Supplementary material for Ratnaswamy *et al*. (February 20, 2001) *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.041452598

Materials and Methods

Cloning. The DNA encoding WT human plasma gelsolin residues 134–250 and 134–266 was amplified with the PCR by using full-length plasma gelsolin cDNA (GenBank accession no. X04412) as the template. The primers to clone the 134–266 fragment from the full-length gelsolin cDNA were of the sequences 5'-AAAAAACATATGACTTCAA-GTCTGGCCTGAAGTACAAGA-3' (forward) and 5'-AAAAAAGGATCCTCAAAGC-ATCCTCCTTGGCGG-3' (reverse). Those for 134–250 were 5'-ATCGCTCATATGTT-CAAGTCTGGCCTGAAGTACAAGAAA-3' (forward) and 5'-AGACTAGGATCCTTA-TTACTTGGGGCCCAGCACCTGGAG-3' (reverse). Site-directed mutagenesis on the WT S2 and S2loop constructs was carried out by the Quickchange method (Stratagene) to convert Asp-187 into either a Tyr (G654T) or an Asn (G654A) residue. D187N mutations were introduced by using primers of sequence 5'-GTCTCAACAATGGCAACTGCTT-CATCCTGG-3' (forward) and 5'-CCAGGATGAAGCAGTTGCCATTGTTGAAGC-3' (reverse) and D187Y with 5'-GCTTCAACAATGGCTACTGCTTCATCCTGG-3' (forward) and 5'-CCAGGATGAAGCAGTAGCCATTGTTGAAGC-3' (reverse).

The PCR products were cloned into the *Nde*I/*Bam*HI restriction sites of a pET-3a vector (Novagen) and transformed into *Escherichia coli* BL21(DE3) pLysS cells (Stratagene) for protein expression.

Protein Expression. A 5-ml overnight culture of BL21 (DE3) Epicurean Gold *E. coli* cells containing the S2 plasmid of interest was used to inoculate 1.5 liters of LB containing chloramphenicol (34 μg/ml) and ampicillin (100 μg/ml). The cultures were incubated with shaking at 37 °C. When the optical density (600 nm) of the cultures reached 0.6 absorbance units, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce the translation of gelsolin. After 5–6 h, the cells were harvested at 5,000 rpm for 10 min by preparative centrifugation at 4°C. The cell pellets were resuspended in PBS (pH 7.4) and stored at –80°C.

Protein Purification. *Isolation of the inclusion bodies.* The cells from a 3-liter culture were thawed and incubated with 1.5 mg of lysozyme per ml of cell suspension for 30 min at 4°C. The resulting lysate was sonicated for 2 min and incubated at room temperature for 30 min with DNase (final concentration of 10 μg/ml). A solution of 60 mM EDTA, 1% Triton-X 100, and 1.5 M NaCl was added to the lysate (1 ml solution/1 ml of lysate), and the resulting solution was stirred on ice for 30 min. The insoluble material was collected by centrifuging the cell lysate at 18,000 rpm for 20 min at 4°C. The pellet was washed twice by resuspension in 40 ml of 0.1 M Tris•HCl, pH 8.0/20 mM EDTA and subjected to centrifugation as above.

Inclusion body solubilization. The pellet was incubated in 15 ml of 6 M GdmCl/0.1 M Tris•HCl (pH 8.0) at 25°C, in the presence of 100 mM DTT and 1 mM EDTA, and largely dissolved within 2 hrs. The pH of the solution was lowered to 3 by the dropwise addition of 1 M HCl, and the remaining insoluble cell debris was removed by

centrifugation. The DTT was removed by two dialysis steps against 500 ml of 4 M GdmCl and 10 mM HCl for 2 h at 25°C. To effect complete removal of DTT, the solution was also dialyzed overnight against 1 liter of 4 M GdmCl at 4°C. All the GdmCl solutions were sparged with N_2 for 20 min before use.

Refolding and oxidation. The concentration of the reduced denatured protein resulting from dialysis was determined by using the Coomassie Plus Protein Assay reagent (Pierce). Oxidation was carried out in a redox buffer composed of 0.1 M Tris•HCl (pH 8.0), 1 mM EDTA, 20% glycerol, 1 M GdmCl, 0.3 mM cystine, and 3 mM cysteine. The protein was rapidly diluted into an appropriate volume of the buffer (typically 2 liters) at 15°C to yield a final protein concentration of up to 75 μg/ml. The buffer was sparged with N_2 and preincubated at 15°C before use. The solution was stirred for 5 h at 15°C and then filtered through a 0.45-μm filter to remove large aggregates. The GdmCl was removed by diafiltration against a 2-liter solution of 20% glycerol in 0.1 M Tris•HCl (pH 8.0) at 4°C using an Amicon Ultrafiltration system (Model CH2/CH2A) equipped with an S3 spiral-wound membrane cartridge, during which the concentration of the protein remains unchanged. After diafiltration the solution was concentrated to 700 ml and filtered through a 0.2-μm filter.

Cation exchange chromatography. 350 ml of the filtered S2 solution was loaded onto a HiTrap SP 5 ml cation exchange column (Amersham Pharmacia) with a peristaltic pump at a flow rate of 5 ml/min on an AKTA FPLC system (Amersham Pharmacia). The protein was eluted at 5 ml/min by using a linear salt gradient from 0.2 M to 1 M NaCl over 20 column volumes in 50 mM sodium phosphate buffer at pH 7.4 . Gelsolin eluted when the NaCl concentration reached 0.6 M.

Gel filtration chromatography. The fractions containing gelsolin were pooled and concentrated to 15 ml. The protein was loaded onto a Superdex-75 XK 50 (Amersham Pharmacia) gel filtration column having a fractionation range of 10 to 75 kDa. The protein was eluted from the column with PBS at pH 7.4 at a flow rate of 5 ml/min, and the purity established by SDS/PAGE analysis was >98%.

WT and the two FAF variants of both constructs were expressed and purified by using the above procedure. The D187Y S2 construct was prone to aggregation even in the presence of glycerol and was therefore not suitable for biophysical characterization.

Determination of Protein Concentration. The molar extinction coefficients at 280 nm (ε_{280}) of all the gelsolin constructs were determined spectrophotometrically by using the procedure described by Pace *et al.* (1).

Reverse-Phase HPLC and Mass Spectrometry Analysis. Two samples (200 μl) of each gelsolin construct (25 μM) were prepared in PBS at pH 7.4. One sample of each pair was treated with 30 mM reduced DTT. The samples were incubated at room temperature for 30 min before analysis. All the samples were chromatographed on a C_{18} reverse-phase analytical column (Vydac, Hesperia, CA, 4.6 mm ID × 250 mm l) by using a 5–80% linear gradient of solvent B over 45 min employing absorbance detection at 280 nm. (Solvent A: 94.9% water, 5% acetonitrile, 0.1% TFA. Solvent B: 94.9% acetonitrile, 5%

water, 0.1% TFA.) The eluted peptides were collected, dried in a speed vac (Savant Instruments), and subjected to electrospray mass spectrometry analysis.

Gel Filtration Assay for Domain 2 Function. The gel filtration assay outlined by Heiss and Cooper was used to evaluate phosphatidylinositol 4,5-bisphosphate (PIP_2) binding to the refolded gelsolin constructs (2). The PIP_2 molecule is amphiphilic and forms micelles in aqueous solutions above a concentration of 86 μM. The micelles have a relative molecular mass of 93 kDa (aggregation number of 82). The PIP_2 micelles were prepared by dissolving 1 mg of the lyophilized potassium salt of PIP_2 in 1 ml of water (100 μM) followed by sonication for 2 min. The purified gelsolin construct of interest (100 μl of a 25 μM solution) was added to 100 μl of the PIP_2 micelle solution and incubated on ice for 30 min. The resulting 200-μl solutions were chromatographed on a Superose 12 HR 10/30 gel filtration column (Amersham Pharmacia) on an AKTA FPLC system. The column was calibrated by using proteins of known molecular masses (RNase A, MW 13 kDa & Albumin, MW 67 kDa). In addition, the elution profiles of the gelsolin proteins without ligand were obtained. Fractions (0.5 ml) were collected, dried in a SpeedVac, and analyzed for the presence of gelsolin by Coomassie staining of SDS/12.5% PAGE gels.

Ultracentrifugation Analysis. Sedimentation equilibrium data were collected on a temperature-controlled Beckman XL-I analytical ultracentrifuge (Beckman Instruments) equipped with an An-60Ti rotor and a photoelectric scanner. The protein samples (25 μM) were loaded into a double sector cell equipped with a 12-mm Epon centerpiece and quartz/sapphire optical windows.

All data were monitored at 280 nm with a step size of 0.001 cm and a rotor speed of 20,000 rpm. Samples came to equilibrium (as determined by overlaying duplicate scans taken at 3-h intervals) after 24 h of centrifugation. Twenty scans were averaged for the final data set of the centrifugation run. The resulting data was regressed by using nonlinear least-squares regression on the ORIGIN software package (Microcal Software, Northhampton, MA). The data best fit to a single ideal species model described by Eq. **6** (3).

$$A_r = \exp[\ln(A_0) + (M\omega^2(1-\bar{\upsilon}\rho)/2RT)(x^2 - x_0^2)] + E \qquad \textbf{[6]}$$

A_r is the sample absorbance at radius x; A_0 is the absorbance at a reference radius x_0; $\bar{\upsilon}$ is the partial specific volume of the protein (ml/g); ρ is the density of the solvent (g/ml); ω is the angular velocity of the rotor (radian s^{-1}); E is a baseline error correction factor; M is the molecular weight; R is the universal gas constant (8.314×10^7 erg/mol); and T is the temperature (K). The partial specific volume $\bar{\upsilon}$ of all constructs of gelsolin was calculated based on the amino acid sequence according to the method of Cohn and Edsall calculated by using the SEDNTERP program (4). The value used for ρ was 1.0 g/ml. The goodness of fit was evaluated on the basis of the randomness and the magnitude of the residuals, expressed as the difference between the theoretical curve and the experimental data, and by checking the parameters against physical reasonability.

References:

1. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G. & Gray, T. (1995) *Protein Sci.* **4,** 2411–2423.

2. Heiss, S. G. & Cooper, J. A. (1991) *Biochemistry* **30,** 8753–8758.

3. Ralston, G. (1993) *Introduction to Analytical Ultracentrifugation* (Beckman Instruments, Fullerton, CA).

4. Philo, S. J. (1994) *Modern Analytical Ultracentrifugation* (Birkhauser, Boston).