Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray diffraction data of the complex of recombinant tick anticoagulant peptide (rTAP) and bovine factor Xa

Anzhi Wei,[a] Angela Smallwood,[a] Richard S. Alexander,[a] Jodie Duke,[b] Harold Ross,[b] Stuart A. Rosenfeld[b] and Chong-Hwan Chang[a]*

[a]Department of Chemical and Physical Sciences, The DuPont Pharmaceuticals Company, DuPont Experimental Station, PO Box 80353, Wilmington, DE 19880, USA, and [b]Department of Applied BioTechnology, The DuPont Pharmaceuticals Company, DuPont Experimental Station, PO Box 80353, Wilmington, DE 19880, USA

Correspondence e-mail: chong-hwan.chang@dupontpharma.com

Received 14 September 1998
Accepted 15 December 1998

The complex of bovine factor Xa and recombinant tick anticoagulant peptide (rTAP) was crystallized in two different crystal forms using polyethylene glycol as a precipitant. Form I belongs to space group $P4_22_12$ with unit-cell dimensions $a = b = 133.1$, $c = 68.8$ Å. It contains one complex per asymmetric unit and diffracts to 3.0 Å resolution. Form II belongs to $P4_12_12$ (or $P4_32_12$) with dimensions $a = b = 126.5$, $c = 146.7$ Å; it contains two complexes per asymmetric unit and diffracts to 2.5 Å. The crystals of both forms consist of factor Xa ($M_W = 45.3$ kDa) and rTAP ($M_W = 6.7$ kDa).

1. Introduction

Recombinant tick anticoagulant peptide (rTAP) is a specific and potent inhibitor of factor Xa (fXa), a central enzyme in the blood-clotting cascade (Waxman *et al.*, 1990; Mao *et al.*, 1995). The discovery of rTAP, which inhibits only factor Xa (Waxman *et al.*, 1990), has produced significant amounts of data ranging from the structure and function of a novel serine-protease inhibitor to the importance of factor Xa in the thrombotic process (Vlasuk, 1993).

The rTAP binds its target protease, fXa, by a mechanism which is unique among serine-protease inhibitors (Jordan *et al.*, 1990; Laskowski & Kato, 1980). Earlier biochemical and mutagenesis studies (Sardana *et al.*, 1991; Jordan *et al.*, 1992) demonstrated that rTAP binds to fXa in a two-step process: the enzyme and the inhibitor rapidly construct a low-affinity complex and the complex then slowly rearranges, gaining additional contacts, to yield the final high-affinity enzyme–inhibitor complex. The amino-terminus of rTAP plays a critical role in the formation of the high-affinity fXa–rTAP inhibitory complex (Jordan *et al.*, 1992). The postulated binding interaction between rTAP and fXa most closely resembles the mechanism of inhibition exhibited by hirudin with thrombin (Jordan *et al.*, 1992; Rydel *et al.*, 1990). The structure of rTAP observed by solution NMR (Lim-Wilby *et al.*, 1995; Antuch *et al.*, 1994) is drastically different from the structure of hirudin, which consists of a long (39 Å) carboxyl-terminal extended domain. The carboxyl-terminal domain of hirudin makes numerous interactions with an anion-binding exosite of thrombin (Jordan *et al.*, 1992). Since rTAP does not have the long tail carboxyl-terminal domain, it is prudent to expect that the secondary binding determinant of rTAP interacts with the exosite of fXa rather than an extended S′ subsite as found in the hirudin–thrombin complex.

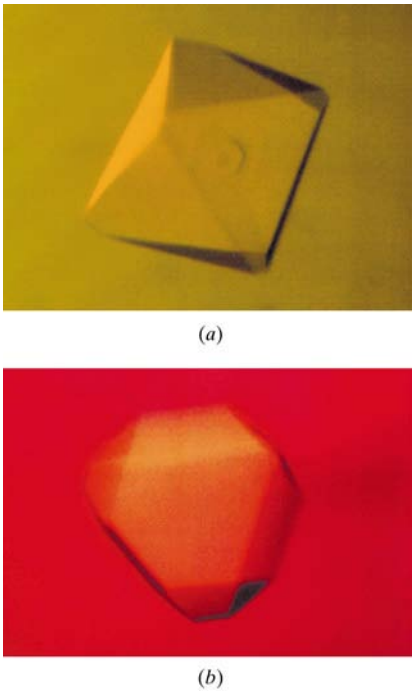
To enhance the understanding of the binding mechanism of the complex, many biochemical and mutagenesis studies (Sardana *et al.*, 1991; Jordan *et al.*, 1992) have been performed. We decided to conduct a structural study in order to reveal the detailed binding mode of the rTAP–fXa complex. The complex structure will provide a model to explain the perplexing results of mutagenesis studies on the rTAP–factor Xa interaction. It may be possible to use the structure for the design of new selective inhibitors of fXa.

2. Materials and methods

2.1. Complex preparation

Bovine factor Xa was obtained from Enzyme Research Labs. The enzyme is prepared from homogeneous bovine factor X using the activating enzyme from the Russels' viper venom. Complete activation was observed using 10% SDS–PAGE gels. The activated enzyme contained approximately equal amounts of two forms of factor Xa (α and β) and was used without further purification. The protein in 10 m*M* Tris–HCl and 0.1 *M* NaCl at pH 7.5 was dialyzed against 20 m*M* HEPES (pH 7.5) and 0.15 *M* NaCl overnight at 277 K.

The rTAP gene, DMY6, constructed and cloned into *Saccharomyces cerevisiae* strain (*mata*/*a*, *ade*1, *ura*3-52, *his*3 *D*::*Gal*10p-*Gal*4-*ura*3, *leu*2-2, 112/*leu*2-04, *cir*0 *cir*0) (Neeper *et al.*, 1990) was provided by R. Ellis, Merck Research Laboratories. A modified procedure of a previously reported method was used to



(*a*)

(*b*)

Figure 1
Bovine factor Xa–rTAP complex crystals: (*a*) form I and (*b*) form II. Crystals of both forms grew in two months to dimensions of 0.25 × 0.25 × 0.15 mm.

select a single colony, to express and to purify the rTAP (Lehman *et al.*, 1993).

The purified rTAP (>98% by HPLC) was desalted and concentrated by a batch adsorption step with a reverse-phase packing POROS R/M (Perkin Elmer) and lyophilized. A pink pigment was removed by charcoal treatment as described (Lehman *et al.*, 1992). The lyophilized rTAP was extensively dialyzed against water before formation of the complex with factor Xa.

The rTAP–factor Xa complex was prepared by incubating equimolar mixtures in 20 m*M* HEPES (pH 7.5) and 0.15 *M* NaCl for 2 h at 298 K, followed by 2 d at 277 K, before setting up the crystallization trials.

2.2. Crystallization

Crystals of the rTAP–fXa complex were grown by the hanging-drop vapor-diffusion method from buffered PEG 8000 solution at 277 K. Typically, 2 µl of protein (10 mg ml^{-1}) mixed with an equal volume of precipitant was used for screening. Small tetragonal prism crystals appeared after four weeks. Useful crystals of 0.25 mm dimensions grew in about two months (Fig. 1).

The form I crystals belong to space group $P4_22_12$ with unit-cell dimensions of $a = b = 133.1$, $c = 68.8$ Å; they were grown by the hanging-drop vapor-diffusion method from 50 m*M* potassium/sodium phosphate (pH 7.0), 0.1 *M* sodium chlorate and 12% PEG 8000 at 277 K. Form I contains one complex per asymmetric unit and diffracts to 3.0 Å resolution. Further screening of crystallization conditions yielded the form II crystals, which belong to space group $P4_12_12$ (or $P4_32_12$) with unit-cell dimensions $a = b = 126.5$, $c = 146.7$ Å. The form II crystals were grown by the hanging-drop vapor-diffusion method from 50 m*M* potassium/sodium phosphate (pH 6.0), 0.2 *M* sodium citrate and 10% PEG 8000 at 277 K. They diffract to 2.5 Å resolution and there are two complexes per asymmetric unit.

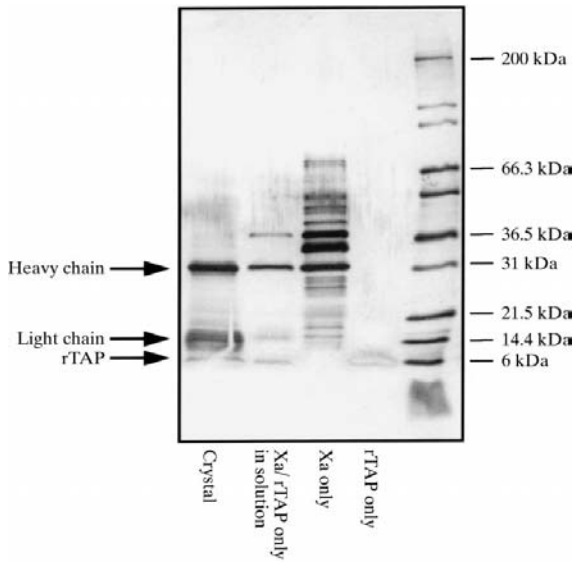


Figure 2
The SDS–PAGE analysis of the factor Xa–rTAP complex. Notice the bands of heavy and light chains of factor Xa and of rTAP both in solution and in crystals.

2.3. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis confirmed that the crystals consisted of a complex of rTAP and factor Xa. Crystals of both forms were harvested, washed with reservoir solution and dissolved in Tris–glycine SDS sample buffer (Novex) with β-mercaptoethanol. The solution containing the dissolved crystals was run on 10–27% Tris–glycine gel. The gel was silver stained using a kit from Novex. The expected bands corresponding to rTAP and factor Xa appeared on the gel (Fig. 2).

2.4. Data collection

X-ray data were collected using an R-AXIS II image-plate detector mounted on a Rigaku RU200 rotating-anode generator operating at 50 kV and 100 mA. Complete data were obtained at 277 K for both crystal forms I and II.

For the form I crystal, the crystal-to-detector distance was 98.8 mm, the oscillation range was 2° and the exposure time was 60 min frame^{-1}. The total scan range for the tetragonal crystal covered 70°. The data were integrated, scaled and reduced with *DENZO* and *SCALEPACK* (Otwinowski, 1993). The $R_{\rm merge}$ was 11.4% and the completeness was 83%.

For the form II crystal, the crystal-to-detector distance was 90.4 mm, the oscillation range was 2° and the exposure time was 40 min frame^{-1}. The total scan range for the tetragonal crystal covered 80°. The $R_{\rm merge}$ was 8.3%, with a completeness of 79.4%.

3. Results and discussion

Both crystal forms consist of rTAP and factor Xa, as confirmed by polyacrylamide gel electrophoresis with dissolved crystals. We were able to determine the crystal structure of form I at 3.0 Å (Wei *et al.*, 1998). The determination of the structure of form II is in progress. The structure of the rTAP–factor Xa complex provides a key to understanding its novel mode of binding and clearly demonstrates the existence of a multistep pathway for the inhibition of fXa by rTAP, wherein rTAP interacts with fXa at both the active site of fXa and a site distinct from the active site. It also opens the way for structural studies of the rTAP–fXa complex and may be of possible use in the design of new selective inhibitors of fXa.

We thank R. Ellis for providing the rTAP strain, S. Brenner and R. Wexler for continued support and helpful discussions and I. DeWees and W. Saxe for proofreading the manuscript.

References

Antuch, W., Guntert, P., Billeter, M., Hawthorne, T., Grossenbacher, H. & Wuthrich, K. (1994). *FEBS Lett.* **352**, 251–257.
Jordan, S. P., Mao, S. S., Lewis, S. D. & Shafer, J. A. (1992). *Biochemistry*, **31**, 5374–5380.
Jordan, S. P., Waxman, L., Smith, D. E. & Valsuk, G. P. (1990). *Biochemistry*, **29**, 11095–11100.
Laskowski, M. Jr & Kato, I. (1980). *Annu. Rev. Biochem.* **49**, 593–626.

Lehman, E. D., Joyce, J. G., Freymeyer, D. K., Bailey, F. J., Herber, W. K. & Miller, W. J. (1993). *Biotechnology*, **11,** 207–212.
Lehman, E. D., Schaefer, T. F., Przysiecki, C. T., Joyce, J. G., Bailey, F. J. & Schulman, C. A. (1992). *J. Chromatogr.* **574**, 225–235.
Lim-Wilby, M. S. L., Hallenga, K., Maeyer, M. D., Lasters, I., Vlasuk, G. P. & Brunck, T. K. (1995). *Protein Sci.* **4**, 178–186.
Mao, S. S., Huang, J., Welebob, C., Neeper, M. P., Garsky, V. M. & Shafer, J. A. (1995). *Biochemistry*, **34**, 5098–5103.
Neeper, M. P., Waxman, L., Smith, D. E., Schulman, C. A., Sardana, M., Ellis, R. W., Schaffer, L. W., Siegl, P. K. & Vlasuk, G. P. (1990). *J. Biol. Chem.* **265**, 17746–17753.
Otwinowski, Z. (1993). *Proceedings of the CCP4 Study Weekend. Data Collection and Processing*, edited by L. Sawyer, N. Isaacs & S. Bailey, pp. 56–62. Warrington: Daresbury Laboratory.
Rydel, T. J., Ravichandran, K. G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C. & Fenton, J. W. II (1990). *Science*, **249**, 277–280.
Sardana, M., Sardana, V., Rodkey, J., Wood, T., Ng, A., Vlasuk, G. P. & Waxman, L. (1991). *J. Biol. Chem.* **266**, 13560–13563.
Vlasuk, G. P. (1993). *Thromb. Haemost.* **70**, 212–216.
Waxman, L., Smith, D. E., Arcuri, K. E. & Vlasuk, G. P. (1990). *Science*, **248**, 593–596.
Wei, A., Alexander, R. S., Duke, J., Ross, H., Rosenfeld, S. A. & Chang, C.-H. (1998). *J. Mol. Biol.* **283**, 147–154.