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CRYSTALLIZATION NOTES

Purification, Characterization and Crystallization of *Acanthamoeba* Profilin Expressed in *Escherichia coli*

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Profilin (isoform I) from *Acanthamoeba castellani* was expressed in *Escherichia coli* using a bacteriophage T7-based expression vector. The recombinant material is similar to authentic profilin from *Acanthamoeba*-based on fluorescence monitored urea denaturation, circular dichroism, actin-nucleotide exchange rate and the K_d for rabbit skeletal actin. This recombinant material crystallized from 80% saturated sodium potassium tartrate, yielding monoclinic crystals, space group $C2$, $a = 91{\cdot}4$ Å, $b = 37{\cdot}4$ Å, $c = 34{\cdot}7$ Å, $\beta = 109{\cdot}6°$. These crystals contain one molecule in the asymmetric unit and diffract to 2·0 Å.

Keywords: profilin; cytoskeleton; crystallization; expression; protein structure

Profilins are small monomeric proteins, 12 to 15 kDa, found in vertebrates, invertebrates, fungi and plants (reviewed by Haarer & Brown, 1990). Profilin was originally described as a G-actin-binding protein (Carlsson *et al.*, 1977) and ascribed the function of regulating the equilibrium between G and F-actin (reviewed by Pollard & Cooper, 1986). *In vitro* experiments demonstrate that profilin can increase the rate of actin-nucleotide exchange in a catalytic fashion (Mockrin & Korn, 1980; Nishida, 1985; Goldschmidt *et al.*, 1991*a*) and thus may control the steady-state concentration of various actin-nucleotide species. This finding is significant in view of the fact that the rate of monomer addition to an actin filament is dependent upon the identity of the bound nucleotide (Pollard & Cooper, 1986). In addition, *in vitro* studies show that profilin can bind to micelles and vesicles containing phosphatidyl inositol phosphates (e.g. PIP and PIP_2) (Goldschmidt-Clermont *et al.*, 1990; Machesky *et al.*, 1990). Furthermore, PIP_2 dissociates the actin–profilin complex (Lassing & Lindberg, 1985; Schutt *et al.*, 1989), providing a possible link between the phosphoinositide signaling pathway and the actin-based cytoskeleton. Recent *in vitro* reconstitution studies indicate that profilin might be directly involved in the phosphoinositide pathway by controlling the catalytic activity of phospholipase C_γ (Goldschmidt-Clermont *et al.*, 1991*b*).

Isoform I of profilin (PI‡) from *Acanthamoeba castellani* (Reichstein & Korn, 1979) was over-expressed using a modification of the bacteriophage T7 expression system. Appropriate restriction sites were introduced into the coding sequence of PI by PCR and the modified DNA was ligated into the *Nde*I-*Stu*I sites of pMW172 (Way *et al.*, 1990). This construct was transformed into the *Escherichia coli* strain BL21(DE3), which harbors a lysogen containing the T7 polymerase under IPTG-inducible control. Protein was purified by a modification of the published procedure used for authentic profilins (Kaiser *et al.*, 1989). Briefly, cells were grown in LB containing 100 μg/ml ampicillin until the culture reached an A_{600} between 0·8 and 1·4, at which time IPTG was added to a final concentration

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‡ Abbreviations used: PI, *Acanthamoeba* profilin isoform I; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; TK8, 10 mM Tris, 40 mM KCl (pH 8·0).

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of 0·6 mM. Cells were harvested by centrifugation (8000 r.p.m., 11,325 *g*, 10 min, Beckman JA-10 rotor). The cell pellet was resuspended in 8 M urea 100 mM Tris, 40 mM KCl, 1 mM PMSF (pH 8·0) and sonicated. This solution was centrifuged for 60 minutes at 37,000 r.p.m. in a Ti-45 rotor. The resulting supernatant was dialyzed against 10 mM Tris, 40 mM KCl, pH 8·0 (TK8). During dialysis varying degrees of precipitation occurred and the solution was cleared by centrifugation. The supernatant was applied to a DE-52 (Whatman) column equilibrated in TK8. The flow through was subsequently applied to a poly-L-proline affinity column equilibrated in TK8. After loading was complete the column was washed with two volumes of TK8 and then TK8 containing 3 M urea. The profilin was eluted with TK8 containing 8 M urea. Urea was removed by dialysis against TK8. Profilin was generally greater than 99% pure as judged by SDS-PAGE. The final yield ranged from 50 to 100 mg/l of culture. When necessary the profilin was concentrated to 5 to 10 mg/ml by Amicon ultrafiltration.

Biophysical assays of recombinant profilin show that it is comparable to bona fide material isolated from *A. castellani*. Circular dichroism spectra of recombinant and authentic profilin were collected on an AVIV spectropolarimeter from solutions containing 0·1 mg/ml protein in TK8 at 22°C. These spectra are virtually identical, indicating similar secondary structural composition. Urea denaturation of authentic and recombinant profilin was monitored by fluorescence (exciting at 280 nm and monitoring at 340 nm). The assays contained 10 μM profilins in TK8 and various concentrations of urea at 22°C. Both authentic and recombinant profilins showed a mid-point for the urea induced transition at approximately 3·5 M.

The recombinant profilin is also indistinguishable from authentic material, on the basis of functional assays. The rate enhancement of actin-nucleotide exchange was measured by following the signal of the fluorescent nucleotide analog etheno-ATP (Goldschmidt-Clermont *et al.*, 1990). Assays were initiated by adding a small volume of G-actin (final concentration 2 μM) to a solution of 10 mM Tris, 0·1 mM $CaCl_2$ (pH 8·0) containing 50 μM etheno-ATP, 5 μM profilin. The binding and dissociation of etheno-ATP was followed by recording the fluorescence as a function of time, exciting at 365 nm and monitoring at 410 nm. In the absence of profilin, the half-life characterizing the process of etheno-ATP binding to actin is approximately 1500 seconds. Under the conditions of this assay, the rate of this process increased by almost an order of magnitude ($t_{\frac{1}{2}} = \sim 160$ s) after the addition of either authentic or recombinant PI. The addition of 1 mM ATP caused the dissociation of etheno-ATP from G-actin with $t_{\frac{1}{2}}$ of 24 s. Both profilins accelerate this process about eightfold.

The K_d for the complex of recombinant profilin with rabbit skeletal actin monomers were determined using pyrene-labeled actin, which shows a significant change in fluorescence upon formation of the complex (Lee *et al.*, 1988). Scatchard analysis yielded a K_d of 3·5 μM, which is in good agreement with values determined by a number of techniques for other profilins (Kaiser *et al.*, 1986; Goldschmidt-Clermont *et al.*, 1991*a*).

The rationale for producing the *Acanthamoeba* profilins in *E. coli* was motivated, in part, by the difficulty encountered with crystals of the authentic *Acanthamoeba* profilin (Magnus *et al.*, 1986). This protein crystallizes from ammonium sulfate in the monoclinic space group $C2$, $a = 110·4$ Å, $b = 31·7$ Å, $c = 33·5$ Å, $\beta = 112·2°$. These crystals have one molecule in the asymmetric unit and diffract to better than 2·0 Å. It has been difficult to obtain good heavy-atom derivatives, perhaps because this small protein has no sulfur (i.e. no Cys or Met) and only a single histidine. Site-directed mutagenesis would allow for the introduction of unique cysteines, which could yield interpretable single-site mercury derivatives (Forest & Schutt, 1992). Interestingly, wild-type recombinant profilin is resistant to crystallizing from ammonium sulfate, even with seeding. However, a new crystal form was obtained by hanging-drop vapor diffusion. The recombinant profilin (5 to 10 mg/ml) in 10 mM Tris, 40 mM KCl (pH 8·0) was combined with an equal volume (5 to 10 μl) of unbuffered 1·5 M sodium potassium tartrate and equilibrated over a well of 1·5 M sodium potassium tartrate at 18°C. These crystals are monoclinic, space group $C2$, $a = 91·4$ Å, $b = 37·4$ Å, $c = 34·7$ Å, $\beta = 109·6°$, and can only be grown reproducibly with seeding. Crystals begin to appear after one or two days, and reach maximum dimensions of 0·5 mm × 0·5 mm × 0·2 mm in about two weeks time. These crystals contain a single molecule in the asymmetric unit and diffract to at least 2·0 Å. Although crystals are readily obtained, it has been difficult to produce diffraction quality crystals, as the vast majority have proven to be severely twinned. Despite this difficulty, a native data set has been collected to 2·5 Å ($R_{sym} =$ 5·3%) using a Siemans area detector with a Rigaku Ru-200 rotating anode generator. Interestingly, we have been unable to crystallize the authentic profilin in this new form.

The different crystal forms might be explained on the basis of different covalent modifications. In PI, lysine 103 is trimethylated, a modification that does not take place in *E. coli*. Furthermore, amino acid composition and sequencing shows that approximately 90% of the recombinant profilin has a blocked amino-terminal methionine, which is absent from the authentic material. One of these modifications (or perhaps an unidentified one) may be responsible for the different crystal forms. Despite these differences, the physical and functional properties of the recombinant profilin described above demonstrate that it is a relevant species with which to conduct structural studies.

Three mutants have been produced in which a serine residue has been changed to a cysteine (S76C, S87C, S93C). Two of these mutants, S76C and S93C,

have been shown to react stoichiometrically with the thiol titrant *p*-chloro-mercuribenzoate, indicating that the cysteines are exposed and reactive (Boyer, 1954). These same two mutants have been purified and produce crystals with a morphology similar to that of wild type. Concurrent with this mutagenic work, ethyl mercury phosphate and $K_2Au(Cl)_4$ have been identified as potential heavy-atom derivatives and are being pursued.

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