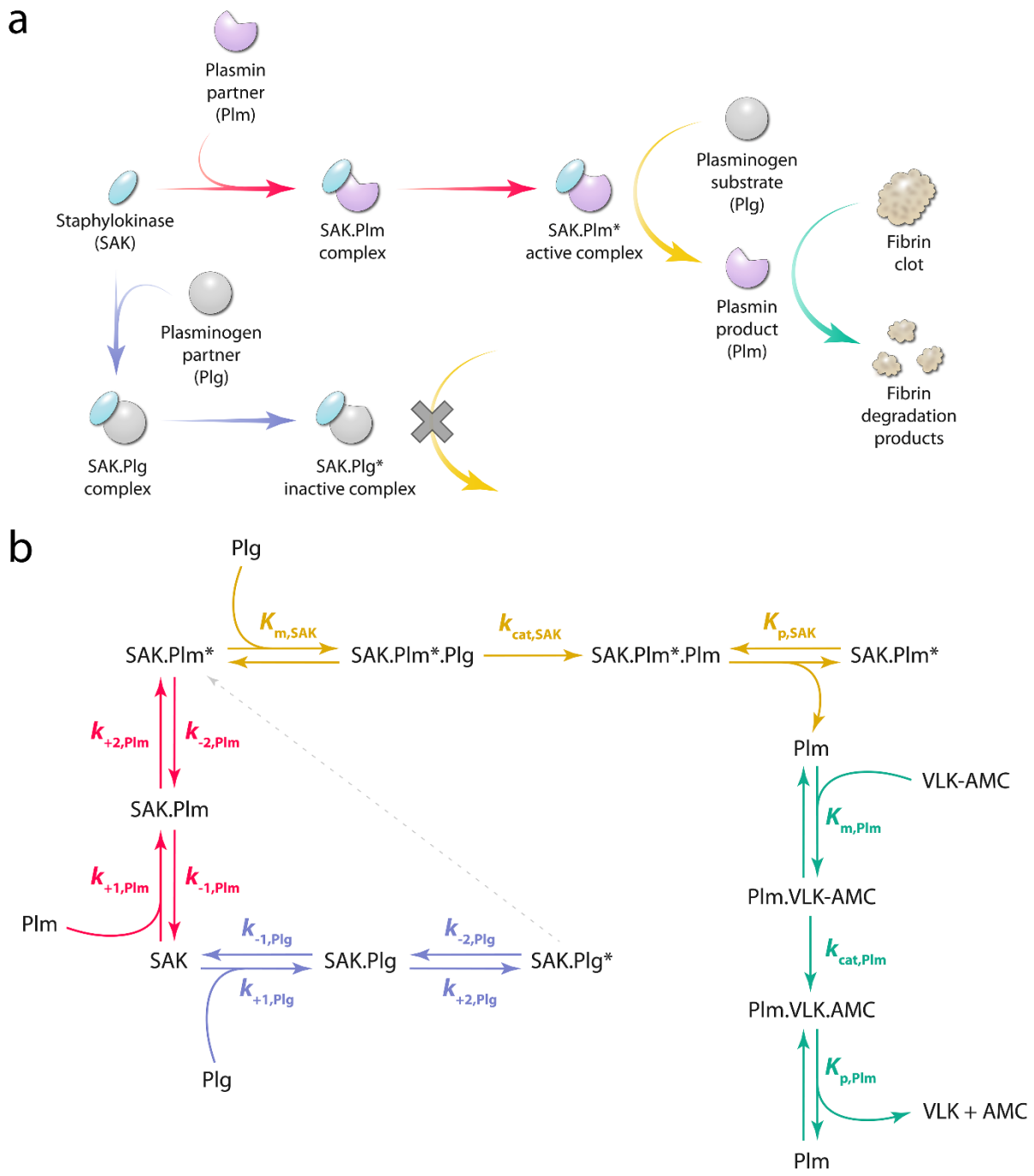

Figure 4. Extended mechanism of staphylokinase (SAK). (a) Simplified graphical schematic of the mechanism, and (b) detailed kinetic pathway with all analyzed steps and parameters are provided for clarity. Compared to the general mechanism (Figure 1), the extended mechanism newly includes binding of staphylokinase (SAK) to plasminogen (Plg) to form an inactive complex, preventing plasminogen-to-plasmin (Plg-to-Plm) conversion and having a significant effect on the overall effectivity. The mechanism further includes a newly identified two-step induced-fit binding mechanism for both Plm and Plg binding by SAK. SAK.Plg* to SAK.Plm* conversion by Plm, reported previously[14] and marked with a grey dotted arrow, was not included in the kinetic model because its rate was very slow and not significantly detectable during the experimental time window. Forward and reverse rate constants of the *i*-th step are denoted with the symbols k_{+i} and k_{-i}, respectively. The steady-state kinetic parameters K_m, k_{cat} and K_p correspond to the Michaelis constant, turnover number and product inhibition constant, respectively.

confidence contour analysis (details in Materials and Methods) showed that all the obtained kinetic parameters were well constrained by the data (Table 2, Supplementary Table S3, Supplementary Figure S5) and confirmed the statistical significance of including the event of Plg binding by SAK in the extended SAK mechanism represented in Figure 4.

The simplified guide facilitates quantification of key processes influencing the overall SAK effectivity. To account for the complex biochemical mechanism and additional effects that influence the overall SAK fibrinolytic effectivity, the herein presented global numerical data analysis is the preferred option. However, the advanced kinetic

methodology requires extensive prior knowledge. Thus, we propose a user-friendly upgrade of the simple conventional data fitting. The upgraded guide (Table 3) allows for easy and reliable quantification of four key processes that influence the overall SAK fibrinolytic effectivity by their respective parameters: (i) plasmin binding, (ii) plasminogen binding, (iii) plasmin selectivity, and (iv) catalytic efficiency. The effect of the key processes is schematically illustrated in Supplementary Figure S6.

Table 2. Kinetic constants describing the extended mechanism of staphylokinase.[a]

Parameter	Value	Standard error (S.E.)	Confidence intervals
STAPHYLOKINASE.PLASMIN STEADY-STATE KINETICS			
$K_{m,SAK}$ [μM]	56	4	48–65
$k_{cat,SAK}$ [s^{-1}]	117	9	107–134
$K_{p,SAK}$ [μM]	> 20	n.a.[c]	> 20
PLASMIN STEADY-STATE KINETICS			
$K_{m,Plm}$ [μM]	400	30	370–450
$k_{cat,Plm}$ [s^{-1}]	0.96	0.06	0.90–1.03
$K_{p,Plm}$ [μM]	310	30	250–490
PLASMIN BINDING KINETICS			
$k_{+1,Plm}$ [$\mu M^{-1}.s^{-1}$]	0.000979	0.000006	0.000919–0.001025
$k_{-1,Plm}$ [s^{-1}]	0.0252	0.0001	0.0229–0.0257
$k_{+2,Plm}$ [s^{-1}]	0.00214	0.00008	0.00184–0.00219
$k_{-2,Plm}$ [s^{-1}]	0.0013	0.0004	0.0010–0.0016
PLASMINOGEN BINDING KINETICS			
$k_{+1,Plm}$ [$\mu M^{-1}.s^{-1}$]	0.0011	0.0005	0.0007–0.0014
$k_{-1,Plm}$ [s^{-1}]	0.031	0.002	0.022–0.043
$k_{+2,Plm}$ [s^{-1}]	0.0014	0.0005	0.0007–0.0023
$k_{-2,Plm}$ [s^{-1}]	< 0.00016	n.a.[c]	0–0.00016

[a]The parameters and their statistics were obtained by simultaneous global fitting of full-progress curves using numerical integration and by confidence contour analysis with a χ^2 threshold of 0.95. The parameters were derived from kinetic experiments performed in physiological phosphate-buffered saline (PBS) pH 7.4 at 25 °C. [b]n.a. = not applicable.

DISCUSSION

SAK is a promising thrombolytic drug with many advantageous properties, including high fibrin specificity and low production cost.[6,7] However, its administration is connected with an immune response and its biocatalytic effectivity is not significantly better than current thrombolytic drugs. Thus, there is a need for its improvement.[2,3] The success of rational protein engineering relies on proper understanding and characterization of the studied system and especially identification of its main limitation.[29,30] In this study, we addressed the challenge that improper SAK kinetic characterization can cause significant underestimation of the catalytic activity and misleading assumptions that the low activity is the limiting factor of the overall fibrinolytic effectivity. By applying rigorous global kinetic analysis, we showed that the activity of the enzymatic SAK.Plm complex is high, but the inefficient formation of this complex is the main limiting factor.

We validated our kinetic data by first applying a conventional data analysis approach and obtained comparable values of underestimated steady-state kinetic parameters as those reported previously.[19,24,25,28,34–39] It should be noted that some studies have reported values of k_{cat} approximately 10-fold higher and out of the range of other consistent results.[22,23,26,27,43] However, those data were collected at elevated temperatures where exponentially higher rates are expected. Therefore, those results are not in conflict with our observations. Conventional analytical fitting of SPR curves of Plm and Plg binding yielded comparable values of dissociation constants K_d as reported previously for the same experimental conditions,[19,40,41] confirming the validity of the data used in this study. Similarly, some studies have reported significantly lower values of K_d[16,23,26,44–46] but these experiments were performed under different conditions, making comparison irrelevant. Most of these studies were based on immobilization of SAK instead of Plm via its amine groups, including the N-terminus. Such arrangement is not optimal since the crystal structure of the SAK.μPlm complex[18] and mutagenesis functional studies[17,40,47] have shown that the N-terminal part of SAK must be accessible for its interaction with Plm.

Although many prior reports have focused on the kinetic characterization of SAK and its modified variants,[19,22–28,34–39,43] we were unable to find a single study reporting the analysis of SAK kinetics by a combination of steady-state and binding affinity datasets. Some studies conducted both types of experiments[19,23,26,40] but did not combine the data to obtain global estimates of kinetic parameters. We employed global numerical analysis of kinetic data to consider not only an initial equilibrium fraction of the active SAK.Plm complex but also assume dynamic changes in this equilibrium due to the ongoing accumulation of Plm during the steady-state kinetic experiment.

Our results showed a significant 10,000-fold underestimation of the turnover number (k_{cat}) value when the conventional analysis approach was applied. A quick correction to obtain a more realistic value of k_{cat} can be made by applying a known equilibrium constant of the SAK.Plm complex to the simple equation provided in the Results section. Furthermore, the global data analysis revealed a detailed extended mechanism of SAK, including (i) an induced-fit mechanism for both Plm and Plg binding and (ii) an inhibitory effect of Plg binding on the overall thrombolytic effectivity of SAK. These processes should be considered when characterizing and comparing the effectivities of SAK variants. Conversion of the inactive SAK.Plg complex into the active SAK.Plm complex, reported previously,[14] was not assumed in our mechanism because it was much slower than other steps of the SAK mechanism and did not alter the data fit. Our detailed analysis with additional pathway steps identified that future modifications of SAK should be focused on improving the Plm selectivity, i.e., either increasing Plm binding or decreasing Plg binding.

While global numerical data analysis without simplifying approximations will always be the preferred option, it is not always easy to use this methodology without extensive prior experience. As an alternative, we propose a simple

Table 3. Upgraded guide for reliable quantification of SAK kinetics.

Key parameter[a]	Determination/calculation	Effect on the overall effectivity
Plasmin binding	Determine $K_{a,Plm}$ (= $1/K_{d,Plm}$) of the active SAK.Plm complex (e.g., by an SPR experiment) to obtain information about SAK affinity toward Plm.	A higher affinity with higher $K_{a,Plm}$ leads to an increased fraction of active SAK.Plm, and thus higher overall effectivity.
Catalytic efficiency	Calculate the ratio $k_{cat,SAK}/K_{m,SAK}$ using the conventional approach and the affinity-corrected value of k_{cat} based on known $K_{d,Plm}$ and Equation 1.	A higher value of the correctly estimated ratio leads to increased catalytic conversion capability of the pure SAK.Plm complex, and thus higher overall effectivity.
Plasminogen binding	Determine $K_{a,Plg}$ (= $1/K_{d,Plg}$) value of the inactive SAK.Plg complex in an analogical way as for plasmin binding.	A higher affinity with higher $K_{a,Plg}$ leads to an increased fraction of inactive SAK.Plg, shifting the equilibrium toward less SAK.Plm, and thus lower overall effectivity.
Plasmin selectivity	Calculate the ratio of the above-determined plasmin(ogen) binding constants $K_{a,Plm}/K_{a,Plg}$ to determine the preference for Plm over Plg.	A higher value of the ratio leads to increased SAK preference toward Plm rather than Plg and formation of more SAK.Plm, and thus higher overall effectivity.

[a]Each parameter quantifying the key process can be estimated in a simplified manner as described in the table without the need for expertise in advanced methods of global kinetic data analysis.

upgrade of the widely used conventional data analysis method to obtain reliable estimates of SAK kinetic parameters. The upgraded guide requires only a few additional steps to determine four key parameters that are critical to fully understand SAK functionality and to correctly interpret the effect of modifications during its engineering.

SAK is only one of many examples of complex multimolecular biochemical systems whose analysis by conventional data fitting might lead to oversimplified results.[31–33] We believe that our study provides an example of how global numerical analysis of kinetic data can be used to better understand the mechanism and limiting factors of complex biochemical processes.

CONCLUSIONS

An extended molecular mechanism of SAK was identified by applying a modern global numerical analysis of kinetic data with three major findings: (i) both Plm and Plg "partner" molecules bind SAK via a two-step induced-fit mechanism; (ii) the steady-state kinetic parameters are significantly underestimated if SAK.Plm and SAK.Plg complex formation is not considered; and (iii) the major limitation of SAK biochemistry is the inefficient formation of the catalytically active SAK.Plm complex. Reliable estimates of key parameters can be obtained using the upgraded conventional analysis. The proposed simplified guide may help to easily track and correctly interpret contributions of individual steps to the overall SAK effectivity when designing improved molecules rationally. The results show that future protein engineering of SAK should target improved selectivity of Plm binding and efficiency of SAK.Plm complex formation.

ASSOCIATED CONTENT

Supporting Information

Materials and Methods; Supporting Figures and Tables: staphylokinase biophysical characterization and protein quality check, results of alternative tested staphylokinase kinetic mechanisms, additional values of kinetic constants and fitted parameters, results of statistical and constraint analysis of kinetic constants and additional fitted parameters, schematic of the effect of key identified processes on staphylokinase thrombolytic effectivity; Supporting Notes: details of rigorous kinetic analysis of binding mechanisms. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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