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*Published in:*  
Thrombosis and Haemostasis

*DOI:*  
[10.1160/TH11-03-0204](https://doi.org/10.1160/TH11-03-0204)

2011

[Link to publication](#)

*Citation for published version (APA):*  
Seron, M. V., Plug, T., Marquart, J. A., Marx, P. F., Herwald, H., de Groot, P. G., & Meijers, J. C. M. (2011). Binding characteristics of thrombin-activatable fibrinolysis inhibitor to streptococcal surface collagen-like proteins A and B. *Thrombosis and Haemostasis*, 106(4), 609-616. <https://doi.org/10.1160/TH11-03-0204>

*Total number of authors:*  
7

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## **Binding characteristics of Thrombin-Activatable Fibrinolysis Inhibitor to streptococcal surface collagen-like proteins A and B**

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Word count of the abstract: 200 words

Word count of the text: 2527 words

Running title: binding site of TAFI for SclA and SclB

Keywords: TAFI, collagen-like proteins, protein binding, *Streptococcus pyogenes*

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Financial support: This work was supported by the Netherlands Organization for Scientific Research (grant number ZonMW 91207002, to J. C. M. Meijers and P. G. de Groot).

## Summary

*Streptococcus pyogenes* is the causative agent in a wide range of diseases in humans. Thrombin-Activatable Fibrinolysis inhibitor (TAFI) binds to collagen-like proteins SclA and SclB at the surface of *S. pyogenes*. Activation of TAFI at this surface redirects inflammation from a transient to chronic state by modulation of the kallikrein/kinin system. We investigated TAFI binding characteristics to SclA/SclB. 34 overlapping TAFI peptides of ~20 amino acids were generated. Two of these peptides (P18: residues G205-S221, and P19: R214-D232) specifically bound to SclA/SclB with high affinity, and competed in a dose-dependent manner with TAFI binding to SclA/SclB. In another series of experiments, the binding properties of activated TAFI (TAFIa) to SclA/SclB were studied with a quadruple TAFI mutant (TAFI-I1YQ) that after activation is a 70-fold more stable enzyme than wild-type TAFIa. TAFI and TAFI-I1YQ bound to the bacterial proteins with similar affinities. The rate of dissociation was different between the proenzyme (both TAFI and TAFI-I1YQ) and the stable enzyme TAFIa-I1YQ. TAFIa-I1YQ bound to SclA/SclB, but dissociated faster than TAFI-I1YQ. In conclusion, the bacterial proteins SclA and SclB bind to a TAFI fragment encompassing residues R214-S221. Binding of TAFI to the bacteria may allow activation of TAFI, whereafter the enzyme easily dissociates.

## **Introduction**

*Streptococcus pyogenes* is an important human Gram-positive pathogen that mainly causes throat and skin infections, such as pharyngitis, impetigo and cellulitis. Although the majority of streptococcal infections are superficial, some cases may progress into invasive and life threatening diseases with an extremely rapid progression, such as streptococcal septic shock and necrotizing fasciitis (1). In order to infect the human host, *S. pyogenes* expresses a number of virulence factors that mediate adhesion to host tissues, enable dissemination of bacteria, and/or that modulate the immune response (2-4).

The streptococcal collagen-like surface proteins A and B (SclA and SclB), also known as Scl1 and Scl2, are two related proteins with a similar structure motif, including a C-terminal region that is attached to the cell wall-membrane (CWM) via a LPATG anchor. This is followed by a central region composed of a collagen-like domain (CL) with long segments of repeated GXY amino acids, a sequence considered a defining feature of collagens. Next to the CL domain, an amino terminal variable domain (V) is present located. In addition, SclA, but not SclB, contains a linker (L) region between the CWM and the CL domain. Both proteins are organized into a “lollipop-like” structure, where the CL domain forms a triple helical-stalk and the V domain folds into a globular head. The genes encoding SclA and SclB are located at different sites of the bacterial chromosome and are differently regulated. While SclA is up-regulated by the Mga regulon, the transcription of SclB is down-regulated by the same protein (5-10).

Recently, we reported the binding of Thrombin-Activatable Fibrinolysis Inhibitor (TAFI) to the surface of a group A streptococci (M41 serotype) and its subsequent activation at

the bacterial surface via plasmin and thrombin-thrombomodulin (11). Furthermore, activation of TAFI on the surface of *S. pyogenes* evoked inflammatory reactions by modulating the kallikrein/kinin systems (12). Identifying the molecular determinants of Scls-TAFI interaction may well offer possibilities for prevention of diseases caused by inflammatory reactions induced by *S. pyogenes*.

TAFI is a zinc-dependent procarboxypeptidase that is synthesized in the liver (13). It is thereafter released into the bloodstream, where it circulates at a concentration in the range of 70-275 nM (14). Cleavage of the TAFI zymogen by enzymes such as thrombin or plasmin results in the formation of activated TAFI (TAFIa), which attenuates fibrinolysis. TAFIa prevents accelerated plasmin formation by removing C-terminal lysine residues from partially degraded fibrin that augment the efficacy of plasminogen activation. Besides a function in fibrinolysis, TAFIa also plays a role in inflammatory processes by hydrolysis of bradykinin, osteopontin and the anaphylotoxins C3a and C5a (15).

The present study was undertaken to 1) identify the TAFI binding site involved in the interaction with the SclA, SclB expressed by *S. pyogenes* and 2) establish the binding properties of TAFIa to SclA and SclB. Using synthetic TAFI peptides, we demonstrated that TAFI binds to both Scl proteins via residues G205 to D232. In addition, we determined that TAFIa is able to bind SclA and SclB but that it rapidly dissociates from the bacterial proteins.

## **Materials and methods**

### **Proteins**

34 overlapping TAFI peptides were synthesized by Peptide 2.0 Inc. (Chantilly, VA). Surface Plasmon Resonance (SPR) experiments with all 34 TAFI peptides were performed with crude peptides. In SPR experiments with peptide 18 (P18) and peptide 19 (P19) and in solid-phase binding assays, peptides were 95 % pure as determined by high-pressure liquid chromatography analysis, and their identity was confirmed by mass spectrometry. Recombinant SclA and SclB from the *S. pyogenes* AP41 strain were produced and purified as described elsewhere (11). TAFI was purified as previously described (16). The rTAFI-IYYQ mutant, which harbours the Thr325→Ile, Thr329→Ile, His333→Tyr and His335→Gln mutations, and the active form of the mutant (rTAFIa-IYYQ) were generated and expressed as described previously (17,18). A polyclonal antibody specific for TAFI was obtained in rabbits as described elsewhere (19). HRP (horseradish peroxidase)-labeled swine anti-rabbit immunoglobulin G (IgG) was purchased from Dako.

### **SPR (Surface Plasmon Resonance) binding assays**

All SPR measurements were performed at 25°C using a BIAcore 2000 biosensor System (GE Healthcare). Recombinant AP41 SclA and SclB were immobilized to a CM5 sensor chip using the amine coupling kit according to the supplier's recommendation (GE Healthcare). SclA and SclB were applied in 10 mM NaAc (pH 3.1). Immobilization of SclA on the chip resulted in an increase of the resonance signal by ~400 RU (resonance

units) and with SclB of ~440 RU. Binding studies were done in 10 mM Hepes, 150 mM NaCl, 0.005% Sulfactant P20 (pH 7.4), at a flow rate of 30  $\mu$ l/min. Different concentrations of rTAFI-IIYQ, rTAFIa-IIYQ (0-200 nM) or 5  $\mu$ M TAFI peptides were injected for 2 or 3 minutes. The dissociation was followed for a period of 1 or 10 minutes and the ligand surface was regenerated with a 30-s injection of 1/3 or 1/5 ionic buffer (92 mM KSCN, 366 mM MgCl<sub>2</sub>, 184 mM urea, 366 mM guanidine) followed by equilibration with flow buffer at the end of each binding cycle. The association ( $k_a$ ) and dissociation ( $k_d$ ) rate constants were determined using the BIAEvaluation Software (Version 4.1; Biacore). The equilibrium dissociation constants ( $K_D$ ) were calculated from the ratio of the measured kinetic rate constants  $k_d/k_a$ .

### **Solid-phase binding assays**

Ninety-six-well NUNC MaxiSorp™ plates (Nalge Nunc International) were coated overnight with 100  $\mu$ l of recombinant SclA or SclB (46 nM) in NaHCO<sub>3</sub> (pH 9.6) at 4°C. Following three washes with Tris-buffered saline supplemented with Tween [50 mM Tris, 150 mM NaCl, 0.1% Tween-20 (pH 7.4)], plates were blocked with 150  $\mu$ l of blocking solution [1.5 % w/v bovine serum albumin (BSA) in Tris-buffered saline] for 1 hour at 37°C. Wells were washed as described above, and 100 nM TAFI in the presence or absence of P18, P19 or P29 (0 to 150  $\mu$ M) diluted in blocking buffer was applied to the wells. The assay mixture was incubated for 1 hour at room temperature. Following the competition step, microtiter plates were washed three times, and 100  $\mu$ l of anti-TAFI antibody was incubated in blocking buffer for 1 hour at room temperature. Next, the plates were washed three times and incubated with HRP-labeled swine anti-rabbit IgG

diluted 1:5000 with blocking buffer. After four washes, the reactions were developed by the addition of 100  $\mu\text{l}$  *o*-phenylenediamine (Sigma-Aldrich) substrate [8 mM  $\text{Na}_2\text{HPO}_4$  (pH 5.0) 2.2 mM *o*-phenylenediamine, 3%  $\text{H}_2\text{O}_2$ ]. Colour development was stopped by the addition of 50  $\mu\text{l}$  of 1M  $\text{H}_2\text{SO}_4$ , and the plates were read at 490 nm using a Thermomax microplate reader (Molecular Devices Corp.). Data were corrected for binding to empty microtiter wells.

## Results

### Characterization of TAFI binding to SclA and SclB

We have previously shown that TAFI binds to SclA and SclB (11). In the present study, we examined the TAFI region involved in SclA and SclB binding. To this end, 34 overlapping TAFI peptides of approximately 20 amino acids comprising the complete TAFI molecule were generated (► Table 1). SPR measurements were carried out to determine binding between TAFI peptides and the Scls. The recombinant bacterial proteins, and BSA as a negative control, were immobilized on the surface of the biosensor chip in three separate flow cells. As shown in ► Figure 1A, only two synthetic peptides (Gly<sup>205</sup>-Ser<sup>221</sup>, P18, and Arg<sup>214</sup>-Asp<sup>232</sup>, P19) were able to bind immobilized Scls. The other synthetic peptides did not show appreciable binding to the bacterial proteins. In addition, P18 and P19 bind to SclA and SclB in a dose-dependent fashion (► Fig. 1B). Next, we evaluated the affinity of P18 and P19 towards the Scls. For SclA we calculated a  $K_D$  of 912 nM (P18) and 540 nM (P19) and for SclB we calculated a  $K_D$  of 971 nM (P18) and 520 nM (P19). These results suggest that the TAFI sequence Gly<sup>205</sup>-Asp<sup>232</sup> contains important residues involved in the interactions with SclA and SclB.

To further analyze the relative affinity of TAFI for SclA and SclB compared to the synthetic peptides, we tested P18 and P19 for their ability to interfere with the interactions between TAFI and the immobilized Scls. TAFI binding to SclA and SclB was competed with various concentrations of P18 or P19. P18 and P19 displayed a dose-

dependent inhibition of TAFI binding to immobilized SclA (►Fig. 2A) and SclB (►Fig. 2B).

These data imply that P18 and P19 have a similar binding region as TAFI for binding to SclA and SclB.

### **Binding analysis of TAFIa to SclA and SclB**

Next we used SPR to investigate the binding characteristics of TAFIa to SclA and SclB. Because TAFIa is a labile enzyme that is inactivated by a conformational change in a temperature-dependent way (16) we used a TAFI quadruple mutant, rTAFI-IIYQ, which has a 70-fold more stable active form (rTAFIa-IIYQ) than the wild type.

Both SclA and SclB were immobilized onto the sensor chip surface, and binding curves were recorded for both rTAFI-IIYQ and rTAFIa-IIYQ variants at 6 different concentrations between 0 and 200 nM. Representative curves for rTAFI-IIYQ and rTAFIa-IIYQ binding to SclA and B are shown in ►Figure 3.

Fitting the SPR profiles to interaction models yielded kinetic and affinity information for the different TAFI-Scls interactions. The responses for rTAFI-IIYQ were well described by a 1:1 interaction model, however, the responses for rTAFIa-IIYQ were not. Instead, the profiles for the active form could be better described by a heterogeneous surface model, which assumed two or more different classes of binding sites. We found similar association rates of rTAFI-IIYQ and rTAFIa-IIYQ towards the bacterial proteins. In

contrast, the dissociation curves for rTAFI-IIYQ and rTAFIa-IIYQ were different; rTAFIa-IIYQ presented a faster dissociation phase towards both SclA and SclB.

Based on the best data fit, rTAFI-IIYQ bound the Scls with affinities in the nanomolar range (rTAFI-IIYQ-SclA:  $K_D = 3.5$  nM. rTAFI-IIYQ-SclB:  $K_D = 4.0$  nM)..These values were lower than previously established for plasma TAFI (11). We also investigated binding of plasma TAFI to SclA and B and found  $K_D$ 's of 5.4-6.6 nM respectively (data not shown). This indicated that rTAFI-IIYQ and plasma TAFI had similar binding kinetics. However, it is unknown why the values for plasma TAFI were lower than in our earlier study.

Compared to rTAFI-IIYQ, a lower affinity was measured for rTAFIa-IIYQ, suggesting that the conformation of the Scl-recognition domain had been slightly changed upon TAFI activation. Kinetic and affinity values obtained for the bacterial proteins with both TAFI variants are shown in ► Table 2. Taken together, the results imply that rTAFIa-IIYQ dissociates faster from SclA and SclB compared to rTAFI-IIYQ.

## Discussion

In the present study we investigated the binding interactions of TAFI and activated TAFI to streptococcal collagen-like surface protein A and B. By using TAFI peptides we identified the binding region involved in the interaction with SclA and B within amino acids 205 to 232 (partially overlapping peptide 18 and 19) of TAFI. P18 bound to the same extent to SclA ( $K_D = 912$  nM) and SclB ( $K_D = 971$  nM). Affinity of P19 to SclA and SclB was similar ( $K_D$  540 nM and  $K_D$  520 nM, respectively). The region of Gly205 to Asp232 is located distally from the TAFI catalytic site (►Fig.4) within helix  $\alpha$ -4 and is surface exposed and does not interfere with the region known to influence TAFIa stability (Arg302, Arg320, Arg330, and Thr/Ile325) (20-22), neither with the residues involved in substrate binding (Gly336, Tyr341, and Glu363) (23).

Although different binding partners have been identified for TAFI, such as plasminogen and fibrinogen (24), the region described here as involved in SclA and SclB binding has not been shown to be overlapping with another TAFI-protein interaction.

We demonstrated that P18 and P19 have the potential to compete with TAFI for binding to the streptococcal proteins. TAFI binding to SclA was inhibited 55% and 76% by P18 and P19 respectively. In contrast, TAFI binding to SclB was inhibited 44% and 26% by P18 and P19 respectively. The partial contribution of P18 and P19 to inhibit TAFI-SclB interaction may be attributed to the fact that SclA and SclB contain different sizes of the variable region. In addition, it is likely that the recognition between the bacterial proteins

and TAFI is dependent on the three-dimensional conformation of the protein, which is not optimally represented by the linear peptides.

Recent findings that the physiological activity of TAFIa is not inhibited by SclA or SclB (11), and the ability of activated TAFI on the surface of *S. pyogenes* to evoke inflammatory reactions by modulating the kallikrein/kinin system (12), prompted us to investigate the TAFIa binding properties to SclA and SclB. Here we provide evidence that activated TAFI binds to SclA and SclB and, in contrast to the TAFI proenzyme, is rapidly dissociated from the bacterial proteins. It is tempting to speculate that this constitutes a mechanism whereby the bacteria attract TAFI to their surface and localize it there. After activation however, the activated TAFI can dissociate and act on substrates elsewhere. These findings suggested that the activation peptide may play a role in the TAFI binding to SclA and SclB. However, we found that the TAFI binding to SclA and SclB was mediated by a region within the TAFI catalytic domain. The crystal structure of TAFI showed that TAFIa stability is directly related to the dynamics of a 55-residue segment (residues 296-350) of the active site (17, 25). Release of the activation peptide increases dynamic flap mobility and in time this leads to conformational changes that expose the cleavage site at Arg302. It cannot be ruled out that binding of TAFIa to SclA and SclB is influenced by the conformational change in TAFIa.

In summary, this study demonstrates that binding of TAFI to SclA and SclB can be inhibited by TAFI peptides. We have identified the region on TAFI, encompassing residues 205-232, that bind to the bacterial proteins. In addition, it was shown that TAFIa

rapidly dissociates from SclA and SclB. A better understanding of the molecular mechanisms behind host/bacteria interactions has the potential to discover important targets in the human host and ultimately new therapeutic approaches for treatment of severe infectious diseases.

## **Abbreviations**

TAFI, thrombin-activatable fibrinolysis inhibitor; TAFIa, activated-thrombin activatable fibrinolysis inhibitor; SclA and SclB, streptococcal surface collagen-like proteins A and B; SPR, surface plasmon resonance.

## **Acknowledgements**

None

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## **Legends to figures and tables**

**Figure 1: Binding of TAFI peptides to immobilized SclA and SclB.** A) 34 overlapping TAFI peptides were used to assess the binding to immobilized SclA and SclB by SPR-based assay. The bars represent the responses in resonance units (RU) corrected for non-specific binding to BSA. B) SPR analysis of dose dependent binding of P18 and P19 to immobilized SclA and SclB.

**Figure 2: Competitive binding of TAFI to immobilized recombinant SclA and SclB with TAFI peptides P18 and P19.** Binding of TAFI to SclA (A) or SclB (B) onto 96-well plate was measured in the presence of various concentrations of P18, P19 and P29. The assays were repeated three times, and results of a representative experiment with duplicate samples are shown.

**Figure 3: Binding of TAFI-IIYQ and TAFIa-IIYQ to immobilized SclA and SclB.** SPR analysis of binding of TAFI-IIYQ to SclA (A) and SclB (B) and binding of TAFIa-IIYQ to SclA (C) and SclB (D). SclA and SclB (~400 RU for SclA, 440 RU for SclB) were immobilized to a CM5 sensor chip and increasing concentrations (0, 5, 20, 50, 100, and 200 nM) of TAFI-IIYQ or TAFIa-IIYQ were applied to the chip.

**Figure 4: P18 and P19 shown in the overall structure of TAFI.** Ribbon drawing of TAFI with the activation peptide shown in blue, the catalytic domain in green, and residues 205-232 representing the partially overlapping peptides 18 and 19 in red.

**Table 1: Overlapping synthetic peptides of TAFI.**

The one-letter code for amino acid residues is used for the sequence. The positions of the amino acids in TAFI protein are shown.

**Table 2: Kinetic and affinity parameters for interactions between TAFI-IIYQ,**

**TAFIa-IIYQ and SclA, SclB.** The parameters were determined by surface plasmon resonance measurements using immobilized SclA and SclB as the ligand, and TAFI-IIYQ or TAFIa-IIYQ as the analyte.  $k_a$ , association rate constant;  $k_d$ , dissociation constant;  $K_D$ , equilibrium dissociation constant. Values are means  $\pm$  S.D.

Figure 1

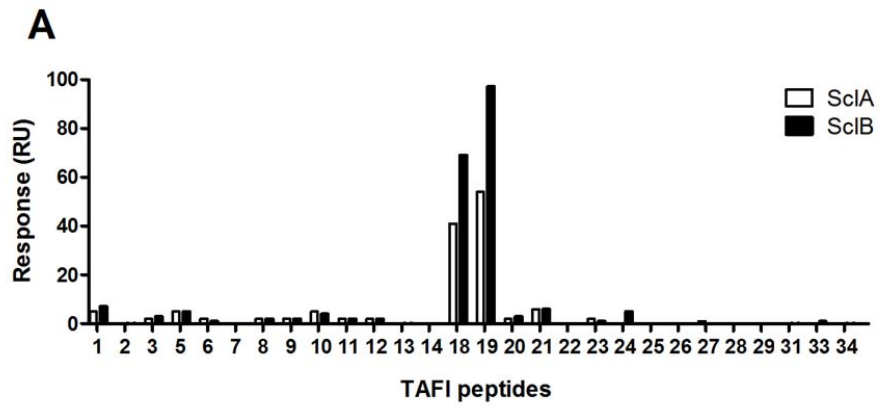


Figure 2

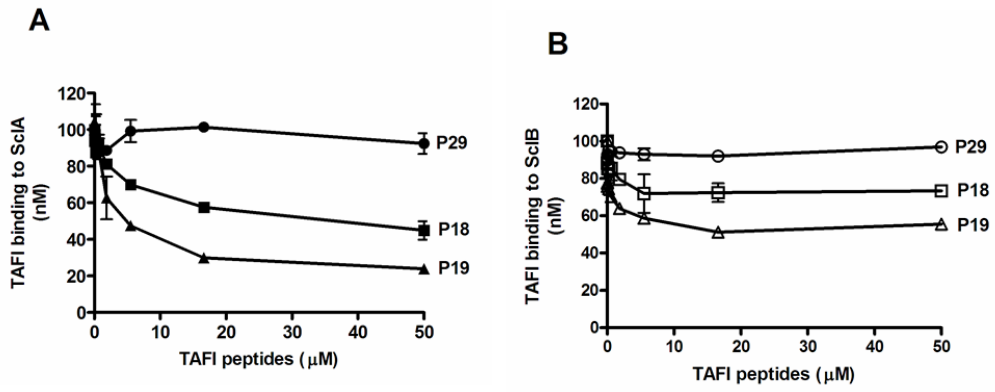


Figure 3

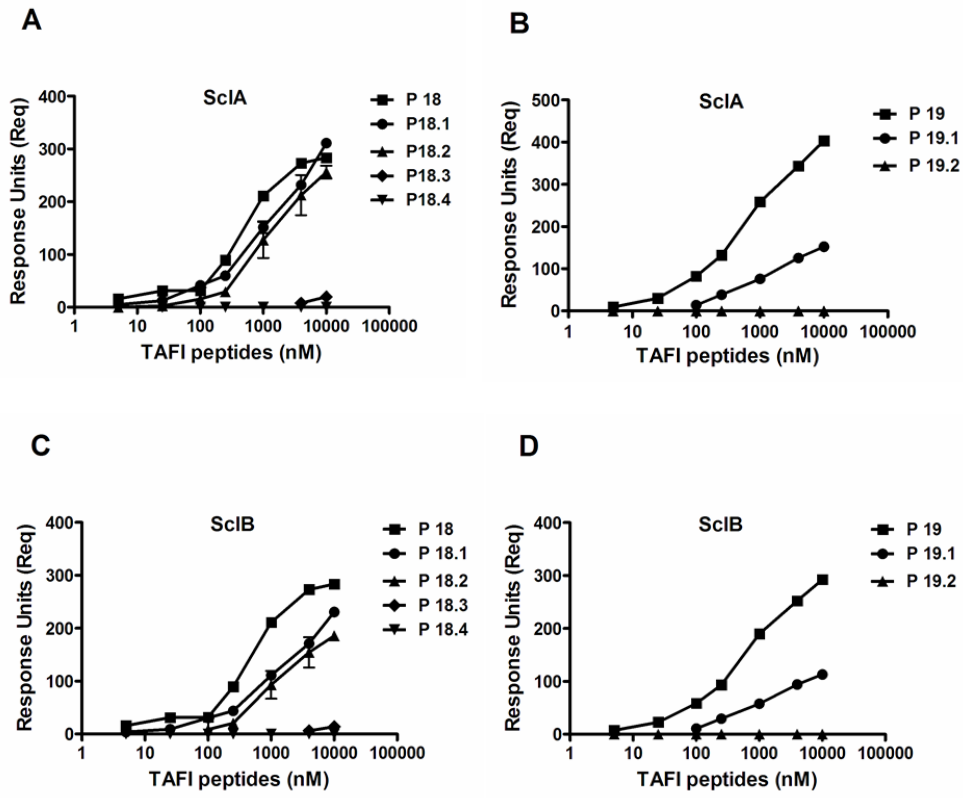
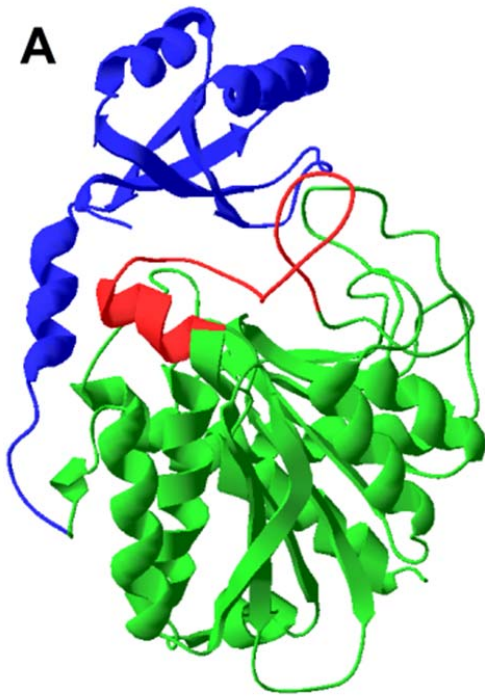


Figure 4



**Table 1**

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Peptide name	Sequence	Position
1	FQSGQVLAALPRTSRQVQ	F1 - Q18
2	RTSRQVQVLQNLTTYE	R12 - E26
3	TYEIVLWQPVTADLIVKKKQ	T26 - Q45
4	TADLIVKKKQVHFFVNAS	T36 - S53
5	HFFVNASDVDNVKAHLN	H47 - N63
6	DNVKAHLNVSGIPCSVLLA	D56 - A74
7	IPCSVLLADVEDLIQQQISN	I67 - N86
8	DVEDLIQQQISNDTVSPR	D75 - R92
9	DTVSPRASASYEYEQYHSLNE	D87 - E106
10	EQYHSLNEIYSWIEFITERH	E99 - H118
11	TERHPDMLTKIHIGS	T115 - S129
12	SFEKYPLYVLKVSQKEQTAK	S130 - K149
13	GKEQTAKNAIWIDCGIHARE	G143 - E162
14	HAREWISPAFCLWFIGH	H159 - H175
15	GHITQFYGIIGQYTN	G174 - N188
16	GQYTNLLRLVDFYVM	G184 - M198
17	DFYVMPVVNVVDGYDYSWKKK	D194 - N213
18	GYDYSWKKNRMWRKNRS	G205 - S221
19	RMWRKNRSFYANNHCIGTD	R214 - D232
20	ANNHCIGTDLNRNFASKHW	A224 - W242
21	DLNRNFASKHWCEEGASSSS	D232 - S251
22	SETYCGLYPESEPEVKAVA	S253 - A271
23	ESEPEVKAVASFLRRNINQ	E262 - Q280
24	RRNINQIKAYISMHSYSQH	R275 - H293
25	HSYSQHIVFPYSYTRSKSKD	H288 - D307
26	PYSYTRSKSKDHEELSLVAS	P297 - S316
27	KDHEELSLVASEAVRAIEKT	K306 - T325
28	EAVRAIEKTSKNTRYTHGHG	E317 - G336

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**Table 2**

Analyte/ligand	$k_{a1} (M^{-1} s^{-1})$	$k_{d1} (s^{-1})$	$K_{D1} (nM)$	$k_{a2} (M^{-1} s^{-1})$	$k_{d2} (s^{-1})$	$K_{D2} (nM)$
TAFI-IIYQ/SclA	$4.5 \pm 0.0 \times 10^4$	$1.6 \pm 0.1 \times 10^{-4}$	$3.5 \pm 0.3$			
TAFI-IIYQ/SclB	$4.0 \pm 0.1 \times 10^4$	$1.6 \pm 0.0 \times 10^{-4}$	$4.0 \pm 0.0$			
TAFIa-IIYQ/SclA	$2.0 \pm 0.0 \times 10^6$	$9.8 \pm 0.1 \times 10^{-3}$	$4.9 \pm 0.1$	$3.4 \pm 0.1 \times 10^4$	$4.3 \pm 0.2 \times 10^{-4}$	$12.6 \pm 0.3$
TAFIa-IIYQ/SclB	$1.1 \pm 0.0 \times 10^6$	$7.3 \pm 0.1 \times 10^{-3}$	$6.6 \pm 0.0$	$2.7 \pm 0.0 \times 10^4$	$3.0 \pm 0.0 \times 10^{-4}$	$11.1 \pm 0.2$