Crystallization of blood coagulation factor XIII by an automated procedure

Rolf Hilgenfeld[1], Alexander Liesum[1], Rüdiger Storm[1], Hubert J. Metzner[2] and Hermann E. Karges[2]

[1]*Hoechst Aktiengesellschaft, P.O.B. 800320, D-6230 Frankfurt 80* and [2]*Behringwerke Aktiengesellschaft, P.O.B. 1140, D-3550 Marburg, FRG*

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Both recombinant blood coagulation factor XIII *a*-chain and factor XIII isolated from human placenta have been crystallized using a novel robotic system for the automatic screening of crystallization conditions. The monoclinic and orthorhombic crystals obtained are suitable for X-ray analysis.

Blood coagulation factor XIII; Transglutaminase; Protein crystallization; Crystallization robotic; X-ray crystallography

1. INTRODUCTION

Factor XIIIa (FXIIIa; fibrinoligase activated fibrin-stabilizing factor, EC 2.3.2.13 [protein-glutamine: amine γ-glutamyltransferase]) is the last enzyme activated in the blood coagulation cascade [1,2]. Being a transglutaminase, it catalyzes the formation of intermolecular γ-glutamyl-ϵ-lysyl isopeptide bonds between fibrin molecules [3] and between fibrin and α_2-plasmin inhibitor [4], thereby stabilizing the blood clot and confering resistance to proteolytic degradation by plasmin. Other substrates of FXIIIa include fibronectin and collagen [5,6], explaining why the enzyme also plays an important role in wound healing [7].

In blood plasma, the enzyme exists as a tetrameric zymogen designated factor XIII (FXIII), composed of two 83-kDa *a*-subunits and two 85-kDa *b*-subunits (a_2b_2) [1–3]. Activation under physiological conditions occurs via a series of events involving (*i*) cleavage by thrombin of the bond between Arg-37 and Gly-38 of the *a*-chain(s) and release of the NH_2 (terminal 37) residue activation peptide [8]; (*ii*), binding to fibrin [9]; (*iii*), dissociation of the noncatalytic *b*-chains from the tetramer to yield the a_2-dimer [1]; and (*iv*), a conformational change induced by binding of Ca^{2+} ions to the *a*-subunits.

The corresponding zymogens isolated from platelets or placenta lack the *b* subunits and consists of a_2-homodimers. Presumably, their *a*-chains are identical to those of the plasma proenzyme [2,7,10], and the steps of the Ca^{2+}-dependent conversion to FXIIIa by thrombin cleavage are also the same as described above, except for step (*iii*) [1–3,8, 11–14].

Correspondence address: R. Hilgenfeld, Central Research G 864, Hoechst AG, P.O.B. 800320, D-6230 Frankfurt 80, FRG

The primary structure of the catalytic *a* subunit has been reported recently [10, 15, 16], and the human gene coding for it has been characterized [17]. Except for its relationship to some tissue transglutaminases [17], for which FXIII *a*-chain is a prototype, the amino acid sequence of the enzyme is unique. However, a short segment around the putative active site residue Cys-314 [18] exhibits limited similarity to the active center of cysteine proteases such as cathepsins B and H, papain [19] and calotropain [20].

In order to shed light on the mechanism of this important enzyme, we have crystallized both recombinant human FXIII *a*-chain and the zymogen isolated from human placenta by a novel technique and initiated the elucidation of its three-dimensional structure by X-ray crystallography.

2. MATERIALS AND METHODS

2.1. *Protein purification*

Factor XIII from placenta was isolated and purified as described in [13]. For preparation of recombinant FXIII *a*-subunit (rFXIII), cDNA coding for the placental factor [15] was cloned and expressed in yeast (*S. cerevisiae*). Isolation of rFXIII from the crude cell extract to more than 95% purity was achieved by salt precipitation, ion-exchange and gel filtration chromatography (to be published elsewhere). In SDS-PAGE rFXIII and FXIII isolated from placenta showed identical molecular weights. Also, both could be activated by thrombin to comparable specific activities.

2.2. *Crystallization procedure*

The technique of isothermal vapour diffusion in its 'hanging drop' variant [21] was used to screen for crystallization conditons. Handling of all solutions during set-up of the experiments was performed by a microcomputer-controlled robotic system described in [22] and manufactured by ICN Biomedicals, Horsham, PA, USA. Sixteen crystallization experiments were performed in a 4 × 4 array on Linbro depression plates (Flow Laboratories, McLean, VA, USA). Solution pH was varied in the columns and precipitant concentration in

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the rows. Citrate/phosphate or tris(hydroxymethyl)aminomethane (Tris) HCl, were used as two-component buffer systems to maintain solution pH. The volume of each component necessary to yield a given pH was taken from data tables stored previously in the microcomputer. Precipitants used were ammonium sulfate, ammonium acetate, or polyethyleneglycol 4000 (PEG); their appropriate dilutions were calculated from the given concentration in stock solutions. The computed volumes of buffer and precipitant solutions were automatically dispensed into individual wells of the crystallization plates. Each well was filled to 1 ml by addition of doubly distilled water. After the wells were filled, 4 μl droplets from each well were transferred to 22 mm diameter plastic coverslips (Flow Laboratories), positioned on a plexiglas template next to the x,y translation table of the system. Finally, a 4 μl droplet of protein solution (12 mg/ml) was added to each buffer droplet on the coverslips which were then manually inverted over the appropriate well and sealed in place by a layer of silicon grease. Crystallization dishes were stored at room temperature or at 4°C and inspected daily.

2.3. *X-ray data collection*

Diffraction qualities of crystals were inspected and three-dimensional data sets collected using a FAST television area detector system with a CAD4 four-circle goniostat mounted on a FR 571 rotating anode X-ray generator, operated at 4.5 kW, with 0.3 mm focus (all X-ray equipment from Enraf Nonius, Delft, The Netherlands). The radiation used was monochromatized CuK_α. Data were collected on the detector as a series of discrete electronic images, each comprising an oscillation of 0.1°. Data acquisition and evaluation were carried out under the control of the MADNES software [24]. Unit cell constants and Bravais lattice type were determined by an autoindexing [25] procedure written by Dr. P. Tucker and confirmed by checking the equivalence of symmetry-related intensities.

Partial diffraction data sets were also collected at the EMBL Outstation at DESY, Hamburg, FRG, using synchrotron radiation of wavelength $\lambda = 0.965$ Å at the high intensity beam line X-11 of the DORIS storage ring (collaboration with Drs. C. Betzel and K.S. Wilson, to be published elsewhere). The detector was a novel imaging plate system developed by Dr. J. Hendrix et al. (EMBL).

3. RESULTS

Automated screening of crystallization conditions for both placenta and recombinant FXIII was initiated by experiments in which solution pH and precipitant concentration were varied over wide ranges. Using ammonium sulfate as a precipitant, thin needle-like crystals were obtained in a wide array of conditions. Since these needles were not useful for X-ray diffraction experiments, no attempt was as yet made to improve their size by refining the crystallization conditions. When 4000 MW polyethylene glycol (PEG) was used as the precipitating agent, crystals exhibiting three different types of morphology could be distinguished already in early screening experiments. At pH 6.9, PEG concentrations around 3.5% (w/v) led to the formation of needles after 1–3 days, whereas skewed hexagons were observed at pH 6.5 – 7.6 and 5.0 – 8.0 % (w/v) PEG. Finally, lower PEG concentrations (2%) yielded crystals of a more irregular, prismatic shape at pH 6.3 – 6.5. Crystals exhibiting the latter two habits tended to appear simultaneously in the same droplet in the intermediate precipitant concentration range and were indeed shown by X-ray diffraction experiments to have identical unit cells (see below; subsequently called crystal form I). All crystals were accompanied by a bulk protein precipitate. The observations described above were identical for both rFXIII and the factor isolated from human placenta.

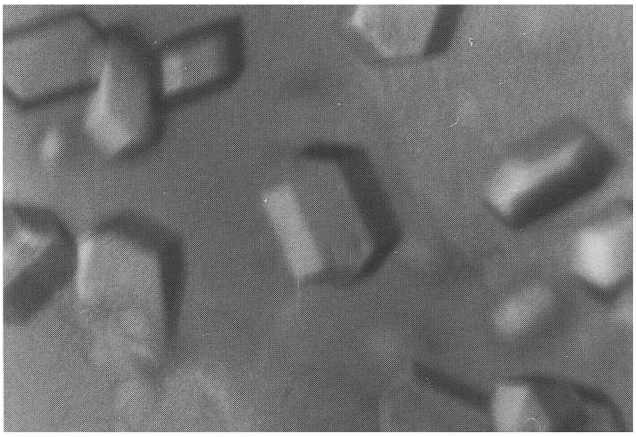

Fig.1. Form I crystals of factor XIII, obtained from 6.5 % (w/v) polyethylene glycol 4000.

In a separate series of experiments which used ammonium acetate as a precipitant, we obtained yet another crystal form with both placenta and recombinant FXIII. The shape of these crystals was again prismatic but they were crystallographically clearly distinct from the form I prisms observed at low PEG concentrations. This crystal form will be designated form II in the following. It is noteworthy that these crystals were accompanied by less amorphous precipitate than those grown from PEG.

The strategy adopted to improve the crystals of both forms was that of successive grid searches as described by Cox and Weber [22,23]. Smaller ranges of pH and precipitant concentration were centred about grid points that yielded crystals in the previous experiment. Thus, the optimum conditions for growth of the form

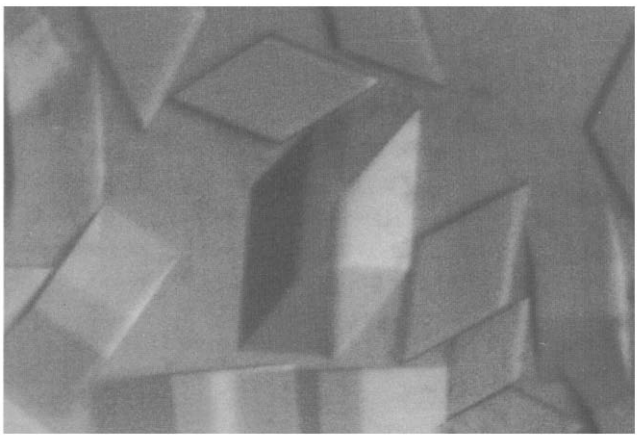

Fig.2. Form II crystals of factors XIII, obtained from 10.5 % (w/v) ammonium acetate.

I crystals were established as 6.5% (w/v) PEG, pH 7.2, room temperature. These crystals reached a maximum size of 0.3 × 0.3 × 0.15 mm³ (Fig. 1). Conditions for form II crystals were refined to 10.5% (w/v) NH_4OAc, pH 7.4. These crystals grew to a size of 0.3 × 0.3 × 0.2 mm³ (Fig. 2).

Nearly complete three-dimensional data sets were collected from crystals of both forms using a FAST television area detector [24]. The skewed hexagons (form I) diffracted to a resolution limit of 2.8 Å, which could be improved to 2.7 Å when synchrotron radiation and an imaging plate detector were used (details to be published elsewhere). The cell constants were determined as a = 101.0(1), b = 72.3(2), c = 133.2(2) Å, β = 106.6(1)° (SDs from a total of five crystals), and the space group was monoclinic, $P2_1$. The cell axes agreed to within 0.2 Å between crystals of recombinant and placenta FXIII. Assuming two molecules, i.e. one *a2* dimer, per asymmetric unit, the packing density of these crystals was calculated as V_m = 2.79 Å³/Da, which is well within the range observed for most protein crystals [26]. The solvent content of these crystals was estimated to be 56%.

The prismatic crystals of form II exhibited a diffraction limit of 3.5 Å resolution and were less stable in the X-ray beam. A resolution limit of 3.0 Å was achievable with synchrotron radiation. The space group was orthorhombic, most probably $P2_12_12_1$, with cell constants a = 95.4(2), b = 101.0(2), c = 181.4(4) Å (only determined for form II crystals of the recombinant material). Assuming one FXIII a_2 dimer per asymmetric unit, the packing density was calculated as V_m = 2.63 Å³/Da, again well within the range normally observed [26]. The solvent contents of these crystals was 53%.

4. DISCUSSION

Obtaining sufficiently large single crystals of proteins is a prerequisite for the determination of their three-dimensional structures by X-ray crystallography. Nucleation and growth of protein crystals is influenced by a large number of parameters the interplay of which is poorly understood, thus making systematic approaches difficult and leading to the common notion that protein crystallization has remained an art rather than become a science [27]. However, in recent years physicochemical studies of the processes occurring prior to and during crystallization have provided a better understanding of the role of at least some of the crystallization parameters [28], and robotic systems aiming at more systematic investigations of crystallization conditions have been developed [22].

Using the automated procedure described by Cox and Weber [22,23], we were able to grow X-ray grade crystals of the FXIII a_2-dimer. The amount of protein needed to establish optimum growth conditions for two different crystal forms was about 20 mg. This figure could have been decreased considerably by use of protein droplet volumes smaller than 4 μl. Therefore, it seems that automatization of protein crystallization trials indeed not only results in a substantial increase in experimental precision, accuracy and, hence, reproducibility but also allows to systematically scan much larger ranges of crystallization conditions with limited amounts of protein material than possible with the traditional trial-and-error methods.

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