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Structural stability of glucose oxidase encapsulated in liposomes to inhibition by hydrogen peroxide produced during glucose oxidation

Makoto Yoshimoto *, Mitsunobu Sato, Shaoqing Wang, Kimitoshi Fukunaga, Katsumi Nakao

Department of Applied Chemistry and Chemical Engineering, Faculty of Engineering, Yamaguchi University, 2-16-1 Tokiwadai, Ube 755-8611, Japan

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Abstract

The glucose oxidase (GO) consists of two identical subunits each of which contains noncovalently bound flavin adenine dinucleotide (FAD) cofactor. GO is known to be inactivated due to hydrogen peroxide (H_2O_2) produced in the oxidation of glucose. In our previous paper, the liposomal GO showed a much higher stability to H_2O_2 than the free enzyme. In this work, to deduce the structure and state of the liposomal GO, the fluorescence properties of the tryptophan residue and FAD cofactor in free GO during the glucose oxidation were measured for its tertiary structure and redox state, respectively. The tryptophan fluorescence data revealed that the initial glucose concentration lower than 0.6 mM resulted in almost no alteration in the tertiary structure, while the higher concentration did in a remarkable change in the structure due to the increase in catalytic turnover. On the other hand, the FAD fluorescence data showed that the reduced FAD was accumulated in the initial stage of the reaction. When glucose was completely consumed, the FAD restored the initial oxidized form for the initial glucose concentrations lower than 0.6 mM, whereas for the higher concentrations the reduced FAD tended to form an inactive complex with H_2O_2 leading to the deactivated enzyme. In the case of the liposomal GO at even such a high initial glucose concentration as 10 mM, the glucose concentration inside liposome was previously estimated to be lower than 0.2 mM due to its low permeability to glucose. Consequently, the formation of the inactive complex was proved to be effectively depressed in the liposomal GO reaction.

Keywords: Liposomal glucose oxidase; Tryptophan fluorescence; FAD fluorescence; Enzyme tertiary structure; Enzyme inactivation; Glucose permeability

1. Introduction

Enzyme molecules encapsulated in the phospholipid vesicles, i.e., liposomes are shown to be modified in their reactivity and stabilized compared to free enzyme [1]. We reported the highly stable liposomal glucose oxidase in catalyzing a prolonged oxidation of glucose producing gluconic acid and hydrogen peroxide (H_2O_2) [2]. In the active glucose oxidase (GO) molecule, two tightly bound flavin adenine dinucleotide (FAD) molecules are stabilized through their interactions with the particular aromatic amino acid residues in GO [3]. H_2O_2 is known to competitively inhibit the activity of GO through forming the inactive complex between the reduced form of FAD cofactor and H_2O_2 produced in the catalytic turnover of GO [4]. Recently, we reported the stable liposomal GO in catalyzing the oxidation of glucose [5]. The mechanism for the highly stabilized liposomal GO was revealed mainly focusing on the role of the catalase contained in the commercially available GO. The catalase was stabilized by encapsulating in liposomes and catalyzed the decomposition of H_2O_2 produced continuously as opposed to free CA. It was also reported that, when the rate of the liposomal GO reaction was increased through enhancing the glucose permeability across the liposome membrane, the inhibition of the GO activity became pronounced due to the increased accumulation of H_2O_2. The mechanistic details on the above deactivation of GO has been remained unknown at the enzyme molecular level.

Since the liposomal aqueous phase is very small in volume and surrounded by fragile lipid bilayer membranes, the structure of enzyme present in liposomes are difficult to directly observe. One of the effective approaches for analyzing the glucose oxidation catalyzed by liposomal enzyme would be to examine the free enzyme reaction which proceeds under a similar condition to the liposomal system. For the liposomal GO-catalyzed oxidation of glucose, the lower glucose concentration inside liposomes compared to that outside liposomes was suggested to be the

* Corresponding author. Tel.: +81 836 85 9271; fax: +81 836 85 9201.
E-mail address: yosimoto@yamaguchi-u.ac.jp (M. Yoshimoto).

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additional critical feature in analyzing the structural stability of the GO molecule [6].

In the present work, the fluorescence measurements [3,7] were performed to evaluate the redox state of FAD cofactor as well as the local tertiary structure around the FAD molecules in GO molecules during the free GO-catalyzed glucose oxidation. In particular, the effects of the glucose concentration on the fluorescence properties of the enzyme were dynamically examined in order to elucidate the high stability of the liposomal GO to H_2O_2 on a GO molecular basis.

2. Materials and methods

2.1. Materials

Glucose oxidase (GO) from *Aspergillus niger* (EC 1.1.3.4) was purchased from Toyobo Co. Ltd. (Osaka, Japan). POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). β-D-Glucose was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

2.2. Preparation of glucose oxidase-containing liposomes (GOL)

The GOL was prepared by the extrusion technique with the 50 mM Tris–HCl/100 mM NaCl buffer at pH 7.4 (denoted as Tris buffer) as reported previously [2,5]. The enzyme-free liposomes were also prepared.

2.3. Oxidation of glucose catalyzed by glucose oxidase (GO)

The oxidation of glucose was initiated at 27 °C in the Tris buffer of pH 7.4 by adding glucose solution to the GO solution (1.3 mg GO/mL solution) or GOL suspension ([lipid] = 1.0 mM, 1.1 mg GO/mL suspension) in a test tube to give the initial glucose concentration of 10 mM. The total reaction volume was 1.5 mL. The time course of the glucose conversion and the remaining GO activity were then measured. For the liposomal GO reaction, the glucose conversion was calculated from the remaining glucose concentration measured with the enzymatic method. This method was applicable to the sample containing the negligible amount of H_2O_2 because it measures the H_2O_2 produced from glucose [5]. The assay samples from the liposomal GO reaction were reported to contain almost no H_2O_2 due to the liposome-stabilized catalase activity in the commercially available GO [5]. For the free GO reaction accumulating H_2O_2, in which the moles of glucose consumed were almost equal to moles of H_2O_2 accumulated, the conversion was calculated from the H_2O_2 concentration measured with the titanium sulfate method [4]. For the remaining GO activity measurements, the free GO or GOL was separated from the reaction solution containing glucose as well as the products H_2O_2 and gluconic acid by using the GPC. The GO activity was measured in the same way as previously described [2,5,8]. The intrinsic GO activity in the GOL was measured after solubilization of liposomes with an excess amount of cholate. The remaining GO activity was determined as the intrinsic activity at the certain reaction time relative to that before initiating the reaction.

2.4. Measurement of tryptophan fluorescence

The intrinsic tryptophan fluorescence was measured for the free GO during catalyzing the glucose oxidation in the Tris buffer of pH 7.4 at 27 °C. The emission spectra of 1 mL of the GO reaction solution above at the fixed GO concentration of 1.3 mg/mL were periodically recorded between 300 and 400 nm at the excitation wavelength of 280 nm [9] using a spectrofluorometer (JASCO FP-750). Time course of the tryptophan fluorescence spectra during the GO-catalyzed glucose oxidation was observed for the different initial glucose concentrations ranging from 0.05 to 20 mM. All the measurements were carried out in the cuvette thermostatted at 27 °C using the perche-type temperature controller (JASCO ETC-272T). The amount of catalase contained in the commercially available GO was much smaller than that of GO so that catalase had negligible effect on the fluorescence measurements of GO.

2.5. Measurement of flavin adenine dinucleotide (FAD) fluorescence

A GO molecule contains two FAD molecules of which oxidized state is essential for the catalytic activity of GO. To estimate the redox state of the FAD within GO molecules during the glucose oxidation, the time course of the emission fluorescence spectra of FAD was measured between 480 and 560 nm at the excitation wavelength of 365 nm [9] at the fixed GO concentration of 1.3 mg/mL for the different initial glucose concentration in the cuvette. The intact FAD fluorescence spectrum of the GO was measured at 24 h after incubation of GO in the Tris buffer of pH 7.4 at 27 °C in the absence of glucose. The reaction, i.e., measurement temperature was controlled at 27 °C throughout the experiment in the same way as in the tryptophan fluorescence measurement above.

3. Results and discussion

3.1. Activity of free and liposomal glucose oxidase during catalyzing glucose oxidation

Fig. 1(a) and (b) shows the time courses of the remaining GO activity and glucose conversion during the prolonged oxidation of glucose catalyzed by the free GO (1.3 GO mg/mL solution) and by the liposomal GO ([lipid] = 1.0 mM, 1.1 mg GO/mL suspension), respectively, at the initial glucose concentration (G_0) of 10 mM. It is seen in the figure that more than 90% of free GO is deactivated within 24 h, while more than 90% of the activity of liposomal GO is maintained during the reaction period of 10 days. The glucose conversion is 85% at 24 h for the free GO reaction and 83% at 10 days for the liposomal GO reaction. It was reported that the free GO deactivation was caused by the accumulation of an inactive complex formed between the