Detection of Escherichia coli Enoyl-ACP Reductase Using Biarsenical-Tetracysteine Motif

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Detection of *Escherichia coli* Enoyl-ACP Reductase Using Biarsenical-Tetracysteine Motif

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Although the tetracysteine (TC) motif has been used as a tag, the binding stability between TC motif and biarsenical reagent against extreme conditions as well as its capacity as a quantitative tag remains not well developed. To reveal these problems, we chose enoyl−acyl carrier protein reductase (FabI), which was involved in the final step of elongation in the bacterial fatty acid biosynthesis, to be tagged by the TC motif. Taking enhanced green fluorescent protein (EGFP) tagged FabI as a control, we investigated the activities of various TC tagged FabIs (N-terminus, C-terminus, or both N- and C-terminus TC motif). The results showed that all the TC tagged FabIs had high enzyme activities while the EGFP tagged FabI exhaustively lost the activity. Beside this, the characteristics of the tag, including labeling stability against extreme conditions, capacity for quantitative analysis, and ability for in-cell labeling, were also investigated. We demonstrated for the first time that the binding between FlAsH reagent and TC motif was stable against high pressure, high field strength, high temperature, and ultrasound. Furthermore, we verified the potential of TC motif for quantitative analysis of target protein by different approaches, including SDS−PAGE, spectrofluorometry (SPF), and capillary zone electrophoresis (CZE).

INTRODUCTION

Analysis of target proteins within living cells is of fundamental importance for detailed understanding of their molecular mechanisms and biological processes (*1, 2*). Most strategies aimed at realizing this objective are based on the usage of biological or chemical tags that could image or quantify target proteins in living cells or tissues, directly or indirectly (*3*).

Green fluorescent protein (GFP) and its variants are the most classic biological tags that have been widely used (*4*−*6*). Fusion of a protein of interest with an autofluorescent protein is a widely adopted strategy for live cell imaging (*7, 8*). However, this strategy has several limitations. First, since the GFP size is relatively large (up to 25 kDa), it may affect the physiological function of the fused proteins, especially the small proteins (*9*). Another disadvantage is that the spectral properties of each autofluorescent protein have been restricted to the fluorophores and require optical forms of readout (*10*), and their modest photostability has not matched well with the requirement of some applications (*11*). To overcome these drawbacks, researchers have been increasingly employing the quantum dots as an alternative because of their high quantum yields, wide spectral range, enhanced photostability, and long fluorescence lifetime (*12*). However, as nanoparticles, the quantum dots are less biocompatible and are too large for the proper functions of relative small proteins. Furthermore, because they are nongenetic, they would be diluted along with the fast cell division from one generation to another (*13, 14*). Therefore, much effort had been made to exploit smaller molecular tags that would label proteins with minimal functional perturbation (*15, 16*).

Many selective small molecule chemical tags for labeling of proteins in living cells have been developed recently, including O6-alkylguanine-DNA alkyltransferase (hAGT) tag, peptide carrier proteins (PCPs) tag, and acyl carrier proteins (ACPs) tag. Although they are advantageous because of the availability of many commercial fluorescent reagents, they still exhibit certain disadvantages. For examples, the human hAGT tag is among the most promising emerging fluorescent tags for cellular imaging in vivo and in vitro, but its usage has been limited by difficulties in obtaining of hAGT-deficient cell lines (*17, 18*). The employing of PCPs (*19*) as well as ACPs (*20*) as tags to monitor the changes in living cells is of considerable current interest, but their usage is restricted to cell surface studies (*21*). To circumvent these drawbacks, the biarsenical-tetracysteine (TC) system first developed by Roger Tsien and co-workers appears to be particularly promising (*22*). It is based on the subnanomolar affinity between a biarsenical variant fluorescein compound such as 4′,5′-bis(1,3,2-dithioarsolan-2-yl)fuorescein (FlAsH) or resorufin (ReAsH) and a genetically encoded short TC motif of only six amino acids (CCXXCC), where C presents cysteine and X is any amino acid except cysteine (*23*). In its freedom situation, the biarsenical dye is premixed with an excess of 1,2-ethanedithiol (EDT). And in its active situation, it becomes highly fluorescent by binding to the cysteines of the TC motif. The main shortcoming of this tag may come from the cell toxicity of biarsenical dye (*24*) and the relatively high background fluorescence (*25*). However, it has displayed outstanding advantages over other large fusion tags attributing to its relatively small size and potential to avoid loss of bioactivity or structural obstruction (*26*). More importantly, the TC motif is so small that it can be introduced into either terminus of target protein directly through cloning primers (*27*).

Although TC-fusion protein has been used as detectors for in-cell research (*28, 29*), the binding stability between TC motif and biarsenical reagent against extreme conditions and its capacity as a quantitative tag has been rarely reported. Herein, much effort has been done surrounding these problems relying on the enoyl−acyl carrier protein (ACP) reductase (FabI) from *Escherichia coli*, which is involved in the final step of elongation in the bacterial fatty acid biosynthesis (*30*) and is an attractive target for the development of new antibacterial agents (*31*). FabI is the only enoyl-ACP reductase in *E. coli* fatty acid synthesis, and the activity of this enzyme displays a prominent role in the cycle of fatty acid elongation. The understanding of its real-

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Table 1. Bacterial Strains, Plasmids, and Oligonucleotides Used in This Work

strain/plasmid	relevant characteristics	source or reference
E. coli strain		
MG1655	*Escherichia coli* K12 wild type	lab collection
BL21(DE3)	*omp*T *hsd*S B ($rB^- mB^-$) (λ DE3)	Invitrogen
Hh31	BL21(DE3) expressing strain, which contains pET-Wt-FabI	this work
Hh32	BL21(DE3) expressing strain, which contains pET-Nt-FabI	this work
Hh33	BL21(DE3) expressing strain, which contains pET-Ct-FabI	this work
Hh34	BL21(DE3) expressing strain, which contains pET-Bt-FabI	this work
Hh35	BL21(DE3) expressing strain, which contains pET-Gt-FabI	this work
plasmid		
pBAD24	Amp*R* cloning vector for introduction of TC motif encoding sequence	ref *33*
pEGFP	Amp*R* *egfp* cassette vector	Invitrogen
pET28a(+)	Kan*R* expressing vector with a T7 promoter	Novagen
pB-lumio[a]	annealed oligonucleotides (pB-lumio-S and pB-lumio-A), which contains a TC motif and a *Nde*I recognition site, was ligated into the *EcoR*I and *Nco*I sites of pBAD24	this work
pB-lu-FabI	MG1655 FabI cloned into the *Nde*I and *Nco*I sites of pB-*lumio* with primers pB-*lu-FabI*-F and pB-*lu-FabI*-R	this work
pET-Wt-FabI	FabI was excised from pB-lu-FabI and inserted in the *Nde*I and *Sal*I sites of pET28a(+)	this work
pET-Nt-FabI	lu-FabI was excised from pB-lu-FabI with *EcoR*I and *Sal*I and inserted in pET28a(+)	this work
pET-Ct-FabI	lu-FabI was amplified from pB-lu-FabI with primers Ctag-F and Ctag-R[b] and inserted in the *Nde*I and *Sal*I sites of pET28a(+)	this work
pET-Bt-FabI	lu-FabI was amplified from pB-lu-FabI with primers Ctag-F and Ctag-R and inserted in the *EcoR*I and *Sal*I sites of pET28a(+)	this work
pET-Gt-FabI	*egfp* was amplified from pEGFP with primers GL-F and GL-R and inserted in the *Nde*I and *Nhe*I sites of pET-Nt-FabI	this work

oligonucleotide	sequence (5′−3′)
pB-lumio-S	AAAGAATTCATGTGTTGTCCTGGCTGTTGCCATATGCTCGAGCCATGGAA
pB-lumio-A	TTCCATGGCTCGAGCATATGGCAACAGCCAGGACAACACATGAATTCTTT
pB-lu-FabI-F	ATTAA ACATA TGGGT TTTCT TTCCG
pB-lu-FabI-R	CATCT AGATT ATTTC AGTTC GAGTT CG
Ctag-F	TCTCC ATACC CGTTT TTTTG GGCTA GC
Ctag-R	AAAGTCGACTTAGCAACAACCAGGGCAACAAAATTTCAGTTCGAGTTCGTTC
GL-F	AATCG CTAGC ATGGT GAGCA AGGG
GL-R	GGCCG AATTC CTTGT ACAGC TCGTC

[a] lumio represents TC motif (-CCPGCC-) encoding sequence TGTTGTCCTGGCTGTTGC. [b] Ctag-R contains another TC motif (-CCPGCC-) encoding sequence GCAACAACCAGGGCAACA.

time activity and content within living cells is of considerable importance for mastering the status of bacterial growth and multiplication and developing correct administration strategy. In the current study, we investigated the enzyme activity and quantization of FabI from *E. coli*, employing the biarsenical-tetracysteine system. Furthermore, the properties of the tag, including labeling stability against extreme conditions, capacity for quantitative analysis, and ability for in-cell labeling, were also investigated comprehensively for the first time.

EXPERIMENTAL SECTION

Materials. The restriction endonuclease *Nde*I, *Nco*I, *EcoR*I, *Xba*I, *Hind*III, *Sal*I, and *Nhe*I were purchased from TaKaRa (Dalian, Liaoning, China). The PCR cleanup kit, plasmid miniprep kit, and DNA gel extraction kit were purchased from AxyGen (Hangzhou, Zhejiang, China). Primers for PCR amplification and sequencing verification were obtained from Sunbiotech (Beijing, China). The lumio green in-gel detection kit was purchased from Invitrogen (Carlsbad, CA). The Ni-NTA resin for His6 affinity purification was purchased from GenScript (Nanjing, Jiangsu, China). All other chemicals were the purest grade commercially available.

Construction of Recombinant FabIs. FabI was genetically fused with six amino acids (CCPGCC), which was the optimized motif of CCXXCC (*32*). The recombinant proteins employed in this work were engineered with N-terminus or C-terminus or both N- and C-terminus TC motif or N-terminus EGFP tag or without any tag. The five corresponding expression plasmids pET-Nt-*fabI,* pET-Ct-*fabI*, pET-Bt-*fabI*, pET-Gt-*fabI*, pET-Wt-*fabI* were constructed (Table 1). The creation procedures are detailed in Figures S-1−S-6, and the sequence of each recombinant proteins is detailed in Supporting Information (SI). The recombinant proteins were overexpressed in the *E. coli* strain BL21(DE3) and utilized for further research in vitro and in vivo.

Protein Expression and Purification. The recombinant protein was expressed in the *E. coli* strain BL21(DE3) in standard LB medium. Protein expression was induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) when an optical density of 0.6−0.8 was reached and incubated for another 4 h to allow expression. Purification was achieved by using its His6 tag following the general protocol of nickel nitrilotriacetic acid column and washing and eluting in an imidazole concentration of 20 and 250 mM, respectively. Collected fractions were dialyzed against 20 mM Tris-Cl, pH 8.0, containing 1 mM DTT, concentrated in the Bradford assay using BSA as a standard, and then stored in −70 °C.

In Vitro FlAsH Labeling. The labeling procedure for TC-tagged FabIs closely followed the method described previously with a few modifications (*32*). The FlAsH reagent (the structure is shown in Figure S-7) was supplied precomplexed to 1,2-ethanedithiol (EDT), which not only decreased the nonspecific binding of As(III) to endogenous thiols but also stabilized and solubilized the biarsenical reagent. TC-tagged FabIs were dissolved in 20 mM Tris-Cl, pH 8.0, containing 1 mM DTT which was used to keep deoxidation of the thiol group in the cysteine residues and improve labeling efficiency. The TC-tagged FabIs were reacted with 1 μM FlAsH-EDT_2 reagent at room temperature for 2 h to ensure complete labeling. The

labeled samples were analyzed by kinetic assay, high performance liquid chromatography (HPLC), and capillary electrophoresis (CE). For SDS−PAGE and spectrofluorometer analysis, the labeling procedure was replaced by heating at 70 °C for 10 min, which was not only time-saving but also efficient.

In Vivo FlAsH Labeling. Intracellular FlAsH labeling of TC-tagged protein was carried out according to the protocol described by Kottegoda et al. (*34*) with some modifications. The BL21(DE3) cells expressing the TC-tagged proteins were washed twice with extracellular buffer (ECB, 135 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.4) before FlAsH labeling. Then the cells were incubated at 37 °C for 2 h in the presence of 1 μM FlAsH reagent. The labeling efficiency was confirmed by LSM 510 META (Carl Zeiss, Jena, Germany) confocal system after washing the FlAsH labeled cells twice with the ECB buffer. The cells were then lysed in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) for CE analysis. As a control, wild-type BL21(DE3) cells were also labeled with FlAsH reagent in parallel.

Kinetic Assay of Tagged FabIs. The kinetic assay of tagged FabIs was performed to explore the distinction of different tags. Kinetic parameters were studied for pro- and post-FlAsH-labeled FabIs. The reaction system for enzyme assay was prepared strictly as described (*35*) with a few modifications. Control experiments for both pro- and post-FlAsH-labeling were conducted simultaneously in identical conditions. After completion of both labeling and enzyme assay, samples were stored in −70 °C prior to further analysis.

Fluorescence Scan. After FlAsH labeling, the TC tagged protein underwent fluorescence scan analysis. Continuous scanning was carried out by fixing excitation wavelength at 505 nm and reading the fluorescence intensity from 519 to 560 nm.

HPLC Analysis of TC-Tagged FabIs. A HPLC system (Waters Ltd., Watford, Hertfordshire, U.K.) with a photodiode array (PDA) detector in tandem with a fluorescence detector was used to analyze the TC-tagged FabIs. An injection of 10 μL of reaction mixture was analyzed on a Proteomix SAX-NP5 column (Sepax, Shanghai, China) using online gradient programming from 100% solution A (20 mM phosphate, containing 1 mM DTT, pH 8.0) to 100% solution B (20 mM phosphate, containing 1 mM DTT and 0.5 M NaCl, pH 8.0) in 30 min with a delay of 20 min.

Capillary Electrophoresis Analysis of TC-Tagged FabIs. A CE system (Beckman Coulter, Fullerton, CA) with a laser-induced fluorescence (LIF) detector was used for analyzing the FlAsH labeled samples. All the separations were completed in an uncoated silica capillary, with 75 μm i.d., effective length of 50 cm with an overall length of 62 cm, at voltage strength of 25 kV. Separations were performed by capillary zone electrophoresis (CZE) for all the samples with an injection pressure of 0.5 psi for 5 s. The running buffer consisted of 1 mM DTT and 20 mM borate at pH 8.4. The presence of DTT in the running buffer is to maintain a reducing environment during the electrophoresis. The LIF detector condition is Ex/Em = 488 nm/535 nm.

SDS−PAGE Analysis. The fact that the TC motif can function with high specificity and affinity opened the way for the development of an in vitro system for quick monitoring of the integrity of target proteins in traditional denaturing gel electrophoresis (*36*). The FlAsH labeled recombinant proteins were mixed with SDS−PAGE loading buffer before electrophoresis. Then the TC-tagged protein complexes were visualized directly under UV illumination (Syngene Gene Genius, Frederick, MD), followed by Coomassie blue staining to visualize the total proteins.

RESULTS AND DISCUSSION

Stability of FlAsH Labeling. We first tested the stability of FlAsH reagent binding to TC motif because it is one of the most important attributes of a tag that can be used in the labeling study. A strong and stable binding force could ensure high reproducibility and credibility. Herein, the binding stability between FlAsH reagent and TC motif was examined for the first time under various extreme conditions including high electric field strength, high pressure, high temperature, and ultrasonic and microwave treatments.

Capillary electrophoresis has been proved to be a powerful tool for investigation of protein and polypeptides (*37*). Moreover, the excitation and emission wavelengths of the TC-tagged complex (Ex/Em = 505 nm/535 nm) were compatible with commonly used detection schemes of CE-LIF (Ex/Em = 488 nm/535 nm). Therefore, a CE-LIF system was employed to test the binding force between FlAsH reagent and TC motif in the condition of high electric field strength. We first tested the Nt-FabI, which was labeled with FlAsH followed by being injected into a capillary and electrophoresed immediately. The resulting electropherograms revealed a singlet at 4.017 ± 0.021 min (n = 13, RDS = 0.52%), while the parallel electropherograms of Nt-FabI and the FlAsH reagent alone produced no corresponding peaks (Figure 1A). And the corresponding peak areas of the repeated injections were found to be $(3.50 \pm 0.10) \times 10^6$ (RDS = 2.8%). These data suggested that the binding of FlAsH reagent with Nt-FabI is strong enough to survive under high field strength. This result also indicated that the capillary zone electrophoresis was a useful tool for the analysis of TC-tagged protein with high resolution and low background signal.

To examine the binding stability against high temperature, the FlAsH labeled FabIs were heated at 70 °C for 10 min before spectrofluorometric (SPF) analysis. Figure 1B showed that the heating procedure did not change the optical qualities of the TC-tagged FabIs/FlAsH complex (for detailed information, see Table S-1), which was consistent with the claim of Invitrogen Co. The influence of microwave and ultrasound on the stability of the fluorescent complex was also tested. The reaction mixture was incubated at 70 °C for 10 min to allow complete labeling, and then microwave (wp750L23-6, Galanz Ltd., Foshan, Guangdong, China) and ultrasound (KQ-100DE, Kunshan, Jiangsu, China) procedures following a time gradient strategy were carried out. The power of the microwave oven was set at 750 W, and the parameters for ultrasound treatment were controlled at 25 °C and 80 W. The scanning results showed that the ultrasonic treatment did not affect the fluorescent complex (Figure 1C), while the microwave displayed a time-dependent destruction effect to the complex (Figure 1D). The destruction effect was very weak when treatment time was shorter than 5 min. When the treatment was extended to 15 min, the emission spectrum of the fluorescent complex changed greatly and the maximum emission wavelength was generalized.

HPLC was used to examine the binding force between the FlAsH reagent and the TC motif against high pressure. The resulting chromatograms revealed a peak area of $(7.17 \pm 0.36) \times 10^6$ μV•s on the PDA detector (RDS = 5.0%) and $(6.27 \pm 0.31) \times 10^8$ μV•s on the fluorescence detector (n = 5, RDS = 4.8%). Those results indicated that the FlAsH dye could keep conjugating to the TC motif stably under high pressure of up to 760 ± 15 psi (Figure 1E). In the study, a fluorescence detector was placed in tandem after a PDA detector, so the singlet observed on the fluorescence detector was delayed compared with that of PDA detector (Figure 1F). Taken together, the above data indicated that the binding between FlAsH reagent and TC

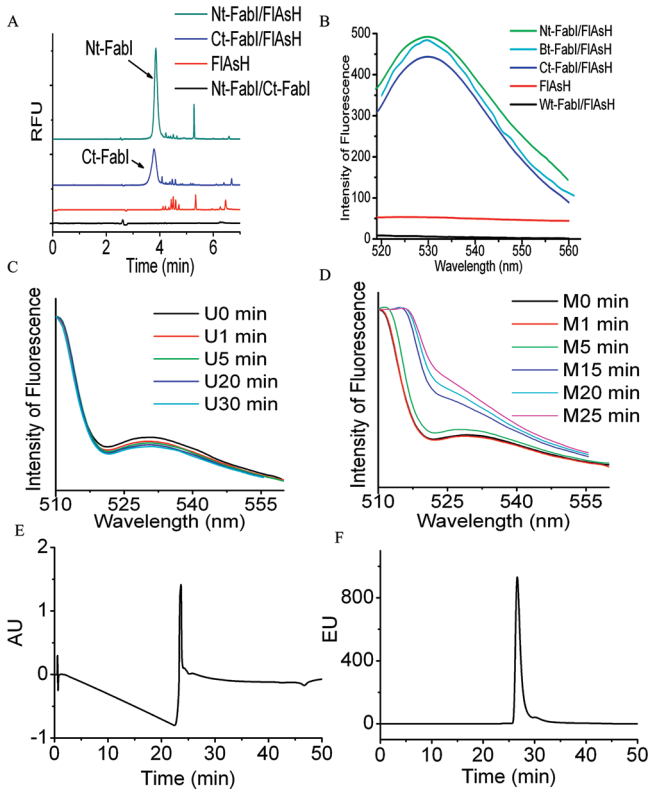

Figure 1. Stability of TC-tagged FabI/FlAsH complex. (A) The TC-tagged FabIs/FlAsH complex was stable under high electric field strength conditions on CZE. The TC-tagged Nt-FabI and Ct-FabI were labeled with FlAsH reagent for up to 2 h at room temperature, and then the samples were analyzed on CE. The separation of all the samples were conducted in an uncoated silica capillary, 75 μm i.d., effective length 50 cm with an overall length of 62 cm. run buffer, 20 mM borate buffer with 1 mM DTT, pH 8.4; voltage, 25 kV; LIF detection condition, Ex/Em = 488 nm/535 nm. (B) The TC-tagged FabIs/FlAsH complex was examined by fluoroscence scanning after being incubated at 70 °C for 10 min. The excitation wavelength was fixed at 505 nm, and each sample got five repeats. The influence of ultrasound treatment (C) and microwave (D) on the binding stability of the Nt-FabI/FlAsH complex was then tested. The graph showed that the ultrasonic treatement did not have an obvious influence on the stability of the fluorescent complex ($p < 0.01$), while the destructive effects of microwave increaseed greatly along with the elongation of the treating time. (E) HPLC separation of the Nt-FabI obtained from PDA detector at 214 nm and (F) the corresponding chromatogram obtained from fluorescence detector under the condition of Ex/Em = 505 nm/535 nm. All the samples labeled with FlAsH reagent were analyzed on a Proteomix SAX-NP5 column (5 μm, 4.6 mm × 50 mm, Sepax, Shanghai, China) by linear gradient elution at a flow rate of 0.75 mL min^{-1}. Mobile-phase composition was changed from 100% solution A (20 mM phosphate, containing 1 mM DTT, pH 8.0) to 100% solution B (20 mM phosphate, containing 1 mM DTT and 0.5 M NaCl, pH 8.0) in 30 min with a delay of 20 min.

motif was stable under extreme conditions, such as high field strength, high temperature, high pressure, and strong ultrasonic treatment.

Influence of Different Tags on the Enzyme Activity. It is critical to choose a proper optical molecular tag for a detailed understanding of the protein dynamics both in vitro and in vivo. To this end, current studies take it as the key criterion for checking whether the structure and function of the target protein are compatible with the tag or affected by the tag. We investigated the influence of the TC tag on the structure and function of the FabI by assaying its enzyme activity in vitro, taking EGFP tag as a control. The activities of TC-tagged FabI with crotonoyl-CoA as substrate were determined at various pH ranging from 6.0 to 8.5. We found that the FabI showed the highest activity at pH 7.6, which was adopted for all subsequent kinetic measurements. The typical kinetic curves for the FabI catalyzed reaction before FlAsH labeling are shown in Figure S-8. Detailed Michaelis−Menten constants κ_{cat} and K_m were determined for the modified FabIs under the same conditions (Table 2). Before FlAsH labeling, all of the TC tagged FabIs exhibited much higher enzyme activity than the EGFP tagged FabI. This suggests that the larger-sized EGFP tag imposed a greater disturbance on tagged FabI than the smaller-sized TC tag. Among the TC tagged FabIs, little variance of the κ_{cat} values was observed, and the K_m values varied smoothly. Compared with the K_m value observed for Wt-FabI, the κ_{cat} value was 2-fold smaller for Nt-FabI and nearly 1-fold greater for Bt-FabI, respectively. The κ_{cat} values have a slight tendency to decrease after the labeling procedure. In contrast, the K_m values tended to increase to a small extent. These data indicated that the presence of the biarsenical dye exhibits very little impact to the reaction mediated by FabI. After the FlAsH labeling, the K_m values of TC tagged FabIs were similar. However, the κ_{cat} value of Ct-FabI was slightly greater than that of Nt-FabI and Bt-FabI by about 0.68-fold and 0.90-fold, respectively. As expected, the reagent imposed a greater effect on Wt-FabI, resulting in a 2.59-fold greater K_m value than that of control, which may due to the higher concentration of nomadic dye in the reaction mixture (*24*). Previous studies evaluating the influence of the TC tag on an enzyme involved in bacterial fatty acid synthesis have indicated that these short peptides did not prevent the bioactivity of FabI from catalyzing the last step reaction of elongation. Furthermore, different modes of introducing the TC tag into the target protein displayed similar effectiveness. The smaller-sized six-amino acid TC tag, labeled or not labeled, displayed tiny disturbance on the structure of target protein. In contrast, the EGFP tagged FabI exhibited a total loss of activity. Therefore, the TC tag is a better protein tag than EGFP because of the smaller size.

Quantitative Analysis of TC Motif FabIs. Although more and more attention has been paid to use the TC motif to study complicated and delicate dynamics in living cells (*38*), little attention has been paid to develop its capacities for quantitative analysis. To expand the application of this system instead of merely being used for living cell imaging, in the current study, we made the first effort to investigate the capacity of TC tag for quantitative analysis comprehensively. The FabI involved in the synthesis cycle of fatty acid in *E. coli* is an important enzyme composed of 263 amino acids with a p*I* of 5.58 and a molecular weight of 27.8 kDa. The final recombinant proteins Nt-FabI, Ct-FabI, and Bt-FabI possessed molecular masses of 28.6, 28.5, and 29.3 kDa, respectively. The TC-tagged FabIs were purified by His6 tag and then labeled with FlAsH reagent. SDS−PAGE analysis was performed to confirm that the biarsenical reagent remained binding to its motif with high specificity (Figure 2A). Experiments to assess the sensitivity of labeling in denaturant gel indicated that the TC tag could specifically bind to the TC motif with high sensitivity over competing proteins under the complicated matrix. The lowest quantity of labeled protein that can be detected directly in gel was less than 1 ng (about 3.49 × 10^{-11} M), while 25 ng of Coomassie blue stained protein was needed to be detected in the same gel (Figure 2B). SDS−PAGE was regarded to be a fast and convenient way for protein quantitative analysis with different staining strategies, such as silver staining (*39*), negative staining (*40*), and fluorescence staining (*41*). It has been reported that the TC motif exhibited drawbacks for quantitative analysis in gel, including unspecific binding to endogenous cysteine-rich proteins (*42*) and unspecific binding of arsenites for either mono- or dithiol compounds (*43, 44*), even when the TC motif was separated as two moieties in the folded interior of a protein (*45*). However, these problems were not present in our

Table 2. Steady-State Kinetic Parameters of FabI Enzymes with Different Modification Pre- and Post-FlAsH Labeling

	pre-FlAsH labeling			labeling control			post-FlAsH labeling		
name	$\kappa_{cat} \times 10^{-3}$ S^{-1}	$K_m \times 10^3$ M	$\kappa_{cat}/K_m \times 10^{-6}$ S^{-1} M^{-1}	$\kappa_{cat} \times 10^{-3}$ S^{-1}	$K_m \times 10^4$ M	$\kappa_{cat}/K_m \times 10^{-6}$ S^{-1} M^{-1}	$\kappa_{cat} \times 10^{-3}$ S^{-1}	$K_m \times 10^4$ M	$\kappa_{cat}/K_m \times 10^{-6}$ S^{-1} M^{-1}
Wt-FabI	2.1 ± 0.10	1.08	1.98 ± 0.09	1.52 ± 0.07	3.65	4.17 ± 0.19	1.05	9.46	1.11
Nt-FabI	3.32 ± 0.20	0.29	11.16 ± 0.68	2.52 ± 0.18	6.92	3.64 ± 0.26	2.12	8.23	2.57
Ct-FabI	5.67 ± 0.15	0.71	7.98 ± 0.02	4.02 ± 0.25	6.35	6.32 ± 0.39	3.55	8.39	4.23
Bt-FabI	2.37 ± 0.23	2.00	1.18 ± 0.12	2.26 ± 0.16	6.67	3.39 ± 0.27	1.87	8.76	2.14
Gt-FabI	nd[a]	nd[a]	nd[a]	nd[a]	nd[a]	nd[a]	nd[a]	nd[a]	nd[a]

[a] nd: not detected.

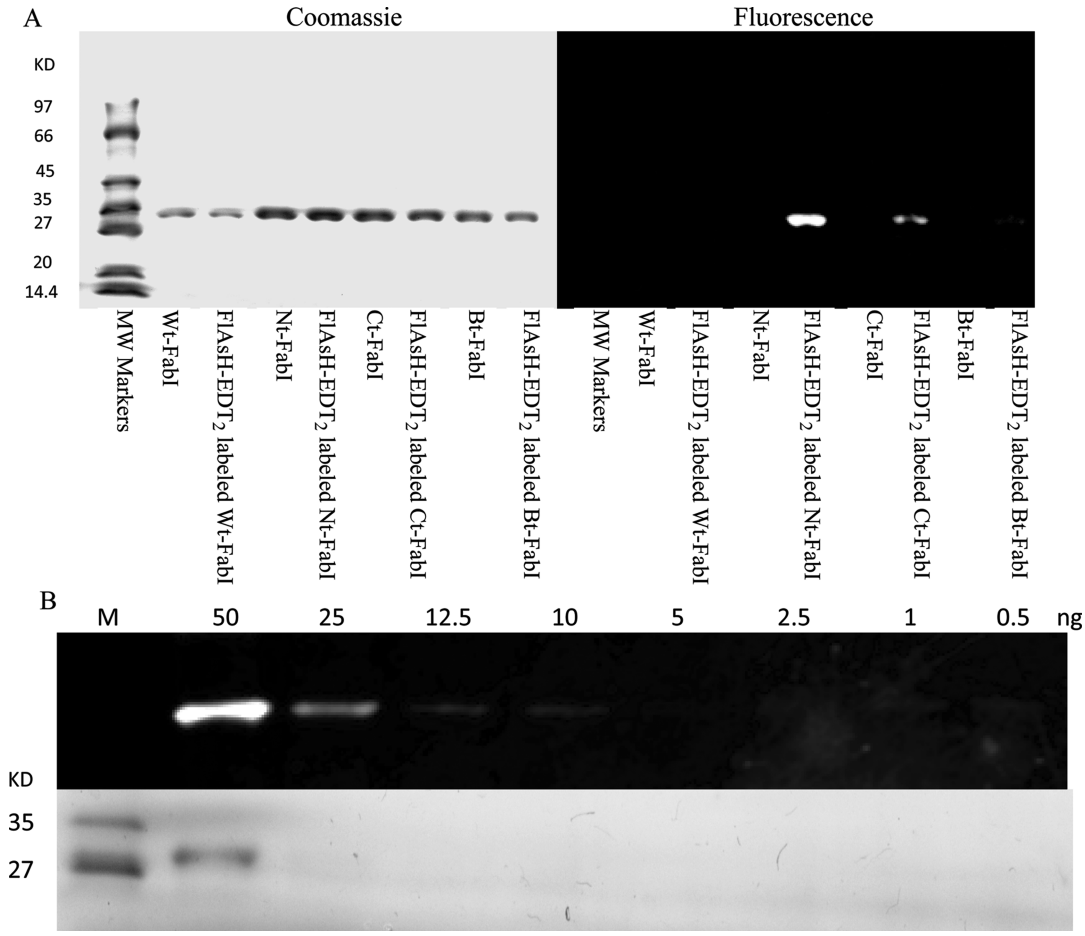

Figure 2. Specificity and sensitivity of TC-tagged FabIs labeled with FlAsH-EDT_2 revealed by SDS−PAGE. (A) Proteins eluted from the Ni-NTA column were labeled directly with FlAsH-EDT_2 reagent in a sample buffer for SDS−PAGE. When the electrophoresis was completed, the gel was visualized first by a 302 nm transilluminator to obtain a fluorescence image and then stained with Coomassie blue. (B) The sensitivity of FlAsH labeling was assayed in SDS−PAGE gel. Dosages of 50, 25, 12.5, 10, 5, 2.5, 1, 0.5 ng labeled Nt-FabI were injected. An injection dosage of as low as 0.5 ng was detected in the gel directly (B, top, the last lane), exhibiting a vivid comparison with the results discovered by the coomassie stained gel (B, bottom).

investigation with purified FabIs. The cysteines within FabI are not adjacently located and could not form closely located parallel pairs in space structure.

On the basis of our previous work, the detection limits for Nt-FabI and Ct-FabI were tested on CZE (Figure 1A). The presence of a single TC motif ensured a better interpreting of its capacity for protein labeling and detection on CE-LIF system. The electropherograms of Ct-FabI displayed an obvious peak at 3.887 ± 0.021 min ($n = 13$, RDS = 0.54%). And the corresponding peak areas of the repeated injections were found to be $(4.31 \pm 0.06) \times 10^5$ (RDS = 1.4%). In contrast, a sample of the FlAsH reagent alone was also injected into the CE column. The resulting electropherogram revealed no corresponding peaks at that time point but displayed many small background peaks. No fluorescence peaks appeared in the analysis of unlabeled TC-tagged FabIs. Assuming complete reactions were achieved, the detection was found to be linear over a range of 2.00×10^{-12} to 1.21×10^{-10} M for Nt-FabI and 1.32×10^{-12} to 1.25×10^{-10} M for Ct-FabI. The detection limits for the TC-tagged FabIs/FlAsH complex were found to be 3.49×10^{-16} M for the Nt-FabI/FlAsH complex and 8.51×10^{-16} M for the Ct-FabI/FlAsH complex which is somewhat higher than that of other researchers obtained by using synthesized peptides (*34*). Although these values were calculated based on the broadened peaks which should be further improved by optimization of the separation conditions, these data demonstrated that the TC motif was a feasible and reliable labeling tag for protein analysis on CZE. The ability of TC tag for quantitative assay was also tested according to the fluorescence intensity by fixing the excitation wavelength at 505 nm on a spectrofluorometer. In this experiment, the labeling procedure was reinforced by incubating at 70 °C for 10 min. The results

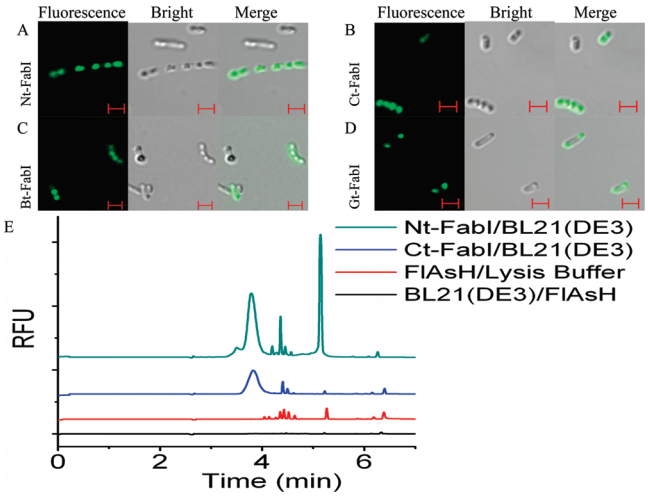

Figure 3. In vivo FlAsH labeled cells displayed minimum cellular background fluorescence. FabI protein was fused with TC-tag at its N-terminus, C-terminus, both N- and C-terminus, or fused with EGFP-tag at its N-terminus. *E. coli* BL21(DE3) cells expressing fusion proteins were grown to logarithmic phase and labeled with FlAsH-EDT_2 reagent at room temperature, followed by imaging with a LSM 510 META confocal microscope system. The BL21(DE3) cells expressing EGFP-tagged protein were microscoped directly. (A) image of cells expressing Nt-FabI; (B) image of cells expressing Ct-FabI; (C) image of cells expressing Bt-FabI; (D) image of cells expressing Gt-FabI. Bar, 2 μm. (E) Electropherograms of FlAsH labeled cell lysate. After confocal imaging, the BL21(DE3) cells expressing the TC-tagged FabIs were lysed and the 100-fold dilution of cell lysate samples were analyzed on the CE system. Cell lysate of wild-type BL21 (DE3) cells preincubated with FlAsH was used as control.

showed that the detection limit of the TC-tagged FabIs in either terminus was degraded for about one order of magnitude by adding another TC tag, and correspondingly, the linear range was extended for a number of folds. That was, the detection limits for Nt-FabI, Ct-FabI and Bt-FabI were $(7.33 \pm 0.38) \times 10^{-12}$ molar, $(6.32 \pm 0.14) \times 10^{-12}$ molar and $(3.41 \pm 0.07) \times 10^{-13}$ molar, and the linear ranges for the three were from 2.42×10^{-11} to 7.34×10^{-9} molar, from 2.49×10^{-11} to 3.12×10^{-9} molar and from 3.41×10^{-11} to 5.72×10^{-19} molar, respectively.

Efficiency and Life Span of FlAsH Labeling in Vivo. The property that the nonfluorescent small biarsenical dye can penetrate through cellular membrane freely along with its various available colors makes it a powerful multicolor and multiuse toolbox for assessing protein location and function (*46, 47*). On the basis of our observation that the TC motif can be used in the detection of the tagged protein in vitro with high specificity, we hypothesize that specific FlAsH labeling of a target protein in vivo is also controllable. As expected, an efficient FlAsH labeling was observed with uniform fluorescence throughout cells when we performed in-cell FlAsH labeling (Figure 3A−D), which was further confirmed by CE analysis of the cell lysate (Figure 3E). To our surprise, no cellular toxicity was observed after labeling with FlAsH reagent for up to 16 days; at this time point the fluorescence still existed and the cells were still alive (data not shown).

The BL21(DE3) cells, after confocal imaging, were then lysed and sampled by 100-fold dilution for CE analysis. The electropherograms of the wild-type cells labeled with the FlAsH reagent and the reagent alone did not display definitive peaks ($n = 4$). In contrast, the electropherograms obtained from the FlAsH labeled cell lysate samples of BL21(DE3) expressing the TC-tagged proteins exhibited a major peak ($n = 6$). In addition, the electropherogram of cell lysate sample of BL21(DE3) expressing Nt-FabI was similar to that of purified Nt-FabI in Figure 1A. The doublet peak for the Nt-FabI/FlAsH complex was the isomeric complex, for the two As(III) atoms of the FlAsH reagent can be bound in more than one orientation with the four thiols of the TC motif (*23, 32*). We also showed that the electropherograms of cell lysate samples from BL21(DE3) expressing Nt-FabI and Ct-FabI were very similar, which may be a result of the similar mass and charge of the two TC-tagged FabIs. Therefore, the data from cell lysate demonstrated the feasibility of employing the CZE technique to analyze TC-tagged protein without purification. To understand whether there is any difference when fusing the TC tag to a different terminus of the protein genetically, we found that the Nt-FabI and Ct-FabI have similar kinetic parameters (see Table 2), which indicated that the two TC-tagged FabIs are similar in many aspects. In other words, the TC tag fused to either terminus of the enzyme exhibited similar minor perturbance on the conformation and functions of fused proteins. Taken together, all these data demonstrated that the TC motif, fused to either terminus of the target protein, could function well with minimal disturbance and background fluorescence during the analysis on CZE.

CONCLUSIONS

In the present study, we demonstrated that the cell-permeable color-facultative small dye kept adhering covalently to the TC motif under extreme conditions. We have also displayed that the small TC tag was more suitable for nondestructive protein labeling than the EGFP tag. It was clear that the small tag could function quite well in target protein quantitative analysis, which may trigger a large number of applications. A capillary zone electrophoresis system equipped with a laser-induced fluorescence detector had a detection limit of 10^{-16} M for the labeled proteins. The attempt to expand the utility of TC tag associated with the HPLC system was of critical dominance, which offered another powerful tool for the detection of tagged proteins. In a word, the biarsenical-tetracysteine motif would have promising prospects for protein labeling and detection both in vitro and in vivo.

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Supporting Information Available: Sequences of recombinant proteins, plasmid construction procedures, structure of FlAsH reagent, and kinetics data and SPF analysis of FabIs. This material is available free of charge via the Internet at http://pubs.acs.org.

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