

Supporting Information

Construction of a thiamin sensor from the periplasmic thiamin binding protein

Hanes, Jeremiah W.¹; Chatterjee, Debasree¹; Soriano, Erika V.¹; Ealick, Steven E.¹;
Begley, Tadhg P.*²

¹Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14853

*²Department of Chemistry, Interdisciplinary Life Sciences Building, 3474 TAMU, Texas
A&M University, College Station, Texas 77843

begley@chem.tamu.edu

Supporting Information

Mutagenesis of TbpA

Standard methods were used for DNA manipulations^{1,2}. Plasmid DNA was purified with a Qiagen Miniprep kit. *Escherichia coli* strain MachI (Invitrogen) was used as a recipient for transformations during plasmid construction and for plasmid propagation and storage. Site-directed mutagenesis was performed by a PCR protocol using PfuTurbo DNA polymerase as per the manufacturer's instructions (Invitrogen) and DpnI (New England Biolabs) to digest the methylated parental DNA prior to transformation.

Preparation of TbpA and thiaminase I

Recombinant *E. coli* TbpA and *Bacillus thiaminolyticus* thiaminase I were over-expressed and purified as previously documented^{3,4}. The mutants of TbpA were treated identically to the wild-type protein. Briefly, the expression and purification scale and procedures for TbpA were as follows: Transformed *E. coli* cells were grown in 1.5 L of LB broth (Sigma-Aldrich) containing 50 µg/mL kanamycin. The 1.5 L cultures were grown at 37 °C with shaking at 200 RPM until an OD₅₉₅ of approximately 0.6. The culture was induced using 1 mM isopropyl-β-D thiogalactoside and grown overnight at 15 °C and the cells were pelleted and stored at -80 °C.

All the purification steps were carried out at 4 °C. The cell pellets were resuspended at room temperature in 35 mL of the lysis buffer (50 mM Tris-HCl, pH 7.6, 300 mM NaCl, 20 mM imidazole, 2 mM tris(2-carboxyethyl)phosphine, 0.1% v/v Triton X-100). Lysis was carried out by sonication. The crude extract was then centrifuged at 17000g for 30 min. After centrifugation, the cell debris was discarded and the clarified lysate was purified according to the method previously described³. The protein concentration was determined using the Pierce Coomassie Plus Assay Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions using bovine serum albumin as a standard (included in the assay kit). The purity of TbpA was determined to be >90% by SDS-PAGE analysis (Figure 1).

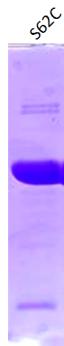


Figure S1. Representative SDS-PAGE analysis of the S62C mutant of TbpA.

Labeling of mutant TbpA

The cysteine mutants of TbpA were labeled with N-[2-(l-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide (MDCC) or N-(1-pyrene)maleimide (pyrene) (stock dissolved in DMSO) in the presence of 2 mM TCEP. A five-fold excess of fluorophore over that of the mutant TbpA was used. The buffer system was: 50 mM Tris-HCl pH 7.6, 100 mM NaCl, 8 mM MgCl₂ and 2 mM TCEP. The fluorophore was added to the protein at room temperature in three aliquots over the course of approximately 10 min while stirring gently using a magnetic stir plate and bar. Mixing was only performed during the addition. The conjugation reaction was carried out for 1 h at room temperature. The labeled protein was then separated from the free dye using an Econo-Pac 10DG (Bio-Rad Laboratories) desalting column equilibrated with the same buffer according to the manufacturer's instructions. Labeling efficiency for the S62C mutant was typically in the range of 95-100% as determined by comparing the concentration of the fluorophore using a UV/Vis spectrophotometer to that of the protein. The extinction coefficients used for MDCC and pyrene were 46,800 cm⁻¹M⁻¹ at 430 nm and 40,000 cm⁻¹M⁻¹ at 338 nm, respectively.

Fluorescence Titration and Transient Kinetic Experiments

The affinity of TbpA for thiamin was studied at room temperature by fluorescence titration using a KinTek Corporation stopped-flow apparatus (model SF-2004) equipped with an optional titration module (model TMX-1000). The buffer used in these studies was: 50 mM Tris-HCl pH 7.6, 100 mM NaCl, 8 mM MgCl₂ and 2 mM TCEP. The same stopped-flow apparatus and buffer conditions were used for the kinetic measurements. Control experiments were performed to inspect for the presence of photobleaching by closing the shutter on the stopped-flow instrument for a significant portion of time during the reactions. This data was then compared to data obtained from reactions where the sample was exposed continuously and if there were detectable photobleaching artifacts present, the lamp intensity was reduced.

Data analysis

Linear and nonlinear regression analysis was performed using the program GraFit 5 (Erihacus Software Ltd., Horley, Surrey, UK) by the least squares method. Titration data were fitted by nonlinear regression analysis to the following quadratic equation:

$$\text{Fluorescence} = F_o + \Delta F \cdot \frac{P_o + L + K_d - \sqrt{(P_o + L + K_d)^2 - 4 \cdot P_o \cdot L}}{2 \cdot P_o}$$

where, F_o is the initial fluorescence; ΔF , is the overall fluorescence change; P_o , is the initial protein concentration; L , is the ligand (thiamin) concentration; and K_d is the apparent dissociation constant. The kinetic data were biphasic and were therefore fitted using the following equation:

$$\text{Fluorescence} = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} + C$$

where, A_1 , is the amplitude of the fast phase; k_1 , is the observed rate of the fast phase; A_2 , is the amplitude of the slow phase; k_2 , is the observed rate of the slow phase; and C , is the offset.

References

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