J. Chim. Phys. (1998) **95**, 443-446

A NMR and MD study of the active site of factor Xa by selective inhibitors

B.T. Doan[1,4,*], F. Fraternali[1,5], Q.T. Do[1,6], R.A. Atkinson[1,7], P. Palmas[1,8], V. Sklenar[1,9], P. Wildgoose[2], P. Strop[3] and V. Saudek[1,10]

[1] *Marion Merrell Research Institute, HMR, 67080 Strasbourg, France*
[2] *Hoechst AG, HMR, Central Pharma Research, 65926 Frankfurt, Germany*
[3] *Selectide Corporation, HMR, Tucson, AZ 85737, U.S.A.*
[4] *LCSOB, RMN, UPMC Paris 6, 75252 Paris, France*
[5] *EMBL, 6900 Heidelberg, Germany*
[6] *Tripos Associates, 92167 Antony, France*
[7] *ESBS, 67400 Illkirch, France*
[8] *CEA, 37260 Monts, France*
[9] *Masaryck University, 611 37, Brno, Czech Republic*
[10] *Synthélabo Biomoléculaire, 67080 Strasbourg, France*

* *Correspondence and reprints.*

RÉSUMÉ

L'étude structurale d'inhibiteurs du facteur Xa, une enzyme de coagulation, obtenus par chimie combinatoire : Ac-Tyr-Ile-Arg-Ile-NH_2, Ac-(4-amino-Phe)-(Cyc.-Gly)-NH_2, a été réalisée par RMN NOE de transfert et modélisation moléculaire. Les structures ont été calculées sous contraintes RMN : géométrie de distance, recuit simulé et minimisation, affinées par une recherche conformationnelle et recuit de l'inhibiteur placé dans le site actif et optimisées par simulation de dynamique moléculaire du complexe dans l'eau. L'inhibiteur présente une structure compacte positionnée dans le site d'interaction hors d'accès du site catalytique. Ce modèle permet d'expliquer le mode d'action, l'affinité et la spécificité des peptides.
mots-clés : NOE de transfert, recuit simulé, Facteur Xa, inhibiteurs.

ABSTRACT

The structure of two selective inhibitors obtained by the screening of a vast combinatorial library, Ac-Tyr-Ile-Arg-Ile-NH_2 and Ac-(4-amino-Phe)-(Cyc.-Gly)-NH_2, in the active site of the blood clotting enzyme factor Xa was determined using transferred NOE NMR and simulated annealing (SA) under NMR constraints. The refined structures of the inhibitors were docked in the active site and SA was performed inside the enzyme which has been kept as a rigid charged template. The final structures were optimised by molecular dynamics simulation of the complexes in water. The inhibitors assume a compact, very well defined conformation embedded in the binding site without blocking the catalysis. The model allows to explain the mode of action, affinity and specificity.
Key-words : transfer NOE, Simulated Annealing, Factor Xa, inhibitors.

Blood coagulation enzymes are serine proteinases responsible for the activation, maintenance regulation and clearance of blood clot formation[1]. Factor Xa (FXa) is a 45kD serine proteinase located at a key cross-road of the coagulation. Recently, a family of tri- to pentapeptide acting as competitive inhibitors of FXa has been identified by screening millions of peptides in a combinatorial library.

To better understand the mode of action and selectivity of these inhibitors, and their interaction with FXa active site, we undertook the study of their active conformation by selecting two representative peptides : **A**: Ac-Tyr-Ile-Arg-Ile-NH2 (Kd=1.6μM) and **B**: Ac-(4-amino-Phe)-(Cyc.-Gly)-NH2 (Kd=0.3μM). Crystals of the complexes could not be obtained to date, on the other hand NMR spectroscopy may be applied directly to study low molecular weight medium affinity ligands in interaction with their macromolecular receptors by measuring transferred NOEs[2].

EXPERIMENTAL

Human FXa was purchased from Enzyme Research Laboratories Inc. It was washed with a 50 mM phosphate buffer pH=7 by ultracentrifugation. The inhibitory peptides were prepared by solid phase synthesis and introduced at 2 mM concentration in 0.02 to 0.1 mM FXa (inhibitor:enzyme ratio 20:1 to 100:1) in 0.5 ml of 50 mM pH=7 phosphate buffer in H_2O:D_2O 90:10.

NMR experiments were carried out on a Bruker DMX 600 MHz spectrometer. A full range of NMR spectra (COSY, TOCSY, ROESY, off-ROESY, NOESY in the States-TPPI mode) were recorded at 37, 24 and 10°C. In order to prevent small temperature variations of proton chemical shifts, a CITY[3] pulse train was introduced before each sequence except TOCSY to preheat the sample. Since FXa is active at neutral physiological pH, water suppression applying saturation was avoided and WATERGATE sequence, with water Z flip-back, was applied instead. To reduce water signal broadening in ω1, radiation damping was attenuated by weak bipolar gradients. The spectra were analysed using AURELIA software package. The assignments were obtained using standard procedures, the NOE build-up provided inter-proton distances with methylene groups for calibration.

Calculations were performed using the molecular modelling software package SYBYL 6.2 (Tripos Associates, St. Louis, MO,USA) according to the scheme :

Distance Geometry (DIANA) 1000 structures
Simulated Annealing (100*(heating 1000K, 5ps 1000K, 10ps to 0K, 100 structures)
Energy Minimisation (no electrostatics for ligand, 200steps simplex, 5000 Powell iterations)
Docking into the active site
Systematic search of angles χ1-4, of the Arg3 in 5° : 94 structures
SA(heating to 1000K in 10ps, steps 0.5ps, 20ps at 1000K, 10steps of 0.5ps to 0K), **EM**
2 models for the binding : **Model P1** **Model P2**
Molecular Dynamics 65 ps in water
Model PW1 **Model PW2**
Analysis -> final model

RESULTS

For the peptides alone, almost no cross-peaks appeared in the NOESY spectra; ROESY and off-ROESY showed only a limited number of NOEs expecting no overall conformation. In the presence of the enzyme, the NOESY displayed a great number of well resolved transferred NOEs (peptide A: 14 intra and 54 inter-residue distances). Only positive tr-ROE cross-peaks were observed indicating no indirect protein mediated intermolecular NOEs. Effects of viscosity was also checked. The NOEs were suppressed after the addition of dansyl-Glu-Gly-Arg-NH_2 specific covalent inhibitor demonstrating the specific interaction of the peptide. The high number of NOEs indicates that the enzyme forces the peptides to adopt a compact conformation.

The conformation of both peptides is very well defined (local backbone RMSD < 0.5Å). A high degree of the global and local structure definition is never achieved with low molecular weight peptides free in solution (here $RMSD_{ROE}$ = 4 Å). The inhibitors are then located inside the active site of the enzyme which induces a restrained motion. The peptide was then docked into the binding site S1 via the arginine forming a salt bridge through its guanidine end group and Asp 189 of the FXa[1]. The position of the guanidine group was placed as the Arg51' of the A-chain of crystallised FXa in the S1 specificity pocket[4]. After SA and EM, two conformational families were identified labelled P1 and P2 for peptide **A**. Model P2 was selected because model P1 presents a more exposed N-terminal Tyr1-Ile2 fragment whereas the model P2 has a completely buried N-terminus. In particular, Ile2 of P2 is embedded in the hydrophobic region formed by Tyr99, Phe174 and Trp215. Model P2 has a lower RMSD (0.98 vs 1.22Å) and lower intrinsic peptide

energy. More importantly, the intra complex energy ($E_{\text{sphere around the ligand}}$) and inhibitor-enzyme energy (E_{total}-E_{ligand}-E_{enzyme}) are respectively 27 and 23 kcal.mol^{-1} in favour of P2. To evaluate the energy of the two models in a force field as complete as possible, a 65ps MD run in water (1200 H_2O, periodic boudary conditions) was performed on the lowest energy representative. The interaction energy is more favourable to PW2 whereas the interaction energy with the solvent is more favourable to PW1. The inhibitor in PW1 exhibits a large number of hydrogens bonds with water (10) and less with the enzyme (2), in contrast, model PW2 shows respectively 7 and 10 H-bonds. Futher evidence in favour of model 2 comes from the analysis of QSAR (sequence requirement vs inhibitory activity) which shows that the hydrophobic residue 2 is essential for the inhibition and the peptides may be truncated after Arg3. The refined structure of tripeptide B is well superimposed to peptide A in model PW2 in the binding site of FXa.

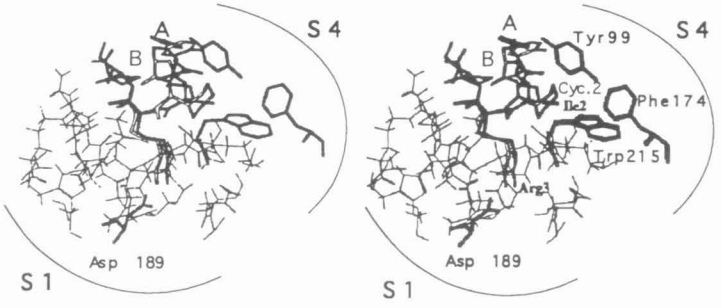

Figure : Peptides A and B docked into S1 S4 FXa sites

The inhibitors assume a 3D compact structure well adapted to the shape of the active site of FXa. They are embedded in a way that hinders the access of the catalytic residues to the cleavable peptide bond. Arg3 is inserted in the pocket S1 and the aliphatic hydrophobic side chain in the sequence position 2 is enveloped by the first residue which closes the cavity and hydrophobic S4 pocket. The selectivity is mainly exerted by the site S4, which seems to be a very attractive target in the design of further selective inhibitors for FXa and enzymes of blood coagulation cascade.

1 E.W. Davie, K. Fujikawa, W. Kiesel (1991) *Biochem.* 30, 10603.
2 F. Ni, Prog. (1994) *NMR. Spectroscopy.* 26, 517.
3 J. Briand, R.R. Ernst (1991) *Chem. Phys. Lett.* 185, 276.
4 K. Padmanabhan, K.P. Padmanabhan, A. Tulinsky, CH. Park, W. Bode, R. Huber, DT. Blankenship, AD. Cardin, W. Kisiel (1993) *J. Mol. Biol.* 232, 947.