Supporting Online Material

Supporting Material 1: Composition of Cell Culture Broths

Proteins used in this work were expressed in bacteria grown in media similar in composition, but not identical to standard Luria-Bertani broth or M9a media. The media referred to as modified Luria-Bertani broth contains 12 g tryptone, 10 g NaCl, 1 g glucose and 5 g yeast extract per liter of broth. LBH broth contains 10 g tryptone, 5 g NaCl, 1 ml of 1 N NaOH and 5 g yeast extract per liter of broth. The version of M9a media used for these experiments contains 7 g $Na_2HPO_4 \cdot 7H_2O$, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl mixed with one liter of water and autoclaved, followed by sterile addition of 10 mL 20% glucose, 0.4 mL 0.25 M $CaCl_2$, 1 mL 1 M $MgSO_4$, and 2 mL 0.5 mg/mL thiamine.

Supporting Material 2: Production of L99A, L99G/E108V, and A98L mutants

The L99A, L99G/E108V, and A98L mutants were expressed and purified by a modified version of the protocol described by Poteete, *et al*. (*1*). For clarity, the entire procedure is listed here. *E. coli* strain RR1 containing lysozyme-producing plasmids were streaked from frozen cultures onto modified LB-agar plates containing 100 μg/mL ampicillin. Single colonies were used to inoculate 100 mL cultures of LBH broth (Supporting material 1) containing 200 μg/mL ampicillin, and were incubated overnight at 32° C. The 100 mL culture was diluted into four 1 L cultures of LBH broth in Fernbach flasks with aeration. Protein expression was induced by an IPTG concentration of 180 mg/L at an optical density of 0.6. After 90 minutes the cultures were pelleted and resuspended in 100 mL of 50 mM Tris-HCl, pH 7.5, 10 mM Na_3HEDTA, 0.1% triton X-100 buffer with 1 protease inhibitor tablet (Complete Mini, EDTA-free Protease Inhibitor Cocktail Tablets,

Roche Applied Science, Indianapolis, IN, USA). The viscosity due to the bacterial genomic DNA was reduced by DNase I treatment, sonication or the use of a French press. The products from the DNase I treatment were removed by dialysis (Spectra/Por 10 kDa MWCO, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA). The dialyzed solution was then loaded onto a 2.5 x 5 cm CM-sepharose column, pre-equilibrated with 50 mM Tris-HCl, 1 mM sodium EDTA, pH 7.25 and gradient eluted using a 0 to 0.3 M NaCl gradient in the same buffer. Proteins were dialyzed into 0.025 M $NaPO_4$, pH 5.8, then concentrated using SP sephadex and stepped off with 0.55 M NaCl, 0.1 M $NaPO_4$, 1 mM sodium EDTA, 0.01% sodium azide, pH 6.6, and stored in the same buffer (*1*).

Supporting Material 3: Production of Seleno-methionine Containing Mutants

The V149G mutant was isolated using a modified version of the inclusion body protocol described by Vetter *et al*. (*2*). Protein expression was induced at 37 ˚C. Following expression, the culture was pelleted by centrifugation at 4,700 x g. The bacterial pellet was resuspended in a buffer composed of 50 mM Tris-HCl, 10 mM sodium EDTA, pH 8.0 plus 1 protease inhibitor tablet (Roche) and then sonicated. The resuspended mixture was then centrifuged for 30 minutes at 27,200 x g to pellet the cellular debris. The pellet was resuspended in 50 mM Tris-HCl, 10 mM sodium EDTA, 50 mM NaCl, 1 mM phenyl methane sulfonyl fluoride, 2.5 mM benzamide, 0.1 mM DTT, pH 8.0 (*2*). One tenth of the volume of the resuspension buffer of 2% Triton X-100 in 50 mM Tris, 10 mM sodium EDTA, pH 8.0 was added to the resuspended bacterial pellet solution (*2*). The suspension was stirred overnight at 4 ˚C, and then centrifuged at 27,200 x g for 30 minutes. Ten volumes of 2.5% octyl-β-D-glucopyranoside in 50 mM

Tris, 10 mM sodium EDTA, pH 8.0, per apparent volume of pellet were added and the suspension was stirred at 4 ˚C for 4 hours. Following centrifugation at 27,200 x g for 30 minutes, the pellet was washed with double-deionized water. The pellet was then suspended in freshly made 4 M urea (unbuffered, pH 6 to 7). The pH of the urea solution was lowered to 3-3.5 by the addition of 10 mM glycine, 2 N phosphoric acid, then mixed well for several minutes, and centrifuged at 12,000 x g for 15 minutes. The supernatant, containing the protein, was dialyzed against a 50 mM citrate, 10% glycerol, pH 3.0 buffer at 4 ˚C overnight, and then dialyzed against a 50 mM citric acid buffer, 10% glycerol, pH adjusted to 5.5 using NaOH at 4 ˚C for 8-16 hours. The solution was centrifuged at 12,000 x g for 25 minutes. The procedure yields at least 40 mg of protein, estimated to be approximately 99 % pure.

Supporting Material 4: Production of Seleno-methionine Containing Mutants

A seleno-methionine containing variant of the L99A mutant (Se-Met L99A) was prepared in a methionine deficient, seleno-methionine rich growth medium using an adaptation of the procedure by Van Duyne *et al*. (*3*). Frozen stocks of *E. coli* strain RR1 (*4*) containing an L99A expressing plasmid were streaked onto modified LB-ampicillin agar plates and grown overnight. Single colonies were used to inoculate 200 mL modified LB cultures containing 200 μg/mL ampicillin. The 200 mL cultures were grown for approximately 8 hours at 37 ˚C. Each 200 mL culture was used to inoculate a 1.25 L culture of M9a media (7 g $Na_2HPO_4 \cdot 7H_2O$, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl mixed with one liter of water and autoclaved, followed by sterile addition of 10 mL 20% glucose, 0.4 mL 0.25 M $CaCl_2$, 1 mL 1 M $MgSO_4$, and 2 mL 0.5 mg/mL thiamine). Each 1.25 L culture was shaken at 250 rpm at 37 ˚C overnight. The next day, each 1.25 L

culture was transferred to a 4 ˚C cold room for 30 minutes. Methionine biosynthesis was halted by adding 50 mL of M9a media containing an amino acid mixture of 100 mg lysine hydrochloride, 100 mg threonine, 100 mg phenylalanine, 50 mg leucine, 50 mg isoleucine and 50 mg valine to each 1.25 L culture. The 1.3 L cultures were shaken at 30 ˚C for approximately 30 minutes at 150 rpm. Next 50 mg/L of L-(+)-selenomethionine (Anatrace, Maunee, OH) was added and protein expression was induced by addition of 45 mg of IPTG per 1.3 L culture. Induction was allowed to proceed for 4 hours.

Following protein expression the cell cultures were pelleted at 5,000 x g for 10 minutes. The cell pellet was resuspended in 50 mM NaCl, 50 mM Tris pH 7.5 buffer with 15 mM methionine to prevent oxidation, and lysed by sonication for 7 minutes. The cellular debris was pelleted by centrifugation at 17,000 x g for 20 minutes. The supernatant was loaded onto a column containing a 2.5 cm bed of CM sepharose. This procedure yields approximately 70 mg of Se-Met L99A per 4 L of culture.

Supporting References

1. Poteete, A. R., Sun, D.-P., Nicholson, H. and Matthews, B. W. (1991) Second-site revertants of an inactive T4 lysozyme mutant restore activity by restructuring the active site cleft, *Biochemistry 30*, 1425-1432.

2. Vetter, I. R., Baase, W. A., Heinz, D. W., Xiong, J.-P., Snow, S. and Matthews, B. W. (1996) Protein structural plasticity exemplified by insertion and deletion mutants in T4 lysozyme, *Protein Sci. 5*, 2399-2415.

3. Van Duyne, G. D., Standaert, R. F., Karplus, P. A., Schreiber, S. L. and Clardy, J. (1993) Atomic structures of the human immunophilin FKBP-12 complexes with FK506 and rapamycin. *J. Mol. Biol. 229*, 105–124.

4. Muchmore, D. C., McIntosh, L. P., Russell, C. B., Anderson, D. E. and Dahlquist, F. W. (1989) Expression and nitrogen-15 labeling of proteins for proton and nitrogen-15 nuclear magnetic resonance. *Methods Enzymol. 177,* 44–73.