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Genetically-encoded nanosensor for quantitative monitoring of methionine in bacterial and yeast cells

Mohd. Mohsin, Altaf Ahmad *

Molecular Ecology Laboratory, Department of Botany, Jamia Hamdard (Hamdard University), New Delhi 110062, India

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ABSTRACT

Metabolic engineering of microorganisms for production of biological molecules represent a key goal for industrial biotechnology. The metabolic engineering requires detailed knowledge of the concentrations and flux rates of metabolites and metabolic intermediates *in vivo*. Genetically-encoded fluorescence resonance energy transfer (FRET) sensors represent a promising technology for measuring metabolite levels and corresponding rate changes in live cells. In the present paper, we report the development of genetically-encoded FRET-based nanosensor for methionine as metabolic engineering of microbial strains for the production of L-methionine is of major interest in industrial biotechnology. In this nanosensor, methionine binding protein (MetN) from *Escherichia coli* (*E. coli*) K12 was taken and used as the reporter element of the sensor. The MetN was sandwiched between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). Specificity, affinity, pH stability and metal effects was analyzed for the *in vitro* characterization of this nanosensor, named as FLIPM. The FLIPM is very specific to methionine and found to be stable with the pH within the physiological range. The calculated affinity (K_d) of FLIPM was 203 μM. This nanosensor successfully monitored the intracellular level of methionine in bacterial as well as yeast cell. The data suggest that these nanosensors may be a versatile tool for studying the *in vivo* dynamics of methionine level non-invasively in living cells.

1. Introduction

Owing to the excellent progress in metabolic engineering and emergence of the concept of system metabolic engineering, industrial biotechnology revolutionizes the conventional chemical manufacturing (Yadav et al., 2012). At present, the amino acid industry has come to occupy an important role in world chemical industries. Annual demand for amino acids used in feed additives and pharmaceutical products is huge (Ma et al., 2009). Every year, more than two million tonnes of L-glutamate and L-lysine (Eggeling and Bott, 2005; Burkovski, 2008), a few thousand tons each of L-threonine, L-leucine and L-valine are produced in industries by using genetically engineered bacterial strains (Eggeling, 2001). The development of strains for the production of L-methionine is of major interest as around 500 kt are produced annually from petrochemicals at present (Ikeda, 2003) because methionine is universally recognized as the most limiting amino acid in broiler diets on corn and soybean meal. The supplementation of broiler feeds with this amino acid in its crystalline forms is very common in the poultry industry (Café and Waldroup, 2006). This action makes possible the reduction of protein level of the diet, reducing nitrogen synthesis excretion, and can typically reduce the cost of feed. Since microbial production of methionine is a key goal for industrial biotechnology, metabolic engineering can help in developing suitable strain for the synthesis of methionine. However, the inadequacy of the metabolic engineering is the analysis of metabolic flux in the biosynthetic pathway. As such, metabolic engineering emphasizes metabolic pathway integration and relies on metabolic fluxes as determinants of cell physiology and measures of metabolic control. The combination of analytical methods to quantify fluxes and their control with molecular biological techniques to implement genetic modifications is the essence of metabolic engineering.

Mass spectrometry and nuclear magnetic resonance based metabolomics approaches are being exploited recently to analyze and screen production strains (Bennett et al., 2009; Sreekumar et al., 2009; Fendt et al., 2010). However, most techniques neither measure metabolite changes in real time nor account for likely variations in local metabolite concentration at the single-cell level. Genetically-encoded nanosensors offer the potential to transform information about such a small and specific metabolite into an optical output. These sensors can sense and respond to changing levels of metabolites within the host cell, enabling the researchers to monitor and optimize native and introduced metabolic pathways (Okumoto et al., 2005; Mohsin et al., 2013). A range of sensors have been developed for the detection of various metabolites (Miyawaki et al., 1997;

* Corresponding author. Tel.: +91 1126059688x5540.
E-mail address: aahmed@jamiahamdard.ac.in (A. Ahmad).

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de Lorimier et al., 2002; Gu et al., 2006; Mohsin et al., 2013), however, no sensor has been developed so far for the measurement of methionine at cellular level.

Here, we report on the construction of a genetically-encoded nanosensor, which allows the intracellular detection and quantification of L-methionine at single-cell resolution. This sensor uses the spatial dependence of FRET between a donor and acceptor fluorescent protein pair. Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) act as donor and acceptor fluorescent proteins, respectively. When the CFP is excited with short wavelength radiation, it emits light at a longer wavelength that is suitable to excite YFP (Mohsin et al., 2013). Methionine binding protein (MetN) from *E. coli* K12 was flanked by CFP and YFP at N and C-terminus respectively. This chimeric protein was expressed in *E. coli* BL21 (DE3) and purified. Binding of methionine to MetN induces conformational change in the sensor protein, resulting in FRET between fluorescent proteins with different emission and excitation wavelengths. We also demonstrated that this nanosensor can be used to measure steady-state concentrations and to monitor flux of methionine in bacteria, using microplate fluorescence readers. The nanosensor was named as fluorescent indicator protein for methionine (FLIPM). The beauty of this sensor is that it can be introduced in any cell type and analysis of methionine can be carried out as many times as required in living cell. As a proof of concept, this nanosensor was expressed in yeast successfully, and real-time monitoring of methionine was carried out.

2. Materials and methods

2.1. Plasmid construction

A cassette was constructed by fusing the CFP and YFP in pGEM®-T easy vector (Promega, USA). CFP and YFP sequences taken from pDH18 (generously gifted by YRC, Washington, USA) were amplified by polymerase chain reaction (PCR) after shortening the 5′ ends and 3′ ends to four codon and two codon each, respectively. The CFP and YFP sequences were cloned into the pGEM®-T easy vector. MetN sequence was retrieved from Kyoto Encyclopedia of Genes and Genomes (KEGG) database and structure of MetN protein was retrieved from the protein data base (PDB). First 26 residues (signal peptide) were removed using SignalP 3.0 server. All oligonucleotides were synthesized from Sigma, USA. The forward primer p1 was: 5′-CCCAAGCTTAGATTAAA GTTGCCGTTGTCGGCGCGAT-3′ with a *Hind*III restriction site (underlined sequences) and the reverse primer p2 was: 5′-AG CACTAGT GGCTGCCGTGGATGAACCGTCGGCGTG-3′ with *Spe*I restriction site (underlined sequences). STOP codon was removed from 3′ end. Amplified fragment was ligated into the CFP–YFP cassette generates the CFP–MetN–YFP construct in pGEM®-T easy. CFP–MetN–YFP sequence was excised from pGEM®-T easy and subcloned into the bacterial expression vector pRSET-B (Invitrogen, USA) at *Bam*HI and *Sac*I sites. This adds an in frame $(His)_6$ tag at the amino terminus of the sequence. The resultant DNA plasmid was referred to as pRSET-FLIPM. Fidelity of expression vector was verified by sequencing analysis (Document S1). All restriction enzymes used were purchased from Fermentas (GmbH, Germany). The CFP_MetN_YFP sequences were shuttled to yeast expression vector pYES-DEST52 (Invitrogen, USA) through gateway cloning technology using LR-clonase II according to the manufacturers instruction, resulting pYES-DEST–CFP_MetN_YFP. *S. cerevisiae/URA*3 strain BY4247 was used and maintained on Yeast Extract Peptone Dextrose (YEPD) agar medium and grown in liquid YEPD medium at 30 °C with aeration on a shaker. *Saccharomyces cerevisiae/URA*3 was transformed by pYES-DEST–CFP_MetN_YFP construct.

2.2. Expression and purification of sensor protein

pRSET-FLIPM sequences were transformed to the *E. coli* BL21 (DE3) by the heat shock method. Cells were grown in theLuria–Bertani (LB) broth supplemented with 100 μg/ml ampicillin at 20 °C for 24 h until the $OD_{600}=0.6$ was reached. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Fermentas, GmbH, Germany) to final concentration of 1 mM was then added to induce the cells for protein expression and grown at 20 °C for next 48 h in the dark since fluorophores are light sensitive. The bacterial cells were harvested by centrifugation at 4000 rpm for 20 mins and resuspended in 20 mM Tris–Cl (pH 8.0); cells were disrupted by sonication (Sonics, USA). A cell-free extract was obtained by centrifugation at 10,000 rpm for 1 h and by filtering through a 0.45 μm filter (Millipore, MA, USA) before it was loaded onto a column (BioRad, CA, USA). Recombinant protein was purified by nickel-nitrilotriacetic acid (Ni-NTA) (Novagen, Madison, USA) His-tag affinity chromatography resin. Protein binding to the resin was performed at 4 °C for 4 h, washed in the column with 20 mM Tris–Cl, 10 mM imidazole (pH 8.0) and eluted with 20 mM Tris–Cl, 50 mM Imidazole. Protein was stored at 4 °C for 16 h to refold in its native conformation. Purity of the chimeric protein was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. in vitro characterization of nanosensor

Characterization of sensor was initially carried out at various buffer systems with pH changes. Twenty mM of each phosphate buffer saline, 3-(N-morpholino) propanesulfonic acid (MOPS) and Tris buffer saline were taken for this study. The purified sensor protein was diluted 20 times by the respective buffers. It was found that sensor protein was showing least variation in FRET ratio with pH of MOPS buffer. In another study stability of the sensor protein was tested with MOPS buffer in the pH range of 5.0–8.0, with addition of methionine and in the absence of methionine. The change in the FRET ratio with respect to change in pH and buffer was monitored by using monochromator microplate reader (DTX880, Beckman Coulter, USA). Emission spectra were recorded on the fluorometer (LS50B, Perkin Elmer, USA) in 20 mM MOPS buffer (pH 7.25). Fluorescence emission spectra were taken by exciting CFP at 435 nm and the emission was observed in the range of 450–580 nm.

Specificity of the sensor protein was tested by measuring FRET with other amino acids like glutamine, cysteine and leucine with concentrations 0 and 10 mM each. To find out the effect of the metal ions Na^+, K^+, Ca^{2+} and Mg^{2+} on the fluorescence, 535/485 ratio was taken by adding methionine with metal ions. 1 mM from each NaCl, KCl, $MgCl_2$ and $CaCl_2$ was added in the solution, mixed with 10 mM methionine in different microplate wells. FRET measurement was carried out by using the microplate reader. The excitation filter was 430/20 nm and emission filters for CFP and YFP were 485/20 nm and 535/25, respectively. Typically 180 μl of sensor protein diluted in MOPS buffer and 20 μl of methionine prepared in MOPS buffer were taken in each well.

To determine the K_d of each FLIPM sensor, sensor protein was mixed with different concentrations of methionine in 20 mM MOPS buffer (pH 7.25) and YFP/CFP ratio was recorded. The K_d of the constructs was determined by fitting the ligand titration curve in simple binding isotherm: $S=(r-r_{apo})/(r_{sat}-r_{apo})=[L]/(K_d+[L]$, where S is saturation; $[L]$, ligand concentration; r, ratio; r_{apo}, ratio in the absence of ligand and r_{sat} ratio at saturation with ligand. All measurements were performed from at least three independent protein extracts. FRET was used to characterize the sensor protein with different parameters. The FRET was calculated as fluorescence emission intensity at 535 nm (YFP) divided by fluorescence