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The mechanism of inactivation of glucose oxidase from *Penicillium amagasakiense* under ambient storage conditions

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ABSTRACT

Glucose oxidase (GOx) from *Penicillium amagasakiense* has a higher specific activity than the more commonly studied *Aspergillus niger* enzyme, and may therefore be preferred in many medical and industrial applications. The enzyme rapidly inactivates on storage at pH 7.0–7.6 at temperatures between 30 and 40 °C. Results of fluorimetry and circular dichroism spectroscopy indicate that GOx inactivation under these conditions is associated with release of the cofactor FAD and molten globule formation, indicated by major loss of tertiary structure but almost complete retention of secondary structure. Inactivation of GOx at pH < 7 leads to precipitation, but at pH ≥ 7 it leads to non-specific formation of small soluble aggregates detectable by PAGE and size-exclusion chromatography (SEC). Inactivation of *P. amagasakiense* GOx differs from that of *A. niger* GOx in displaying complete rather than partial retention of secondary structure and in being promoted rather than prevented by NaCl. The contrasting salt effects may reflect differences in the nature of the interface between subunits in the native dimers and/or the quantity of secondary structure loss upon inactivation.

1. Introduction

Glucose oxidase (GOx) has been studied and exploited for over half a century, and will continue to be so due to its widespread industrial, medical, and research applications. Thus far, the most important application of GOx has been in medical diagnostics, specifically the diagnosis and control of diabetes [1], but it also has a wide range of other applications (see below). The practical importance of GOx has led to much research being carried out on the enzyme, with a particular focus on the enzyme's stability and on approaches to stabilisation, e.g. through immobilisation [2–9]. GOx from fungi is a glycoprotein with a molecular mass of ~160 kDa [10]. It is a homodimer of ~80 kDa monomers [11], each of which folds into two structural domains, one containing a tightly, but non-covalently, bound FAD cofactor, and the other housing the substrate binding site [12]. The protein displays an ellipsoidal 'butterfly' shape with the monomers, which form the 'wings', being connected via a long, narrow, depressed, contact area [13,14].

Previous results suggest that denaturation of GOx generally results in FAD release from the enzyme, with concomitant changes in the absorption and emission spectra of the protein [15–17]. Melting temperature studies indicate that GOx from *Aspergillus niger* forms a molten globule upon inactivation at elevated temperature in a buffer of around neutral pH, with dissociation of FAD from the holoenzyme and loss of most tertiary structure, but with retention of approximately 60–70% of secondary structure [18,19]. It has been shown that the presence of NaCl or K_2SO_4 increases the stability of GOx at elevated temperatures [18]. Previous studies on the rate of inactivation at elevated temperatures (55 °C and above) have led to the conclusion that inactivation of GOx follows first order kinetics under these conditions [10,18], though a study on the inactivation of GOx from *Penicillium adametzii* led to the conclusion that inactivation at elevated temperature follows biphasic kinetics [20]. It has also been shown that GOx aggregates on inactivation at elevated temperature, but there is disagreement on the mechanism of this process. Gouda et al. [18] concluded from size exclusion chromatography (SEC) results that the enzyme aggregates non-specifically, whilst Zoldák et al. [19] inferred from cross-linking studies that thermally denatured GOx forms oligomers no larger than tetramers.

While most of the work described above was carried out on the *A. niger* enzyme, the much less studied GOx from *Penicillium amagasakiense* is the subject of the work reported here. The primary structures of the two variants show ~65% identity, and 79% simi-

Abbreviations: ANS, 1-anilino-8-naphthalene sulphonate; GOx, glucose oxidase; kDa, kilodaltons; MRE, mean residue ellipticity; RFI, relative fluorescence intensity; SEC, size exclusion chromatography; Trp, tryptophan.

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larity [12,21], with a high degree of sequence conservation in the FAD-binding site and active site. Despite these similarities, GOx from *P. amagasakiense* has a glucose turnover number approximately twice that of *A. niger* GOx [22].

The fact that *P. amagasakiense* GOx is a more efficient catalyst means it may be more applicable to some of the lesser-known industrial and medical uses of GOx, such as removal of glucose during food processing [23], gluconic acid production [24], and the production of hydrogen peroxide in wound dressings [25]. With practical application in mind, we have studied the inactivation of the enzyme over time at temperatures up to 40 °C and at pH around neutral. These are conditions under which, for instance, an *in vivo* glucose monitor [26,27] or wound dressing would be required to operate. This is in contrast to the previous assessments of GOx stability and inactivation, which have studied changes at higher temperatures (50–70 °C).

2. Materials and methods

2.1. Reagents

Glucose Oxidase (EC. No. 1.1.3.4) from *P. amagasakiense* was purchased from Biocatalysts Limited, U.K. Size exclusion chromatography showed a single large peak with a relative molecular mass of 159 kDa, corresponding to native GOx dimer. 3,3′,5,5′-Tetramethylbenzidine (TMB) and 8-anilino-1-naphthalenesulfonic acid (ANS) were purchased from Sigma–Aldrich, analytical reagent grade anhydrous D-glucose from Fisher Scientific, and lactoperoxidase from DMV International, The Netherlands.

2.2. Inactivation of GOx

Solutions of 5.2 μM GOx were incubated in a Grant GR150 precision stirred thermostatic bath at the required temperature. Aliquots were removed at specific time-points and refrigerated immediately. The residual activity of GOx was then measured in the samples no longer than 48 h after the refrigeration.

2.3. Activity assays

GOx was assayed at room temperature using the following method. The sample, 10 μl, (diluted in citrate-phosphate buffer to a GOx concentration of 20 nM) was added to 96-well plate, and 195 μl of assay mixture (consisting of 10% (w/v) D-glucose, 50 μg/ml lactoperoxidase and 150 μg/ml TMB in 0.1 M pH 5 phosphate-citrate buffer) was added to each well. A Thermo Scientific Multiskan Ascent microplate spectrophotometer was used to measure absorbance at 630 nm at intervals of 2, 3, 4, and 5 min after assay mixture was added. For each set of assays a number of standards of known GOx concentration were also assayed. The resulting calibration curve consistently gave a linear trend with an R^2 value >0.99.

2.4. Fluorescence measurements

Fluorescence measurements were carried out on a Photon Technology International fluorimeter. An excitation and emission band width of 2 nm, data pitch of 1 nm, and response time of 1 s were used for all scans. Emission spectra of tryptophan residues and FAD cofactor were observed upon excitation at 290 and 450 nm respectively. Binding of the external chromophore, ANS, to GOx was assessed using an excitation wavelength of 390 nm. All fluorescence scans were performed using a 5 mm × 5 mm square cuvette. All spectra presented here are the average of 4 consecutive scans, and are expressed as relative fluorescence intensity (RFI).

For continuous monitoring of the change of fluorescence during inactivation, 270 μl of 0.1 M pH 7.5 citrate-phosphate buffer in a 5 mm × 5 mm square cuvette was heated in the fluorimeter cell to 40 °C. The temperature of the cell was maintained at 40 °C by circulation from an attached RTE-200 waterbath. To start the inactivation, 30 μl of 52 μM GOx in the same buffer as above was added to the cuvette, diluting the GOx to a concentration of 5.2 μM (as used for the corresponding steady state fluorescence scans). This allowed rapid heating of the GOx to the required temperature. Fluorescence was continuously recorded using excitation and emission wavelengths of 290 and 335 nm respectively for tryptophan (Trp) fluorescence, and 450 and 520 nm respectively for FAD fluorescence. Monitoring by the fluorimeter was initiated before the GOx was added to the cell, and the moment that the fluorimeter cell lid was closed was taken as time-zero.

2.5. CD measurements

CD scans were performed on a Jasco J-815 CD spectrometer. A band width of 1 nm, data pitch of 0.5 nm, and response time of 1 s were used for all scans. All spectra presented here are the average of 16 consecutive scans. Far-UV CD scans in the range 260–190 nm were performed using a 1 mm path length cuvette, and near-UV CD scans in the range 320–240 nm were performed using a cuvette of 10 mm path length.

For continuous monitoring of the change in CD at 270 nm during inactivation, 1980 μl of 0.1 M pH 7.5 citrate–phosphate buffer (in a 10 mm path length cuvette) was heated to 40 °C in the CD spectrometer cell. The temperature of the cell was maintained at 40 °C with a Jasco PFD-4255/15 Peltier block. To start inactivation, 220 μl of 52 μM GOx in the same buffer as above was added to the cuvette, giving a final concentration of 5.2 μM. As for fluorescence analysis, continuous monitoring was started before the addition of GOx, and time zero was taken as the moment the cell lid was closed after the GOx was added.

2.6. Size exclusion HPLC

Size exclusion HPLC measurements were carried out using a Phenomenex Biosep S2000 column (7.8 × 300 mm) with a guard column, on an Agilent 1200 system. An injection volume of 10 μl and a flow rate of 0.45 ml/min was used, with a protein concentration of 5.2 μM GOx in 10 mM pH 7.0 phosphate buffer with varying concentrations of NaCl. Elution of samples was carried out isocratically using a 25 mM phosphate buffer, 150 mM NaCl, pH 6.2 mobile phase. Detection was carried out at 280 nm.

2.7. Data processing

Curve fitting was performed using the SigmaPlot 9.0 application. Secondary structure prediction from circular dichroism data was performed using the online Dichroweb application [28]. The CDSSTR SVD analysis method [29] was used with the SP175 Reference data set [30].

Activity, fluorescence and CD data were normalised, where appropriate, using the following equation:

$$X = \left(\frac{(P_\alpha - P_t)}{(P_\alpha - P_0)} \right) \times 100 \quad (1)$$

X, residual enzyme (%) with native parameter at time t; P_α, final parameter value; P_t, parameter value at time t; P_0, parameter value at time-zero

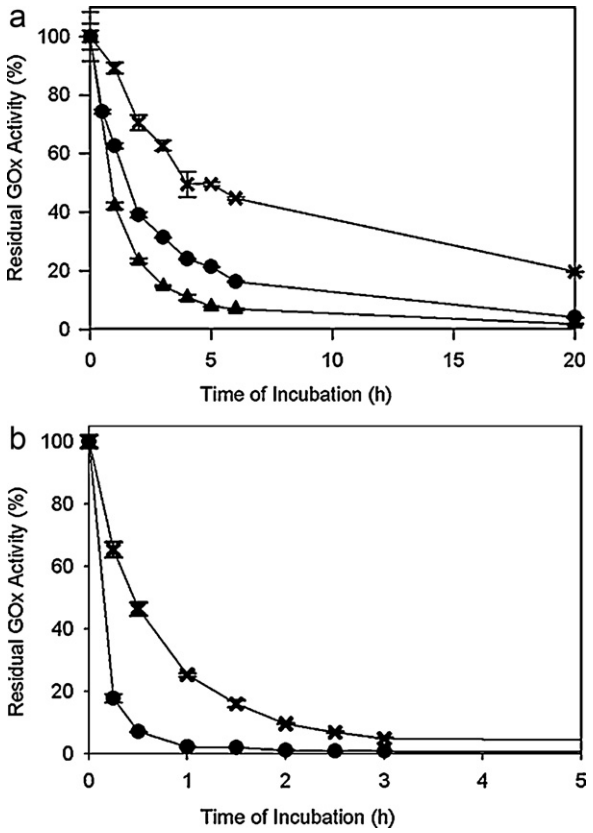

Fig. 1. Inactivation of GOx on incubation at 30 °C and 40 °C. GOx activity was determined as a function of incubation time in 0.1 M citrate–phosphate buffer; (a) 30 °C (b) 40 °C; ×, pH 7.2; ●, pH 7.4; ▲, pH 7.5; ■, pH 7.6.