

Supplementary Material

Supporting Online Material for:

Structural adaptation of a thermostable biotin-binding protein in a psychrophilic environment

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The PDF includes:

Supplementary Table 1

Supplementary Figures 1 and 2

Supplementary Experimental Procedure of the expression , purification, crystallization and data collection.

Supplementary Table 1: Primers used for mutagenesis of rhizavidin and shwanavidin.

Mutant	Sequence 5' - 3'
Shwanavidin F43A	cta tat taa tcg tgc ggc ggg cgc tgc gtg cca gaa cag
Shwanavidin C45A	ggg ctt tgc ggc cca gaa cag ccc gta tcc g
Shwanavidin C74A	gcg tgg aaa gcg cca aca gca tta cca gct gga g
Rhizavidin C50A	ccc agg gca ccg gag ccc aga act cgc c
Rhizavidin C79A	aac aat tcg acg gag aac gcc aat tcc gca acc gga tg

Protein	Vol. (ml)	MW (kDa)
Aldolase (A)	12	156
BSA (B)	12.77	67
Ovalbumin (O)	13.52	43
Chymotrypsin	14.9	25
Shwanavidin W.T. +biotin	14.55	26.2
Shwanavidin F43A +biotin	14.63	25.0
Shwanavidin C45A/C74A +biotin	14.7	24.0
Ribonuclease A (R)	15.16	13.7
Fasciculin (F)	17	7

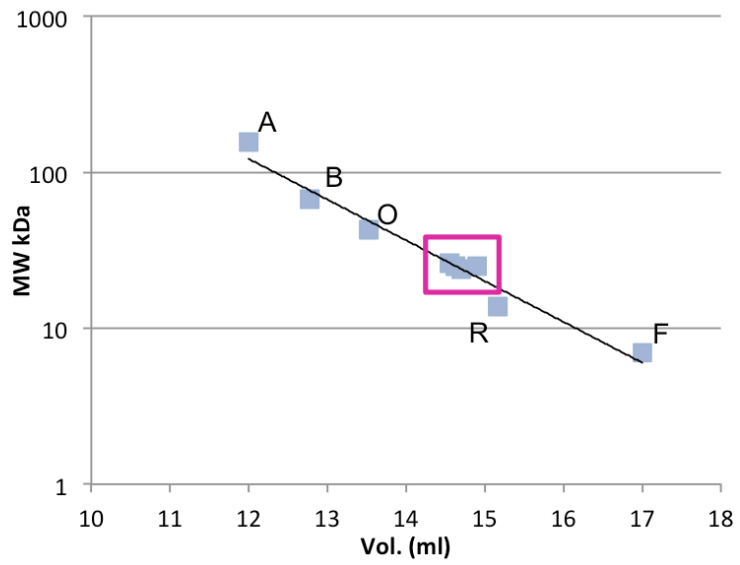


Figure 1: Gel filtration profiles of the shwanavidin (wild type and mutants) in comparison to other proteins with known size using a Superdex 75 analytical column (right) where the molecular weights were plotted versus the elution volume. Several proteins listed in the left table were used as molecular weight markers for calibration. It is obvious that all shwanavidins (indicated by the magenta box) examined have a molecular weight corresponding to a dimeric structure.

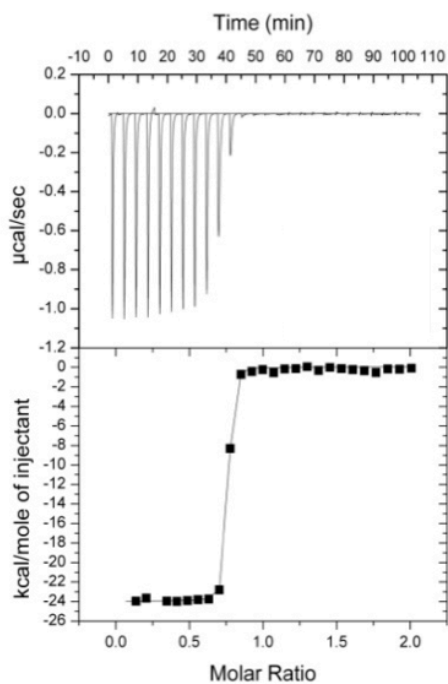


Figure 2: Biotin-binding studies of shwanavidin by ITC: The binding enthalpy (ΔH) of shwanavidin–biotin interaction at 25° was -23.96 kcal/mol and entropy was highly negative. Measuring the binding constant was beyond instrument’s sensitivity, and K_a could not be calculated. Yet, the saturation of shwanavidin by biotin is clearly observed in the titration curve. Each point in the graph indicates a 10 μ l aliquot of 120 μ M biotin titrated into the protein solution, resulting in change of μ calories over time. The molar ratio (x-axis) indicates the degree of saturation, and reached 0.73.

Experimental Procedure

Expression and Purification of wild type, C45A/C74A and F43A mutants of shwanavidin

His-shwanavidin was expressed in *E.coli*, strain BL21 DE3 pLysS (Novagen), which was cultured in 2xYT medium (consisting of 16 gr of trypton, 10gr of yeast extract, and 5gr of NaCl in 1 liter) as well as 100 µg/ml ampicillin (Sigma-Aldrich) and 34µg/ml chloramphenicol at 23°C, with rotation (225 rev./min). The 2xYT medium also contained autoinduced medium¹ (AM) (NPS: (50mM KH₂PO₄, 50mM Na₂HPO₄, 25mM (NH₄)₂SO₄), 5052: (0.05% glucose, 0.5% glycerol, 0.2% α-lactose), and 1mM MgSO₄) in order to increase expression. When OD ($\lambda=600$ nm) reached a value of 0.7, 0.4 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) was added for induction. Cultivation was continued for approximately 16 h at 23°C. The expression conditions for F43A and C45A/C74A mutants were similar to those of the His-shwanavidin, where only the 2^xYT medium contained 0.2mM IPTG, without autoinduced medium.

Attempts to express the core shwanavidin at pLysS strain yielded undetectable amounts of recombinant protein. The protein was thus transformed into *E.coli* strain BL21 DE3 Origami (Novagen), in order to sustain the disulfide bridges and avoid protein aggregation. Cells were cultured in 2^xYT +AM medium (+100 µg/ml ampicillin (Sigma-Aldrich), 30µg/ml kanamycin, 12.5µg/ml tetracyclin) at 26°C, with rotation (225 rev./min). When the OD ($\lambda=600$ nm) reached the value of 0.6, 0.1 mM IPTG was added for induction. Cultivation was continued for approximately 16 h at 26°C yielding 6 mg protein per liter.

For the purification stage of shwanavidin and His-shwanavidin, cells were lysed in a lysis buffer containing 50mM tris pH 8, 0.3M NaCl, 10% glycerol, 0.5% triton and 0.2mg/ml lysozyme. The His-shwanavidin was initially isolated using Ni²⁺-NTA open column (ADAR biotech), which was primarily calibrated with a buffer solution containing 50mM Tris buffer pH 8.0, 0.3 M NaCl, 10% glycerol, 100mM NaH₂PO₄ and 10mM imidazole. Cell lysate was equilibrated for 45 minutes, and then washed using a washing buffer containing 20mM imidazole. Elution was conducted with same buffer and 250mM imidazole. Shwanavidin was isolated by affinity chromatography on a 2-aminobiotin column (Pierce), and subsequently eluted with 0.1 M acetic acid pH 3, as previously described ². Further biochemical studies were conducted with the core protein only.

The His-shwanavidin C45A/C74A and F43A mutants were lysed by lysis buffer containing 50mM tris pH 8, 0.5M NaCl, 10% glycerol, 0.5% triton, 10mM Imidazole and 200µg/ml lysozyme. A purification protocol similar to that of the shwanavidin was used, where imidazole gradient, 30-125mM, was used as a washing buffer before protein elution at 250mM imidazole. The hexahistidine tag was later removed by TEV protease, (0.7mg/ml), in 0.75M NaCl, 50mM tris pH 8 and 10% glycerol buffer, and incubated for approximately 72 hours. Shorter incubation time (less than 24 hours) resulted in incomplete cleavage of the hexahistidine tag. Cleaved protein was then purified from TEV protease and cleaved hexahistidine tag by Ni⁺² column. The unbound fraction contained the core protein, and was dialyzed against 0.75M NaCl, 50mM tris pH 8, 10% glycerol buffer.

Crystallization and Data Collection

Expression and purification of shwanavidin (wild type and mutants) in *E. Coli* are described in the Supplementary Material segment. Crystals of the apo shwanavidin (wild type and F43A) were obtained by the microbatch method at 20°C. A 1- μ l drop initially contained equal amounts of 3.0 mg/ml protein in 0.1 M in acetic acid pH 3 mixed with 170 mM Bis-Tris pH 6-6.8, 2.0 M sodium formate. Cubic shaped crystals appeared within several hours and reached a final size of 0.05 mm within 3-5 days. The shwanavidin-biotin complex was obtained by incubating the protein in saturated solution of *d*-biotin (1.5mg in 1 ml 0.1M acetic acid) for 30 minutes at 4°C. Crystals of the complex were obtained using the micro batch method at 20°C. The 1- μ l drop contained equal amounts of 3.2 mg/ml protein in 0.1M Acetic acid pH 3 mixed with 1.0M Na/K phosphate buffer at a pH range of 6.6-6.9. Crystals of F43A mutant complexed with complex were obtained via the microbatch method using a crystallization solution containing 1.5M ammonium sulfate, 50mM tris pH 8.3. Identical conditions also resulted in crystals of the apo F43A mutant. Diamond shaped crystals appeared within few hours and reached a final size of 0.1 mm within 1-5 days. Prior to freezing, all crystals were briefly suspended in a cryoprotectant solution containing 25% glycerol with crystallization solution. Crystallographic data for all crystals were collected at the European Synchrotron Radiation Facility (ESRF), Grenoble, France, at 100°K, using an Oxford Cryosystem Cryostream cooling device. All data described here were processed and scaled using the HKL2000 suite³.

References

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3. Otwinowski, Z., Minor, W. (1997). Processing of X-ray diffraction data collected in oscillation mode. *Methods in Enzymology*.