2022 年 11 月 22 日
Year/Month/Day

# 学 位 論 文 の 内 容 の 要 約
Summary of doctoral dissertation content

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| 氏　　名<br>Name | 山下　有紀 |
| 学位の種類<br>Doctoral degree | 博士（工学） |
| 学府又は研究科・専攻<br>Graduate School / Major | 大学院工学府　生命工学専攻 |
| 指 導 を 受 け た 大 学<br>University | 東京農工大学 |
| 学位論文題目<br>Thesis title | Improvement of substrate specificity and analysis of direct electron transfer pathway of direct electron transfer type glucose dehydrogenase as the enzyme for glucose sensor application |

【論文の内容の要約】[Summary of the contents of the doctoral dissertation]

A tight glycemic control to keep blood glucose levels in near normal range is important for managing diabetes to reduce the risk of diabetic complications such as microvascular disease and hypoglycemia. Thus, monitoring of blood glucose in daily life is important in diabetes therapy. Currently, there are two types of techniques available for monitoring blood glucose, continuous glucose monitoring (CGM) and self-monitoring of blood glucose (SMBG). In both techniques electrochemical detection of glucose by using either glucose oxidase (GOX) or glucose dehydrogenase (GDH) as its sensing component are employed.

The direct electron transfer (DET) is an ideal principle for electrochemical detection of glucose, in which the electrons generated by the glucose oxidation are transferred directly from the enzyme to the electrode. It enables the sensor to operate in a lower redox potential, closer to the enzyme's co-factor, reducing the effect of interfering substance in the samples. Moreover, the avoidance of toxic artificial mediators is attractive for implantable sensor applications, such as CGM systems.

The flavin adenine dinucleotide (FAD) dependent glucose dehydrogenase (FADGDH) from *Burkholderia cepacia* is the first naturally available DET-type glucose oxidizing enzyme. FADGDH consist of three subunits: the catalytic subunit, the electron transfer subunit which is a cytochrome *c*, and a small subunit which is a hitchhiker protein. The electron transfer subunit enables this enzyme to transfer electrons generated from

glucose oxidation to the electrode. This enzyme has attractive features as a glucose sensor component, such as high catalytic activity and not reacting to oxygen. The final goal of this study is to engineer FADGDH to be an optimal glucose sensing enzyme for DET-type glucose sensor. However, the substrate specificity of FADGDH is broad and improvement is necessary. Moreover, for future application of this enzyme to DET-type glucose sensors the improvement of DET-efficiency will be useful. Therefore, in this thesis, engineering of the substrate specificity of FADGDH was conducted and a DET-type disposable glucose sensor strip was developed with the obtained mutant. Moreover, the elucidation of intra/inter-molecular electron transfer pathways of the electron transfer subunit was conducted as a first step to realize the future engineering of the DET-efficiency by protein engineering of FADGDH.

In Chapter 1 “Introduction”, the background of this research and the objective of this thesis was described.

In Chapter 2 “Improvement of substrate specificity of FADGDH”, site directed mutagenesis was carried out in the catalytic subunit of FADGDH aiming to lower the activity toward maltose. The target residues for mutation were selected by 1) comparison of amino acid sequence of the substrate binding site of two reported GOXs and 2) comparing the residues in the vicinity of glucose in the predicted structural model of FADGDH and the crystal structure of *Aspergillus niger* GOX. Among the mutants constructed, Ser326Gln/Ser365Tyr (QY) double mutant was revealed to possess the most improved substrate specificity. In this mutant, the substrate specificity was improved by 30-fold compared to the wild type (WT) FADGDH while retaining high activity towards glucose. The results of mutagenesis were further discussed by comparing the recently elucidated crystal structure of FADGDH, which was obtained after this study was conducted. It was revealed that the mutations in the residues that are in the cavity opening leading to the substrate binding site had significant effect on maltose activity, maybe hindering the maltose entry to the substrate binding site. We also succeeded in constructing a direct electron transfer type disposable glucose sensor strip employing either WT or the QY mutant. Both sensors showed clear correlation in the response current with the increase of glucose concentration. Moreover, the QY employed sensor showed accurate readings even with the presence of maltose in the sample. Thus, we have succeeded in constructing a DET-Type disposable sensor strip which was unaffected by maltose. The QY mutant seemed promising for application to glucose sensors. Also, by combining the information obtained by site-directed mutagenesis in this study with

the structural information will contribute greatly for further engineering of FADGDH as a glucose sensor component.

In Chapter 3 “Elucidation of the electron transfer pathway of the cytochrome *c* subunit of FADGDH”, site-directed mutagenesis was conducted to the sixth ligand of the existing three hemes in the electron transfer subunit of FADGDH. By comparing and analyzing the amino acid sequence of the electron transfer subunit and the cyt *c* subunits of the reported hetero-oligomeric flavocytochrome dehydrogenases, the sixth ligand for each of the three heme irons was predicted and were substituted to His. The three hemes were named, heme 1, heme 2 and heme 3 in the order of the appearance of the heme binding motif in the amino acid sequence. The catalytic activities of the WT and mutant enzymes were compared by investigating their dye-mediated dehydrogenase activities and their DET abilities toward the electrode. It was revealed that heme 1 is responsible for the electron transfer with external electron acceptor in solution, heme 2 is responsible for DET, and heme 3 seemed to be the electron acceptor from the catalytic subunit, since the mutation negatively affected not only the dye-mediated dehydrogenase activity but also the DET ability.

Based on the results obtained, two electron transfer pathways were proposed; (1) electrons are transferred from the catalytic subunit to heme 3, then to heme 2, to heme 1 and finally to electron acceptors in solution, (2) in case where the enzyme complex is immobilized on the electrode, electrons are transferred from the catalytic subunit to heme 3 and then passed to the electrode mainly from heme 2. Combined with the previous studies and results of this study, the intra- and inter-molecular electron transfer pathway of FADGDH was unveiled. These findings will give valuable insights to understand the DET mechanism of FADGDH and will contribute greatly for further engineering of this enzyme for improvement of its performance and its application toward CGM systems and other DET-based glucose monitoring devices.

In Chapter 4 “Conclusion”, achievement in this thesis was summarized, and future perspectives of this study were described.