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学位論文題目	Design of novel biosensing systems for ligand molecules by combination of fluorescent unnatural mutant binding proteins and ligand analogues (蛍光性非天然変異体結合タンパク質とリガンド類似体の組み合わせによるリガンド分子に対する新しいバイオセンシングシステムの設計)
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Abstract

In this study, two types of biosensing systems for small ligand molecules were designed by combination of a fluorescent unnatural mutant binding protein and ligand analogues. The pairs of fluorescent unnatural mutant streptavidin and biotin analogues were used as the model systems. Molecular biosensing systems for free biotin detection were designed by the application of fluorescence quenching of the mutant streptavidin with quencher-modified biotin, or by the application of fluorescence enhancement of the mutant streptavidin with spacer-modified biotin. These results suggested that combination of fluorescent unnatural mutant binding proteins and ligand analogues might be very useful for simple and sensitive detection of free small ligand molecules.

Biotin is a water-soluble important vitamin. It is not only an essential component for synthesis of vitamin C, but also works as a coenzyme in the synthesis of fatty acids, isoleucine, and valine, and plays an important role in gluconeogenesis. Biotin deficiency induces alopecia, conjunctivitis, dermatitis, depression, lethargy, hallucination, numbness and tingling of the extremities. Therefore, more rapid convenient methods for the determination of biotin are further required.

The incorporation of functional unnatural amino acids into proteins is a powerful and versatile technique for designing functional proteins for biosensor fabrication and for analyzing protein structure and function. A number of researchers have synthesized proteins containing unnatural amino acids at desired positions by using an amber suppression technique. Alternatively, four-base codon method can perform the site-directed introduction of single or multiple functional unnatural amino acids that possess special functions such as fluorescence and oxidation-reduction to provide them to the proteins. In this method, four-base codons can be introduced into assigned positions of a protein gene. The full-length protein containing the unnatural amino acids could be produced when the four-base codons are successfully decoded by the unnatural aminoacyl-tRNAs having the corresponding four-base anticodons. On the other hand, when the first three bases of the four-base codon are decoded as a three-base codon by a cognate naturally occurring aminoacyl-tRNA, a frame-shift occurs and causes the emergence of a stop codon resulting in the termination of peptide elongation. Therefore, the full-length protein could be obtained only when the four bases are successfully decoded as a single codon. The four-base codon method is advantageous over the amber codon suppression technique, because even more than three unnatural amino acids can be introduced into a single protein.

Streptavidin is a homo-tetrameric biotin binding protein which has extremely high dissociation constant (Kd) in the order of 10^{-14} mol/L for biotin. Typical example of the conventional methods for biotin determination is fluorescence polarization measurement during competitive binding reaction of native biotin and fluorescent dye-modified biotin to streptavidin. In this study, deferent designs of molecular biosensing system for biotin with only a simple fluorescence spectrophotometer were proposed here.

Trp120 of the neighbor subunit in streptavidin tetramer is placed within 15 Å from the carboxyl terminal of biotin. A fluorescent unnatural amino acid, BODIPY-FL-aminophenylalanine (BFLAF), which was prepared by chemical modification of p-aminophenylalanine with the BODIPY-FL, was incorporated at the Tyr54, Tyr83 or Trp120 position of a streptavidin molecule to synthesize three different monoclonal fluorescent mutant streptavidins by using four-base codon method and to apply them into molecular biosensor for biotin. On the other hand, carbazole was modified to the carboxyl terminal of biotin as a quencher for BFLAF. Fluorescence of the Trp120BFLAF mutant streptavidin was certainly quenched by binding of carbazole-labeled biotin to the mutant streptavidin to make complex. Whereas, in the presence of free biotin was existed in the assay solution, fluorescence quenching of the Trp120BFLAF by the binding of carbazole-labeled biotin was suppressed because of the competitive binding reactions of natural biotin the fluorescent mutant streptavidin. By this competitive binding assay, free biotin was determined in the concentration range from 20 nM to 100 nM.

On the other hand, it was recently reported that the tryptophan near the fluorophore which was modified to the target protein could quench the fluorescence from the fluorophore. In this study, we further discovered that fluorescence of the Trp120BFLAF mutant streptavidin was enhanced when the biotin analogue with a $(AC_5)_2$-hydrazide tail was bound to the fluorescent mutant protein, whereas natural biotin binding did not induce the fluorescence intensity change. We then speculate that the fluorescence quenching of Trp120BFLAF by Trp79 or Trp108 of the neighbor subunit may be existed and disturbed by the long spacer tail of the biotin analogue. Finally, we applied this fluorescence enhancement of the Trp120BFLAF mutant streptavidin by binding of biotin-$(AC_5)_2$-hydrazide to the molecular biosensing system for free biotin by measuring the suppression of the fluorescence enhancement under the competitive binding reaction of free biotin and biotin-$(AC_5)_2$-hydrazide to the fluorescent mutant streptavidin.. This method also performed sensitive detection for free biotin in the concentration range from 20 nM to 100 nM.

These results demonstrated that various useful molecular biosensing systems for small ligands might be constructed by combination of position-specifically designed fluorescence mutant binding proteins with the corresponding quencher-labeled ligand analogues or spacer-modified ligand analougs.

博士学位論文審査結果の要旨

平成２６年２月７日に、朱　先偉氏の博士学位論文の公聴会を開催し、５名の審査員による博士論文審査および最終試験を行い、ともに合格と判断した。

氏は、本博士学位論文で、生体内で重要な働きをする低分子や薬物を簡単に検出、定量する新しいバイオセンシングシステムの設計を目指した。具体的にはビタミンとして重要なビオチンに対する結合タンパク質であるストレプトアビジンに注目し、蛍光性非天然アミノ酸を部位特異的の導入した蛍光性変異タンパク質を合成した。また、消光団をカルボキシル基末端に結合したビオチン類似体も自作した。そして、消光団を修飾したビオチン類似体の結合に伴うこの非天然ストレプトアビジンの蛍光消光あるいは、スペーサー分子を修飾したビオチン類似体と非天然ストレプトアビジンとの結合に伴う蛍光増強を計測した。そして、最終的には、いずれの系でも競争結合反応を利用して、測定対象の遊離ビオチンを検出、定量できることを示した。

本研究の成果から、今後、蛍光性非天然変異体結合タンパク質とリガンド類似体の組み合せによって、測定対象となるリガンド分子を簡単に検出、定量する種々のバイオセンシングシステムの設計が可能であることが示唆された。

また、その成果の一部は、Xianwei Zhu, Hiroaki Shinohara, Fluorescence Enhancement of Fluorescent Unnatural Streptavidin by Binding of a Biotin Analogue with Spacer Tail and Its Application to Biotin Sensing, The Scientific World Journal, Volume 2014, Article ID165369, 6 pagesとして掲載予定である。

本学位論文は、以上の成果により、リガンド分子に対するセンサータンパク質とそれを用いたセンシングシステムの新たな設計方針を示したものであり、十分に博士学位論文としての価値が認められた。