Irreversible thermal denaturation of Glucose oxidase from *Aspergillus niger*

is the transition to the denatured state with residual structure

by

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Running title: Thermal denaturation of GOX

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Summary

Glucose oxidase (GOX, β-D-glucose:oxygen oxidoreductase) from *Aspergillus niger* is a dimeric flavoprotein with a molecular mass of 80 kDa/monomer. Thermal denaturation of glucose oxidase has been studied by absorbance, circular dichroism spectroscopy, viscosimetry and differential scanning calorimetry. Thermal transition of this homodimeric enzyme is irreversible and, surprisingly, independent of GOX concentration (0.2 – 5.1 mg/ml). It has an apparent transition temperature of 55.8±1.2 °C and an activation energy of ~280 kJ/mol, calculated from the Lumry-Eyring model. The thermally denatured state of GOX after re-cooling has the following characteristics: (i) it retains ~ 70% of the native secondary structure ellipticity; (ii) has a relatively low intrinsic viscosity; 7.5 ml/g, (iii) binds ANS; (iv) has a low Stern-Volmer constant of tryptophan quenching, and (v) forms defined oligomeric (dimers, trimers, tetramers) structures. It is significantly different from chemically denatured (6.67M GdmHCl) GOX. Both the thermal and the chemical denaturation of GOX cause dissociation of the flavin cofactor; however, only the chemical denaturation is accompanied by dissociation of the homodimeric GOX into monomers. The protein concentration is independent of the transition temperature and the properties of the thermally denatured protein indicate that thermally denatured GOX is a compact structure, a form of molten-globule like apoenzyme. GOX is thus an exceptional example of a relatively unstable mesophilic dimeric enzyme with residual structure in its thermally denatured state.

*Abbreviations*: CD, circular dichroism; DSC, differential scanning calorimetry; ANS, 8-anilino-1-naphthalenesulfonate; GdmHCl, guanidinium hydrochloride; GOX, glucose oxidase
Introduction

Glucose oxidase (GOX, β-D-glucose:oxygen oxidoreductase, EC 1.1.3.4) is a flavoenzyme that catalyzes oxidation of β-D-glucose by molecular oxygen to δ-gluconolactone, which subsequently hydrolyzes spontaneously to gluconic acid and hydrogen peroxide. The enzyme contains one tightly, noncovalently bound flavinadenin dinucleotide phosphate (FAD) cofactor per monomer and is a homodimer with a molecular mass of 160 kDa, depending on the extent of glycosylation (1). Glucose oxidase from *Aspergillus niger* is glycosylated by neutral sugars (mostly mannose-like sugars) and by amino sugars (2). Several reports find that the sugar content may vary from 11 up to 30% (3-5).

GOX is of considerable commercial importance. The enzyme has applications in the food and fermentation industry, the textile industry and as a molecular diagnostic and analytical tool in medical and environmental monitoring applications (6-10).

The study of GOX and its applications is limited by its conformational instability. A significant effort has been made to increase the stability of GOX. It is known, that both the thermal stability and the dynamic properties of the enzyme depend on its redox state (11, 12). Protein glycosylation affects the conformational dynamics of the active site and thus the activity of the enzyme (13). However, the way in which glycosylation affects the stability of GOX, is not known. On the other hand, modification of the glucose oxidase surface by artificial long polyethylene-glycol chains results in an increased melting temperature (14). Externally added polyols like sorbitol or glycerol significantly increased the melting temperature of glucose oxidase (15). The activity and conformational stability have also been studied in the immobilized state (16, 17), the crosslinked state (18), and in the presence of different additives such as mono- and divalent salts, and lysozyme (19-21).

Despite the progress in understanding the thermal denaturation/inactivation of GOX, the mechanism of the irreversible thermally induced denaturation is not clear. Moreover,
there are inconsistencies in the reported basic thermodynamic parameters, e.g. the transition
temperature of the thermal denaturation of GOX.

In the present work we have analyzed the thermal denaturation of GOX using a kinetic
model of denaturation proposed by Lumry and Eyring (22, 23). Obtained results enable us to
(i) characterize the thermally denatured state; (ii) suggest a modified mechanism of thermal
denaturation and (iii) indicate a possible source of inconsistencies in the reported values that
characterize the thermal transition of glucose oxidase.
Materials and methods

Materials

Chemicals of high purity grade were obtained from Merck, Sigma and Lachema. Glucose oxidase was purchased from Merck and Sigma (type VII-S). Before each assay the protein was dialyzed overnight against 50 mM sodium phosphate buffer, pH 7.2, at 4°C. The concentration of glucose oxidase was calculated using the extinction coefficient \( \epsilon_{450} = 28,200\, \text{M}^{-1}\cdot\text{cm}^{-1} \) (3). SDS PAGE gels stained with Coomassie Brilliant Blue showed a single band with an apparent molecular mass of 80 kDa. The concentrations of guanidinium hydrochloride were determined from refractive index measurements using an Abbe Refractometer AR3-AR6 (24). The pH values of the solutions were measured with a Sensorex glass electrode. The solvent accessible surface area (probe radius of 1.4 Å²) was calculated using Surface Racer (25).

Circular dichroism

CD measurements were performed on a Jasco J-810 (Tokyo, Japan) spectropolarimeter with 5-15 \( \mu\text{M} \) of glucose oxidase at 20°C in 50 mM sodium phosphate buffer, pH 7.2, and a given concentration of GdmHCl. A cuvette with a 1 mm pathlength was used for the peptide region and a 1 cm pathlength cuvette was used for the aromatic and visible regions. Each spectrum is the result of the averaging of 4-6 consecutive scans. The thermal transitions were recorded at 222 nm at a constant scan rate of 1 K/min and with different concentrations of enzyme. The temperature of the sample was controlled with a PTC–348 WI Peltier block.
Absorbance

Spectrophotometric measurements were carried out on a Shimadzu 3000 spectrophotometer. The difference spectra were collected at various temperatures in different spectral regions (aromatic and visible). Changes in the absorbance at 280-320 nm are due to changes of the environments of the tryptophans and the flavin. The flavin absorbance was measured in the spectral region of 350-550 nm. The scan rate was 1 K/min and the cuvette was heated by an external thermal bath. The temperature inside the cuvette was controlled with a digital thermometer connected to a computer. The concentration of glucose oxidase was 5-30 $\mu$M in 50 mM phosphate buffer, pH 7.2.

Fluorescence

Fluorescence measurements were performed on a Shimadzu RF 5000 and a RF 5301-PC spectrofluorophotometer. Changes in the emission spectra of the internal chromophores, Trp and FAD, were observed upon excitation at 290 and 450 nm, respectively. The protein concentration was 15 $\mu$M in 50 mM sodium phosphate, pH 7.2, in a final volume of 2.5 ml. Binding of the ANS probe (20 mM stock dissolved in ethanol) to the various states of glucose oxidase was measured as the change in the ANS emission spectra upon excitation at 390 nm.

Fluorescence quenching

Quenching experiments were performed with non-charged (acrylamide) and charged (CsCl, KI) quenchers. Freshly prepared stock solutions of quenchers at a 2 M concentration were prepared in 50 mM sodium phosphate buffer, pH 7.2. The measurements were performed with 5-10 $\mu$M protein. Quenchers were added to the cuvette in small, 5-20 $\mu$L, aliquots. The emission spectra were recorded after excitation at 290 nm (Trp) or 450 nm (FAD). Data were analyzed using the Stern-Volmer equation (1) assuming a single population of fluorophores (26):

$$\frac{F_0}{F} = 1 + K_{sv}[Q]$$

(1)
where \( K_{SV} \) is the Stern-Volmer quenching constant. The Stern-Volmer plots were analyzed by linear regression using computer software Grafit 3.00. The Stern-Volmer quenching constants were obtained from the linear regression analysis of the experimental data (10-15 points) for various quencher concentrations. The coefficient of linearity, \( r>0.98 \) for FAD and \( r>0.96 \) for tryptophans, was an additional criterion confirming a single fluorophore population.

**Viscosimetry**

Viscosimetric measurements were performed on VISCODENS using a defined shear rate \( \gamma \) of 60 \( \text{s}^{-1} \) (27). The volume of the sample was 1.6 ml. The concentration of glucose oxidase was 22.8 \( \mu M \). Samples were dialyzed overnight against 50 mM phosphate buffer, pH 7.2, in the absence and presence of 6.67 M GdmHCl. Filtered buffer used for the overnight dialysis was used as a reference solvent for viscosimetry measurements. The thermal transitions were measured with a scanning rate of 20°C/hour. The specific viscosity was calculated from:

\[
\eta_{\text{spec}} = \frac{\eta - \eta_0}{\eta_0}
\]

where \( \eta \) is the relative viscosity of protein and \( \eta_0 \) is the relative viscosity of the reference buffer. The reduced viscosity \( \eta_{\text{red}} \) [ml/g] can be calculated by applying the following equation:

\[
\eta_{\text{red}} = \frac{1}{c} \times \eta_{\text{spec}}
\]

where \( c \) is the concentration of the protein in g/ml. Calculation of the intrinsic viscosity \([\eta]\) was possible from the reduced viscosity by the assumption of a highly diluted solution of the protein.

\[
[\eta] = \lim_{c \to 0} \eta_{\text{red}}
\]
Cross-linking Using Glutaraldehyde

Various states, native, thermally and chemically denatured, of glucose oxidase were treated by glutaraldehyde as previously described (20) with minor modifications. The modifications were: (i) final concentration of glutaraldehyde was 5% and (ii) time of incubation was 10 minutes. Protein, upon glutaraldehyde crosslinking, was dissolved in 0.1 M Tris-HCl, pH 8.0, 1% SDS and 50 mM β-mercaptoethanol and heated to 95 °C for 5 minutes. Resulting samples were analyzed by 8% SDS/PAGE. To obtain reproducible results a fresh solution of glutaraldehyde was made before experiments.

Sedimentation Velocity

Sedimentation experiments were performed in the Center for Analytical Ultracentrifugation of Macromolecular Assemblies located in the Department of Biochemistry at The University of Texas Health Science Center at San Antonio (USA, http://www.cauma.uthscsa.edu). Sedimentation velocity studies were performed in a Beckman XLA ultracentrifuge at 20 °C using optical absorption detection at 280 nm. Absorbance scans were collected at 50000 rpm. The sedimentation velocity data were analyzed by the method of van Holde and Weischet (28). The sedimentation coefficients, $s_{20,w}$, were calculated using the UltraScan (Version 6.2.0.) computer software (http://www.ultrascan.uthscsa.edu) as previously described (29, 30).

All studies were performed on 3.2 µM GOX (Sigma) dialyzed for 12 hours against 50 mM sodium phosphate buffer, pH 7.2. The molecular weight, $M_w$, of the GOX was calculated from Svedberg’s equation:

$$M_w = \frac{s_{20,w}RT}{(1 - \bar{v}\rho_o)D_{20,w}}$$

(5)

where $s_{20,w}$ is the sedimentation coefficient corrected for density and viscosity; $\rho$ is the solvent density; $D$ is the diffusion coefficient; $\bar{v}$ is the partial specific volume of the protein. The
partial specific volume was 0.714 cm$^3$/g for GOX in buffer (31) and 0.704 cm$^3$/g for GOX in buffer with GdmHCl (32). The viscosity and density correction of the aqueous solution of GdmHCl was made using published data (33). The diffusion coefficient was 4.12 x 10$^{-7}$ cm$^2$/sec (3).

**Analysis of thermal denaturation**

The thermal transition of glucose oxidase was analyzed using a two-state equation:

\[
y_{\text{obs}} = \frac{y_N + m_N \cdot T + (y_U + m_U \cdot T) \exp \left( \frac{\Delta H_{\text{eff}}}{R} \left( \frac{1}{T} - \frac{1}{T_{\text{trs}}} \right) \right)}{1 + \exp \left( \frac{\Delta H_{\text{eff}}}{R} \left( \frac{1}{T} - \frac{1}{T_{\text{trs}}} \right) \right)}
\]

(6)

where $y_{\text{obs}}$ is the observed spectral parameter, $y_N$ and $y_U$ are spectral parameters of the native (with slope $m_N$) and denatured form (with slope $m_U$), $T$ is temperature (K), $R$ is the gas constant 8.314 J/K/mol, $\Delta H_{\text{eff}}$ is the apparent van’t Hoff enthalpy and $T_{\text{trs}}$ is the apparent temperature of transition, i.e. 50% of the population is in the native form. The data were fitted with computer software Grafit 3.0, using a simple weighted data nonlinear regression analysis.

**Calorimetry**

Calorimetric (DSC) measurements were performed on a DASM-4 microcalorimeter at heating rates between 0.12-2 K/min. The measuring cell volume was of 0.4786 cm$^3$. The protein concentration was determined using the flavin absorbance at 450 nm. All DSC traces were corrected for baseline (34). The thermal transition of glucose oxidase was irreversible in all of the measured conditions. Nevertheless, the area under the DSC peak was used to calculate the enthalpy of the processes that accompanied the protein thermal denaturation. The transition temperature and the reversibility of the thermal transitions were not dependent on the protein (3.5-37.0 µM) and flavin concentration (0-3.6 mM). The pH values of the
solutions were measured before and after cooling the sample. Samples with pH changes less than 0.2 pH units were used.
Results

Thermally induced denaturation of glucose oxidase

Changes in the absorption spectra of thermally and chemically denatured GOX are illustrated in Figure 1. Although the observed changes were relatively small, they are well-defined in both the absolute and differential mode.

Flavin absorbance in the visible part of the spectra was used to follow the thermally induced changes in the active site of the protein at various protein concentrations (inset A in Figure 2). The transition temperature of the thermal denaturation observed in the region of 350-550 nm was 55.7 °C. Upon cooling to 20 °C, the heat-induced changes in the GOX spectrum were irreversible (inset B in Figure 2). Dialysis of the thermally denatured protein resulted in loss of the flavin molecule as revealed by the absence of the flavin fluorescence and absorbance (data not shown). Thus, the dissociation constant for the flavin molecule was strongly perturbed upon thermal denaturation and the flavin cofactor dissociated from the active site as was previously suggested (3, 35). This is in contrast with some other reports (36, 37). Surprisingly, the transition temperature of thermal denaturation determined from the flavin absorbance was independent of the glucose oxidase concentration (inset A in Figure 2). It should be pointed out that the samples were dialyzed before the thermal denaturation measurements. In contrast, an apparent concentration dependence was observed for undialyzed protein, probably due to the presence of stabilizers in the dry sample.

Spectrophotometric measurements were also performed in the aromatic region (250-320 nm) where the tryptophan absorbance predominates over the contribution from the flavin cofactor. The denaturation curve, obtained from the difference spectra, was sigmoidal in shape without any apparent deviations, indicating a two-state transition (Figure 2). The
transition temperature of the thermal induced denaturation observed in this wavelength region was 54.6 °C and was also irreversible.

The process of GOX thermal denaturation was also analyzed by circular dichroism (Figure 3). CD measurements were performed at 222 nm, i.e. a wavelength that reflects changes in secondary structure. The observed changes led to nearly the same melting curve as in the case of absorbance spectroscopy thus indicating that the secondary and tertiary structural changes proceeded at the same time with $T_{tr} 54.3$ °C. The analysis of fit was performed using a two-state approximation. Randomly scattered residual graphs for the nonlinear regression strongly indicate the absence of intermediates monitored by ellipticity changes at 222 nm (insets in Figure 3).

Changes in the quaternary and tertiary structures were monitored by viscosimetry. Viscosimetry is sensitive to the overall shape of the protein and the surrounding hydration layer. The transition temperature obtained from viscosimetry equaled 53.8 °C (the thermal denaturation curve is shown in Figure 13).

**Dependence of thermal transition on protein and cofactor concentrations and scan rate**

The different methods used required the use of different protein concentrations. The GOX concentration in the present work varied ~25–fold but this concentration variation did not affect the denaturation temperature of the protein (Figure 4A). The independence of the thermal transition of the homodimeric GOX on its concentration was surprising. In the case of the reversible transition of the dimeric (multimeric) proteins with $m$ monomeric units there is a dependence of the melting temperature on the total concentration of the protein $[N]$ (38, 39):

$$\frac{\partial 1/T_{tr}}{\partial \ln [N]} = \frac{R}{\Delta H_{m1} \times (m - 1)}$$
In the case of the irreversible process of the multimeric proteins the plot ln[N] vs. 1/T_{trs} is also linear as it is for the equilibrium process. The slope of this plot for the irreversible transition is equal to (23):

\[ \frac{\partial 1/T_{trs}}{\partial \ln[N]} = -\frac{R \times m}{E_a \times (m-1)} \]

where \( R \) is the gas constant and \( E_a \) is the activation barrier for the irreversible step. For the irreversible step, a lack of dependence of the transition temperature on the protein concentration indicates that the dissociation of the monomers is not part of the rate determining irreversible reaction.

Similarly, the thermal transition measured by DSC was independent of cofactor concentration. Even a 360 fold change in FAD concentration had no effect on the thermal transition or the reversibility of glucose oxidase (Figure 4B).

The irreversible processes associated with the thermal denaturation of the protein are generally sensitive to the scan rate because they are under kinetic control. However, when the thermal transition precedes the irreversible step, the denaturation is independent of the scan rate (23) as in the case of thermophilic EF-Ts (40). The transition temperature of the irreversible thermal denaturation of GOX was dependent on the scan rate. Increasing the scan rate resulted in an increased melting temperature (Figure 5). DASM-4 offers the possibility to vary the scan rate from 0.125 to 2 K/min (Figure 6). Generally, an increase in the scan rate leads to an increase in the transition temperature up to a so-called high scan limit, where the irreversible step does not affect the hot side of the thermal peak. At this limit, the transition temperature versus the scan rate reaches a plateau (41). Due to the intrinsic limitation of the scan rates used in DSC, the plateau was experimentally unachievable in our measurements (inset Figure 6).

Different temperature slopes from various scan rates enabled us to analyze the irreversible step (42). Plotting the data ln \( v/T_{trs}^2 \) vs. 1/T_{trs} allowed us to determine the
activation energy $E_a$ from the slope of the dependence (Figure 6). The activation energy of the irreversible step in the thermal denaturation of GOX is ~280 kJ/mol (linear correlation coefficient $r=0.999$).

**Cross-linking studies**

The quaternary structure of the thermally and chemically denatured proteins was compared by performing glutaraldehyde cross-linking experiments. Glutaraldehyde (bis-aldehyde homobifunctional cross-linker) may react by several routes to form covalent cross-link with amine-containing molecules (43). Reactions with proteins proceed through the formation of Schiff bases or Michael-type additions. Figure 7 shows the results of the cross-linking of the native and the thermally and chemically denatured GOX. For the native GOX the protein band of glutaraldehyde cross-linked samples corresponding only to dimers was observed (Figure 7). For GOX treated with 6.67M GdmHCL only protein bands corresponding to monomers were observed in accordance with a recent study (20). For the thermally-denatured GOX three protein bands corresponding to dimers, trimers, and tetramers were observed. No significant amount of higher oligomers of the protein was observed (Figure 7).

**Sedimentation velocity**

The integral distribution of $s_{20,w}$ of GOX was examined in three different experimental conditions (Figure 8). GOX in 50 mM Na-phosphate buffer, pH 7.2 (triangles) was highly homogeneous with $s_{20,w}$ of 7.8S. The molecular weight of the native GOX was calculated using Svedberg’s equation (equation 5 in Experimental procedures) and found to be 157,000 Da, a value in excellent agreement with previously published data (1, 2, 32). Sedimentation velocity data of GOX in buffer containing 6.67 M GdmHCl (circles) yields an apparent sedimentation coefficient of $s_{obs}=0.95S$, which corresponds to the $s_{20,w}=2.8S$. Although, the diffusion coefficient for GOX in GdmHCl is not known and, therefore, the molecular weight
could not be calculated from sedimentation velocity data. However, based on previously published data for GOX in GdmHCl (20), we believe that the $s_{20,w}$ value reflects the unfolded monomeric form of GOX. GOX was much more heterogeneous after temperature denaturation. The sedimentation coefficient across the sedimentation boundary varied between 3.6 and 11.0S, which indicates formation of both smaller and larger molecules than dimeric GOX. Molecular weights for these $s_{20,w}$ would be $\sim 73000$ (3.6S) to 230000 (11S).

**Circular dichroism of native and denatured states**

Glucose oxidase is a flavoprotein that binds one flavin molecule per monomer in the buried active site. Interaction of the flavin molecule and the tryptophan residues makes circular dichroism a very suitable tool for the study of conformational changes in the active site of glucose oxidase. In the native state, glucose oxidase possesses a relatively strong CD signal in the visible, near-UV and far-UV regions. The CD spectra show a positive Cotton effect at 375 nm that corresponds to the flavin moiety. There was no peak observed at 450 nm where the flavin has its maximum in the absorbance spectrum, probably due to the well-known canceling effect in the CD spectra (44). The rotatory strength in the aromatic region is the highest for the band at 280 nm, probably due to the coupling of FAD and tryptophans in the active site, as in other flavin containing proteins (45). The far-UV region is characterized by the peptide $n\rightarrow\pi^*$ electronic transition, which is sensitive mainly to the conformation of the secondary structure with some contribution from the aromatic side chains. The CD spectrum of native GOX in the far-UV region is typical for a protein containing $\alpha$-helices and $\beta$-sheets.

The thermal transition monitored by ellipticity at 222 nm showed the presence of the residual secondary structure even at 90 °C (Figure 9A) indicating the extreme stability of the protein secondary structure. Re-cooling of the sample resulted in $\sim 70\%$ restored ellipticity at 222 nm suggesting a relatively high reversibility of the protein secondary structure (Figure
9A). In the visible and near-UV regions, the difference between the native and the thermally denatured states was more significant. The thermally denatured state had no signal in the near-UV or in the visible part of CD spectrum due to the dissociation of FAD from the protein. The CD spectrum of the protein chemically unfolded by 6.67M GdmHCl showed a typical positive value of ellipticity in the far-UV region, most likely due to the presence of the polyprolin II conformation (46). Due to the dissociation of FAD, no CD signals were observed in the near-UV and the visible parts of the CD spectrum for the protein denatured by GdmHCl (Figure 9B, C).

The difference in ellipticity of the thermally denatured and chemically denatured GOX was also detected at high temperatures (>80°C) (Figure 10). The difference in the ellipticities at 90°C of both states clearly indicates the presence of the residual structure. In fact, an increase in the GdmHCl concentration in the samples causes a decrease in the difference of ellipticity of the denatured states (Figure 10).

GOX contains one reduced cysteine residue in the structure that is not exposed to solvent after thermal denaturation (21). In fact, heating of the protein in the presence of iodoacetamide had no significant effect on the reversibility of the transition or on the presence of the residual structure (data not shown).

**Fluorescence and fluorescence quenching**

All of the fluorescence quenching experiments were performed at 20 °C (Table 1). The irreversibility of the thermal denaturation of glucose oxidase made it possible to analyze the thermally denatured state at 20 °C. The tryptophan fluorescence of glucose oxidase is the result of the contributions of 10 tryptophans per monomer (Trp 111, 122, 131, 133, 232, 350, 376, 402, 426, 503). All of the tryptophans are inaccessible to solvent molecules as indicated by the solvent accessibility data. Only Nε1 of Trp133 is slightly exposed and accessible to solvent molecules, with an accessible surface area of 4.4 Å². One can expect that the protein
matrix will strongly slow the penetration of the quencher molecules and thus lead to a low Stern-Volmer constant. Charged quenchers are used to analyze the polarity of the environment close to tryptophan residues (26). Iodide is a very effective negatively charged quencher of tryptophan fluorescence in glucose oxidase. From the quenching experiments on glucose oxidase, summarized in Table 1, we found that the GdmHCl induced unfolded state is more “open”, and the tryptophans are more accessible to the solvent than in the thermally denatured state. The native protein has a very low Stern-Volmer constant for iodide: $K_{SV} = 1.1 \pm 0.1 \text{ M}^{-1}$. After the irreversible thermal transition, glucose oxidase is in a state with disrupted tertiary structure with tryptophans that are more exposed to the solvent, as indicated by the increased Stern-Volmer constant, $2.0 \pm 0.1 \text{ M}^{-1}$. The accessibility of the tryptophans increased even more in the presence of GdmHCl. The Stern Volmer constants indicate that the accessibility of the tryptophan fluorophores increases in order from native, to the thermally denatured and then to the chemically denatured state of glucose oxidase. A similar situation is observed for the cesium cation and for acrylamide (Figure 11A). The high linear correlation coefficients justify the analysis of the quenching experiments with a simple Stern-Volmer analysis (equation 1).

The presence of a single molecule of FAD compared with 10 tryptophans simplifies interpretation of the experimental data. The obtained Stern-Volmer constants for various quenchers for GOX in different states follow the pattern observed for tryptophans, i.e. the flavin in the denatured states (GdmHCl, and T) is always more accessible to the solvent than in the native state of GOX. Unexpectedly, the Stern-Volmer constant of FAD for iodide in the thermally denatured state is higher than for the chemically denatured state. However, if the viscosity effect of the solvent is properly taken into account, the difference between the thermally and chemically denatured states is very low with respect to the Stern-Volmer constants of FAD quenching. To correct for the viscosity at 6.67 M GdmHCl, $K_{SV}$ was
multiplied by a factor of 1.55 (47), and this results in nearly the same Stern-Volmer constants for both denatured states of glucose oxidase (Figure 11B). However, the flavin moiety is not a sensitive probe for discriminating between two differently denatured states. FAD is released to the solvent in the process of chemical and thermal denaturation, thus in both denatured states FAD is free in solution and hence is not sensitive to different conformations of the active site of GOX. The $K_{SV}$ (for KI) is 27.8 M$^{-1}$ for FAD free in solution, and is very similar in the thermally and chemically denatured GOX. This further supports the observation that the FAD being dissociated into solution is due to the denaturation of the protein (Table 1).

*Binding of ANS*

Extrinsic fluorophores like ANS are unique, simple probes for detecting molten-globule states (48, 49). The native and chemically denatured states do not bind the ANS molecule. On the other hand, the thermally denatured state of GOX binds ANS as is evident from the emission spectrum of the probe. An increase in the quantum yield and a blue shift of the emission spectrum were observed in the thermally denatured glucose oxidase (Figure 12). The binding of ANS to the thermally denatured glucose oxidase indicates that the protein is in a molten-globule like state.

*Viscosity of native, thermally and chemically denatured states of glucose oxidase*

The intrinsic viscosity of a protein reflects the shape of the protein together with tightly bonded water molecules. The intrinsic viscosity of glucose oxidase was calculated from the reduced viscosity assuming highly diluted protein (equation 4). The intrinsic viscosity of the native protein at 20 °C was 4.12 ml/g, which closely agrees with the values reported previously (32, 50). The thermally denatured protein at 20 °C has an intrinsic viscosity of 7.5 ml/g and the chemically denatured protein has an intrinsic viscosity of 29.5 ml/g at the same temperature. This is in excellent agreement with the previously reported
value of 30 ml/g for the chemically denatured state in 6 M GdmHCl (32). All viscosity measurements were done using the same concentration of the protein.

VISCODENS allowed us to measure the temperature dependence in the scanning mode with the precision of ±0.02 K (27). The thermal denaturation of the native conformation was followed at different temperatures with a constant scan rate of 20 K/hour. Higher scan rates were not possible due to the limitations of the viscosimeter. Viscosity was first corrected to the reference solvent viscosity. The reference solvent was the solvent used for overnight dialysis of the sample. Upon denaturation, the viscosity increased ~2-fold (Figure 11). Re-cooling of the sample showed that the transition was irreversible and the increased viscosity of the solution at 20 °C reflects a partial unfolding of the protein. A second heating scan led to an increase of viscosity without a sigmoidal shape. The viscosity of the first and second scans overlapped at high temperatures. In the presence of 6.67 M GdmHCl, the corrected viscosity is much higher than that of the thermally denatured state over the whole temperature range. The thermally denatured state was thus more compact than the GdmHCl denatured state. This difference cannot be assigned to the difference in the solvation layer but must be associated with the presence of a more expanded unfolded state in the presence of GdmHCl. The difference between the viscosity of the thermal and GdmHCl denatured protein at all experimental temperatures was positive and slightly decreased with temperature. This difference was independent of protein concentration. It should also be mentioned that the concentrations of protein used in the viscosimeter were relatively high. At a concentration of glucose oxidase about ~50 µM, there was a distortion observed in the post transition region of the thermal transition, probably due to aggregation of the protein (not shown). At lower protein concentrations, the hot side of the thermal transition was not affected.
Discussion

The thermal transition of GOX is close to a two-state transition

In the present work different biophysical methods were applied to study the thermal and chemical denaturation of the glucose oxidase. It was shown that thermal denaturation of glucose oxidase is irreversible transition to the compact denatured with defined oligomeric structure that is significantly different from the chemically denatured state of GOX - unfolded monomer.

Data obtained by DSC and viscosimetry reflect global changes in the protein structure. Ellipticity in the far-UV region reflected alterations within the secondary structure. Because GOX is a \(\alpha/\beta\) protein, the changes in ellipticity at 222 nm monitors the global changes related to the \(\alpha\)-helical secondary structure. Similarly, absorbance spectroscopy in the aromatic region reflects local changes in the environment around the aromatic amino acids, mainly tryptophan residues. However, the 10 tryptophans of GOX are distributed all over the protein and give information about the global rather than the local properties, related to tertiary structure. The probe used to monitor the local properties of the active site was absorbance spectroscopy in the visible region. Interestingly, despite the fact that GOX is a relatively large dimeric protein (80 kDa/monomer), the transition temperature of the thermal denaturation of GOX is in a narrow range from 54 to 58.5 °C with the center at 55.8 °C. There was no apparent dependence on protein concentration (Figure 4A), suggesting that the thermal transition of GOX has a two-state character. It should be pointed out that dissociation of FAD during the thermal denaturation is most likely the reason for the transition irreversibility although the addition of excess FAD had no effect.

The transition temperature of GOX, as a homodimeric protein, should depend on its concentration (23) because the homodimeric structure is expected to dissociate due to the
thermally induced denaturation. Surprisingly, a more than 25 fold difference in protein concentration resulted in basically the same transition temperature (Figure 4A). Therefore, GOX does not change its oligomeric state in the process of thermal transition, i.e. it neither dissociates into monomers nor forms large aggregates. This conclusion is strengthened by the cross linking experiments that showed the thermally denatured state of GOX forms maximally tetramers (Figure 7), in agreement with a previous report (37). Moreover, analytical ultracentrifugation experiments confirmed that the thermally denatured state of GOX forms maximally trimeric structures and an insignificant amount of monomers (less than 10%) (Figure 8). This finding is contrary to the suggestion that dissociation of FAD induces GOX monomerization (15, 50). Recently presented results indicates that after the dissociation of FAD from the holoenzyme, the apoenzyme is not in a monomeric state but tends to form aggregates (21). An alternative explanation of the reported gel-filtration experiments would be the formation of an extended molten-globule like structure. Our cross-linking and analytical ultracentrifugation experiments support these results and indicate formation of oligomeric structures up to tetramers.

Thermally-denatured GOX – compact homodimeric molten-globule like apoenzyme

Our results strongly indicate the presence of a residual structure without the apparent formation of large aggregates. Aggregation of the protein usually results in a distortion of the post-denaturation line. Aggregation also causes significantly asymmetric peaks with a characteristic dropped postdenaturation baseline in the DSC experiments. Lack of such changes in our results indicate the absence of the large aggregation processes induced by thermal denaturation. We have compared thermally denatured GOX with chemically denatured GOX. The chemically denatured GOX was characterized recently in a detailed way as the monomeric state of GOX (20). However, our results show that, at temperatures above the denaturation temperature, GOX contains secondary (Figures 9, 10), tertiary and/or
quaternary structures (Figures 8, 13) that are dissimilar from the monomeric chemically
denatured GOX. Since the thermal transition of GOX was irreversible, it was possible to
analyze thermally denatured GOX after re-cooling and to compare it with the chemically
denatured state. Thermally denatured GOX, after re-cooling, had the typical properties of a
molten-globule state: (i) ~70% preserved $\alpha$-helical secondary structure with a perturbed
tertiary structure (Figure 9), and (ii) the ability to bind the ANS probe (Figure 12). Moreover,
the Stern-Volmer constant, reflecting the solvent exposure of tryptophan residues of the
thermally denatured state, was significantly lower than that of the chemically denatured state
(Figure 9A). This, together with previous observations, indicate a partially preserved tertiary
structure, probably due to the lower dynamics of the thermally denatured state after re-
cooling. These results further reveal significant differences between the thermally denatured
states of GOX, at low and high temperatures, and the chemically denatured monomeric GOX.

Viscosity measurement is sensitive to the overall dimension of the protein. Thermally
and chemically denatured states differ in intrinsic viscosity and therefore they differ in overall
dimensions. Proteins in 6 M GdmCl are close to the random coil (51). The presence of
residual structure restricts the torsional angles in the polypeptide chain. Increasing the
temperature liberates rotations and conformation of the random coil shifts toward random coil
with free rotation (52). Slope $\eta$/dT is negative for random coils. Tanford suggested that
such slope should be the criterion for existence of a disordered conformation along the entire
length of a polypeptide chain (52). Glucose oxidase in the thermally denatured state had ~3
fold lower negative slope of $\eta$/dT (-0.037 ± 0.002 ml/g/K, $r$=-0.956) than glucose oxidase
in 6.67 M GdmHCl (-0.1176 ± 0.002 ml/g/K, $r$=-0.996). This indicates that there is a
significantly smaller amount of the disordered structure in the thermally denatured state in
comparison with the unfolded protein in the presence of 6.67 M GdmHCl.

Mechanism of thermal denaturation of GOX
The following results enabled us to propose a mechanism for the thermal denaturation of GOX: (i) Randomly scattered residuals in the residual graph for the nonlinear regression using a two-state approximation; (ii) nearly the same transition temperature reflected by different probes distributed all over the protein structure and (iii) the irreversibility of the thermal transition. The results indicate that the transition is in agreement with the Lumry-Eyring model, discussed in detail by Sanchez-Ruiz (23), and may be described by the following scheme:

\[
N_2 \leftrightarrow I_2 \rightarrow D_2 \quad \text{(scheme 1)}
\]

where \(N_2\) is the native homodimeric structure, \(D_2\) is the thermally denatured homodimer and \(I_2\) corresponds to an intermediate state, undetectable due to its low concentration. Including the molten globule-like state, \(MG\), and the chemically denatured monomeric state, \(F\), scheme 1 may be extended into the following final equation:

\[
\begin{align*}
\uparrow ? & \quad \downarrow \\
N_2 & \leftrightarrow I_2 & \leftrightarrow D_2 & \leftarrow GdmHCl & \rightarrow 2F
\end{align*}
\]

\[
MG_x \leftrightarrow D_2 \leftarrow GdmHCl & \rightarrow 2F
\]

where \(x = 2, 3, 4\), indicates formation of dimers, trimers, and tetramers of GOX after recooling. Although it is probable that GOX exists in these oligomeric states and also in the thermally denatured state at high temperatures, there is no direct probe for determination of the oligomeric state of the protein at such conditions. The steps shown with a question mark, \(MG_x \rightarrow N_2\) and \(2F \rightarrow D_2\), are theoretically possible but may not be technically feasible. The irreversible steps \(I_2 \rightarrow D_2\) and the \(MG_2 \rightarrow N_2\) are most likely accompanied by dissociation and association of the FAD cofactor, respectively, although the reversibility of the thermal transition was not affected by an addition of excess FAD.

*Flavin cofactor and protein dimer dissociation occur after the rate determining step*
The binding site of flavin cofactor is strongly structurally perturbed in the thermally denatured state as indicated by fluorescence quenching data, dialysis data and spectra of circular dichroism. It is reasonable to expect that the flavin cofactor dissociates during the process of denaturation. In this case, (and also in special conditions of the Lumry-Eyring model) the equilibrium thermodynamics predict that the thermal transition is affected by the presence of the ligand. Increasing the free ligand concentration must lead to the increase of the transition temperature.

Experimentally, excess flavin cofactor does not affect thermal transition of the glucose oxidase (Figure 4B). Reversibility of the thermal transition is also not affected by ligand concentration; there is no endothermic or exothermic peak in second scan. The results obtained by calorimetry could be interpreted by inclusion of a kinetic step in the process of denaturation and analysis of some simple kinetic model such as Lumry-Eyring. For irreversible processes (kinetically driven), lack of dependence of apparent melting temperature on ligand concentration indicates that the ligand dissociates after the rate determining step. Only events before the rate determining step are able to affect the thermal transition. Ligand dissociation occurring before the rate determining step affects thermal transition in a very similar way as is predicted by the equilibrium thermodynamics. In this situation, the plot of ln [Ligand] vs. 1/Ttrs would lead to straight line. The apparent transition temperature was not affected by the protein concentration. From this fact one can conclude that: (i) dissociation of oligomeric protein occurs (if it does occur) after the rate determining step; (ii) the irreversible process shows first order kinetics; and (iii) flavin dissociation occurs after the rate determining step.

Therefore, flavin and protein dimer dissociation are not rate determining steps in the process of thermal denaturation of glucose oxidase from Aspergillus niger. These steps occur after rate determining steps, perhaps in the concerted manner.
The DSC experiments made it possible to characterize, in a more detailed way, one of the irreversible steps in the process of denaturation, i.e. the step $I_2 \rightarrow D_2$ in scheme 2. From the dependence of the transition temperature on the scan rate, it was possible to determine the activation energy, $E_a$, of this step. The value obtained for the activation energy, ~280 kJ/mol, indicates a strong temperature dependence for the irreversible steps. This result, in fact, can partially explain the difference in the reported transition temperatures of GOX in the literature. While the transition temperatures of thermal denaturation of GOX relating to FAD dissociation vary over a relatively narrow range, from 59 °C (21) to 62.6 °C (15), $T_{trs}$ relating to a loss of secondary and tertiary structure monitored by circular dichroism or DSC varies from 62 °C (21), to 66.8 °C (19) to 76 °C (11). The strong dependence of $T_{trs}$ on the scan rate, coupled with the relatively high value for the activation energy, might explain the DSC value reported by Nakamura and Koga (11) – the relatively high value of $T_{trs}$ is probably the result of a high scan rate, 5 K/min. But not all of the differences can be explained by different scan rates. In fact, we have performed parallel experiments with GOX from two different companies, Merck and Sigma, and the transition temperatures differed by ~5°C (e.g., DSC determined $T_{trs}$ were 58.8 °C and 63.7 °C for the protein from Merck and Sigma, respectively), without an apparent effect on any other results presented in this work. As a possible explanation we suggest that the thermal stability of GOX may be dependent on glycosylation. It has been shown that exogenous sugars have a stabilizing effect on GOX (15), and one can, therefore, speculate that covalently attached sugars might have an analogous effect.

**Biological implications of the residual structure of thermally denatured GOX**

The presence of the residual structure in the thermally denatured state of the protein may affect the thermodynamic parameters of the protein. The residual structure is connected with a decrease in the number of solvent exposed amino acids (53) that leads to a decreased
value of excess molar heat capacity, $\Delta c_p$ (54). $\Delta c_p$ is one of the parameters that defines the protein stability curve plotted as the free energy of stability, $\Delta G$, versus temperature, (55) and can contribute to the higher denaturation temperature (54) or shifts of the maximum of the protein stability curve to lower temperatures (40). From plotting $\Delta c_p$ versus the number of amino acids in the protein (53), it is seen that with an increasing numbers of amino acids, the range of temperatures at which the protein is stable, i.e. $\Delta G > 0$, decreases. This is especially critical for large proteins without the domain structure of GOX, which contains 583 amino acids per monomer. Thus, it is possible that a function of the residual structure in GOX is to enlarge the temperature range over which the protein is stable. This strategy is seen in the thermophilic proteins (40, 54, 56) and in some mesophilic single domain proteins (57, 58). Glucose oxidase thus might be one of the first mesophilic homodimeric proteins reported with a residual structure in its thermally denatured state.

Acknowledgments

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References


Figure Legend

Figure 1. Absorbance spectra of glucose oxidase at 20 °C (thick line), 90 °C (thin line) and chemically-denatured, in the presence of 6.67 M GdmHCl, protein at 20 °C (dotted line).

Figure 2. The thermal transition of glucose oxidase in the aromatic (250-320 nm) (●) (3.75 µM), and visible (350-550) (○) (24.6 µM) regions of the absorbance spectrum. Insets: (A) The thermal transition of GOX in the visible region at different concentrations: 4.0 µM (○), 12.6 µM (●), 27.0 µM (□). (B) Thermal transition of glucose oxidase followed in the visible region (○) and the second scan of the same sample, after recooling (●). The thermal transition of glucose oxidase was measured in 50 mM phosphate buffer, pH 7.2. The scan rate was 1 K/min.

Figure 3. Analysis of the thermal transition observed by the far UV-CD at 222 nm using a two-state approximation. The thermal transition of glucose oxidase was analyzed using a two-state equation (5). The concentration of glucose oxidase was 4.5 µM in 50 mM sodium phosphate buffer, pH 7.2. Residuals, differences between experimental and theoretical points, were randomly scattered as confirmed by the residual plot (inset, left up) and the distribution of the residuals (inset, right down). The solid line in the later case represents a Gaussian distribution curve. The residuals are described by the Gaussian distribution function, which indicates random error distribution around the mean value.

Figure 4. (A) Dependence of the transition temperature on the concentration of protein using various methods. Flavin absorbance (○), tryptophan absorbance (●), DSC (Δ), CD (■), viscosimetry corrected for scan rate (□) and measured at a constant scan rate, 1 K/min, using
various concentration of glucose oxidase. (B) Dependence of the transition temperatures determined by DSC in the presence of various concentration of FAD. The concentration of glucose oxidase was 21.7 µM, FAD concentration varied from 0 to 3.6 mM. The thermal transition of glucose oxidase was measured in 50 mM phosphate buffer, pH 7.2. The dashed line indicates the average value of $T_{ms}=55.8$ °C obtained from different methods at different concentrations of GOX shown in the section A.

**Figure 5.** Calorimetric traces of the thermal transition, corrected for baseline, of glucose oxidase from *Aspergillus niger* at various scan rates. The thermal transition of glucose oxidase from *Aspergillus niger* was measured by differential scanning microcalorimetry DASM-4 in sodium phosphate buffer, pH 7.2. Numbers at the curves designate scan rates in K/min. The concentration of glucose oxidase was 21.7 µM.

**Figure 6.** Arrhenius-like plot for the irreversible thermal transition of glucose oxidase from *Aspergillus niger*. Data obtained from DSC curves (Figure 4) yields a linear dependence (coefficient of linearity of $r = 0.9995$). The slope of this plot equals $-\frac{E_a}{R}$. The experimental data are described by the line $y = - (34.1 \pm 0.7) \times 10^{3} x + (91 \pm 2)$. The inset shows the dependence of the melting temperature on the scan rate and the line connecting experimental points is only to guide the eyes.

**Figure 7.** SDS-PAGE profile of glutaraldehyde cross-linked native, chemically denatured and thermally denatured GOX. Lane 1 – native GOX, lane 2 – native cross-linked GOX, lane 3 – chemically denatured (6.67M GdmHCl) cross-linked GOX, lane 4 – thermally denatured cross-linked GOX.
Figure 8. Velocity sedimentation analysis of GOX (3.2µM) in 50 mM Na-phosphate buffer, pH 7.2 (triangles); GOX incubated for 5 min at 90 °C, slowly re-cooled at room temperature and dialyzed for 12 hours (squares); GOX dialyzed for 12 hours against buffer containing 6.67 M GdmHCl (circles).

Figure 9. Circular dichroism of glucose oxidase in the far-UV region (A), near-UV region (B) and visible region (C) of the spectrum. Various forms of glucose oxidase: native at 20 °C (solid line), thermally-denatured at 90 °C (thin solid), re-cooled thermally denatured at 20 °C (broken line); and chemically denatured in 6.67 M GdmHCl (dotted line) in sodium phosphate buffer, pH 7.2 at 20 °C. The protein concentration was 5.25 µM in 1mm cuvette (A), 11.5 µM in 5mm cuvette (B), and 11.5 µM in 10mm cuvette (C).

Figure 10. The thermal transition of glucose oxidase at various concentrations of GdmHCl. The thermal transition of glucose oxidase was measured at different concentrations of GdmHCl (0, 1.0, 1.75, 2.0, 2.75, 3.25 and 6.67 M) in 50 mM phosphate buffer, pH 7.2 at 222 nm. The concentration of glucose oxidase was 1.5 µM. The scan rate was 1 K/min. The arrows indicate the difference between thermally denatured and chemically denatured states of GOX at high temperatures.

Figure 11. The Stern-Volmer plot for fluorescence quenching of tryptophan residues (A) and FAD (B) in native, thermally and chemically denatured glucose oxidase. Fluorescence quenching of tryptophans was measured in the presence of acrylamide and the quenching of FAD fluorescence was performed by iodide in 50 mM sodium phosphate buffer, pH 7.2 at 20 °C. The concentration of glucose oxidase was 1.5 µM. The solid lines are linear fits of experimental data for the native, chemically and thermally denatured states (see Table 1).
broken line represents correction of the Stern-Volmer constant for the viscosity of GdmHCl (40) for the chemically denatured state.

**Figure 12.** Fluorescence of ANS for different forms of glucose oxidase from *Aspergillus niger* in the absence of ANS: native GOX(N), chemically denatured GOX(D) (6.67M GdmHCl), and thermally denatured GOX(T). In the presence of ANS: GOX(N)+ANS, GOX(U)+ANS, GOX(T)+ANS, and free ANS at 20 °C. The emission spectra were measured upon excitation at 390 nm. The concentration of glucose oxidase and ANS were 6.2 μM and 1.6 mM, respectively.

**Figure 13.** Intrinsic viscosity of glucose oxidase from *Aspergillus niger* in various states. The intrinsic viscosity of glucose oxidase (21.7 μM) of the native, chemically and thermally denatured states. Experiments were performed in 50 mM phosphate buffer, pH 7.2. The contribution of the reference solvent was subtracted.
**Table 1.** Tryptophan and flavin fluorescence quenching of GOX in its various states.

<table>
<thead>
<tr>
<th>State</th>
<th>Quencher</th>
<th></th>
<th>Ster-Volmer constant $K_{SV}$ (M$^{-1}$)</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>FAD</td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>acrylamide$^#$</td>
<td>*</td>
<td>2.16 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>CsCl</td>
<td>0.58 ± 0.03</td>
<td>0.42 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>KI</td>
<td>11.3 ± 0.60</td>
<td>1.13 ± 0.08</td>
</tr>
<tr>
<td>Denatured (T)</td>
<td>acrylamide$^#$</td>
<td>*</td>
<td>7.00 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>CsCl</td>
<td>0.92 ± 0.02</td>
<td>0.52 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>KI</td>
<td>25.40 ± 0.30</td>
<td>1.01 ± 0.01</td>
</tr>
<tr>
<td>Unfolded (GdmHCl)$^&amp;$</td>
<td>acrylamide$^#$</td>
<td>*</td>
<td>9.10 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>CsCl$^\perp$</td>
<td>*</td>
<td>2.33 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>KI</td>
<td>26.00 ± 0.30</td>
<td>7.00 ± 0.30</td>
</tr>
</tbody>
</table>

* not determined

$^#$ Acrylamide is very weak quencher of flavin fluorescence.

$^\perp$ Fluorescence quenching of FAD by CsCl in the presence of GdmHCl yields in non-linear dependence and shows significant deviations from linear fits.

$^&$ Stern-Volmer constants were corrected to the change of the relative viscosity in the presence of GdmHCl (47).
Figure 1

![Graph showing absorbance vs wavelength](image1)

Figure 2

![Graph showing fraction of native state vs temperature](image2)
Figure 3

![Graph showing CD [mdeg] vs Temperature (°C).](image)

Figure 4

**A**

![Graph showing T_m vs Protein concentration [µM].](image)

**B**

![Graph showing T_m vs FAD concentration [mM].](image)
Figure 5

Excess molar heat capacity (kJ/K/mol)

Temperature (°C)

Figure 6

$\ln \left( \frac{v}{T_{trs}} \right)$ vs $T_{trs}^{-1}$ (K$^{-1}$)

$T_{trs}$ (°C)

$T_{trs}^{-1}$ (K$^{-1}$)
Figure 11

![Graph A](image1)

**Figure 12**

![Graph B](image2)
Figure 13

![Graph showing intrinsic viscosity vs. temperature for three states: Native, Unfolded (GdmCl), and Denatured (T). The x-axis represents temperature in °C, ranging from 20 to 80. The y-axis represents intrinsic viscosity in ml/g, ranging from 4 to 28.]

Temperature (°C)

Intrinsic viscosity (ml/g)

Native

Unfolded (GdmCl)

Denatured (T)
Irreversible thermal denaturation of glucose oxidase from aspergillus niger is the transition to the denatured state with residual structure
Gabriel Zoldak, Anton Zubrik, Andrej Musatov, Marek Stupak and Erik Sedlak

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