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# THE THERMAL SCANNING FLUORESCENCE STUDY ON THE CONFORMATIONAL STABILITY OF GLUCOSE OXIDASE (GOD) FROM *ASPERGILLUS NIGER*

Review: **Professor Stanisław Wysocki, Ph. D., D. Sc.**

*In this study, the temperature-induced unfolding process of glucose oxidase (GOD) from Aspergillus niger has been investigated by monitoring fluorescence intensity of FAD, which dissociates from the enzyme during denaturation. The temperature, as well as Gibbs free energy of the unfolding process of GOD have been determined. Additionally, the effect of glucose on the flavoprotein thermostability has also been studied. The obtained results suggest that thermal scanning fluorescence method is a sensitive, fast and simple method for recording conformational changes associated with the temperature-induced unfolding process of GOD.*

# 1. Introduction

Glucose oxidase (β-D-glucose: oxygen-1-oxidoreductase, EC 1.1.3.4, GOD) from *A. niger* is a flavoenzyme which catalyses the oxidation of β-D-glucose into δ-gluconolactone which is spontaneously hydrolysed to gluconic acid and hydrogen peroxide.

This enzyme has recently been of a great interest mainly due to its ever increasing commercial importance. The principal application of glucose oxidase in the food industry is the removal of glucose or oxygen from food stuffs. More recently this enzyme has been used as a molecular diagnostic tool in biosensors to quantitatively measure levels of D-glucose in laboratory samples. GOD also has pharmaceutical applications due to the discovery that the enzyme can act as an antibiotic, since it can form peroxide during catalysis [1,2].

GOD from *A.niger* is a homodimeric flavoprotein with a molecular mass of 160 kDa. Having a carbohydrate content of 16% (w/w) it may also be considered a glycoprotein. The presence of two molecules of flavin adenine dinucleotide (FAD) cofactor per molecule of GOD confers redox capabilities to the enzyme and therefore aids the process of catalysis [3,4]. Studies on glucose oxidase and other flavoproteins have reported that the fluorescent prostethic group, FAD, exhibits different spectral characteristics in different flavoproteins reflecting the specific environmental property of isoalloxazine chromophore present in the FAD molecule. For this reason, the FAD group has been used as a natural marker to probe the dynamical microenvironment of the fluorophore in GOD. Each molecule of FAD is tightly bound but not covalently bonded to it's monomeric subunit of GOD. Since the FAD is not covalently bound it can be released from the holoprotein during denaturation as a consequence of partial protein unfolding. The release of FAD molecules from the flavoenzyme is accompanied by the enhancement of FAD fluorescence intensity. Therefore structural changes associated with unfolding of glucose oxidase during denaturation may be deduced from the monitoring of fluorescence intensity corresponding to FAD molecules present in the enzyme. So far, there have been reported several studies on the unfolding of GOD induced by denaturing agents (such as guanidinium chloride, urea, strong acids and bases) using fluorescence techniques [5,6]. Haq and coworkers carried out an investigation of the acid induced unfolding of glucose oxidase from *A.niger* using a combination of both steady-state tryptophan fluorescence and intrinsic FAD fluorescence measurements, among other fluorescent techniques [6].

In our work the fluorescence intensity of FAD in GOD upon increasing temperature has been monitored in order to study the effect of temperature on the structural properties of GOD. The temperature and Gibbs free energy of the

flavoprotein unfolding process has been determined. Additionally, the effect of substrate on the thermal stability of the enzyme has also been studied.

The primary goal of this work is to study structural changes associated with the temperature-induced unfolding of the dimeric enzyme GOD using the thermal scanning fluorescence method.

## 2. Experimental details

### 2.1. Materials

FAD was purchased from Sigma as flavin adenine dinucleotide disodum salt hydrate (molecular mass 829,51 g/mol, purity>96%) and was used without further purification. FAD concentration was determined spectrophotometrically at 450 nm using the absorption coefficient of $11{,}3*10^3$ $M^{-1}cm^{-1}$. For the absorption and fluorescence measurements 9,2 μM FAD solution in 0.1M phosphate buffer (pH 7.0) was used. The fluorescence quantum yield of FAD has been determined using rhodamine 6G in ethanol as a fluorescent standard [7].

Glucose oxidase extracted from *Aspergillus niger* purchased from Serva Feinbiochemic was of the highest purity available and used without further purification. GOD solution of the concentration of 6,25 μM was prepared in 0.1M phosphate buffer (pH 7.0).

### 2.2. Methods

#### 2.2.1. Absorption and steady-state fluorescence measurements

Absorption measurements were obtained using a Nicolet Evolution 300 UV-Vis spectrophotometer (resolution 1nm; A range 0-6) from Thermo Electron Corporation.

Steady-state fluorescence measurements were made using spectrofluorometer Fluoromax-2 (2000 signals/noise) from Jobin Yvon-Spex. Solutions were placed into a 10mm stoppered quartz cuvette and unless otherwise stated temperature was kept constant at 25°C.

#### 2.2.2. Thermal scanning fluorescence

The equilibrium constant K(T) for the transition from folded (native) to the unfolded (denaturated) form of GOD is given by expression:

$$K(T) = \frac{x}{1-x}, \tag{1}$$

where x is the fraction of unfolded (denaturated) GOD and may be expressed as:

$$x = \frac{K(T)}{1 + K(T)} \tag{2}$$

During the thermal fluorescence experiment the fluorescence intensity of the fluorophore (FAD) is monitored upon increasing temperature. In order to obtain equilibrium conditions during the whole time of the thermal scanning fluorescence experiment, the linear heating rate of ß = 0,05 K/min was kept.

The observed fluorescence intensity $F_{obv}$ may be expressed as following:

$$F_{obv}(T) = \alpha \times c_0 \times \left\{ \phi_f(T) \times \varepsilon_f \times (1 - x) + \phi_{unf}(T) \times \varepsilon_{unf} \times x \right\} \tag{3}$$

where: T – temperature $x = \frac{K(T)}{1 + K(T)}$ is the fraction of unfolded (denaturated) GOD;

α – apparatus factor;

$c_0$ – analytical concentration of GOD;

Φ – fluorescence quantum yield of ($\Phi_f$) folded and ($\Phi_{unf}$) unfolded GOD, respectively;

ε – molar extinction coefficient of ($\Phi_f$) folded and ($\Phi_{unf}$) unfolded GOD, respectively.

Confining our analysis to the region of the fraction of denaturated GOD (x ∈ <0,1>), it may be assumed that Gibbs free energy (ΔG) and fluorescence quantum yield for both folded and unfolded form are independent of temperature. For x = 0, the observed initial fluorescence corresponds to the folded GOD:

$$F_{obv} = F_{initial} = \alpha \times c_0 \times \phi_f \times \varepsilon_f \tag{4}$$

Similarly, for x = 1 the only species responsible for the observed fluorescence is the unfolded GOD:

$$F_{obv} = F_{unfolded} = \alpha \times c_0 \times \phi_{unf} \times \varepsilon_{unf} \tag{5}$$

Therefore, taking into account equations (4) and (5), the observed fluorescence intensity $F_{obv}$ (3) may be expressed as:

$$F_{obv} = F_{initial} \times (1 - x) + F_{unfolded} \times x \tag{6}$$

Hence, from the experiment x may be easily calculated from the expression:

$$x = \frac{F_{obv} - F_{initial}}{F_{unfolded} - F_{initial}} \tag{7}$$

The temperature of the unfolding process of GOD may be obtained by determination of the inflection point for the function given by equation (7). It is known that the precondition of the existence of the inflection point is the zero adjustment of the second derivative.

Therefore the first and second derivatives of the x function (given by equation 2) are computed to be:

$$\frac{\partial x}{\partial T} = \frac{1}{(K+1)^2} \times \frac{\partial K}{\partial T} \tag{8}$$

$$\frac{\partial^2 x}{\partial T^2} = \frac{(1+K) \times \frac{\partial^2 K}{\partial T^2} - 2 \times \left[\frac{\partial K}{\partial T}\right]^2}{(1+K)^3} \tag{9}$$

Considering $K = \exp\left[\frac{-\Delta G}{R \times T}\right]$, $\frac{\partial K}{\partial T} = K \times \frac{\Delta G}{R \times T^2}$ and $\frac{\partial^2 K}{\partial T^2} = K \times \left(\frac{\Delta G}{R \times T^2}\right)^2 - \frac{2 \times \Delta G \times K}{R \times T^3}$ equation (9) may be written as following:

$$\frac{\partial^2 x}{\partial T^2} = \frac{\frac{K \times \Delta G}{R \times T^3}\left[\frac{\Delta G}{R \times T} \times (1-K) - 2 \times (1+K)\right]}{(1+K)^3} \tag{10}$$

Setting the calculated in this way the second derivative (10) equal to zero we obtain:

$$\frac{\Delta G}{R \times T_{ip}} = \frac{2 \times (1+K)}{(1-K)} \tag{11}$$

where temperature $T_{ip}$ corresponds to the inflection point.

Defining $\frac{\Delta G}{R \times T_{ip}} = a$, and considering that $K = \exp\left[\frac{-\Delta G}{R \times T_{ip}}\right]$ in equation (11), the equation with one unknown quantity is obtained:

$$a = \frac{2 \times [1 + \exp(-a)]}{[1 - \exp(-a)]} \tag{12}$$

Solving equation (12) the value of $a$ equal to 2,39936 is finally obtained and may be inserted to the equation $\frac{\Delta G}{R \times T_{ip}} = a$. In this way, when the

temperature corresponding to the inflection point of the function given by equation (7) is determined, Gibbs free energy of the unfolding process may be easily calculated from the formula:

$$\Delta G = R \times T_{ip} \times 2{,}39936 \tag{13}$$

# 3. Results and discussion

## 3.1 Thermal scanning fluorescence of FAD dissolved in phosphate buffer

The absorption spectra of FAD shown in Fig. 1 consists of two strong peaks situated at ~220 and 270 nm, respectively, and a weak, structured band with two peaks at 380 and 450 nm. The peak at 450 nm is slightly stronger than the peak at 380 nm.

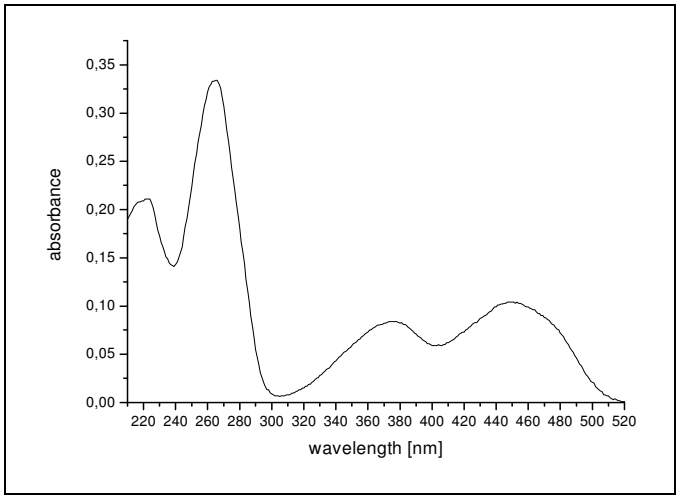


Fig.1. The absorption spectra of FAD dissolved in phosphate buffer

Fig. 2 presents the steady-state fluorescence spectra of FAD dissolved in phosphate buffer. The maximum of fluorescence for FAD excited at 450 nm is 525 nm. These spectral characteristics of FAD are fully consistent with previous studies on FAD dissolved in phosphate buffer [8-10]. The fluorescence quantum yield of FAD using rhodamine 6G in ethanol as a fluorescent standard has also been determined. The value of 0,020 has been obtained.

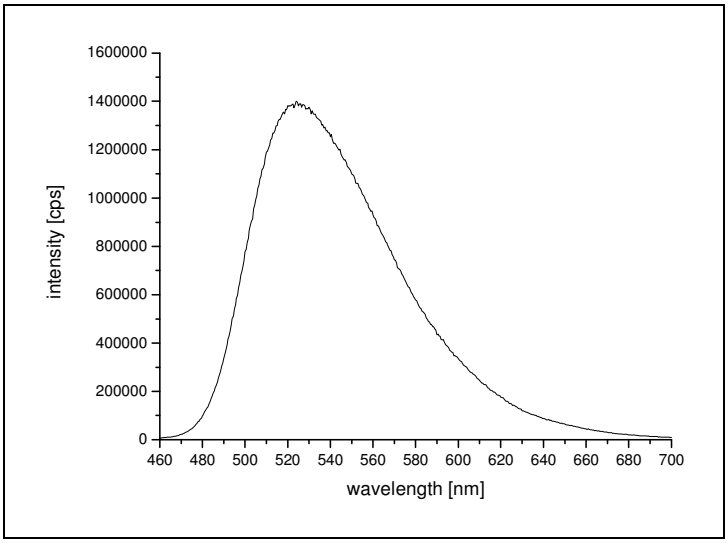


Fig. 2. The fluorescence spectra of FAD dissolved in phosphate buffer

According to the previously reported studies on FAD molecule [8-10], FAD may exist in two conformations: the first one called 'closed' conformation in which isoalloxazine and adenine rings stack to each other and an extended 'open' un-stacked conformation. The un-stacked form of FAD is fluorescent (the fluorescence quantum yield for un-stacked form is about 0,13) whereas the stacked form of FAD is thought to be non-fluorescent (the fluorescence quantum yield for stacked form at pH = 7 is approximately 0,033). It is known that the stacked form of FAD is non-fluorescent due to reductive photo-induced electron-transfer from the adenine moiety to the isoalloxasine moiety. Additionally, molecular dynamics simulations showed the transition from open conformation of FAD to the closed conformation in which the flavin and adenine ring system stack coplanar. Moreover, the simulations in the excited state revealed stacking within the flavin excited-state lifetime [11]. The transition from stacked to un-stacked form of FAD may be induced by the pH value of aqueous solution. The non-fluorescent stacked conformation is found to be dominant in aqueous solution in the pH range from 3,5 to 11, while at higher and lower pH the fluorescent un-stacked conformation dominates [12].

Since not only pH value, but also temperature is expected to be one of the major factor which may induce the stacked-to-un-stacked transition of FAD molecule, the fluorescence spectra of FAD in different temperatures ranging from 15 to 55°C has been recorded (Fig. 3).

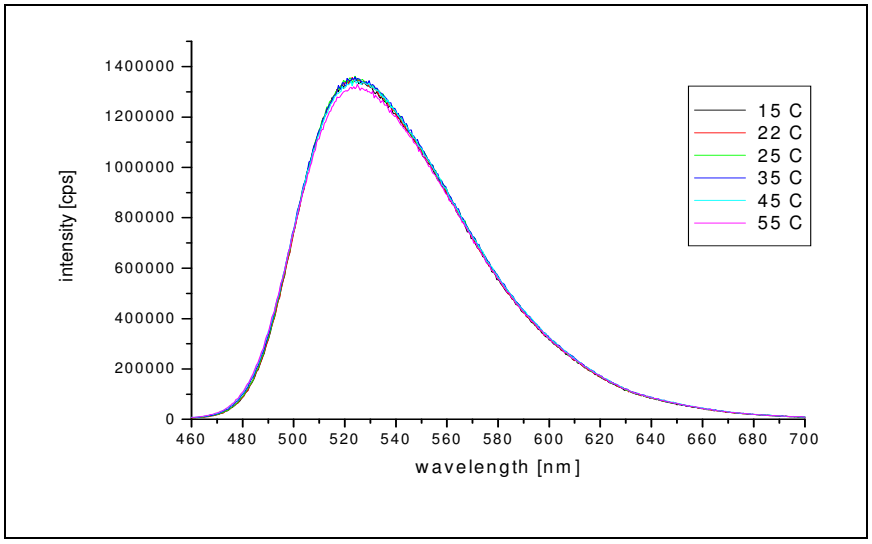


Fig. 3. The effect of increasing temperature on the steady-state fluorescence spectra of FAD, excited at 450 nm

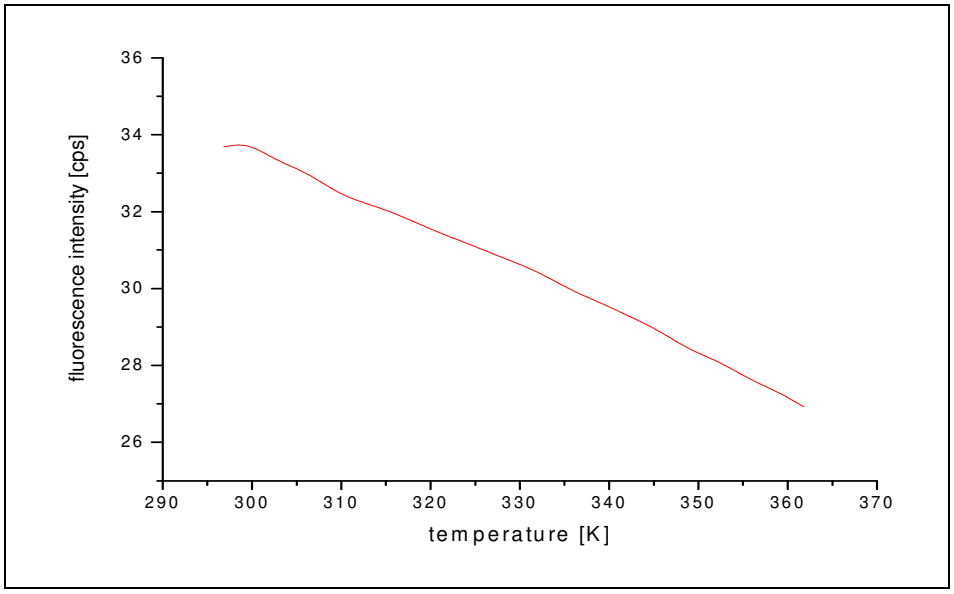


Fig. 4. Changes in FAD fluorescence upon increasing temperature as monitored by fluorescence emission at 525 nm, excitation at 450 nm

For FAD stacked-to-un-stacked transition, if it occurs, a significant enhancement of the fluorescence intensity is expected upon increasing temperature. Whereas, the only change observed in the FAD emission spectra upon increasing temperature is the decrease in fluorescence intensity at 525 nm, which suggests there is no temperature–induced transition from stacked-to un-stacked form of FAD. Due to the surprising nature of this result, the thermal scanning fluorescence experiment for FAD dissolved in phosphate buffer has been carried out.

For this purpose the fluorescence intensity of FAD excited at 450 nm has been monitored at 525 nm upon increasing temperature from 25°C to 91°C. The only change observed is the decrease in fluorescence intensity of FAD as monitored at 525 nm. (Fig. 4). The decrease in fluorescence intensity may result from temperature-activated intersystem crossing from singlet $S_1$ state to tryplet $T_1$ state. The results of the above mentioned experiment confirmed that no stacked-unstacked transition of FAD occurs upon increasing temperature.

## 3.2. The temperature-induced changes in FAD fluorescence intensity of GOD

The absorption spectra of GOD is shown in Fig 5. The absorption band situated at 278 nm corresponds to aromatic aminoacids residues of the protein, whereas the band with the maximum at 450 nm suggest the presence of FAD molecules.

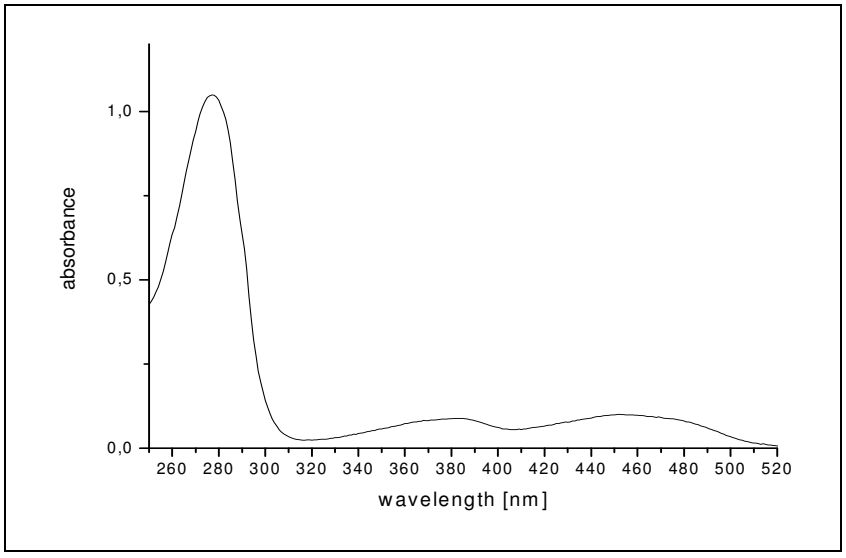


Fig. 5. The absorption spectra of GOD dissolved in phosphate buffer

The steady-state fluorescence spectra of GOD excited at the 278 nm (the maximum of the absorption for aromatic aminoacids) and at 450 nm (the maximum of FAD absorption) is shown in Fig. 6a and Fig. 6b, respectively.

It can be seen that the flavine coenzyme (FAD) embedded in the protein generates very weak, hardly detectable emission unlike the aromatic, aminoacid residues which exhibit a large fluorescence. For native (folded) form of GOD significant tryptophane fluorescence with emission of the maximum at 329 nm was observed. The fluorescence intensity with the maximum at 525 nm, which corresponds to FAD was hardly observed.

The results of recently performed studies on unspecific flavin binding protein dodecine showed that in that enzyme FAD was bound in the form of intramolecularly stacked conformation [13]. Therefore it is expected that FAD molecules also exist in GOD as a non-fluorescent stacked form. The low FAD fluorescence intensity of native GOD may result from the existance of additional mechanism of quanching, most probably the proton-transfer from histidine moiety to the FAD moiety.

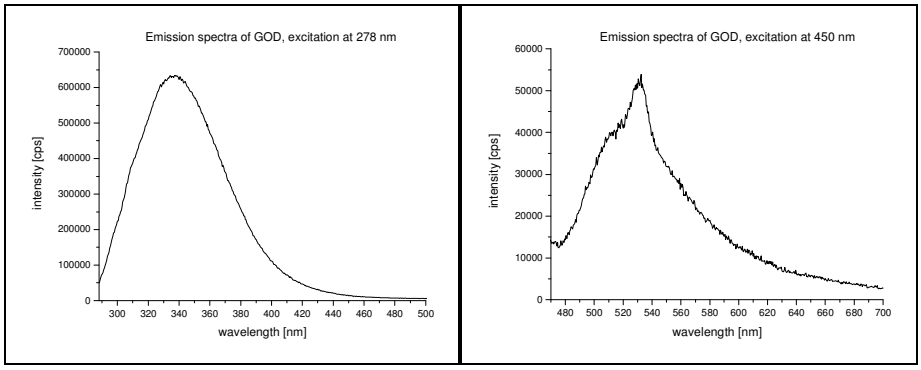


Fig. 6. The steady-state fluorescence spectra of GOD excited at a) 278 nm, b) 450 nm

As mentioned earlier, for denaturated GOD, dissociation of the subunits of the enzyme GOD accompanied by the release of cofactor FAD occurs. The release of FAD from the protein to the solution is associated with the apparent enhancement of the FAD fluorescence. Therefore monitoring fluorescence intensity of FAD may be useful method to study structural changes, which lead to the denaturation of GOD. Fig. 7 illustrates temperature-induced changes in the FAD fluorescence intensity of GOD dissolved in buffer (Fig. 7a) and GOD in buffer with the presence of glucose (Fig. 7b).

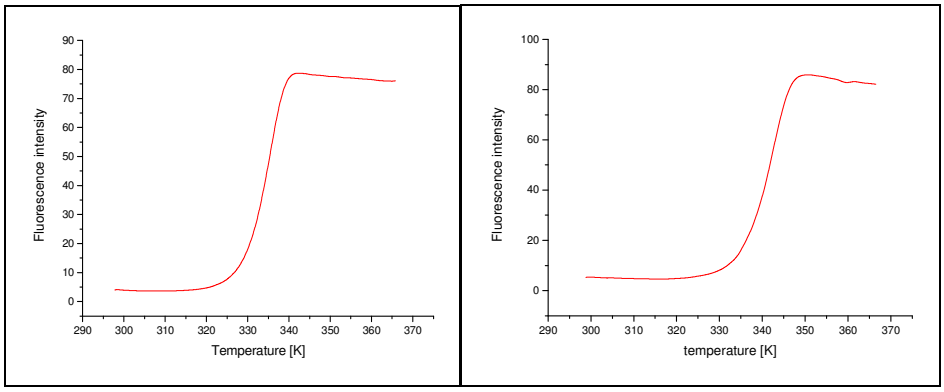


Fig. 7. Changes in FAD fluorescence in GOD upon increasing temperature as monitored by fluorescence emission at 525 nm, excitation at 450 nm. a) GOD dissolved in phosphate buffer, b) GOD dissolved in phosphate buffer in the presence of glucose

A sigmoidal dependence of FAD fluorescence intensity with increasing temperature was observed. An initial slightly decrease in FAD fluorescence intensity may be associated with the intersystem crossing from $S_1$ to $T_1$ state. A large (about 10-fold and 13-fold for GOD, and GOD with glucose, respectively) enhancement of FAD fluorescence intensity between 325 K and 345 K has been associated with dissociation of the FAD molecule from the enzyme as a result of thermal denaturation of the enzyme. The value of the temperature in which the thermal denaturation process of the enzyme occurs may be determined by simple two-fold differentiation of the function I = f(T), where I is the fluorescence intensity of FAD in GOD and T is temperature. In this way, the inflection point of the function which corresponds to the temperature of the transition from folded (native) to the unfolded (denaturated) form of GOD has been determined (Fig. 8).

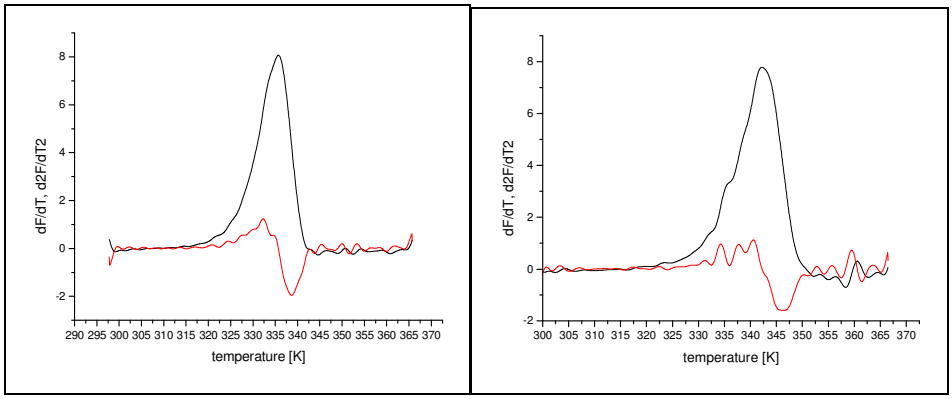


Fig. 8. The determination of the inflection point for the function F = f(T) for GOD in phosphate buffer (a) and for GOD in phosphate buffer with glucose (b)

From the comparison of the temperature corresponding to thermal unfolding transition of GOD (335,73 K) and GOD treated with substrate (342,21 K) it may be concluded that glucose increases stabilization of the enzyme against thermal denaturation.

The calculated from equation (13) Gibbs free energy associated with temperature-induced unfolding process of glucose oxidase is equal to 6697,151 J/mol and 6826,490 J/mol for GOD in phosphate buffer and for GOD in phosphate buffer with glucose, respectively.

## Conclusions

In the current work a systematic investigation of the temperature induced unfolding of GOD from *A. niger* was made using thermal scanning fluorescence method. For this purpose the fluorescence intensity of FAD in GOD upon increasing temperature has been monitored. A large (10-fold) enhancement of FAD fluorescence intensity which is associated with the dissociation of the FAD molecules from the enzyme has been observed.

The temperature of the unfolding process of GOD has been determined to be 335,73 K. Additionally, the thermal scanning fluorescence experiment for GOD treated with glucose has also been performed. In the presence of substrate GOD showed an enhanced stability against thermal denaturation as compared to the native GOD dimer without glucose. The unfolding process of the glucose-stabilized GOD occurs at 342,21 K. The obtained results prove that thermal scanning fluorescence method may be successfully applied to study the conformational changes associated with unfolding process of flavoproteins.

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# BADANIE TERMOSTABILNOŚCI KONFORMACYJNEJ OKSYDAZY GLUKOZOWEJ (GOD) Z *ASPERGILLUS NIGER*

## Streszczenie

W pracy zbadano, wykorzystując metody fluorescencyjne, proces termicznej denaturacji oksydazy glukozowej (GOD) pochodzącej z *Aspergillus niger*. Zachodzącym podczas procesu termicznego rozfałdowania zmianom konformacyjnym GOD towarzyszy oddysocjowanie od enzymu cząsteczek dinukleotydu flawinoadeninowego (FAD). Dlatego też podczas termicznej

denaturacji enzymu zaobserwowano zmiany intensywności fluorescencji FAD. Monitorując intensywność fluorescencji FAD w miarę wzrostu temperatury, wyznaczono temperaturę oraz energię swobodną Gibbsa dla procesu termicznego rozfałdowania oksydazy glukozowej. Ponadto, rejestrując zmiany intensywności emisji FAD w miarę wzrostu temperatury, zbadano także wpływ substratu na termostabilność oksydazy glukozowej. Okazało się, że w obecności glukozy enzym wykazuje zwiększoną odporność na działanie podwyższonej temperatury. Rezultaty przeprowadzonych badań wskazują, że metoda monitorowania intensywności fluorescencji stanowi czułą, szybką i prostą metodę badania zmian konformacyjnych związanych z procesem termicznej inaktywacji enzymu.

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