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ORIGINAL RESEARCH

Thermal inactivation and conformational lock studies on glucose oxidase

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Abstract In this study, the dissociative thermal inactivation and conformational lock theories are applied for the homodimeric enzyme glucose oxidase (GOD) in order to analyze its structure. For this purpose, the rate of activity reduction of glucose oxidase is studied at various temperatures using β-D-glucose as the substrate by incubation of enzyme at various temperatures in the wide range between 40 and 70 °C using UV–Vis spectrophotometry. It was observed that in the two ranges of temperatures, the enzyme has two different forms. In relatively low temperatures, the enzyme is in its dimeric state and has normal activity. In high temperatures, the activity almost disappears and it aggregates. The above achievements are confirmed by dynamic light scattering. The experimental parameter "n" as the obvious number of conformational locks at the dimer interface of glucose oxidase is obtained by kinetic data, and the value is near to two. To confirm the above results, the X-ray crystallography structure of the enzyme, GOD (pdb, 1gal), was also studied. The secondary and tertiary structures of the enzyme to track the thermal inactivation were studied by circular dichroism and fluorescence spectroscopy, respectively. We proposed a mechanism model for thermal inactivation of GOD based on the absence of the monomeric form of the enzyme by circular dichroism and fluorescence spectroscopy.

Keywords Glucose oxidase · Conformational lock · Thermal inactivation · Dimeric form · Monomeric form · Dynamic light scattering

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Introduction

Glucose oxidase (β-D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4) is a flavoprotein that catalyzes the oxidation of glucose by molecular oxygen to peroxide hydrogen and glucono-δ-lactone, which subsequently hydrolyzes spontaneously to gluconic acid [1, 2]. GOD, that was first discovered by Muller (1928) in *Aspergillus niger* extracts [3], is a homodimer enzyme [4], with the dimensions 70 × 55 × 80 Å [5, 6]. This homodimer is composed of two monomers that each contains 583 amino acid residues [7]. A narrow contact area connected the monomers to each other. Each monomer (molecular mass approximately 80 kDa) has one co-enzyme molecule of flavin adenine dinucleotide (FAD) [8]; this co-enzyme acts as an electron receptor during catalysis [9]. Each monomer makes two structural domains; one of them makes the FAD binding site and the other one forms the substrate binding site [5]. GOD is a glycoprotein, most of the carbohydrate moiety of which contains mannose [10, 11]. This enzyme has gained wide application in several industries. It is used as a food preservative and color stabilizer [12], in the production of gluconic acid [13, 14], in the textile industry [15, 16], and as a molecular diagnostic and analytical tool in the medical industry for the control of diabetes [17–19].

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Some functional advantages such as combining functions of different domains, multivalency, high binding strength, increasing the structure stabilization [20], inducing cytotoxicity [21], and functioning as hormone receptor [22] appear from the protein oligomerization.

It is possible to study and explain properties of interfaces in oligomeric enzymes and their influence on catalytic activity by two independent methods, involving the use of the structural and kinetics data [23]. The conformational lock is a theory that was suggested by Poltrak in 1998 for the first time which is applicable for the structural analyzing of the oligomeric enzymes. Poltorak reported that in alkaline phosphatase from different sources, the results from both methods are in reasonable agreement [24, 25]. The conformational lock structure points to contact sites between the subunits in an oligomeric enzyme which they break by thermal increasing step by step until it separate to into subunits [26].

In this study, thermal inactivation and conformational lock were followed by kinetics of inactivation, spectroscopic measurements, and dynamic light scattering (DLS) measurements in order to obtain a putative mechanism for thermal inactivation of GOD.

Materials and methods

Glucose oxidase,-D-glucose, ortho-dianisidine, and 8-anilino-1-naphthalenesulfonic acid (ANS) were products of Sigma; horseradish peroxidase was a product of Merck.

Enzyme activity assay

Glucose oxidase was assayed at 35 °C by peroxidase coupled assay. Peroxidase was added to an *o*-dianisidine and glucose-containing buffer (pH 5.8). GOD solution, appropriately diluted, was added after proper mixing. The complete reaction mixture contained the following: 100 mM potassium phosphate as a buffer, 0.025 μM GOD, 8.2 nM horseradish peroxidase, 0.21 mM *ortho*-dianisidine as a chromogenic dye, and 0.56 M of D-glucose as a substrate. The increase of absorbance at 500 nm was measured for 3 min. at 35 °C by a Shimadzu 3100 UV–Vis spectrophotometer.

Thermal inactivation

Glucose oxidase (0.025 μM) was incubated in sealed vials in a 100 mM phosphate buffer, pH 5.8, at various temperatures (40–65 °C). An aliquot of enzyme solution was removed from the incubated samples, cooled immediately to 27 °C in a water bath (a temperature at which the irreversible inactivation of the enzyme reached zero), and 25 μl of the enzyme solution at 27 °C was assayed for enzyme activity. Cooling on ice was avoided to prevent any irreversible cold-induced conformational change. The activities that were measured were used for drawing the thermal inactivation plots.

Determination of optimum temperature

Optimum temperature (T_{opt}) is defined as the maximum temperature at which the enzyme activity does not change during the incubation time [27]. In order to obtain T_{opt}, the thermal inactivation curves (logarithm of remaining activity percentage versus incubation time) were drawn with an enzyme concentration of 0.025 μM at various temperatures for 25 min.

Particles size measurements

According to the thermal inactivation curve, GOD was incubated in sealed vials in a 100 mM phosphate buffer, pH 5.8, at various temperatures for about 30 min (the time in which the activity of GOD decreased); after cooling instantly to 27 °C in a water bath, the average particle size of the protein samples was measured by the DLS Model of Brookhaven instrument. The measurements of GOD were performed for samples, which were filtered through a 0.22-μm filter, as well as for buffer and deionized water.

Fluorescence spectrophotometry

Fluorescence measurements were followed by binding of the ANS probe to the GOD and the subsequent incubation of the enzyme with ANS at different temperatures. The measurements were performed as the change in the ANS emission spectra upon excitation at 390 nm took place. The concentration of GOD and ANS was 5 μM and 1.5 mM, respectively, in a final volume of 400 μl in sodium phosphate buffer at pH 5.8.

Circular dichroism

Circular dichroism measurements were performed by a Jasco Model 810 spectropolarimeter in the Far-UV-CD region (190–260 nm) using 1-mm path cell with 5.25 μM GOD at 25, 55, 60, and 90 °C in 100 mM sodium phosphate buffer, pH 5.8.

Kinetics of thermal inactivation

The dissociative thermal inactivation theory for oligomeric proteins and its mechanism can be explained according to the following scheme [29]:

$$E_2 \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} 2E_1 \xrightarrow{k_{den}} 2E_{den} \quad (1)$$

where E_2 is the active dimer and E_1 and E_{den} show the monomeric and denatured forms of the enzyme, respectively. k_1, k_{-1}, and k_{den} represent rate constants for forward, backward, and inactivation, respectively. The values of these rate constants can be gained using graph ln(v/v0) versus time under denaturing conditions [28]. The protein concentration around the numerical value of dissociation constant and certain temperatures (about 7 °C above T_{opt}) is necessary for gaining the k values. For dimeric enzymes, the kinetic curve of thermal inactivation under denaturing condition includes two linear phases and an inflection point. These two linear phases are related to the non-steady-state kinetics of dissociation of the active dimer into the deactive monomer and the slow kinetics of irreversible inactivation of the monomers [29].

In some oligomeric enzymes, the thermal inactivation curves may consist of a latency period before two linear phases. This would indicate that several active intermediates are related to the conformational lock [29, 30].

$$E_2 \leftrightarrow E_2^1 \leftrightarrow E_2^2 \leftrightarrow \cdots \leftrightarrow E_2^n \overset{k_{dis}}{\leftrightarrow} 2E_1 \overset{k_{dis}}{\leftrightarrow} 2E_{den} \quad (2)$$

where the "n" value represents different active forms of the dimeric enzyme. It can be obtained from the following equation:

$$n = \frac{0.13 + \delta}{0.13 - 0.0\delta} \quad (3)$$

The δ is a parameter without dimension that is obtained by the plot v/v0 versus time and $\delta = R-1$; the value of R depends on the minimal number of the steps (n) before loss of activity [29, 30].

Results and discussion

Data obtained by the thermal inactivation curve of GOD showed that the enzyme is active up to 55 °C, but its activity decreases at temperatures above 55 °C. Therefore, T_{opt} for the GOD is equal to 55 °C (Fig. 1).

Figure 2 shows that the graph of thermal inactivation of this enzyme has three phases, and the δ value was gained from the second phase of this graph and the n value was also calculated and equal to 2 at 60 °C and 65 °C (Table 1).

Data obtained by DLS shows that GOD is a dimeric form up to 55 °C (the mean diameter of the native GOD molecule is 7 ± 1), but it forms aggregates at incubation temperatures above 55 °C. This finding indicates that GOD does not dissociate into monomers during thermal inactivation (Fig. 3).

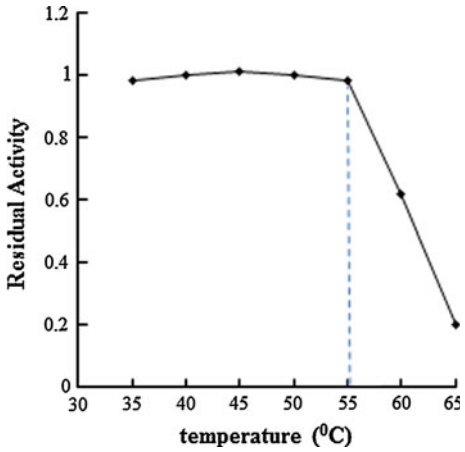

Fig. 1 Residual activity of GOD versus incubation temperatures at 100 mM potassium phosphate buffer, pH 5.8

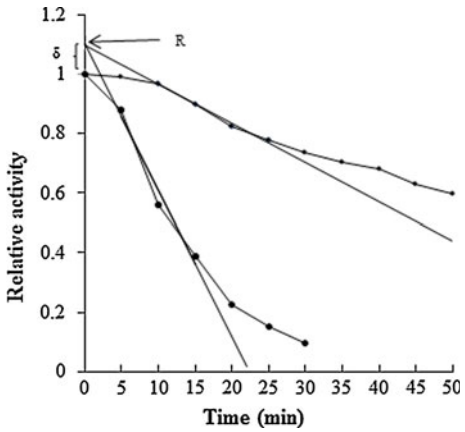

Fig. 2 Thermal inactivation kinetics of GOD at temperatures 60 °C (*filled diamond*) and 65 °C (*filled circle*). The concentration of GOD was 0.025 μM

Table 1 "δ" and "n" values for GOD at the temperatures 60 and 65 °C

Temperature (°C)	δ	n
60	0.095	1.796
65	0.105	1.883

The effect of thermal inactivation on the secondary structure of GOD was followed by changes in ellipticity values at a wavelength of 222 nm at 25, 55, 60, and 90 °C (Fig. 4).

Fluorescence measurements of GOD at different temperatures are shown in Fig. 5. It has been observed that by increasing the temperature, the emission spectrum is increased.

Since glucose oxidase is used in various industries such as pharmaceutical, medical, biologic reactors, and biosensors, it was necessary that the thermal inactivation and conformational lock of GOD from *Aspergillus niger* as an

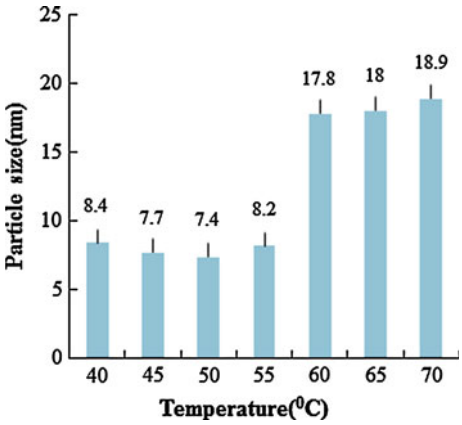

Fig. 3 The distribution of dimensions for glucose oxidase between 40 and 70 °C

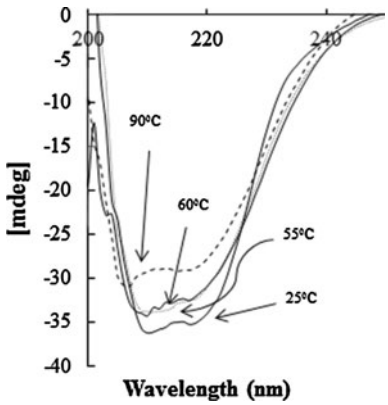

Fig. 4 Circular dichroism of glucose oxidase in the far-UV region at various temperatures. (25, 55, 60, and 90 °C). The concentration of GOD was 5.25 μM

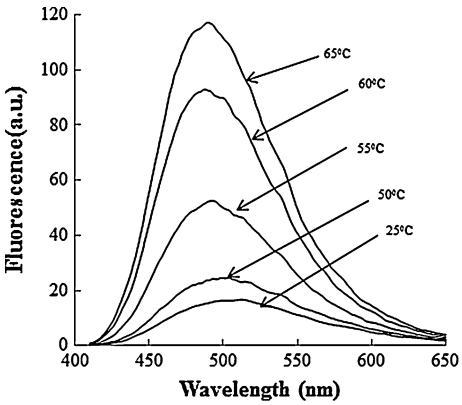

Fig. 5 Fluorescence of ANS for glucose oxidase at ambient temperature and 50–65 °C. The concentration of GOD and ANS were 5 μM and 1.5 mM, respectively

enzyme were studied. The conformation and enzymatic reactions' rate can be changed by the effect of environmental agents such as temperature; therefore, the structural and functional cognition are important for optimum enzymatic stability for industrial and medical application.

Table 2 Contact areas and residues involved in conformational lock for GOD [5]

Contact area	Residues in subunit A	Residues in subunit B
I	Ser75, Asp70, Arg56, Arg58, Glu81, Glu55	Tyr444, Leu445, His447, Asp442, Arg95, His272
II	Asn89	Arg337, Glu487, Asp492, Asn493

In this study, the thermal inactivation of GOD has occurred above 55 °C. Data obtained by the thermal inactivation curve show that GOD contains the conformational lock structure that it opens in two steps. The structure of enzyme, GOD (pdb, 1gal), was also investigated by X-ray crystallography. The crystal structure of the enzyme was solved at 2.3 Å resolution. The holoenzyme is composed of two identical monomers. The crystallographic structure of GOD shows that this enzyme has two contact sites between its monomers. In the first contact area, ser75 forms, with a main chain and a side chain, a hydrogen bond, which is a link to Tyr 444 and Leu 445. This link to the dimer molecule is strengthened by a salt bridge between the neighboring Arg 58 and Asp 442. A second link connects both 75 and 98 regains of the dimer in the middle, where salt bridges between Glu 81 and Arg 95 are formed. A secondary contact area is established by the carbohydrate moiety. The carbohydrate residue attached to Asn 89 at the tip of the 75–98 region fits into a small groove on the dimer molecule, formed by *cis*-proline 490 and is hydrogen-bonded to residues 487, 492, and 493 [5, 6] (see Table 2).

Data obtained by DLS indicated that GOD remains in its dimeric form up to 55 °C, but the aggregate forms start at 60 °C and upper temperatures. This finding is in agreement with the works of Zoldak [31] who reported GOD does not change its oligomeric state in the process of thermal transition and it neither dissociates into monomers nor forms large aggregates, and it is contrary to the suggestion that dissociation of FAD causes GOD monomerization [5]. Based on a previous study, in some oligomeric enzymes, contact sites between their monomers may be involved in their active site and these residues contribute to conformational lock. So, in this case, by thermal inactivation, the destruction of contact sites can result in the destruction of the active site, leading to the explanation that only the dimeric form is active for GOD.

It is worthy to note the circular dichroism results show no changes of spectra at 55–60 °C (Fig. 4), whereas the fluorescence results indicate the spectra changes for the

mate condition. This means that in this range of temperatures, a molten globule-like state [32] occurred for GOD.

Conclusion

Structural analysis of the dimeric GOD shows that the total number of contacts in the conformational lock is two. The results also indicate that during the process of thermal inactivation, GOD as a homodimer enzyme can retain its catalytic activity and dimeric form up to 55 °C, but by increasing the temperatures, due to reducing of the enzymatic activity, the conformation of GOD changed. The hydrophobic surfaces of the enzyme expose to the solvent at 60 °C and upper incubation temperatures that lead to the formation of aggregates. The results obtained in this study enabled us to propose following scheme for thermal inactivation of GOD:

$$\mathrm{GOD}\ (D) \xrightarrow{55\ ^\circ\mathrm{C}} \mathrm{GOD}\ (D) \xrightarrow{>55\ ^\circ\mathrm{C}} \text{moltenglobule-like}$$
$$(55-60^\circ\mathrm{C}) \xrightarrow{\geq 60\ ^\circ\mathrm{C}} \text{aggregation.}$$

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