

Supporting information

Deciphering protein microenvironment by using a cysteine specific *switch-ON* fluorescent probe

Jessy Mariam,^a Anila Hoskere Ashoka,^b Vandana Gaded,^a Firoj Ali,^b Harshada Malvi,^a
Amitava Das^{*b,c} and Ruchi Anand^{*a}

^a Department of Chemistry, IIT Bombay, Mumbai-400076, India. E-mail: ruchi@chem.iitb.ac.in

^b Analytical Science Discipline, CSIR-Central Salt and Marine Chemicals Research Institute, G.B. Marg, Bhavnagar:
364002, Gujarat, India.

^c Department of Chemical Sciences, Indian Institute of Science and Education Research, Kolkata, Mohanpur: 742246,
India. E-mail: amitava@iiserkol.ac.in

TABLE OF CONTENTS

Title	Page No.
Experimental section	S1
Figure S1: Assessment of protein purity by polyacrylamide electrophoresis.....	S2
Figure S2: Fluorescence spectra of purified proteins, buffer, and probe L	S2
Figure S3: Crystal structure of KsgA (PDB ID: 6IFS).....	S3
Figure S4: Fluorescence emission spectra of free probe L at varying temperatures....	S3
Figure S5: Hydrolytic deamination reaction catalyzed by prokaryotic TadA, at wobble position A34 in tRNA ^{Arg2}	S4
Figure S6: Local environment around C83 and C86 in TadA showing co-ordination with a zinc atom.....	S4
Figure S7: Box plot of fluorescence intensity of TadA and its mutants at four representative temperatures	S5
Figure S8: Crystal structure of <i>S.aureus</i> TadA in complex with tRNA (PDB ID: 2B3J).....	S5
Figure S9: Effect of viscosity and polarity on the switch-ON fluorescence of probe L....	S6
Table S1: Comparison of switch-ON fluorescent probes reported for protein unfolding studies.....	S7
References	S8

Experimental section

Cloning and site-directed mutagenesis

BSU00180 (abbreviated as BsTadA) gene was cloned from *Bacillus subtilis* 168 genomic DNA using standard PCR techniques. CGCGGATCCATGACACAAGAT-3' (forward BamH1 primer) and 5'-CGGGGTACCCTATTTCAGACAA-3' (reverse Kpn1 primer) primers were used to facilitate its cloning in the modified pET28a vector. The resulting construct consisted of an N-terminal (His)₆ tag with a TEV (Tobacco Etch Virus) cleavage site. This clone was further used as a template to design three single point cysteine mutants of TadA i.e C63S, C109A, C137S, and a double mutant C63AC109A. Clones were generated using overlap PCR and Phusion DNA polymerase from New England Biolabs. Cloning of KsgA (*Bacillus subtilis*) was performed as described in Bhujbalrao *et al.*¹ The KsgA C119A mutant was produced using overlap PCR with Phusion DNA polymerase. All the clones and constructs were confirmed by Sanger DNA sequencing. The clone of native PurL in pET vector was obtained from Prof. Steven E. Ealick's laboratory at Cornell University.²

Viscosity and polarity effects on switch-ON fluorescence of probe L

To study the effect of viscosity on the switch-ON fluorescence of probe L, fluorescence emission spectra of L in 20% and 70% glycerol prepared in deionized water, were recorded on Cary Varian spectrofluorimeter in the wavelength range of 460-650 nm, with an excitation wavelength of 445 nm. The excitation and emission slit widths were set to 5 nm. The same experiment was performed with 20% and 70% glycerol in 25 mM HEPES, 300 mM NaCl buffer, pH 7.4. To investigate the effect of polarity on the switch-ON fluorescence of probe L, fluorescence emission spectrum of L in dioxane was recorded with the same instrumental parameters.

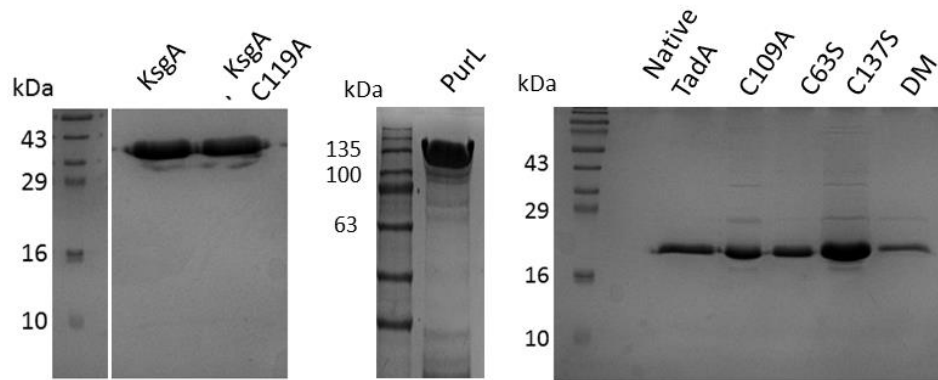


Figure S1: Assessment of protein purity by polyacrylamide gel electrophoresis. SDS PAGE gels depicting the purified proteins KsgA (35 kDa), its cysteine mutant C119A, PurL (140 kDa), TadA, and its cysteine mutants (20 kDa) C63S, C109A, C137S, and double mutant C63AC109A.

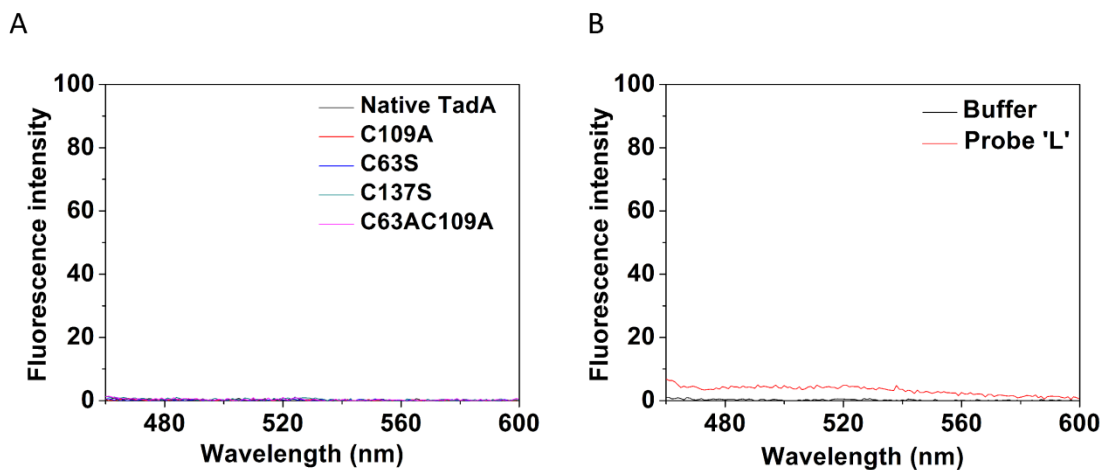


Figure S2: Fluorescence spectra of purified proteins, buffer, and probe L. A) Fluorescence spectra of purified proteins in absence of L. ($\lambda_{ex} = 445$ nm). No background fluorescence signal was observed. B) Fluorescence spectra of buffer (25 mM HEPES 300 mM NaCl, pH 7.4) and 20 μ M of L, $\lambda_{ex} = 445$ nm. Negligible background fluorescence signal observed with probe L.

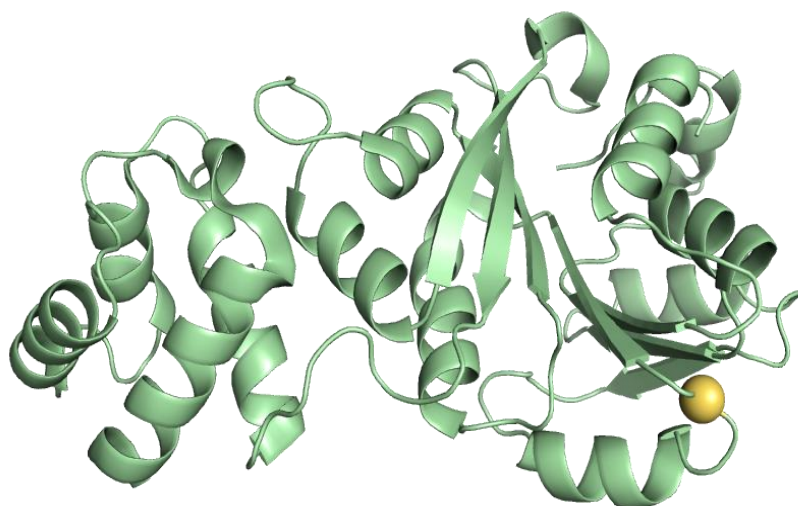


Figure S3: Crystal structure of KsgA (PDB ID: 6IFS)¹ with a single cysteine C119 (golden sphere)

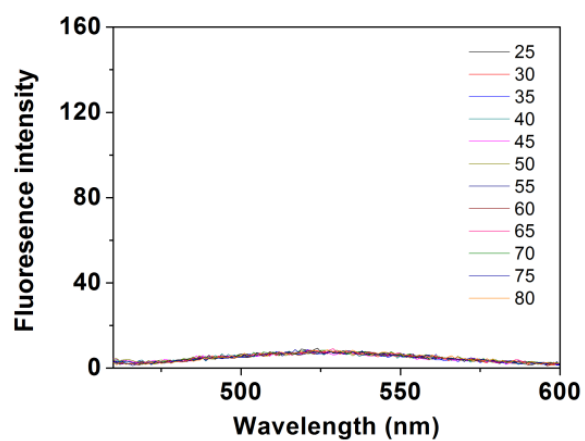


Figure S4: Fluorescence emission spectra of free probe L in the temperature range of 25-80 °C ($\lambda_{ex} = 445$ nm). Temperature does not switch-ON the fluorescence of the free probe and thus does not interfere with thermal unfolding experiments.

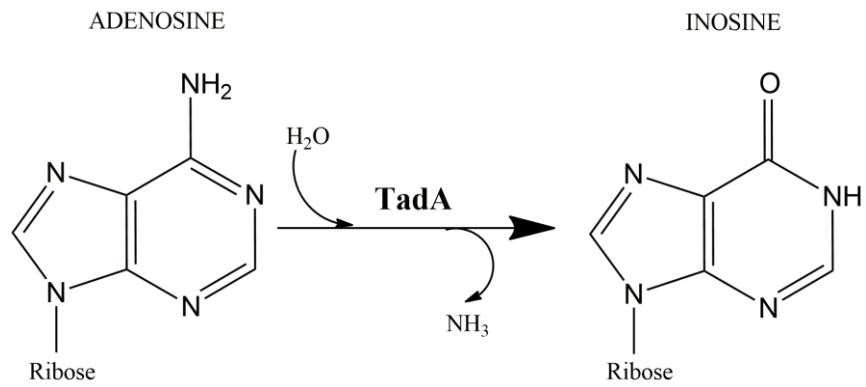


Figure S5: Hydrolytic deamination reaction catalyzed by prokaryotic TadA, at wobble position A34 in tRNA^{Arg2}

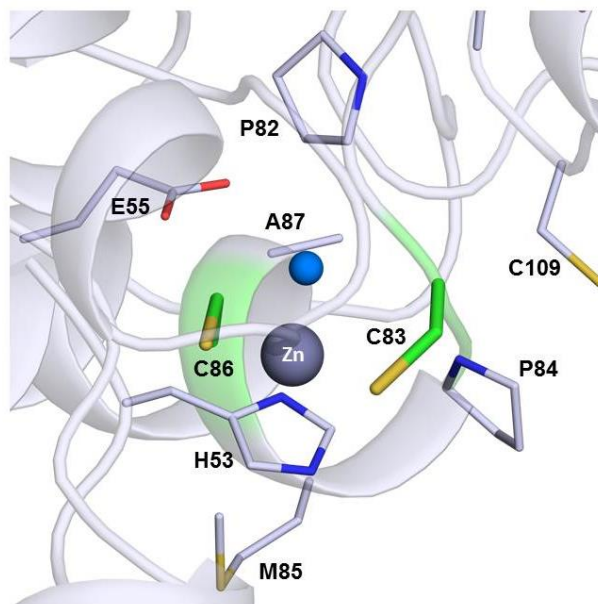


Figure S6: Local environment around C83 and C86 in TadA (PDB ID: 7CPH), showing co-ordination with a zinc atom

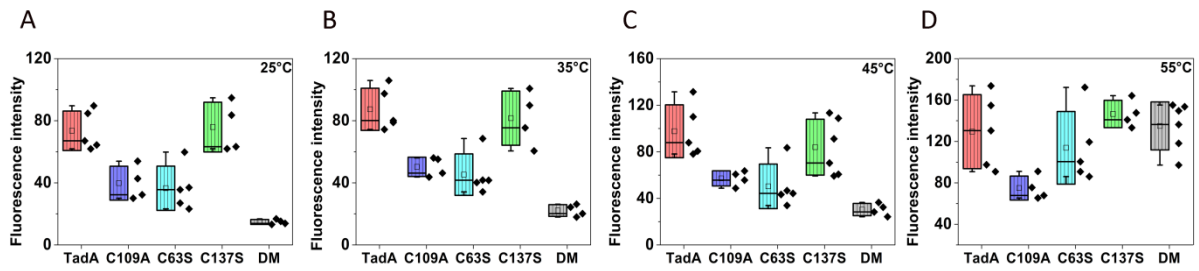


Figure S7: Box plot of fluorescence intensity of TadA and its mutants at four representative temperatures of 25, 35, 45 and 55 °C. The box represents the standard deviation, horizontal line within the box represents median, solid symbols (♦) are the data points, hollow symbols (□) correspond to mean and the whiskers denote minimum-maximum value.

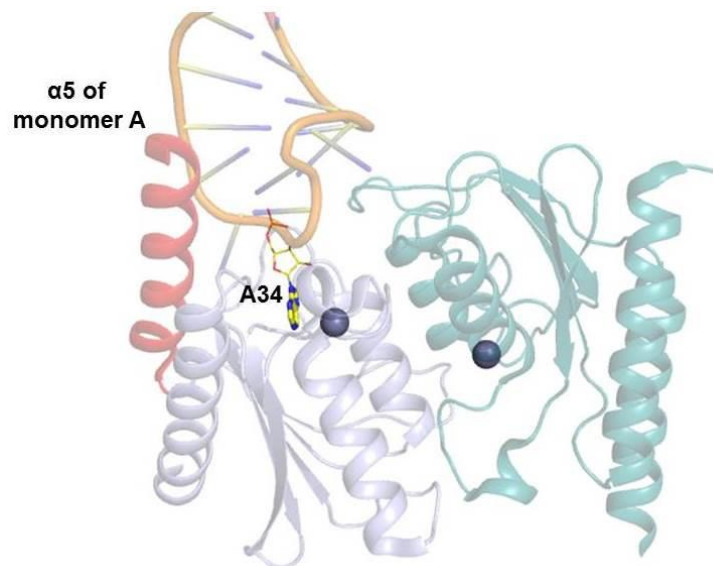


Figure S8: Crystal structure of *S.aureus* TadA in complex with tRNA (PDB ID: 2B3J)³, depicting the importance of $\alpha 5$ (red helix of monomer A) in stabilizing tRNA. The wobble base, A34 of the anticodon is splayed out into the active site of TadA.

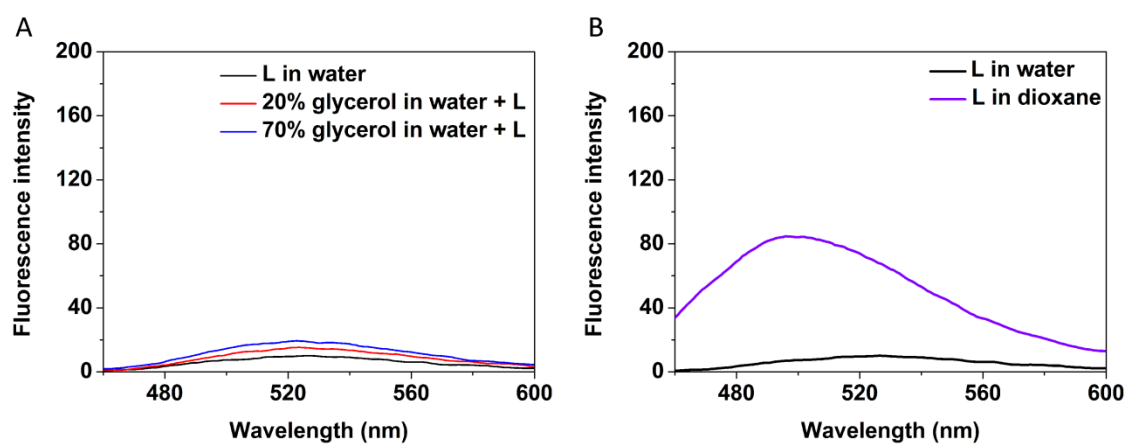


Figure S9: Effect of viscosity and polarity on probe L. A) Effect of glycerol (20% and 70%) on the switch-ON fluorescence of probe L. Similar results were obtained with L in 25 mM HEPES, 300 mM NaCl, 20%/70% glycerol buffer, pH 7.4 B) Effect of dioxane on the switch-ON fluorescence of probe L.

	Switch-ON fluorescent probes	Spectral features	Substrate specificity
1	L ⁴ (coumarin based probe)	$\lambda_{\text{ex}} = 445 \text{ nm}$ $\lambda_{\text{em}} = 515 \text{ nm}$	Specific to Cys over Homocysteine (Hcy) and glutathione (GSH)
2	DCIA (7-diethylamino-3-((4'-(iodoacetyl)-amino)phenyl)-4-methylcoumarin) ⁵	$\lambda_{\text{ex}} = 350 \text{ nm}$ $\lambda_{\text{em}} = 440 \text{ nm}$	Cannot differentiate between modified cysteines
3	TPE-MI (Tetraphenylethene maleimide) ⁶	$\lambda_{\text{ex}} = 350 \text{ nm}$ $\lambda_{\text{em}} = 470 \text{ nm}$	No interference from GSH
4	TPE-NMI (maleimide-functionalized tetraphenylethene (TPE)-derivative) ⁷	$\lambda_{\text{ex}} = 360 \text{ nm}$ $\lambda_{\text{em}} = 505 \text{ nm}$	No interference from GSH
5	SCG [diethyl 2-(4-(pyren-1-yl)benzylidene)malonate] ⁸	$\lambda_{\text{ex}} = 365 \text{ nm}$ $\lambda_{\text{em}} = 383, 400 \text{ nm}$	Specific to Cys over Hcy/GSH

Table S1: Comparison of probe L with other switch-ON fluorescent probes reported for protein unfolding studies.

References

1. R. Bhujbalrao and R. Anand, *J. Am. Chem. Soc.*, 2019, **141**, 1425-1429.
2. R. Anand, A. A. Hoskins, J. Stubbe and S. E. Ealick, *Biochemistry*, 2004, **43**, 10328-10342.
3. H. C. Losey, A. J. Ruthenburg and G. L. Verdine, *Nat. Struct. Mol. Biol.*, 2006, **13**, 153-159.
4. A. H. Ashoka, U. G. Reddy, F. Ali, N. Taye, S. Chattopadhyay and A. Das, *Chem. Commun.*, 2015, **51**, 15592-15595.
5. E. Branigan, C. Pliotas, G. Hagelueken and J. H. Naismith, *Nat. Protoc.*, 2013, **8**, 2090-2097.
6. M. Z. Chen, N. S. Moily, J. L. Bridgford, R. J. Wood, M. Radwan, T. A. Smith, Z. Song, B. Z. Tang, L. Tilley, X. Xu, G. E. Reid, M. A. Pouladi, Y. Hong and D. M. Hatters, *Nat. Commun.*, 2017, **8**, 474.
7. S. Zhang, M. Liu, L. Y. F. Tan, Q. Hong, Z. L. Pow, T. C. Owyong, S. Ding, W. W. H. Wong and Y. Hong, *Chem. Asian J.*, 2019, **14**, 904-909.
8. S. Chatterjee, S. Ghosh, S. Mishra, K. Das Saha, B. Banerji and K. Chattopadhyay, *Biochemistry*, 2019, **58**, 1109-1119.