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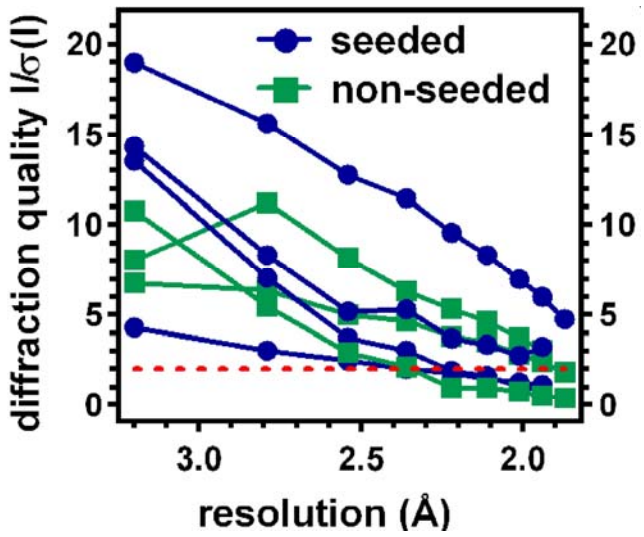
Supporting Information

69451 Weinheim, Germany

Time-Controlled Microfluidic Seeding in nL Droplets to Separate Nucleation and Growth Stages of Protein Crystallization[**]

Cory J. Gerdts[‡], Valentina Tereshko[†], Maneesh K. Yadav[§], Irina Dementieva[±#], Frank Collart[±], Andrzej Joachimiak[±], Raymond C. Stevens[§], Peter Kuhn[∞], Anthony Kossiakoff[†], Rustem F. Ismagilov[*‡]

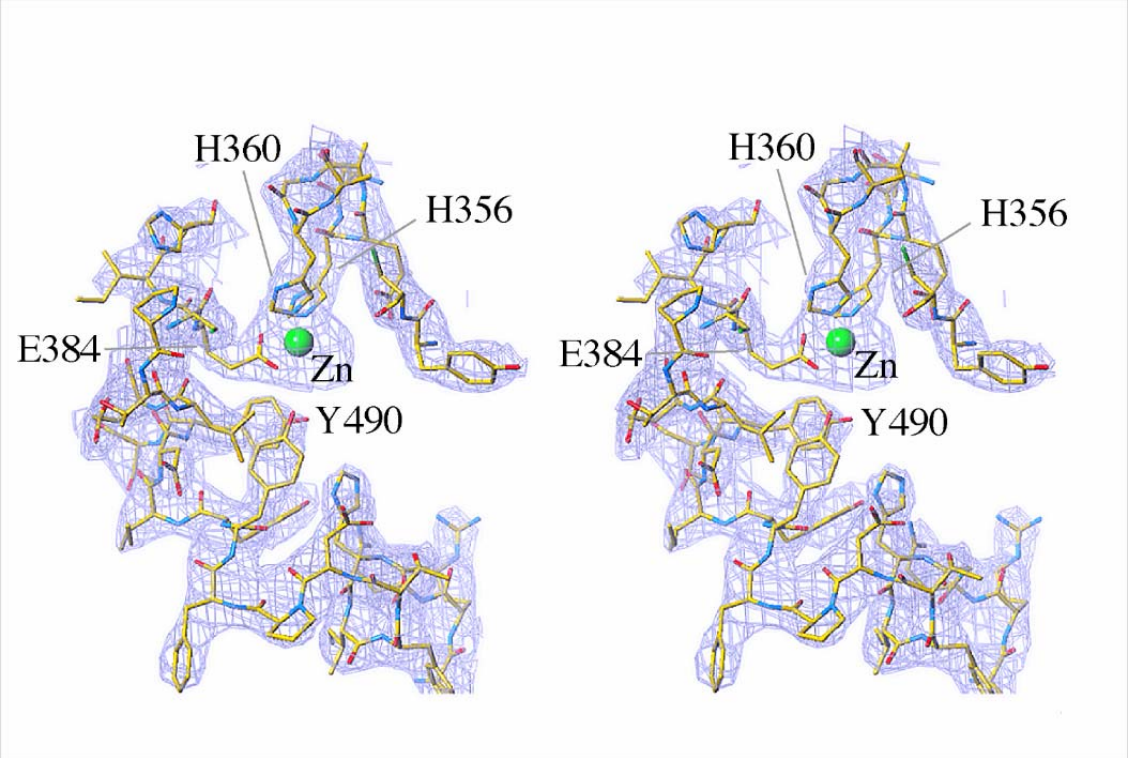
Supplementary Figure 1



Supplementary Figure 1. Comparing quality of seeded and non-seeded Thaumatin crystals. Each line represents one crystal. Data points represent the average signal-to-noise ratio [I/σ(I)] of reflections in the corresponding resolution shell. The red dotted line indicated a signal-to-noise ratio of 2. Data analysis was performed using HKL2000[1].

1. Otwinowski, Z. & Minor, W. in Macromolecular Crystallography, Pt A, Vol. 276 307-326 (ACADEMIC PRESS INC, San Diego; 1997).

Supplementary Figure 2.



Supplementary Figure 2. The stereo view of Sigma-A weighted 2F(obs)-F(calc) electron density map contoured at 1.5 σ level around the Zn-bound region in 3.1 Å X-ray crystal structure of Oligoendopeptidase F obtained by the microfluidic technique.

Experimental Details

Protein sample preparation. Thaumatin protein was purchased from Sigma. "SARS protein" was expressed and purified from E. coli. The cloning, expression and initial screening for crystallization conditions are described in the Supplementary Methods online. Oligoendopeptidase F (putative) from B*acillus stearothermophilus* is MCSG target APC36224. The cloning, expression, purification and screening for crystallization conditions are described in Supplementary Methods online.

Microfluidic Device Fabrication. Polydimethyl siloxane (PDMS) microfluidic devices were fabricated as described previously[1, 2]. For long-term storage, the PDMS devices were interfaced to glass capillaries from Hampton Research that had been surface treated with (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-tricholorsilane[2].

Seeding in Seconds. A two-stage PDMS microfluidic device was fabricated using two-step photolithography. The channel size in the first stage was 0.05 mm wide and 0.05 mm deep and 121.4 mm long. The channel size of the second stage was 0.2 mm wide and 0.2 mm deep. The solutions used in the nucleation stage were as follows: Protein - 65 mg/mL Thaumatin in 0.1 M ADA buffer pH 6.5; Precipitant - 2.0 M Na K tartrate in 0.1 M HEPES buffer pH 7.0; Carrier fluid - a combination of FC-3283 (3M) and 1H,1H,2H,2H-perfluorooctanol (PFO) (Alfa Aesar) at a ratio of 10:1. The flow rates for a nucleation time of 5 seconds were 0.60 μL/min each for the protein and precipitant and 2.40 μL/min for the carrier fluid. The flow rates for a nucleation time of 9 seconds were 0.34 μL/min each for the protein and precipitant and 1.36 μL/min for the carrier fluid. The solutions used in the growth stage were as follows: Protein - 15 or 25 mg/mL Thaumatin in 0.1 M ADA buffer pH 6.5; Precipitant - 1.0 M Na K tartrate in 0.1 M HEPES buffer pH 7.0; Carrier fluid - a combination of FC-3283 and PFO at a ratio of 50:1. The flow rates for a nucleation time of 5 seconds were 9.6 μL/min each for the protein and precipitant and 38.4 μL/min for the carrier fluid. The flow rates for a nucleation time of 9 seconds and also for the control experiments were 5.4 μL/min each for the protein and precipitant and 21.6 μL/min for the carrier fluid. Plugs were formed in stage one and flowed for 5 or 9 seconds (determined by the flow rates) and then combined with the plugs that were being formed in the second stage. A similar process has been described previously[3]. Plugs were collected in 0.2 mm OD glass capillaries (Glass Number 50 - Hampton Research), stored at 18° C and monitored over time.

Seeding in Days. For "SARS protein", a two-stage PDMS microfluidic device was fabricated by connecting two PMDS devices (the first being either 0.1 mm wide and deep or 0.2 mm wide and deep and the second being 0.2 mm wide and deep) with a long piece of 0.2 mm diameter Teflon tubing (Zeus). A third stream of water was used to separate the protein and precipitant until the plug formed. The solutions used in the nucleation stage were as follows: Protein - 4 mM SARS target "SARS protein" in 10 mM Tris-HCl and 100 mM NaCl, pH 7.8; Precipitant - 40.5% PEG 3350, 0.4 M NH_4NO_3, 0.15 M $MgCl_2$, 0.1 M NaAc pH 4.6; Carrier fluid - a combination of FC-3283 and PFO at a ratio of 10:1. The flow rate was 0.5 μL/min for the protein stream, 0.1-0.2 μL/min for the separating water stream, 0.3-0.4 μL/min for the precipitant stream and 1.0 μL/min for the carrier fluid. Once the plugs formed, the flow was stopped until clusters of seed crystals grew. The flow was restarted and flowed into the growth stage. The solutions used in the growth stage were as follows: Protein - 4mM "SARS protein" in 10 mM Tris-HCl and 100 mM NaCl, pH 7.8; Precipitant - 12.15% PEG 3350, 0.13 M

NH_4NO_3, 0.05 M $MgCl_2$, 0.33 M NaAc pH 4.6; Carrier fluid - a combination of FC-3283 and PFO at a ratio of 50:1. The flow rate was 0.5 μL/min for the stream of crystal seeds, 3.0 μL/min for the protein stream, 3.0 μL/min for the precipitant stream and 6.0 μL/min for the carrier fluid. Plugs were formed in stage one at 4° C, the plugs filled the Teflon tubing that connected the 2 PDMS devices, and they were stored in the Teflon tubing (still at 4° C) until seed crystals grew (1-3 days). Once seed crystals grew, the flow was restarted and the seed crystals were injected into the growth stage. The seeded plugs from the growth stage were collected in glass capillaries and stored at 4° C until single crystals grew within a week.

For Oligoendopeptidase F, traditional vapor diffusion trials were setup using 17 mg/mL protein and 60, 65, 70 and 90% tascimate solution in 0.1 M 1,3-Bis[tris(hydroxymethyl)methylamino]propane (Bis-Tris Propane) buffer, pH 7.0 (Hampton Research). Precipitation from a vapor diffusion trial was aspirated and flowed into a PDMS, microfluidic device. The precipitation was combined with 17 mg/mL protein and 90% tascimate solution to generate seeded Oligoendopeptidase F plugs at a final concentration of 8.5 mg/mL protein and 45% tascimate. These plugs were flowed into a 0.2 mm glass capillary for storage at 23° C. Small single crystals grew within 8 days. In order to grow larger crystals, the initial small crystals were used seed further trials[4] in 0.3 mm and then 0.6 mm glass capillaries at the same protein and precipitant concentrations. Two single crystals (~0.25 mm long and ~0.02-0.03 mm thick) from a 0.6 mm capillary yielded the X-ray diffraction data that was used in the structure solution and refinement.

X-ray diffraction Studies. Diffraction studies of seeded Thaumatin crystals versus non-seeded Thaumatin crystals were performed at room temperature while the crystals remained inside the capillaries in which they were grown. This eliminated any extra errors that could have been introduced during cryoprotection or flash freezing. Diffraction data were collected at BioCARS station 14 BM-C of the Advanced Photon Source (Argonne National Laboratory) using 5-second exposures, 1° rotations and an incident wavelength of 0.9 Å. The "SARS protein" crystals were tested at GM/CA Cat station 23ID-D of the Advanced Photon Source (Argonne National Laboratory).

Crystals of Oligoendopeptidase F grown by microfluidic technique were removed from the capillary by attaching a syringe to one end and pumping the crystals into a drop of cryoprotectant. The crystal cluster grown in the hanging drop was first transferred to cryoprotectant, and then a single crystal was separated from the cluster by cutting it off using crystal manipulation tools (Hampton Research). In both cases we used 20% glycerol, 48% tascimate in 0.1 M "Bis-Tris Propane" buffer, pH 7.0 as a cryoprotectant. The crystals were transferred to/from cryoprotectant using CryoLoops (Hampton Research) and flash frozen in liquid nitrogen. The X-ray diffraction experiments were performed at GM/CA Cat station 23 ID-D. The crystals grown by microfluidic and hanging drop techniques were isomorphous and belong to space group $P3_121$ with cell dimensions a = b = 119.5 Å, c = 250 Å. The crystals contained two protein molecules (564 amino acids, 14 Se-Met residues per molecule) in the asymmetric unit with ~70% solvent content. The X-ray diffraction experiments were performed at GM/CA Cat station 23 ID-D of the Advanced Photon Source (Argonne National Laboratory). In addition to very profound Se-peak, the excitation scan showed immediately the presence of Zn-ions in the structure. We used Se-peak energy (λ = 0.9793Å) X-ray data collected on the Se-Met labeled protein. The thickness of the crystal grown in microfluidic capillaries was 0.02-0.03 mm. However, owing to trigonal $P3_121$ symmetry and the sufficient crystal length (0.2 mm), we

were able to collect a complete (>98%) data set of Bijvoet differences. The first two reference frames taken at 0° and 90° indicated that the resolution limit is similar for microfluidic and hanging drop crystals and reaches ~2.9 Å. Anomalous X-ray data (> 98% complete) were collected at 100 K using 2-second exposure times and 0.5° or 1° oscillation widths. Owing to larger size of the crystal grown by hanging drop technique (crystal length & width 0.35mm x 0.05mm) we were able to use two separate parts of the crystal in order to collect two data sets using inverse beam geometry (90 frames with oscillation range 45° were used in the final scaling from each set). For smaller crystals grown by microfluidic technique (crystal length & width 0.2mm x 0.025mm) we have collected two data sets from two different crystals (120 frames with oscillation range 60° and 20 frames with oscillation range 20° for the first and second crystals, respectively). A Dali-search (http://www.ebi.ac.uk/dali/) based on the three-dimensional structure of Oligoendopeptidase F shows that it bears significant similarity to other zinc metallopeptidases: human testicular Angiotensin-converting enzyme (PDB code: 1O86),[5] *E.coli* Dipeptidyl Carboxypeptidase Dcp (PDB code: 1Y79),[6] neurolysin (PDB code 1I1I),[7] and *Pyrococcus furiosus* carboxypeptidase (PDB code 1K9X).[8] None of these structures display detectable sequence similarity to Oligoendopeptidase F.

The Oligoendopeptidase F structure was determined independently for X-ray data sets collected on crystals grown by microfluidic and hanging drop techniques using SAD technique. We have used anomalous X-ray data collected with 0.5° oscillation width from one single crystal for each technique. Owing to substantial radiation damage, the resulting resolution cutoff of X-ray data used for the structure determination was 3.3 Å and 3.0 Å for the crystals grown by microfluidic and hanging drop techniques, respectively. In the refinement we have used the scaled data from one (hanging drop, resolution 3.0 Å) or two (microfluidic, resolution 3.1 Å) crystals. Details of the data processing, structure determination and refinement are provided in Supplementary Methods online. The crystal data, SAD phasing and refinement statistics for crystals obtained from the two methods are compared in Supplementary Tables 1 and 2 online. The Sigma-A weighted 2F(obs)-F(calc) electron density map contoured at 1 σ around the Zn-bound region is shown in Supplementary Figure 2 online. Atomic coordinates and structure factors have been deposited into the PDB with ID 2H1J and 2H1N for the crystals grown by microfluidic and hanging drop techniques, respectively.

SARS nucleocapsid N-terminal domain construct design, cloning, expression, and purification. A construct corresponding to the N-terminal domain (residues 47-175; truncating the first 46 residues predicted to be disordered) of SARS nucleocapsid N-terminal domain (GenBank accession number NP_828858) was amplified by polymerase chain reaction (PCR) from genomic cDNA of the SARS-CoV Tor-2 strain using Taq polymerase and primer pairs encoding the 5' and 3' ends (forward: 5' – ATGAATTCAACTGTGCTTTCCTTCTGTGCTAGGGCCGGCC GGG – 3', reverse: 5' – CCCGGCCGGCCC TACTCTGCGTAGAAGCCTTTTGG – 3'. The PCR product was cloned into the expression vector pMH1f, a derivative of pBAD (Invitrogen). Expression in pMH1F is driven by the *araBAD* promoter, and the recombinant protein is produced with an N-terminal $Thio_6His_6$ tag (MGSDKIHHHHHH).

A sequence verified clone was transformed into the *E. coli* methionine auxotrophic strain DL41. An overnight culture from a fresh transformant was used to inoculate flasks of 2XYT-ampicillin media. The culture was grown at 37°C with vigorous shaking to an OD_{600} of 0.6 to 0.7, induced with 0.2% w/v L-arabinose, and grown at 14°C overnight. The cells were harvested

by centrifugation and lysed by sonication in buffer containing 50 mM potassium phosphate, pH 7.8, 300 mM NaCl, 10% glycerol, 5 mM imidazole, 0.5 mg/mL lysozyme, 100 µl/L benzonase and EDTA-free protease inhibitor (Roche; one tablet per 50 mL buffer). The lysate was clarified by ultracentrifugation at 45000 rpm for 20 min at 4° C and the soluble fraction applied onto a metal chelate column (Talon resin charged with cobalt; Clontech). The column was washed with 20 mM Tris, pH 7.8, 300 mM NaCl, 10% glycerol, 5 mM imidazole and eluted with 25 mM Tris, pH 7.8, 300 mM NaCl, 150 mM imidazole. The eluate was then purified by cation exchange on Poros HS column using a linear gradient of NaCl (0 – 1 M) in 25 Tris-HCl, pH 8.0 followed by size exclusion on a Superdex 75 column equilibrated with 10 mM Tris, pH 7.8, 150 mM NaCl. Pure fractions of SARS nucleocapsid N-terminal domainN were concentrated to 1 mM and either used immediately for crystallization trials or flash frozen in liquid nitrogen for later use.

Crystallization of SARS nucleocapsid N-terminal domain using vapor diffusion. SARS nucleocapsid N-terminal domainN was crystallized using the nanodroplet vapor diffusion method. Drops (100 nL 1 mM protein + 100 nL crystallant) were dispensed into 96-well low profile Greiner plates using an Innovadyne liquid handling robot. After ~1 week at 4° C, harvestable plate-like crystals (~200 µm x ~50 µm x ~20 µm) were obtained in 0.2 M ammonium nitrate, 12% PEG 3350, 0.15M magnesium chloride.

Oligoendopeptidase F protein cloning, expression, and purification. The open reading frame of putative Oligoendopeptidase F protein from B*acillus stearothermophilus* was amplified from genomic DNA with *KOD* DNA polymerase by using conditions and reagents provided by the vendor (Novagen, Madison, WI). The gene was cloned in pMCSG7 vector[9] by using a modified ligation-independent cloning protocol[10]. This process generated an expression clone producing a protein with TEV cleavable His_6-tag fused into N-terminus of the target protein and adds three artificial residues (SerAsnAla) on that end. The fusion protein was expressed in an *Escherichia coli* BL21 (DE3) – Gold (Stratagene) that harbored a plasmid pMAGIC encoding three rare *E. coli* tRNAs (Arg [AGG/AGA] and Ile [ATA]) as described earlier[11]. A selenomethionine (Se-Met) derivative of the expressed protein was prepared as described previously[12] and purified according to standard protocol[13]. The transformed BL21 cells were grown in M9 minimal media at 37°C. M9 medium is supplied with 0.4% sucrose, 8.5 mM NaCl, 0.1mM $CaCl_2$, 2 mM $MgSO_4$, and 1% thiamine. After OD_{600} reached 0.5, 0.01% (w/v) each of leucine, isoleucine, lysine, phenylalanine, threonine, and valine was added to inhibit the metabolic pathway of methionine and encourage Se-Met incorporation. Se-Met was then added at 6% (w/v), and 15 min later protein expression was induced by 1 mM isopropyl-β-D-thiogalactoside (IPTG). The cells were then incubated at 20°C overnight.

The harvested cells were resuspended in 5 volumes of lysis buffer (50 mM HEPES pH 8.0, 500 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol, and 5% v/v glycerol). The cells were lysed by sonication after the addition of inhibitors of proteases (Sigma, P8849) and 1 mg/mL lysozyme. The lysate was clarified by centrifugation at 30,000 *g* (RC5C-Plus centrifuge, Sorval) for 20 min, followed by filtration through a 0.45 µm filter and 0.22 µm filters (Gelman). Immobilized metal affinity chromatography (IMAC-I) using a 5-mL HiTrap Chelating HP column charged with Ni^{+2} ions and buffer-exchange chromatography on a HiPrep 26/10 desalting column (both Amersham Biosciences) were performed using AKTA EXPLORER 3D (Amersham Biosciences). His_6-tag was cleaved using the recombinant TEV protease expressed from the vector pRK508[14]. The protease was added to the target protein in a 1:30 ratio and the

mixture was incubated at 4° C for 48 h. A second Ni-NTA affinity chromatography was performed manually to remove the His_6-tag and His-tagged TEV protease using a 1-mL HiTrap Chelating column (Amersham Biosciences) charged with Ni^{+2} ions. The protein was dialyzed against 20 mM HEPES pH 7.2, 150 mM NaCl, 2 mM DTT, concentrated using a Centricon Plus-20 Centrifugal Concentrator (Millipore) to the concentration of ~17mg/ml and stored in aliquots of 50 μl at the temperature of liquid nitrogen. In addition to 564 naturally occurring amino acids, the Oligoendopeptidase F protein bears three artificial residues (SNA) on the N-terminal end.

Preliminary screening of crystallization conditions for Oligoendopeptidase F. The crystallization conditions for Oligoendopeptidase F protein were obtained with use of commercially available screens (Hampton Research and Emerald Biostructures; total of ~300 conditions were screened) and traditional vapor diffusion technique in hanging drops containing 1 μL of protein solution and 1 μL of crystallization reagent. The droplets were equilibrated at 19° C against the reservoir containing 1 mL of the crystallization buffer. The only condition that resulted in the crystal formation was the SaltRX condition #96 (0.1M "Bis-Tris Propane" buffer, pH 7.0, 60% Tascimate). The crystalline precipitate appeared in 3 days, and then long needle crystal clustered together grew from the precipitate reaching their maximum size in 7-10 days. The optimization of the crystallization conditions did not lead to the formation of single crystals using traditional hanging drop technique. However, one of the drops produced the cluster that had crystalline components that were significantly larger. We were able to cut one single crystal (~0.35 mm long and ~0.05 mm thick) and use it for further X-ray crystallographic study. Our extensive attempts to reproduce the conditions that lead to formation of the crystals of similar size failed.

Oligoendopeptidase F X-ray structure determination. The absorption edge of Se was determined by using an X-ray fluorescence scan of the protein drop, followed by examination of the fluorescence data by using CHOOCH[15]. All X-ray data were processed and scaled with HKL2000[16]. The structure was determined independently by the SAD technique for data sets collected on crystals grown by microfluidic (3.3 Å resolution) and hanging drop (3.0 Å resolution) techniques. Heavy-atom search was performed with SHELXD[17] against all Bijvoet differences up to a resolution of 4.0 Å. In both cases we found 28 sites that show off non-crystallographic 2-fold symmetry. This fact immediately indicated that we have two protein molecules in the asymmetric unit with the high (~70%) solvent content. The SAD phasing utilizing the anomalous signal from sites found in SHELXD was performed with SOLVE[18]. The density modification, non-crystallographic symmetry (NCS) averaging and initial polyalanine model building were performed with RESOLVE[19]. The model building and sequence assignment were completed manually using TURBO_FRODO[20]. In both cases two sites found during heavy-atoms search using anomalous signal collected at Se-edge turned to be Zn-ions (one ion per protein molecule).

Two crystals grown by microfluidic technique were used to obtain 3.1 Å resolution X-ray data set used in the final structure refinement. In the merging we have used 70 frames (35°) from crystal #1 and 20 frames (20°) from crystal #2. To refine the structure obtained by hanging drop technique we have used the same data set as for structure solution (3.0 Å resolution). In refinement the NCS restraints were applied between two molecules in the asymmetric unit. R_{free} was monitored by setting aside 5% of the reflection as a test set. Rigid-body, positional refinement and stimulated annealing were performed in CNS1.1[21]. The model was checked manually against a composite omit map calculated in CNS1.1. Both structures were refined

using maximum likelihood target using amplitudes and phase probability distribution. The positional and temperature factor refinement was completed with the program REFMAC 5.2[14] in CCP4[15]. Two rigid-body refinements of B factors were performed first by using the translation, libration, and screw-rotation (TLS) parameters. Each protein molecule was treated as one TLS rigid-body. The TLS refinement was accompanied by the restrained individual B-factor refinement. A Ramachandran plot calculated with PROCHECK[22] indicates that 99.8% of the non-Gly and non-Pro residues in the final models lie in the most favored and additional allowed regions. Electron density calculated at 1σ is well connected for all main-chain atoms. The density around Zn-bound region is shown in Figure S2. The crystal data, data collection, SAD phasing and refinement statistics for both types of crystals are compared in Supplementary Tables S1 and S2.

The root mean square deviations (rmsd's) for main chain atoms is less than 0.3 Å between the final models refined against X-ray data obtained from both types of crystals.
We have detected the difference electron density at >2.5σ level in the center groove of the molecule. This density appears three times and has pear-like shape. We have used tascimate (the Hampton Research proprietary mixture of organic acids) to crystallize Oligoendopeptidase F. It is likely that one of the organic acids or their mixture enters the groove in the solution and helps to stabilize the closed conformation of Oligoendopeptidase F in the crystal form. We did not attempt to interpret the difference density and used the unknown ligand type (UNL) with occupancy = 0 in the coordinate file 2H1N to indicate this fact.

Equipment and Settings. Figure 1, 2a, and 4 were drawn using Macromedia Freehand 10. Microphotographs in Figures 2, 3, 4 and 5 were compiled in Macromedia Freehand 10. Microphotographs in Figures 2a, 2b, 2c, 4, and 5 were taken using a SPOT camera and each was brightened across the entire image using Adobe Photoshop 6.0. The graphs in Figure 3 were constructed using IgorPro. Figure 6 was constructed using Pymol.

Supplemental Table 1

Table S1. Data collection and refinement statistics.

	Microfluidic seeding		Hanging drop
	Crystal #1	Crystals #1, 2 merged	
Data collection [a)]			
Space group	$P3_121$	$P3_121$	$P3_121$
Cell dimensions			
a, *b*, *c* (Å)	119.5, 119.5, 248.9	119.3, 119.3, 248.7	120.0, 120.0 , 249.8
α, β, γ (°)	90, 90, 120	90, 90, 120	90, 90, 120
Resolution (Å) [b)]	50- 3.3 (3.42-3.30)	50 – 3.1 (3.21-3.10)	50 – 3.0 (3.11-3.00)
R_{merge}	0.068 (0.28)	0.097 (0.39)	0.047 (0.36)
$I/\sigma I$	9.2 (2.3)	9.3 (1.9)	17.6 (2.1)
Completeness (%)	98.9 (98.8)	99.9 (100)	98.7 (98.3)
Redundancy	3.7(3.8)	3.4 (3.4)	5.6 (5.6)
Refinement			
Resolution (Å)	20 - 3.3	20 – 3.1	20 – 3.0
No. reflections	31069	37722	41581
R_{work}/ R_{free}	0.194 / 0.230	0.187 / 0.225	0.185 / 0.210
No. atoms			
Protein	9337	9337	9358
Zn^{+2} ion	2	2	2
Water	-	2	6
B-factors			
Protein	73.2	69.8	63.6
Zn^{+2} ion	73.2	76.0	63.5
Water	-	41.3	49.6

R.m.s deviations			
Bond lengths (Å)	0.011	0.011	0.010
Bond angles (º)	1.03	1.13	1.05

[a] One crystal was used in data collection, structure solution and refinement for hanging drop growing technique. Two crystals were used in the structure determination for microfluidic macroseeding.
[b] Highest resolution shell is shown in parenthesis.

Supplemental Table 2

Table S2. SAD phasing statistics [a] (Supplementary Information)

	Microfluidic seeding Crystal #1	Hanging drop technique
SHELXD Heavy-atom search		
Resolution (Å)	50 – 4.0	50 – 4.0
No. reflections: all/ E>1.2	13284 / 3005	15180 / 3349
Mean $\|E^2-1\|$	0.959	0.948
No. of sites	28	28
Correlation Coefficient : all/weak	49.9 / 20.8	45.0 / 20.4
SOLVE SAD Phasing		
Resolution (Å)	50- 3.3	50 – 3.0
Completeness (%) (F > 2♦)	88.6	91.7
Signal-to-noise ratio	1.7	2.3
Figure-of-merit	0.23	0.29
Phasing power	1.25	1.0

[a] One crystal was used in structure solution for both microfluidic macroseeding and hanging drop experiments.

Supplementary References

1. Song, H. & Ismagilov, R.F. Millisecond kinetics on a microfluidic chip using nanoliters of reagents. *J. Am. Chem. Soc.* **125**, 14613-14619 (2003).
2. Zheng, B., Tice, J.D., Roach, L.S. & Ismagilov, R.F. A droplet-based, composite PDMS/glass capillary microfluidic system for evaluating protein crystallization conditions by microbatch and vapor-diffusion methods with on-chip X-ray diffraction. *Angew. Chem.-Int. Edit.* **43**, 2508-2511 (2004).
3. Gerdts, C.J., Sharoyan, D.E. & Ismagilov, R.F. A synthetic reaction network: Chemical amplification using nonequilibrium autocatalytic reactions coupled in time. *J. Am. Chem. Soc.* **126**, 6327-6331 (2004).
4. Thaller, C. et al. Repeated Seeding Technique for Growing Large Single-Crystals of Proteins. *J. Mol. Biol.* **147**, 465-469 (1981).
5. Natesh, R., Schwager, S.L.U., Sturrock, E.D. & Acharya, K.R. Crystal structure of the human angiotensin-converting enzyme-lisinopril complex. *Nature* **421**, 551-554 (2003).
6. Comellas-Bigier, M., Lang, R., Bode, W. & Maskos, K. Crystal structure of the E-coli dipeptidyl carboxypeptidase Dcp: Further indication of a ligand-dependant hinge movement mechanism. *J. Mol. Biol.* **349**, 99-112 (2005).
7. Brown, C.K. et al. Structure of neurolysin reveals a deep channel that limits substrate access. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 3127-3132 (2001).
8. Arndt, J.W. et al. Crystal structure of a novel carboxypeptidase from the hyperthermophilic Archaeon Pyrococcus furiosus. *Struct. Fold. Des.* **10**, 215-224 (2002).
9. Dieckman, L., Gu, M.Y., Stols, L., Donnelly, M.I. & Collart, F.R. High throughput methods for gene cloning and expression. *Protein Expr. Purif.* **25**, 1-7 (2002).
10. Stols, L. et al. A new vector for high-throughput, ligation-independent cloning encoding a tobacco etch virus protease cleavage site. *Protein Expr. Purif.* **25**, 8-15 (2002).
11. Wu, R.Y. et al. Crystal structure of Enterococcus faecalis SlyA-like transcriptional factor. *J. Biol. Chem.* **278**, 20240-20244 (2003).
12. Walsh, M.A., Dementieva, I., Evans, G., Sanishvili, R. & Joachimiak, A. Taking MAD to the extreme: ultrafast protein structure determination. *Acta Crystallogr. Sect. D-Biol. Crystallogr.* **55**, 1168-1173 (1999).
13. Kim, Y. et al. Automation of protein purification for structural genomics. *Journal of Structure and Functional Genomics* **5**, 9 (2004).
14. Kapust, R.B. & Waugh, D.S. Controlled intracellular processing of fusion proteins by TEV protease. *Protein Expr. Purif.* **19**, 312-318 (2000).
15. Evans, G. & Pettifer, R.F. CHOOCH: a program for deriving anomalous-scattering factors from X-ray fluorescence spectra. *J. Appl. Crystallogr.* **34**, 82-86 (2001).
16. Otwinowski, Z. & Minor, W. in Macromolecular Crystallography, Pt A, Vol. 276 307-326 (ACADEMIC PRESS INC, San Diego; 1997).
17. Schneider, T.R. & Sheldrick, G.M. Substructure solution with SHELXD. *Acta Crystallogr. Sect. D-Biol. Crystallogr.* **58**, 1772-1779 (2002).
18. Terwilliger, T.C. & Berendzen, J. Automated MAD and MIR structure solution. *Acta Crystallogr. Sect. D-Biol. Crystallogr.* **55**, 849-861 (1999).
19. Terwilliger, T.C. Automated main-chain model building by template matching and iterative fragment extension. *Acta Crystallogr. Sect. D-Biol. Crystallogr.* **59**, 38-44 (2003).

20. Cambillau, C. & Roussel, A., Edn. Turbo Frodo version Open GL.1 (University Aix-Marseille II), Marseille, France; 1997).
21. Adams, P.D., Pannu, N.S., Read, R.J. & Brunger, A.T. Cross-validated maximum likelihood enhances crystallographic simulated annealing refinement. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 5018-5023 (1997).
22. Laskowski, R.A., Macarthur, M.W., Moss, D.S. & Thornton, J.M. PROCHECK - a Program to Check the Stereochemical Quality of Protein Structures. *J. Appl. Crystallogr.* **26**, 283-291 (1993).