

Protein-Templated Formation of an Inhibitor of the Blood Coagulation Factor Xa through a Background-Free Amidation Reaction

Mike Jaegle, Torsten Steinmetzer, and Jörg Rademann*

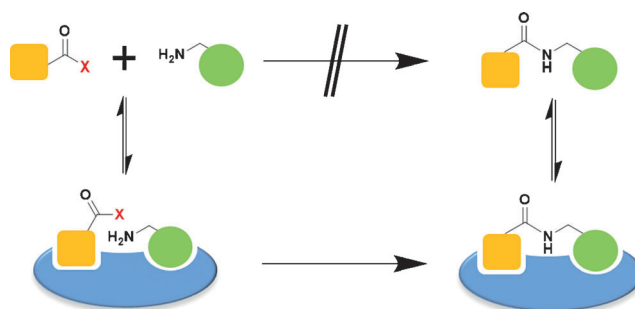
Abstract: Protein-templated reactions enable the target-guided formation of protein ligands from reactive fragments, ideally with no background reaction. Herein, we investigate the templated formation of amides. A nucleophilic fragment that binds to the coagulation factor Xa was incubated with the protein and thirteen differentially activated dipeptides. The protein induced a non-catalytic templated reaction for the phenyl and trifluoroethyl esters; the latter was shown to be a completely background-free reaction. Starting from two fragments with millimolar affinity, a 29 nM superadditive inhibitor of factor Xa was obtained. The fragment ligation reaction was detected with high sensitivity by an enzyme activity assay and by mass spectrometry. The reaction progress and autoinhibition of the templated reaction by the formed ligation product were determined, and the structure of the protein-inhibitor complex was elucidated.

Over the past years, fragment-based drug discovery has been recognized as a powerful bottom-up approach towards potent, selective, and efficient protein ligands.^[1] The development of fragment-based ligands, however, is challenging for several reasons. Bioactive fragments are usually difficult to identify owing to their low, often millimolar affinities.^[2] In most cases, biophysical methods such as NMR spectroscopy,^[3] protein crystallography,^[4] or surface plasmon resonance^[5] have been employed to solve this “detection problem” of fragment-based drug discovery. Once two fragments with adjacent, non-overlapping binding sites have been identified, a suitable linker has to be found to prepare a bioactive fragment combination with increased affinity. This challenge can be denominated as the “linkage problem” and usually requires considerable chemical variation to be solved. The covalent assembly of protein ligands on the protein target, also referred to as protein-templated or target-guided formation of protein ligands, can be a powerful solution for both of these challenges.^[6]

The templated formation of protein ligands from fragments has been realized for both reversible and irreversible ligation reactions. Whereas there are numerous examples of reversible ligation reactions,^[6,7] the investigation of irreversible ligation reactions has been limited to a few reaction types, mostly involving dipolar cycloaddition reactions.^[8–10] In addition, larger protein ligands have been constructed from peptides by native chemical ligation^[11] and alkylation reactions^[12] but these were usually accompanied by considerable background reactions. An extension of the repertoire of protein-templated reactions would be especially desirable for linkages that are found in many bioactive ligands for diverse protein targets. The amide bond belongs to this class of privileged fragment linkages, as revealed by an analysis of the World Drug Index, a database of bioactive molecules.^[13] Our goal was to establish protein-templated amidation reactions that allow for direct, sensitive, and rapid detection of biological activity (not only binding) of the formed fragment combination products in a biochemical assay.

Amide linkages are generally formed from activated carboxylic acid derivatives (“active esters”) and amine nucleophiles.^[14] A clean templated amidation reaction requires the reversible binding of both reactive fragments to the protein and has to proceed from the bound state only, with no background reaction of non-bound fragments (Scheme 1).

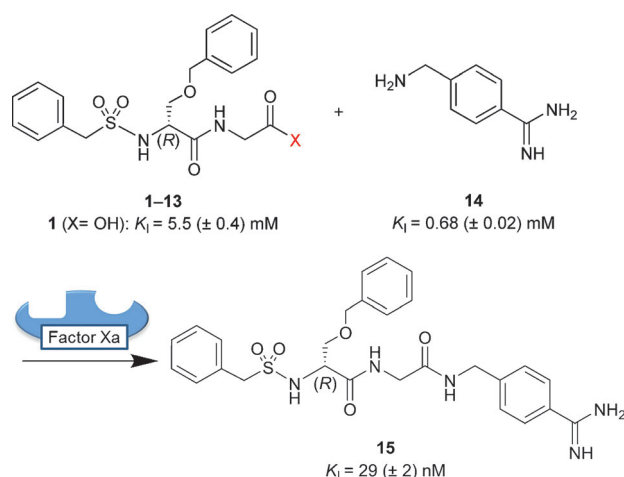
We used the protein factor Xa as a template, a serine protease of the blood coagulation cascade and target of antithrombotic drugs.^[15] The dipeptide *O*-benzyl-*N*-benzylsulfonyl-D-serinyl-glycine (**1**; Scheme 2) was selected as a small-molecule fragment of a reported inhibitor and was expected to bind to the S2–S4 pockets of the enzyme.^[16] To screen active esters for their suitability as substrates in



Scheme 1. Protein-templated amide bond formation. The reaction of an activated carboxylic acid derivative (yellow) with a nucleophilic amine fragment (green) occurs only in the presence of the protein target by a non-catalytic mechanism (templated reaction, bottom) whereas the non-templated background reaction (top) does not proceed.

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Scheme 2. Dipeptide derivatives **1–13** and 4-aminomethylbenzamidinium (**14**) were incubated in the presence of the protein target factor Xa to observe the protein-templated formation of inhibitor **15**.

protein-templated amidation reactions, carboxylic acid derivatives **1–13** were prepared, which cover a broad range of reactivity towards a nucleophilic amine fragment. 4-Aminomethylbenzamidinium (**14**) was employed as the nucleophile and typical S1-binding fragment of trypsin-like serine proteases.^[17] The inhibition of factor Xa was determined in an enzyme activity assay using the fluorogenic substrate 7-(*N*-Boc-leucinyglycyl-arginyl)-7-amino-4-methylcoumarin ($K_M = 76 \mu M$) for detection.^[18] Carboxylic acid **1** was found to be a moderately active competitive inhibitor of factor Xa with a binding affinity (K_i) of 5.5 mM, and the K_i value of **14** was 0.68 mM. Ligation product **15**, however, was highly active with a K_i value of 29 nM. Biolayer interferometry (BLI) was used as an independent biophysical method to confirm the binding kinetics of the inhibitors (see the Supporting Information).^[19]

For the sensitive detection of templated inhibitor formation, each of the carboxylic acid derivatives **1–13** and the nucleophilic fragment **14** was incubated at room temperature with protein factor Xa (14.5 nM) under “templating conditions” for two hours. Then, the fluorogenic substrate was added, and the relative inhibition of the enzyme was determined (Table 1). For comparison, carboxylic acid derivatives **1–13** were incubated for the same time only with the nucleophilic fragment **14** but without the protein under “non-templating conditions”. The inhibition was recorded after adding equal concentrations of substrate and enzyme as before. Nucleophile **14** at a concentration of 0.285 mM and a mixture of **1** (5 mM) and **14** (0.285 mM) were employed as negative controls, resulting in 20% and 32% inhibition, respectively, both under templating and non-templating conditions. Complete inhibition of factor Xa was observed for **14** and 0.285 mM of either acyl fluoride **2**, acyl imidazole **3**, pentafluorophenyl ester **4**, and 4-nitrophenyl ester **5**, indicating the formation of inhibitor **15** with and without the protein and the high reactivity of these activated acid derivatives. Reactions with phenyl thioester **6**, 3-acyl-2-thiono-1,3-thiazolidine **7**, and piperidinyl-1-ester **8** afforded partial inhibition of factor Xa but no templating effect was detected. The corresponding ethyl thioester **9**, pyridin-3-yl ester **10**, and

Table 1: Inhibition of factor Xa through the formation of inhibitor **15**.^[a]

Entry	X =	Inhibition / % →
1		~30
2		100
3		100
4		100
5		100
6		~85
7		~75
8		~55
9		~40
10		~35
11		~25
12		~55] ***
13		~45] ***
14		~20

[a] In the fragment ligation assay, the dipeptide derivatives **1–13** were incubated with 4-aminomethylbenzamidinium (**14**) in the presence (blue) or absence (red) of the target protein (pH 8, 2 h, 20 °C; see the Supporting Information for details). The active esters **12** and **13** displayed a statistically significant templating effect as determined with an unpaired t-test yielding $p = 0.0002$.

methyl ester **11** displayed only weak partial inhibition even at 5 mM when incubated with nucleophile **14**, again without any signs of a templating effect. A clear, statistically significant, and reproducible templating effect was observed for two dipeptide derivatives, namely phenyl ester **12** and 2,2,2-trifluoroethyl ester **13**.

Thus the templated amidation reactions of these two ester fragments were investigated in greater detail. Reactions of **12** or **13** with **14** in the presence of the protein target, factor Xa, were analyzed after 2, 5, and 10 h at 20 °C (RT) or 37 °C (Figure 1). Both the extension of the reaction time and the

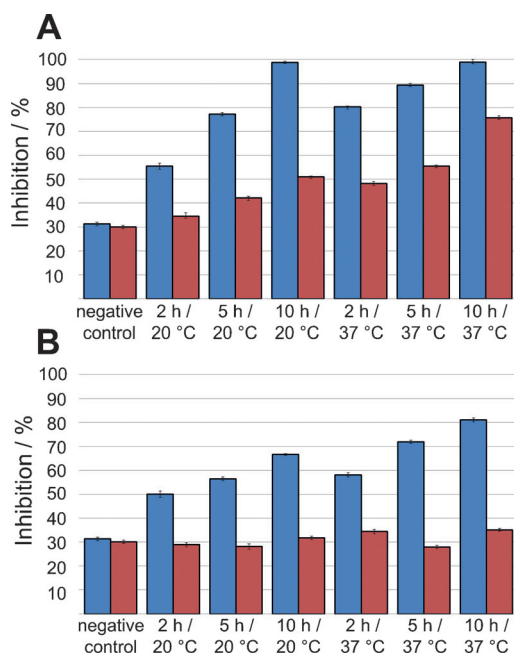


Figure 1. Inhibition of factor Xa by the ligation product of phenyl ester **12** (A) or 2,2,2-trifluoroethyl ester **13** (B; both 5 mM) with 4-amino-methylbenzamidinium (**14**; 0.285 mM). Blue: templated reaction; red: non-templated reaction. A mixture of carboxylic acid **1** and amine **14** was used as negative control.

higher temperature led to markedly increased inhibition for both esters. With phenyl ester **12**, inhibition was slightly enhanced in the non-templated reaction at 37 °C and after prolonged reaction times. For ester **13**, the inhibition remained the same for the non-templated reaction, both at room temperature and at 37 °C, indicating the absence of a non-templated background reaction.

The templated formation of protein ligand **15** was validated by reverse-phase HPLC-MS. After 2 h incubation with the protein, significant amounts of amidation product **15** were detected for both active esters **12** and **13** in the templated reaction at 20 °C, whereas no product was detectable without the protein (see the Supporting Information, Figure S1). To quantify the amount of product formed by the templated reaction, a high-resolution quadrupole time of flight (QTOF) mass spectrometer was employed as a detector (Figure 2). Phenyl ester **12** furnished 26 nM of the inhibitor **15** with a half saturation time ($t_{1/2}$) of 1.4 h; trifluoroethyl ester **13** yielded 10 nM of **15** with $t_{1/2}$ = 1.7 h. Without the protein template, only 4 and 0.15 nM of **15** were detected with no time-dependent background reaction. The templated reactions of both esters were clearly autoinhibited as the highly active product **15** prevented the binding and templated reaction of further fragments.

Finally, it was our goal to rationalize the observed templated reaction on the basis of the protein structure (Figure 3). Crystals of factor Xa in complex with inhibitor **15** were generated by back-soaking (see the Supporting Information for details), and the crystal structure of the complex was solved at 2.2 Å resolution (PDB No. 5K0H). As expected, the benzamidinium occupies the S1 pocket while the *O*-benzyl

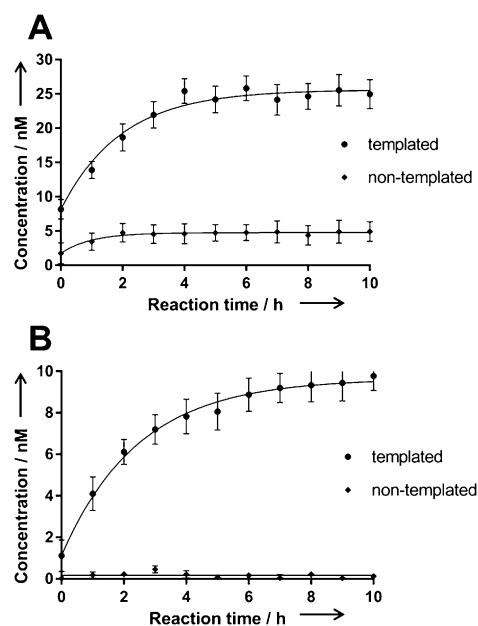


Figure 2. Formation of the factor Xa inhibitor **15** in the templated amidation reaction of **14** (0.285 mM) and phenyl ester **12** (A) or 2,2,2-trifluoroethyl ester **13** (5 mM; B) at room temperature. The concentration of **15** was quantified by extracted ion chromatography HPLC/QTOF-MS, yielding a saturation curve with c_{max} = 25.6/9.6 nM and $t_{1/2}$ = 1.4/1.7 h, respectively.

and *N*-benzylsulfonyl residues are found in adjacent hydrophobic pockets. Starting from the crystal structure, the binding modes of both ester fragments **12** and **13** and benzamidinium **14** were derived. The energy-minimized model of the protein complex with ester **12/13** and the benzamidinium fragment reveals a distance of 3.4 Å between the amine N atom of **14** and the ester carbonyl C atom of **12** and **13** while the van der Waals radii of nitrogen and carbon add up to 3.25 Å. The N-C-O angle between the N nucleophile and the attacked carbonyl double bond amounts to 82° for **12** and 83° for **13**. Thus the structure model supports the feasibility of a templated reaction between ester fragments and amino-methylbenzamidinium **14** to inhibitor **15**.

In summary, we have described the first examples of a background-free protein-templated amidation reaction. Templated formation of the factor Xa inhibitor **15** (K_i = 29 nM) was shown to occur from phenyl ester **12** and 2,2,2-trifluoroethyl ester **13** by direct determination of the enzymatic activity in a kinetic assay and by analysis of the autoinhibited ligation reaction by HPLC-MS. While phenyl ester **12** showed nearly no background reaction with amine **14** at room temperature (20 °C) after two hours, 2,2,2-trifluoroethyl ester **13** was completely unreactive without the protein template even at 37 °C and after ten hours. Applying the equation $\Delta G = -RT \ln K_i$, the K_i values of the fragments correspond to free binding energies of −12.7 and −17.8 kJ mol^{−1} for **1** and **14**, respectively.

Assuming that the free binding energies of the fragments are additive in the ligation product, a free binding energy of −30.4 kJ mol^{−1} would be expected for inhibitor **15**, corresponding to a K_i value of 3.7 μM. Thus the fragment ligation product **15** with an inhibition constant of 29 nM providing

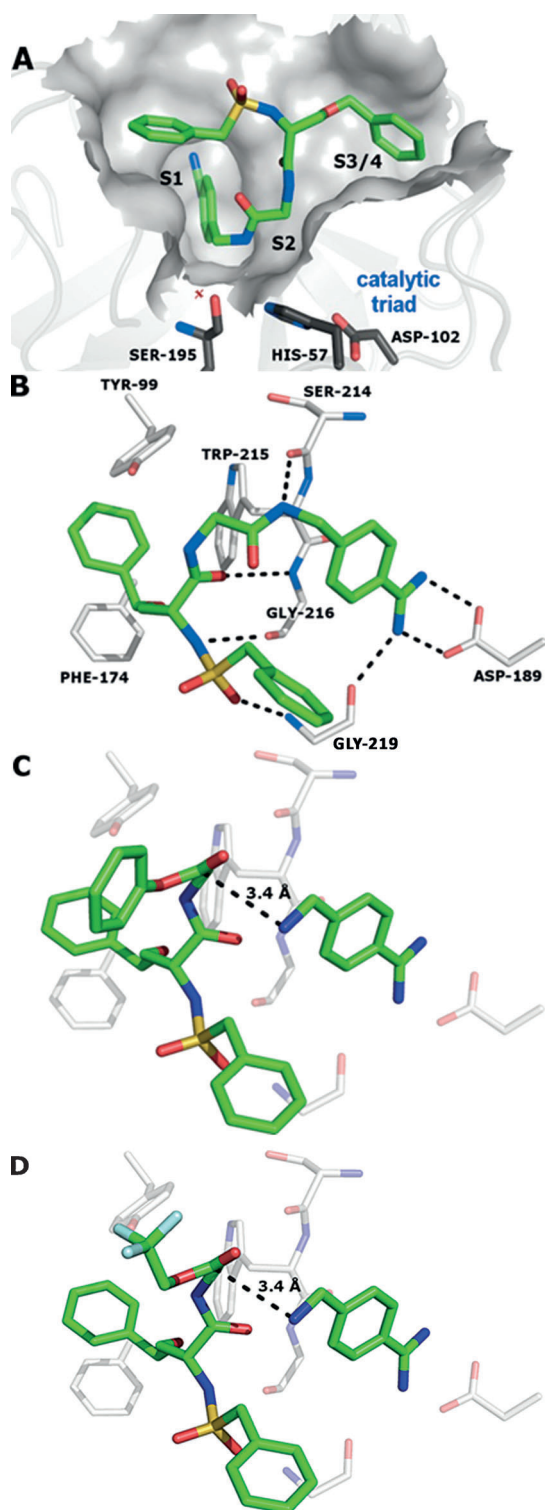


Figure 3. Structure of factor Xa in complex with inhibitor **15** (PDB No. 5K0H) displaying the protein surface (A) and the key interacting amino acids (B). C, D) Calculated complexes of the protein with benzamidine **14** and phenyl ester **12** or 2,2,2-trifluoroethyl ester **13**, respectively.

a free binding energy of $-42.3 \text{ kJ mol}^{-1}$ constitutes an example for a strongly superadditive enhancement effect.^[20] One possible reason for this effect of fragment linking is that the linker between the fragments, in this case the amide bond,

is more favorable for binding than the respective functional groups in the starting fragments. To investigate this possibility, the primary amide derivative of **1** (\rightarrow compound **16**) and the N-acetylated derivative of **14** (\rightarrow compound **17**) were synthesized and tested in the inhibition assay (see the Supporting Information). As **16** and **17** showed slightly reduced affinities to the protein target (**16**: $K_i = 6 \text{ mM}$; **17**: 1.1 mM) compared with their parent compounds, it can be concluded that the linker does not contribute to the superadditive effect of inhibitor **15**. Instead, the results suggest that the binding entropy of the fragment ligation product **15** is less negative than that of the fragments **1** and **14**, giving rise to the increased affinity of the inhibitor.

It should be noted that the reported templated amidation reactions of an amine with stable amino acid esters demonstrate an alternative, non-catalytic mechanism of peptide bond formation without a covalent acyl-RNA or acyl-protein intermediate as in ribosomal and non-ribosomal amidation reactions. It remains to be investigated whether the reported mechanism is relevant in living systems. In principle, however, it should be functional on all protein surfaces that are capable of binding two fragments in an orientation that favors a subsequent ligation reaction. Therefore, the reported results suggest a systematic search for protein sites that are capable of inducing ligand-molding reactions to yield bioactive protein ligands independent of the catalytic function of the protein.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: amide bond formation · enzyme inhibitors · fragment-based drug discovery · peptide bond formation · protein-templated reactions

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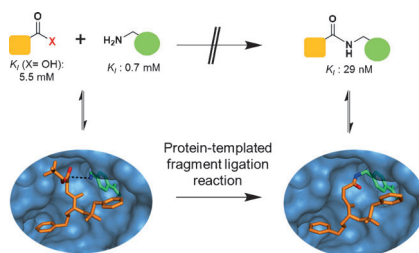
Communications



Protein-Templated Reactions

M. Jaegle, T. Steinmetzer,
J. Rademann* ————— ■■■■-■■■■

Protein-Templated Formation of an
Inhibitor of the Blood Coagulation
Factor Xa through a Background-Free
Amidation Reaction



A protein molds its own killer: The protein factor Xa induces the ligation of two weakly bound fragments into a potent, superadditive inhibitor through amide bond formation (see scheme, bottom). Suitable peptide esters react on the protein surface in a non-catalytic, autoinhibited mechanism without background reaction in the free solution.