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Investigation of structural stability and enzymatic activity of glucose oxidase and its subunits

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ABSTRACT

Glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) catalyzes the oxidation of β-D glucose utilizing molecular oxygen as an electron acceptor to produce D-glucono-1,5-lactoneand hydrogen peroxide, which has applications in food, biotechnology and medical industries. It was known that dimer form was considered to be active and monomer form has inactive conformation. However, there are no evidences at the molecular levels for Glucose oxidase (GOx) inactivation through dissociation. Here, using molecular dynamic simulation, it has been investigated for the first time that why dimer form of the enzyme is active. We have performed a series of molecular dynamics simulations at different forms of GOx (monomer and dimer with and without FAD cofactor). The analysis of tertiary structure showed that monomer is more unstable and has more deviation from the crystal structure. In contrast, dimer has a stable conformation during simulation. These results are in good agreement with experimental data about enzyme inactivation by dissociation. It was also found that when FAD is removed from monomer, it became more unstable in comparison with monomer containing cofactor. This shows essential role of FAD in both activity and stability of the enzyme. According to the MD simulation, enzyme inactivation is associated with changing in secondary structure at the interface. Interestingly, it was found that some secondary structures are destructed while some structures are formed in monomer upon dissociation. The analysis of active site structure during simulation revealed that both dissociation and release of the FAD influence on inactivation of GOx. This study provided novel insight to understand the mechanism of enzyme inactivation upon dissociation, which would be useful for rational enzyme design.

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1. Introduction

The quaternary structure reflects the number and arrangement of the protein subunits. Conformational alterations within individual monomers or reorientation of the subunits could be result in changes in quaternary structure. Multimeric enzymes undergo such changes and perform their physiological function [1]. The quaternary structure of proteins is quite poorly understood at the molecular level [2–4]. Moreover, we do not know much about the structural and dynamic details of multimeric protein and its subunits during dissociation [5–8]. As example, glucose oxidase as a homodimer was found to be catalytically inactive in monomer form [9]. Glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase) catalyzes the oxidation of β-D glucose utilizing molecular oxygen to produce D-glucolactone and H_2O_2 [10]. Glucose oxidase (GOx) has found in the food industry for the glucose removal from dried eggs, for the removal of oxygen from fruit juices, for the production of gluconic acid, and as a source of hydrogen peroxide in food preservation [11]. It has also been used for the determination of glucose in samples such as blood, urine, food and fermentation products [12]. In addition, glucose oxidase may damage cancerous tissue and cells because of H_2O_2 production. Therefore, it is reported as an anticancer drug [13]. Glucose oxidase has been identified from various sources, but only the enzyme from *Aspergillus niger* (*asp*-GOx) and *Penicilliumam amagasakiense* (*pen*-GOx) has been studied in detail. Although most of the biochemical characterization has been carried out with *asp*-GOx (accession number: P13006), it has been shown that *pen*-GOx (accession number: P81156) catalyzes the glucose oxidation more efficiently. *Pen*-GOx has 79% similarity and 66% sequence identity to *asp*-GOx, and belongs to glucose-methanol-choline (GMC) oxidoreductase family. *Pen*-GOx is less stable than *asp*-GOx, however has 10 times higher specificity constant (k_{cat}/K_m) and 6 times higher affinity constant for

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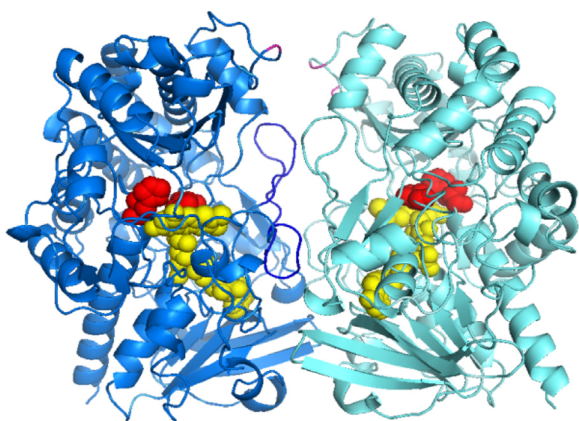

Fig. 1. Cartoon representation of GOx (pdb code: 1GPE). Colors legend: monomers (ice-blue and blue), FAD (yellow) and active sites (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

β-D-glucose than *asp*-GOx [10,11]. Crystallized protein (PDB ID: 1GPE) is a dimeric glycoprotein, composed of two identical subunits. Each subunit contains 587 amino acids and has a molecular weight of 66.7 kDa. The structure of *pen*-GOx contains one mole of tightly but not covalently bound flavin adenine dinucleotide (FAD) cofactor at the active site of each subunit (Fig. 1). Active-site residues of *pen*-GOx are two histidines, His520 and His563, which could act as general bases and general acids in the reductive half-reaction and the oxidative half-reaction, respectively [12–17]. FAD, especially isoalloxazine, is important for the catalytic activity for β-D-glucose. The binding of FAD cofactor also plays a key role on the catalytic activity of flavoproteins for the correct folding, assembly and protein stability [18,19]. The three-dimensional structure of the glucose oxidase was stabilized by FAD, which can act as a redox carrier in catalysis [20,21] .

Several experimental studies have suggested dissociation of glucose oxidase into its monomers. Jones et al. proved that dimeric structure of *asp*-GOx dissociates into two subunits at pH 5 and below in the presence of 25 mM SDS. They reported that at low pH, protonation of negatively charged carboxylate moieties would lead to neutralization of charge and dissociation of *asp*-GOx [22]. Moreover, *pen*-GOx was dissociated into two polypeptide chains at pH 3 with 6 M guanidine hydrochloride [23]. In addition, high concentration of $CaCl_2$ or $MgCl_2$ (≥2 M) induced structural changes in *asp*-GOx. Direct binding of divalent cations to negatively charged groups in GOx, at higher divalent cation concentration, results in dissociation of the native conformation of dimeric enzyme molecule and stabilization of the enzyme monomer [24]. On the other hands, experimental evidence agrees that only the dimeric form of GOx is active. Caves et al. has studied inactivation of the glucose oxidase at temperatures up to 40 °C and at neutral pH for extended times. Their studies showed that the enzyme inactivates on storage at pH 7.0–7.6 at temperatures 30–40 °C. Results suggested that GOx inactivation under these conditions is associated with release of the FAD coenzyme from the dimer and molten globule formation. Inactivation of GOx at pH < 7 and pH ≥ 7 would lead to precipitation and non-specific formation of small soluble aggregates, respectively [9,25].

Despite the experiments on enzyme dissociation, there are not any studies on the structural and dynamic details of different forms of GOx (monomer and dimer) to explain inactivation mechanism of GOx upon dissociation in the atomic-level. Molecular dynamics (MD) simulations can be applied as an effective way to investigate the structural changes on the atomic level [24–26]. MD simulation is a powerful tool for studying bimolecular systems at spatial and temporal scales that are difficult to access experimentally. Simulations can be used to provide insight into specific questions about the properties of a model system [27]. It also was used to explore the relationships between the activity and conformational structure of macromolecule [28], in the presence of ionic liquids [29–31], stabilizer [32] , under an acidic pH condition [33] and in supercritical CO_2 [34,35]. Understanding of GOx dissociation stability is a crucial step to assessing its overall stability. In addition, study of dissociation of enzymes into monomers may help us to understand how a multidomain oligomeric protein, such as glucose oxidase, folds and how its subunits acquire stability. To this end, we have investigated the effect of dissociation on the conformational stability of subunits and activity of GOx. To gain a deep insight into the mechanism of enzyme inactivation induced by dissociation in the molecular level, molecular dynamics simulation was carried out. Moreover, the influence of FAD coenzyme on stability and function of GOx has been investigated. Does the loss of enzyme activity occurring at the dissociation of enzyme subunits depend on the dissociation of FAD or on the change of bonds between enzyme molecules?

2. Molecular dynamic simulation

The MD simulations were carried out by GROMACS 5.0.4 with a GROMOS 43a1 forcefiled [36,37]. Parameters for FAD that were not present in the GROMOS force field were taken from the work of van den Berg et al. [38]. The crystal structure used in the MD simulation was obtained from the Protein Data Bank (PDB) with PDB code of 1GPE [14]. Protein was placed in a cubic box center consisting SPC216 water molecules and the box size was designed by the criterion that the distance of protein atoms from the wall was greater than 1 nm. For each component of the systems Particle Mesh Ewald (PME) algorithm was employed to calculate the total electrostatic energy [39,40]. The other non-bonded interactions were estimate by L-J model with a cutoff distance of 10 Å. To minimize the energy of each system and to relax water and ions, the steepest-descent algorithm was performed. The linear constraint solver (LINCS) algorithm [41] was applied to fix the chemical bonds between the atoms of the protein and SETTLE algorithm [42] in the case of solvent molecules. After minimization, the system heating, equilibration and data sampling were performed. The system heating was carried out from 0 K to the desired temperature in a NVT ensemble, followed by further 1 ns simulation for equilibration and 50 ns simulation for data sampling in a NPT ensemble. The Berendsen coupling algorithm was applied to maintain constant temperature and pressure during simulations [43]. The weak-coupling algorithm was applied for the temperature and pressure regulation with a coupling time of 1.0 ps.

The root mean square deviation (RMSD) of certain atoms in a molecule with respect to a reference structure ref is defined as:

$$RMSD = [\frac{1}{M}\sum_{i=1}^{N} m_i||r_i(t) - r_i^{ref}||^2]^{\frac{1}{2}} \quad (1)$$

Where $M = \sum_{i=1}^{N} m_i$ and r_i (t) is the coordinate of atom i at time t after least-square fitting the structure to the reference structure.

Root mean square fluctuation (RMSF) is a criterion for the deviation between the position of particle i and some reference position and it was obtained from the equation:

$$RMSF = [\frac{1}{T}\sum_{t_j=1}^{T} ||r_i(t_j) - r_i^{ref}||^2]^{2} \quad (2)$$

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