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Engineering glucose oxidase to minimize the influence of oxygen on sensor response

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ABSTRACT

Glucose oxidase (GOx) is an important industrial enzyme and is recognized as the gold standard for monitoring blood glucose. However, due to its inherent oxidase property, the presence of oxygen affects electrochemical measurements of venous blood glucose employing artificial electron mediators. We therefore attempted to engineer *Penicillium amagasakiense*-derived GOx into a dehydrogenase by focusing on the amino acid residues predicted to interact with oxygen. Our rational amino acid substitution approach resulted in the construction of the Ser114Ala/Phe355Leu mutant, which has an 11-fold decrease in oxidase activity and 2.8-fold increase in dehydrogenase activity compared with wild-type GOx. As a result, the dehydrogenase/oxidase activity ratio of the engineered enzyme was 32-fold greater than that of the wild-type enzyme. The enzyme sensor constructed with Ser114Ala/Phe355Leu was considerably less affected by oxygen than the wild-type GOx-based sensor at lower glucose concentrations.

1. Introduction

Glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase, E.C. 1.1.3.4; GOx) is a flavoprotein that catalyzes the oxidation of β-D-glucose to glucono-δ-lactone by using molecular oxygen as the electron acceptor [1]. This reaction is divided into two half-reactions, a reductive half-reaction in which the flavin cofactor is reduced with oxidation of the sugar substrate, followed by the oxidative half-reaction where the cofactor is reoxidized by oxygen [2]. Because this enzyme has high substrate specificity for β-D-glucose, it has long been a major enzyme employed in analytical test kits and biosensors for the determination of glucose in clinical and industrial samples, especially in the monitoring of blood glucose in diabetes patients. However, electron mediator-type enzyme sensors employing GOx are inherently influenced by the amount of oxygen dissolved in the sample. Therefore, oxidases that are less oxygen-sensitive would be greatly beneficial for the development of amperometric enzyme sensors.

We have previously reported on the engineering of fructosyl amino acid oxidase [3] and fructosyl peptide oxidase [4], which can be used to assess treatment effectiveness in diabetes patients by monitoring the levels of glycated proteins. These oxidases were successfully engineered to drastically decrease their oxidase activity while increasing their dehydrogenase activity by disrupting a putative proton relay system that may be responsible for transferring electrons to oxygen. These results motivated us to follow a similar approach on the GOx of *Penicillium amagasakiense*, which has a higher turnover rate and a better affinity for glucose than its *Aspergillus niger* counterpart [5]. The residues responsible for the transfer of electrons to oxygen in the oxidative half reaction were predicted by first constructing structural models of GOx complexed with oxygen based of the oxygen-complexed crystal structure of cholesterol oxidase, which is also a member of the glucose/methanol/choline (GMC) oxidoreductase family [6]. Alanine substitutions were carried out on the residues predicted to interact with oxygen, producing a number of *P. amagasakiense* GOx variants with decreased oxidase activity and increased dehydrogenase activity. The Ser114Ala substitution produced a significant decrease in oxidase activity and was the most promising mutant produced by a detailed saturation mutagenesis study [6]. In this study, we carried out a detailed saturation mutagenesis

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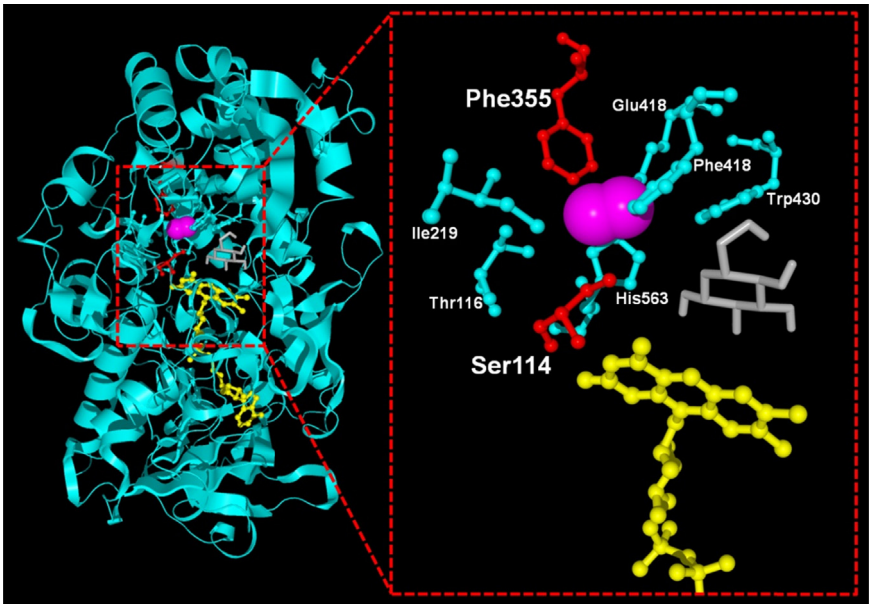

Fig. 1. Predicted 3D structural model of *P. amagasakiense* GOx (PDB ID; 1gpe) complexed to oxygen. The active site region is shown on the right, with the oxygen molecule (magenta) located in the previously predicted location [6]. Also shown are the FAD cofactor (yellow), glucose substrate (gray), the residues modified in this study (Phe355 and Ser114, red), and the other residues predicted to interact with oxygen (cyan). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

investigation of *P. amagasakiense* GOx at position Phe355, the position that had produced the next best alanine-substitution mutant.

2. Experimental

2.1. Bacterial strains and plasmids

Escherichia coli BL21 (DE3) was used as the host strain for the expression of wild-type and mutant GOxs. The gene encoding the mature (i.e., without signal sequence) *P. amagasakiense* GOx was synthesized based on its GenBank nucleotide sequence (ID: AAD01493) and inserted into the pET-22b(+) expression vector (Merck KGaA, Darmstadt, Germany) as described previously [6].

2.2. Enzyme assays

Site-directed mutagenesis and enzyme preparation, from refolded inclusion bodies produced in *Escherichia coli*, was carried out as previously described [6]. Refolded enzyme preparations were assayed for protein concentration and analyzed by SDS-PAGE to ensure comparable levels of refolding. The crude refolded enzymes were assayed at 10 μg/ml protein concentrations.

The glucose oxidase activity was investigated with different concentrations of glucose by measuring the formation of hydrogen peroxide using 1.5 mM 4-aminoantipyrine, 1.5 mM N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline, and 2 U horseradish peroxidase/mL. The formation of the quinoneimine dye was measured at 555 nm (39.2 mM^{-1} cm^{-1} molar absorption coefficient). The dye-mediated glucose dehydrogenase activity of GOx was measured using 0.6 mM methylphenazinium methylsulfate (PMS) and 0.06 mM 2,6-dichlorophenolindophenol (DCIP) as electron acceptors at room temperature in 20 mM potassium phosphate buffer (pH 7.0) and following the reduction of DCIP at 600 nm (39.2 mM^{-1} cm^{-1} molar extinction coefficient). The amount of an enzyme that forms 1 μmol hydrogen peroxide (oxidase activity) or reduces 1 μmol DCIP (dehydrogenase activity) in 1 min is defined as 1 U of enzyme.

2.3. Preparation and operation of enzyme sensors

A mixture of purified wild-type or mutant GOx (200 mU based on dehydrogenase activity) and 2% (w/v) photoreactive polyvinylalcohol (AWP) were applied on a glassy carbon electrode (3.0 mm diameter; Bioanalytical Systems, Inc., West Lafayette, USA) and dried for 1.5 h in the dark at 37 °C. The AWP was then polymerized by exposure to UV for 30 s and equilibrated in 100 mM potassium phosphate buffer (pH 7.0) at 4 °C. The sensor was based on a three-electrode system: an enzyme-based working electrode, a platinum-wire counter electrode, and an Ag/AgCl reference electrode. Measurements were carried out at 25 °C with an applied electric potential of 100 mV, in 10 ml of 100 mM potassium phosphate buffer (pH 7.0), 10 mM 1-methoxy-PMS (mPMS) as electron mediator, and 1, 2, 3, 4, 5, 7, 10, 13, 16, or 20 mM glucose with gentle magnetic stirring (250 rpm). The open reaction chamber was either exposed to normal atmospheric conditions or bubbled with argon for 2 h prior to measurement and surface-sprayed with argon during the measurement period.

3. Results and discussion

3.1. Saturation mutagenesis at Phe355

In our previous experiments with fructosyl amino acid oxidase [3] and fructosyl peptide oxidase [4], in which we drastically increased the dehydrogenase to oxidase activity ratio, we demonstrated that the amino acid residues involved in electron transfer to oxygen and those involved in electron transfer to mediators are found in two very distinct locations. We then attempted a similar approach with GOx and carried out alanine-substitution mutagenesis of residues predicted to interact with oxygen based on an oxygen-complexed structural model of GOx that we had constructed [6]. Our goal was to specifically disrupt the ability of GOx to transfer electrons to oxygen while leaving unaffected its ability to transfer electrons to mediators. Because Phe355 of *P. amagasakiense* GOx is positioned in very close proximity to the predicted location of the oxygen molecule (Fig. 1) and its substitution to Ala had