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A fully functional deletion staphylokinase derivative crystallizes in two non-isomorphous monoclinic modifications

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Two non-isomorphous monoclinic types of diffraction-quality crystals of Δ10Sak, a fully functional staphylokinase derivative lacking the first ten amino acids, have been grown by the hanging-drop vapour-diffusion technique. Type I crystals grow from a solution containing $Zn(OAc)_2$, Tris buffer pH 7.5 and PEG 8000, while type II crystals can be obtained from a solution containing $MgCl_2$, Tris buffer pH 8.5 and PEG 4000. Both crystal types were suitable for data collection (to 2.4 and 2.6 Å resolution, respectively) and further structural investigation.

1. Introduction

Staphylokinase (Sak), a 136 amino-acid protein produced by certain *Staphylococcus aureus* strains, has been shown to be a very potent plasminogen activator (Lack, 1948). Recently, it has emerged as a potent and highly fibrin-specific thrombolytic agent for treatment of patients with acute myocardial infarction or with peripheral arterial occlusion (Vanderschueren, Barrios *et al.*, 1995; Vanderschueren, Stockx *et al.*, 1995; Collen, 1998). Unlike some other plasminogen activators (*e.g.* tissue-type plasminogen activator) Sak has no proteolytic properties by itself, but it rather acts by forming a 1:1 complex with plasmin. Only this Sak–plasmin complex acts as the real plasminogen activator which is able to activate other free plasminogen molecules (Collen *et al.*, 1993; Grella & Castellino, 1997). At this moment, however, a detailed understanding of the mechanism of action is still missing.

In an effort to unravel parts of this mechanism we recently solved the three-dimensional structure of the SakSTAR variant of recombinant Sak (Rabijns *et al.*, 1997). Unfortunately, owing to disorder, we could not observe the first 15 residues of this structure. However, since several biochemical studies have shown that residues 11–15 of Sak are crucial for plasminogen activation (Silence *et al.*, 1995; Gase *et al.*, 1996), knowledge of the exact fold of these residues could be of particular importance to the understanding of the mechanism of complex formation between Sak and plasmin and the subsequent plasminogen activation. In order to obtain more accurate electron-density maps for this functionally important part of the N-terminal region in Sak, residues 1–10 were removed in an attempt to stabilize residues 11–15. The still fully active Δ10Sak derivative (Makino, 1978; Collen *et al.*, 1992) was then subjected to crystallization experiments.

Furthermore, structural analysis of Δ10Sak and its comparison with the structure of full-length staphylokinase might provide an insight in the submolecular conformational changes upon plasmin-mediated N-terminal processing (*i.e.* deleting the first ten amino acids), which seems to be a prerequisite for plasminogen activation (Schlott *et al.*, 1997). Here, we describe the crystallization and the preliminary X-ray analysis of the two crystal forms which could be obtained with the Δ10Sak sample.

2. Production of Δ10Sak

Δ10Sak was prepared in a recombinant form by molecular-biology techniques in *Escherichia coli* as described below. Plasmid DNA was isolated using the BIO 101 RPM kit (Vista, CA). All other methods used for the construction of the expression vector pMEX-Δ10Sak have been described previously (Sambrook *et al.*, 1989). A Δ*10Sak* gene was constructed by the polymerase chain reaction (PCR), using Vent polymerase (New England Biolabs, Leusden, The Netherlands) and the expression vector pMEX.SakSTAR (Schlott *et al.*, 1994) as template. After a denaturation step of 3 min at 367 K, the fragment was amplified by cycling 30 times (1 s at 367 K, 1 s at 323 K, 10 s at 345 K) followed by a final elongation step of 2 min at 345 K, using the 5′-primer LY272 and the 3′-primer 818D.

The sequence of LY272, 5′-CCTCA-TATGAAAGGCGATGACGCGAGTTATTT TG corresponds to the sequence of residues 11–18 of SakSTAR (underlined) preceded by a methionine codon and a few vector nucleotides. The primer 818D, 5′-CAAAA-CAGCCAAGCTTCATTCATTCAGC, corresponds to the 3′ end of the staphylokinase gene in the pMEX.SakSTAR vector. The amplified product was purified, digested with *Hin*dIII

Table 1
Data-collection and reduction statistics.

Values in parentheses indicate data in the highest resolution shell.

	Type I crystals	Type II crystals
Wavelength used (Å)	1.54178	1.10
Resolution limit (Å)	2.4 (2.51–2.4)	2.6 (2.7–2.65)
Total observations	9151 (357)	5600 (185)
Unique reflections	4018 (325)	2787 (111)
Completeness of all data (%)	82.2 (67.0)	75.2 (69.4)
Completeness of data (%) ($I > 2\sigma$)	73.8 (46.1)	72.6 (61.9)
Mean I/σ	14.3 (2.1)	18.5 (10.0)
R_{sym} value (%)	7.01 (28.2)	4.6 (12.4)

and cloned in the *Stu*I–*Hin*dIII digested pMEX.SakSTAR vector (replacing the entire SakSTAR gene with the *Δ10Sak* gene). The sequence of the variant was confirmed by sequencing. The Δ10Sak variant was expressed from transformed *E. coli* WK6 cells grown in Terrific Broth (TB) (Sambrook *et al.*, 1989). A 4 ml aliquot of an overnight saturated culture in LB medium was used to inoculate a 2 l culture in TB containing 100 µg ml^{-1} ampicillin. The culture was grown for 20 h at 303 K and IPTG (200 µg) was added to boost the expression during the last 3 h of culture. The cells were pelleted by centrifugation, resuspended in 200 ml 0.1 *M* phosphate buffer pH 6.0 and disrupted by sonication at 273 K. The suspension was then centrifuged for 20 min at 20000 rev min^{-1} and the supernatant was stored at 277 K.

Recombinant Δ10Sak was purified by chromatography on a 1.6 × 6 cm column of SP-Sepharose, followed by a chromatography on a 1.6 × 8 cm column of phenyl-Sepharose. The Δ10Sak-containing fractions localized by SDS–PAGE were pooled for further analysis.

3. Crystallization and preliminary X-ray analysis of Δ10Sak

Screening for preliminary crystallization conditions was carried out using the sparse-matrix sampling method (Jancarik & Kim, 1991). The sample was first concentrated to a concentration of approximately 20 mg ml^{-1}. Hanging drops containing 1.5 µl protein solution and 1.5 µl precipitant solution were assembled and were set to equilibrate at 277 K using Linbro plates. At the start of crystallization optimization, the Δ10Sak sample gave crystals under conditions (type I) 0.2 *M* $Zn(OAc)_2$, 0.1 M Cacodylate buffer pH 6.5 and 18% PEG 8000 and under conditions (type II) 0.2 M $MgCl_2$, 0.1 M Tris buffer pH 8.5 and 35% PEG 4000. The latter crystallization condition was identical to that used to grow full-length Sak crystals (Rabijns *et al.*, 1997).

3.1. Type I crystals

The crystals grown from 0.2 *M* $Zn(OAc)_2$, 0.1 *M* cacodylate pH 6.5 and 18% PEG 8000 appeared to be good-quality crystals, but their size (0.1 × 0.05 × 0.05 mm) was not sufficient for further crystallographic analysis. Different strategies were followed in an attempt to obtain larger crystals (*e.g.* buffers were changed, different additives were tried), but only the replacement of cacodylate buffer pH 6.5 with Tris buffer at pH 7.5 yielded significantly larger crystals (0.2 × 0.2 × 0.2 mm). In this case, however, visual inspection of the crystals showed that the quality of the crystals had decreased. To overcome this problem, different additives were tested and, interestingly, the addition of glycerol, which has been shown in several studies to promote crystal packing (Arakawa & Timasheff, 1985; Sousa & Lafer, 1990), improved the appearance of the crystals considerably. After optimization, type I Δ10Sak crystals were grown using the hanging-drop vapour-diffusion technique in Linbro plates with a precipitant solution containing 0.2 *M* $Zn(OAc)_2$, 0.1 *M* Tris buffer pH 7.5, 18% PEG 8000 and 4% glycerol. The protein solution had a concentration of 40 mg ml^{-1} and the 3 µl drops were set to equilibrate at 277 K. Since the crystals were sensitive to radiation damage, flash-freezing with liquid nitrogen was used for all subsequent diffraction experiments. Prior to this flash-freezing, the crystals were placed for approximately 10 s in a cryoprotectant solution containing 0.2 *M* $Zn(OAc)_2$, 0.1 *M* Tris buffer pH 7.5, 18% PEG 8000 and 15% glycerol. Preliminary data collection was performed at 140 K using a Rigaku rotating-anode generator (λ = 1.54178 Å, 5 kW, graphite monochromator) and a P3 goniostat coupled to a Siemens X1000 area detector; for data reduction the programs *SADIE* and *SAINT* were used (Siemens Analytical X-ray Systems, 1993). From this, type I crystals were found to diffract up to 2.4 Å and they grow in space group *C*2 with unit-cell parameters a = 59.3, b = 43.0, c = 48.3 Å and β = 90.0°. The unit-cell parameters for these crystals were found to be different from those of full-length Sak crystals, which could be caused by a different packing of the protein molecules in the asymmetric unit. Other data-collection and reduction statistics are shown in Table 1.

3.2. Type II crystals: a deteriorated version of full-length SakSTAR crystals

The best type II crystals of Δ10Sak could be obtained using a hanging-drop vapour-diffusion setup at 277 K with a reservoir solution containing 0.2 *M* $MgCl_2$, 0.1 *M* Tris buffer pH 8.5 and 40% PEG 4000, a crystallization condition very similar to the crystallization condition of full-length Sak. Although initially no complete data set could be collected, owing to the presence of streaky reflection spots for certain crystal orientations (caused by irregular packing), it was possible to determine the space group (*C*2 space group with unit-cell parameters a = 60.5, b = 43.9, c = 50.0 Å and β = 106.8°) from the preliminary data. Finally, after screening of several crystals, a 75.2% complete data set to 2.6 Å with an R_{sym} of 4.6% was collected at the BW7B beamline of the DESY synchrotron (Hamburg, Germany). Data reduction was performed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Detailed statistics for this data set are listed in Table 1.

In conclusion, two crystal forms could be obtained from Δ10Sak samples. The cell parameters for both these crystal forms were found to be different from those of full-length Sak crystals, which could be the result of different molecular interactions. Data were collected on both forms and molecular-replacement tests to solve the Δ10Sak structure are under way.

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References

Arakawa, T. & Timasheff, S. N. (1985). *Methods Enzymol.* **114**, 49–77.
Collen, D., Silence, K., Demarsin, E., De Mol, M. & Lijnen, H. R. (1992). *Fibrinolysis*, **6**, 203–213.

Collen, D., Schlott, B., Engelborghs, Y., Van Hoef, B., Hartmann, M., Lijnen, H. R. & Behnke, D. (1993). *J. Biol. Chem.* **268**, 8284–8289.
Collen, D. (1998). *Nature Med.* **4**, 279–284.
Gase, A., Hartmann, M., Gührs, K. H., Röcker, A., Collen, D., Behnke, D. & Schlott, B. (1996). *Thromb. Haemost.* **76**, 755–760.
Grella, D. K. & Castellino, F. J. (1997). *Blood*, **89**, 1585–1589.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Lack, C. H. (1948). *Nature (London)*, **161**, 559–560.
Makino, T. (1978). *Biochim. Biophys. Acta*, **522**, 267–269.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Rabijns, A., De Bondt, H. L. & De Ranter, C. (1997). *Nature Struct. Biol.* **4**, 357–360.
Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd ed. New York: Cold Spring Harbor Laboratory Press.
Schlott, B., Gührs, K. H., Hartmann, M., Röcker, A. & Collen, D. (1997). *J. Biol. Chem.* **272**, 6067–6072.
Schlott, B., Hartmann, M., Gührs, K. H., Birch-Hirschfeld, E., Pohl, H. D., Vanderschueren, S., Van de Werf, F., Michoel, A., Collen, D. & Behnke, D. (1994). *Biotechnology*, **12**, 185–189.
Siemens Analytical X-ray Systems Inc. (1993). *Software Reference Manual.* Preliminary release. Karlsruhe, Germany.
Silence, K., Hartmann, M., Gührs, K. H., Gase, A., Schlott, B., Collen, D. & Lijnen, H. R. (1995). *J. Biol. Chem.* **270**, 27192–27195.
Sousa, R. & Lafer, E. M. (1990). *Methods*, **1**, 50–56.
Vanderschueren, S., Barrios, L., Kerdsinchai, P., Van de Heuvel, P., Hermans, L., Vrolix, M., De Man, F., Benit, E., Muyldermans, L., Collen, D. & Van de Werf, F. (1995). *Circulation*, **92**, 2044–2049.
Vanderschueren, S., Stockx, L., Wilms, G., Lacroix, H., Verhaege, R., Vermylen, J. & Collen, D. (1995). *Circulation*, **92**, 2050–2057.