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A dual-ligand-modulable fluorescent protein based on UnaG and calmodulin

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ABSTRACT

UnaG is a green-emitting fluorescent protein that utilizes unconjugated bilirubin (BR) as its fluorophore. While BR has captured the attention of physiologists as an important antioxidant that scavenges reactive oxygen species in biological membranes, its excessive accumulation causes several clinical symptoms. Although the optimal regulation of BR concentration would result in clinical therapies for aging as well as reduce risks of clinical symptoms, UnaG hardly releases BR owing to its extremely high affinity for BR ($K_d = 98$ pM). Herein, we engineered the BR binding and fluorescence of UnaG to be Ca^{2+}-sensitive via a genetic insertion of calmodulin (CaM). The resultant UnaG/CaM hybrid protein is a dual-ligand modulable fluorescent protein; binding of the fluorogenic ligand BR is negatively regulated by the other ligand, Ca^{2+} ion. The affinity for BR differed by three orders of magnitude between the Ca^{2+}-free state ($K_d = 9.70$ nM) and Ca^{2+}-saturated state ($K_d = 9.65$ μM). The chimeric protein can release nano- to micromolar levels of BR with Ca^{2+} control, and is thus named BReleaCa (BR + releaser + Ca^{2+}). Such a protein hybridization technique will be generally applicable to change the ligand binding properties of a variety of ligand-inducible functional proteins.

1. Introduction

UnaG belongs to the fatty-acid-binding protein (FABP) family, and is a unique ligand-inducible fluorescent protein [1]. It binds unconjugated bilirubin (BR) non-covalently as a fluorogenic ligand with high specificity and affinity ($K_d = 98$ pM), and the resulting UnaG-BR complex (holoUnaG) emits green fluorescence ($\lambda_{max} = 527$ nm, $\Phi_f = 0.51$) [1]. When expressed in mammalian cells in culture, holoUnaG is presumed to predominate over apoUnaG considering the extremely high affinity for BR and the high BR concentration in bovine-serum-containing culture medium [1]. Such a strong ligand-protein interaction was corroborated by a crystallographic analysis (Fig. 1A and B) that identified fifteen hydrogen bonds in the interface [1].

The physiological significance of BR has been a subject of debate. On the one hand, it is well known that the excessive accumulation of BR causes several clinical symptoms, such as jaundice and kernicterus, and that BR is an important diagnostic index in clinical medicine [2,3]. On the other hand, it has been reported that BR acts as an antioxidant by scavenging reactive oxygen species (ROS) in biological membranes [4,5]. It is also an enigma why eel muscles contain such a large amount of holoUnaG that releases a small quantity of BR due to the high affinity.

In this study, we engineered the BR binding of UnaG to be sensitive to free Ca^{2+} ion. We speculated that the insertion of calmodulin (CaM) near the BR binding pocket of UnaG [6] would result in the production of a novel fluorescent protein that releases the fluorophore in a Ca^{2+}-dependent manner. Recently, To et al. developed a "UnaG-based protein-protein interaction reporter (uPPI)" by using the protein-fragment complementation approach [7]. Their study revealed that the site between residues 84 and 85 of UnaG was tolerant to protein insertion. In addition, as the site is close to Ser80/Asp81 residues, which anchor the propionate carboxylate from the *exo*-vinyl dipyrrinone of BR, the Ca^{2+}-dependent conformational change on the inserted CaM was expected to affect the BR affinity. Remarkably, one of the UnaG/CaM chimeric proteins constructed in this study decreased BR affinity and accordingly the fluorescence intensity (FI) markedly with Ca^{2+}.

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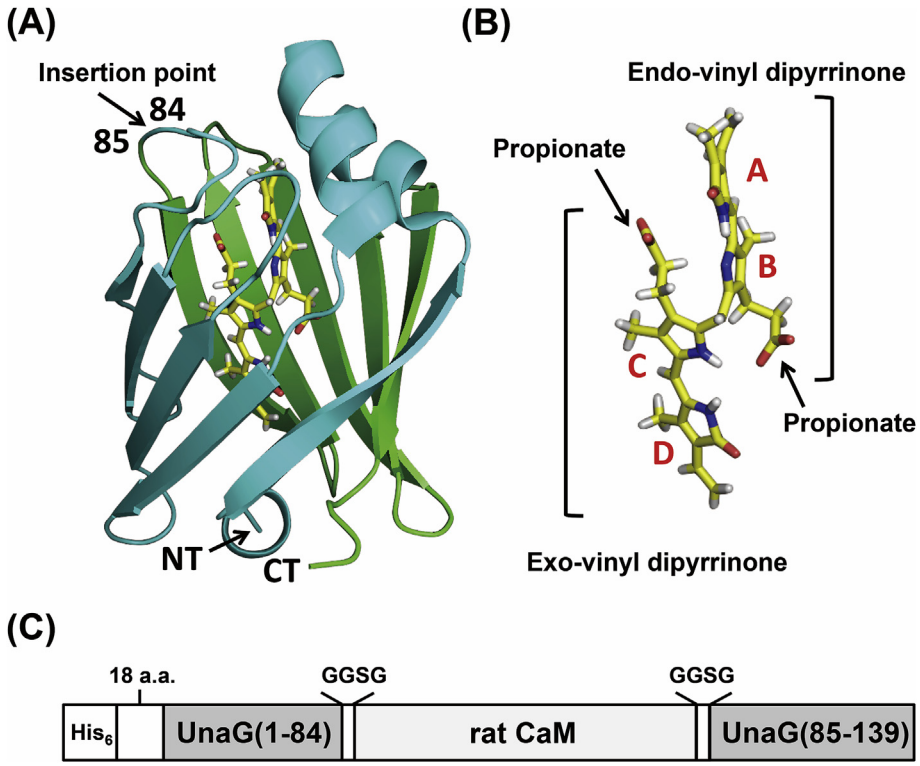

Fig. 1. Crystallographic structure of holoUnaG. (A) Overall structure of UnaG-BR complex. The site between residues 84 and 85 for insertion of foreign peptides is indicated. The image was created using PyMol (DeLano Scientific) from PDB file 4I3B [1]. (B) The bound BR in (A). BR is shown as a stick representation with atoms colored (carbon, yellow; oxygen, red; nitrogen, blue; hydrogen, gray). Rings A/B and rings C/D compose the *endo*-vinyl dipyrrinone and *exo*-vinyl dipyrrinone moieties. NT, amino terminus. CT, carboxyl terminus. (C) Schematic domain structures and sequences of BReleaCa (#3) for expression in *E. coli*. Insertion of the entire protein of rat CaM. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Thus the protein is referred to as BReleaCa (= BR + releaser + Ca^{2+}). We investigated the association/dissociation kinetics between BReleaCa and BR by monitoring FI as the readout and provide mechanical insights into the interplay between BR and Ca^{2+} on the dual-ligand-modulable protein.

2. Materials and methods

2.1. Chemicals

BR (bilirubin), dimethylsulphoxide (DMSO), and ampicillin sodium were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Carbosynth Ltd. (Berkshire, U.K.). HEPES was purchased from Dojindo Lab. (Kumamoto, Japan). Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid tetrasodium salt (EGTA-Na) was purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, U.S.A.). Calcium Calibration Buffer Kit #1 was purchased from Thermo Fisher Scientific (Waltham, MA, U.S.A.). BR was dissolved in DMSO at 1 mM or at 25 mM, and stored at −20 °C until use.

2.2. Preparation of proteins

Calmodulin (*CaM*) and *mCherry* were acquired from Addgene plasmid 40753 and 35687, respectively (Cambridge, MA, U.S.A.). pColdI-UnaG was prepared as described previously [8]. To create *BReleaCa*, *CaM* was inserted into *UnaG* between residues 84 and 85 with both ends employing tetrapeptide linkers (GGSG-CaM-GGSG) at the 19-bp overlapping sequences by a SLiCE reaction [9,10], resulting in pColdI-BReleaCa. Protein expression and purification were performed as described previously [8] with a replacement of K-Pi buffer (20 mM potassium phosphate buffer adjusted to pH 7.5) with HEPES buffer (100 mM HEPES buffer containing 150 mM NaCl, adjusted to pH 7.5).

2.3. Spectroscopic studies (steady state)

Fluorescence measurements were performed using an RF-5300PC spectrophotometer (Shimadzu, Kyoto, Japan) and an F-2500 spectrophotometer (Hitachi Ltd., Tokyo, Japan). Absorption spectra were recorded using a V-660 spectrophotometer (JASCO Corporation, Tokyo, Japan). The reconstitution of BReleaCa with BR was triggered by the addition of a small volume of high concentration BR dissolved in DMSO (1 mM or 25 mM) to 2 ml of BReleaCa solution. The dissociation constant for BR binding in the absence of Ca^{2+}: $K_d^{2+}{}_{(Ca(-))}$ was determined using 10 nM BReleaCa solutions containing 500 μM EGTA-Na. The dissociation constant in the presence of Ca^{2+}: $K_d^{2+}{}_{(Ca(+))}$ was determined using 1 μM BReleaCa solutions containing 500 μM $CaCl_2$. All data were collected at room temperatures at around 25 °C.

2.4. Measurements of k_{on} (stopped-flow analysis)

The measurements of association rate constants were performed using an SX20 Stopped-Flow Spectrometer (Applied Photophysics, Leatherhead, U.K.). Equal volumes of a 2 μM BReleaCa solution and a 2 μM BR solution were rapidly mixed, and thus the total concentrations of BReleaCa and BR ($[BReleaCa]_t$ and $[BR]_t$, respectively) were both 1 μM. Since the fluorescence of BR-BReleaCa is considerably bright, it is possible to reproducibly characterize the rapid increase in FI (dFI/dt), which should be proportional to the initial rate of the generation of BR-BReleaCa (d

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