Thermal Inactivation of Glucose Oxidase: Mechanism and Stabilization Using Additives

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SUMMARY

Thermal inactivation of glucose oxidase (GOD; β-D-glucose: oxygen oxidoreductase), from A. niger, followed first order kinetics both in the absence and presence of additives. Additives such as lysozyme, NaCl and K₂SO₄ increased the half-life of the enzyme by 3.5, 33.4 and 23.7-fold respectively, from its initial value at 60°C. The activation energy increased from 60.3 kcal mol⁻¹ to 72.9, 76.1 and 88.3 kcal mol⁻¹ while the entropy of activation increased from 104 to 141, 147 and 184 cal. mol⁻¹. deg⁻¹ in the presence of 7.1x 10⁻⁵ M lysozyme, 1 M NaCl and 0.2 M K₂SO₄, respectively. The thermal unfolding of GOD in the temperature range of 25 – 90°C was studied using circular dichroism (CD) measurements at 222, 274 and 375 nm. Size exclusion chromatography was employed to follow the state of association of enzyme and dissociation of flavin adenosinedinucleotide (FAD) from GOD. The midpoint for thermal inactivation of residual activity and the dissociation of FAD was 59°C, whereas the corresponding midpoint for loss of secondary and tertiary structure was 62°C. Dissociation of FAD from the holoenzyme was responsible for the thermal inactivation of GOD. The irreversible nature of inactivation was caused by a change in the state of association of apoenzyme. The dissociation of FAD resulted in the loss of secondary and tertiary structure, leading to the unfolding and nonspecific aggregation of the enzyme molecule due to hydrophobic interactions of side chains. This confirmed the critical role of FAD in structure and activity. Cysteine oxidation did not contribute to the nonspecific aggregation. The stabilization of enzyme by NaCl and lysozyme was primarily due to charge neutralization. K₂SO₄ enhanced the thermal stability by primarily strengthening the hydrophobic interactions and made the holoenzyme a more compact dimeric structure.
INTRODUCTION

Glucose oxidase (β-D-glucose: oxygen-oxidoreductase, EC 1.1.3.4) from A. niger, is a flavoprotein which catalyses the oxidation of β-D-glucose to D-glucono-δ-lactone and hydrogen peroxide, using molecular oxygen as the electron acceptor. Glucose oxidase (GOD) finds application in food and fermentation industry apart from being an analytical tool in biosensors for medical applications and environmental monitoring (1 – 3). This protein is a dimer of two identical subunits with a molecular weight of 160 kDa (4). The dimer contains two disulfide bonds, two free sulfhydryl groups (4) and two FAD molecules (tightly bound) not covalently linked to the enzyme (5). The dimer has a high degree of localization of negative charges on the enzyme surface and dimer interface (6). The flavin cofactors are responsible for the oxidation – reduction properties of the enzyme (7). Under denaturing conditions, the subunits of GOD dissociate accompanied by the loss of cofactor FAD (7, 8).

Various additives such as salts, mono- and polyhydric alcohols and polyelectrolytes were used to increase the thermal stability of GOD (9, 10). The effectiveness of additives depended on the nature of enzyme, its hydrophobic/hydrophilic character and the degree of its interaction with the additives (9). Aggregation, the main causative factor for the inactivation of glucose oxidase, could be prevented by modifying the microenvironment of the enzyme (11). The thermal stability of GOD at 60°C could be increased by incorporating lysozyme as an additive during immobilization. The role played by the complimentarity of surface charges of the enzyme and lysozyme appeared to be crucial in the stabilization of GOD (12).

The presence of salt ions (primarily sulfate) is known to increase the stability of the folded conformations of proteins (13). Details of the mechanism are not yet completely
understood, partly because of the presence of several intra- and intermolecular interactions in proteins that may or may not be stabilized by sulfate.

Light is yet to be shed on the mechanism of thermal inactivation of GOD, in spite of several attempts at improving its stability (9-11). An understanding of the thermal inactivation mechanism of GOD could lead to thermostabilization of the enzyme using appropriate additives. With this objective, experiments were carried out on the effect of some selected additives on the thermal stability of GOD. In addition to lysozyme, found earlier by us to increase the stability of GOD, two more salts viz., NaCl and K₂SO₄, which are commonly known to stabilize enzymes through ionic and hydrophobic interactions, respectively, were selected for the thermal stability studies reported here. The mechanism of inactivation and the effect of additives on the thermal stability of the enzyme were followed by kinetics of inactivation, spectroscopic measurements and size exclusion chromatography.

**EXPERIMENTAL PROCEDURES**

GOD (EC 1.1.3.4) from *A. niger* (Type VII-S, 180,000 U/ g solid), FAD, acrylamide, and N, N’-methylene-bis-acrylamide, sodium dodecyl sulfate and lysozyme from hen’s egg and 8-Anilino-1-naphthalenesulfonic acid (ANS) from Sigma Chemical Co., St. Louis, MO, USA., β-mercaptoethanol (β-ME), glycine, TEMED and Horseradish peroxidase (320 PPG U/ mg) from ICN Biomedicals Inc., Ohio, USA, and Shodex® PROTEIN KW - 803 size exclusion column (300 mm x 8 mm) with an exclusion limit of 1.5 x 10⁵ from Showa Denko, Japan were used. All other chemicals and buffer salts used were of analytical grade.

**Purification of GOD**

The traces of catalase, associated with commercial preparations of GOD, were removed by size exclusion chromatography on a Sephacryl S-200 HR column (45 x 2.1 cm) pre-
equilibrated with 20 mM phosphate buffer (pH 6.0). GOD was loaded on the column and 0.5 ml fractions were collected at a flow rate of 10 ml/h. Protein concentration and activity of the fractions were measured. The protein concentration (of GOD) was determined using a value of $A_{280}^{1\%} = 13.8$ (14). The fractions containing GOD were pooled and used.

**Enzyme activity assay**

GOD was assayed at 30°C by peroxidase coupled assay (15). Glucose and peroxidase were added to an o-Dianisidine containing buffer (pH 6.0). GOD solution, appropriately diluted, was added after proper mixing. The increase in absorption at 460 nm was monitored for 4 min at 30°C with a spectrophotometer.

**SDS-PAGE Electrophoresis**

SDS–PAGE experiments were performed on 17.5 % vertical minislab gel (Broviga, Balaji Scientific instruments, Chennai, India) according to Laemmli (16). Gels were fixed using water: methanol: TCA (5:4:1 by volume) and stained with 0.1% w/v coomassie brilliant blue in water: methanol: acetic acid in the same ratio. Gels were destained in water: methanol: acetic acid in the above mentioned ratio until the background was clear. Molecular weight protein markers (Bangalore Genei, India) used were phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20 kDa) and lysozyme (14.3 kDa).

**Thermal unfolding transitions by activity measurements**

The loss of enzyme activity as a function of temperature was followed, in the presence and absence of additives, in 20mM phosphate buffer (pH 6.0). The enzyme samples were incubated for 15 min at different temperatures ranging from 25- 80°C. After cooling to 4°C, the residual activity was measured at 30°C by transferring an aliquot to the assay mixture. The
midpoint of thermal inactivation, $T_m$, at which the activity was diminished by 50%, was calculated from the plot of percent residual activity versus temperature.

**Kinetics of Thermal Inactivation**

Kinetics of thermal inactivation of GOD was studied at different temperatures (56-67°C), both in the absence and presence of selected additives. 100 µl (1mg) of enzyme solution was added to 0.9ml of 20mM phosphate buffer (pH 6.0) and kept in a constant temperature bath at the desired temperatures. 10 µl samples of enzyme solution were withdrawn at periodic intervals and cooled in ice bath prior to assay; the residual activity was measured and expressed as a percentage of initial activity. From a semi-logarithmic plot of residual activity versus time, the inactivation rate constants ($k_r$) were calculated (from the slopes) and apparent half-lives were estimated.

**Activation energy calculations**

Thermal stability of GOD in the presence and absence of selected additives was determined by the inactivation rate constant ($k_r$) as a function of temperature, in the range 56-67°C. The temperature dependence of $k_r$ was analyzed from Arrhenius plot (natural logarithm of $k_r$ versus reciprocal of the absolute temperature); the activation energy ($E_a$) was obtained from the slope of the plot. Activation enthalpy ($\Delta H^*$) was calculated according to the equation

$$\Delta H^* = E_a - RT$$

(Eq.1)

where $R$ = universal gas constant; $T$ is the absolute temperature.

The values for free energy of inactivation ($\Delta G^*$) at different temperatures were obtained from the equation

$$\Delta G^* = -RT \ln \left( \frac{k_r h}{k T} \right)$$

(Eq.2)
where $h$ is the Planck’s constant and $k$ is the Boltzmann constant.

Activation entropy ($\Delta S^*$) was calculated from

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T$$ (Eq.3)

**Effect of Lysozyme on $K_m$ and $V_{max}$ of GOD**

Two kinetic parameters namely, the Michaelis-Menten constant ($K_m$) and Velocity maximum ($V_{max}$) were calculated from the double reciprocal plot in order to study the effect of lysozyme on functional properties of GOD. The kinetics of GOD in 20 mM phosphate buffer (pH 6.0) was studied by varying the initial substrate concentration.

**Circular Dichroism Spectra Measurements**

Circular dichroism measurements were made with a Jasco J-810 automatic recording spectropolarimeter fitted with a xenon lamp and calibrated with $\pm$ d-10-camphor sulphonic acid. Dry nitrogen was purged continuously into the instrument before and during the experiment. The measurements were made at 30°C (unless otherwise mentioned). The light path length of the cell used was 1 mm in the far-UV region, 5 mm in near-UV and 10 mm in the visible regions. The protein concentrations were 0.2-0.25 mg/ml, 0.7-0.8 mg/ml and 2.5 – 3.5 mg/ml in the far-UV, near-UV and visible region, respectively. The samples were prepared in 20 mM sodium phosphate buffer (pH 6.0). For the thermal unfolding measurements, data was collected at 222 nm, 274 nm and 375 nm every sec at a heating rate of 1°C/ min.

The secondary structure of GOD was analyzed using the computer program of Yang et al. (17), which calculates the structural component ratio of secondary structures for the protein, by least squares’ method. The mean residue ellipticity $[\Theta]_{MRW}$, was calculated using a value of 115 for mean residue weight of GOD.
**Steady-state fluorescence measurements**

Fluorescence measurements were made with a Shimadzu RF 5000 spectrofluorophotometer using a 10 mm path length quartz cell. GOD (1.5 µM concentration) in 20 mM phosphate buffer (pH 6.0) was used for measuring the intrinsic fluorescence. The temperature of the cell was maintained at 30°C by circulating water through the thermostatted cuvette holder. The emission spectra of intrinsic protein fluorescence were recorded after excitation at 285 nm. For ANS binding studies, an enzyme solution of 1.5 µM concentration was incubated with 20 µM ANS at 30°C for 1 h and spectra were recorded in the region 400 to 600 nm. The enzyme, in presence of either 0.6 M NaCl or 0.2 M K2SO4, was incubated for 2 h at 30°C before recording the spectra. Appropriate blank spectra of ANS in the corresponding salt concentrations were subtracted to obtain the fluorescence emission caused by ANS binding to protein.

**Size exclusion chromatography**

Gel filtration measurements were carried out using a Shodex® PROTEIN KW-803 column (300 x 8 mm), with the manufacturer’s exclusion limit of 1.5 x 10^5 kDa for proteins, on a Waters HPLC system equipped with a 1525 binary pump and Waters 2996 photodiode array detector. For following the elution profile after thermal denaturation, both in the absence and presence of 0.2 M K2SO4, 20 µl GOD solution (4 – 5 µM) was injected into the column at 30°C before and after heating at 60°C for 15 min. Elution of the sample was carried out isocratically using 20 mM phosphate buffer (pH 6.0) with a flow rate of 0.5 ml/ min at 30°C and detection at 280 and 375 nm by photodiode array detector.

For Stokes’ radius measurements, the column was equilibrated with 20 mM phosphate buffer (pH 6.0), containing the desired salt concentrations, at 30°C. 20 µl of GOD solution (4 – 5
μM), equilibrated in the desired salt concentration (0 – 0.4 M K₂SO₄ in 20 mM phosphate buffer, pH 6.0) was injected into the column and eluted in the same buffer at 0.5 ml/ min flow rate. The absorbance was detected at 280 and 375 nm. Standard proteins from molecular weight marker kit for gel filtration (Sigma Chemical Co., St. Louis, MO, USA.) including alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome C (12.4 kDa) with known Stokes’ radius were used for calibrating the column. Blue dextran at 1mg.ml⁻¹ concentration was used for determining the void volume.

**Experiments with sulfhydryl groups**

The thiol groups exposed during the course of thermal unfolding of GOD were quantified by measuring their reactivity with DTNB as a function of temperature in a Gilford Response II spectrophotometer with an integrated temperature programmer. The transition was followed by increase in absorbance at 412 nm. The heating rate was 1°C/ min.

**RESULTS**

The commercial preparation of GOD was purified by gel filtration on a column of Sephacryl S-200 HR. Homogeneity of the preparations were ascertained by HPLC and SDS – PAGE (Fig.1). The purified enzyme had an absorbance ratio of 11.1 (280/ 450 nm), which is in good agreement with the reported value (4).

*Thermal unfolding transitions by activity measurements*

GOD gets irreversibly inactivated over the temperature range 25 - 80°C. The residual activity of GOD as a function of temperature in presence and absence of additives such as...
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lysozyme, K$_2$SO$_4$ and NaCl is given (Fig. 2). These additives shifted the T$_m$ of GOD from 59°C to 61, 67 and 69°C, respectively (Fig.2).

**Thermal inactivation Kinetics and effect of additives on the thermal stability of GOD**

The thermal inactivation kinetics of native GOD was studied in the temperature range 56-67°C in 20 mM phosphate buffer (pH 6.0). The effect of various additives, viz., lysozyme, NaCl and K$_2$SO$_4$, on the thermal stability of GOD, was followed by measuring the residual activity with time. At all the temperatures studied, inactivation followed an exponential decay. The semi-logarithmic plots (Fig.3A-D) indicated thermal inactivation kinetics followed first order in all the cases. The Arrhenius plots (Fig.3D inset) were linear in the temperature range studied. From this plot and making use of equations 1-3, the activation parameters, viz., free energy ($\Delta G^*$), enthalpy ($\Delta H^*$) and entropy ($\Delta S^*$) of activation were calculated (Table 1). Half-life of GOD was found to increase in presence of each of the additives at all the temperatures studied. Taking a typical case, at 60°C, the half-life increased by 3.5, 33.4 and 23.7-fold, activation energy of GOD increased from 60.3 to 72.9, 76.1 and 88.3 kcal mol$^{-1}$ while activation entropy increased from 104 to 142, 147 and 184 cal deg$^{-1}$ mol$^{-1}$ with 7.1x 10$^{-5}$ M lysozyme, 1 M NaCl and 0.2 M K$_2$SO$_4$ respectively. The corresponding net free energy change, $\Delta G^*$ at 60°C, was 0.9, 2.4 and 2.1 kcal mol$^{-1}$, respectively. The magnitude of free energy of activation reflected the effectiveness of relative stabilization by various additives. The relatively small value of $\Delta G^*$ (24.2 kcal.mol$^{-1}$), for native GOD at 60°C, pointed to the labile nature of enzyme. The difference in the slopes (activation energy) of Arrhenius plot (Fig. 3A inset), in presence of the lysozyme, NaCl and the K$_2$SO$_4$ indicated the differences in mechanism of enzyme stabilization. Significant increase in the activation energy, $E_a$, in the presence of only 0.2 M K$_2$SO$_4$ (88.3 kcal.mole$^{-1}$) compared to 1 M NaCl (76.1 kcal.mole$^{-1}$) and 7.1 x 10$^{-5}$ M lysozyme (72.9 kcal.mole$^{-1}$), indicated that
stabilization of GOD by K$_2$SO$_4$ was of conformational origin. This was further confirmed by CD and size exclusion chromatography measurements. Stabilization of GOD (in terms of increased half-life and activation parameters) by NaCl and lysozyme in acidic pH values indicated the role of ionic interaction between GOD and lysozyme or NaCl. However, activation energy, i.e., the energy required to denature the enzyme, was higher in the presence of 0.2 M K$_2$SO$_4$ when compared to either 1 M NaCl or $7.1 \times 10^{-5}$ M lysozyme. This indicated that hydrophobic interactions play a more dominant role in the stabilization of GOD than ionic interactions. The change in the activation entropy, $\Delta S^*$, in the presence of additives can be explained in terms of an enhancement of the order and compactness of the structure, thus favoring intramolecular stabilizing forces and consequently increasing the stability of the enzyme. Significant change in the activation entropy and the difference in the slopes of the Arrhenius plots in the presence of K$_2$SO$_4$ indicated that the stabilization of GOD was of conformational origin. CD measurements and size exclusion chromatography measurements confirmed this.

**Effect of lysozyme concentration on the stability of GOD**

Detailed studies on the thermal inactivation of GOD were carried out in presence of various concentrations of lysozyme at 60°C in 20 mM phosphate buffer (pH 6.0). As the mole ratio of lysozyme to GOD increased from 0-110, half-life of GOD increased from 13 to 46 minutes (Fig. 4A), thereafter showing a decrease in the thermal stability of GOD. A significant observation (Fig. 4A) was that in the presence of higher concentrations of lysozyme (>150 mole ratio) the half-life of GOD decreased (seen as dotted line). This was due to aggregation of lysozyme observed at higher concentrations. In order to avoid interference (of the aggregated lysozyme) during residual activity measurements, after exposing to 60°C for the specified time, aggregated lysozyme was separated by centrifugation for 15 minutes at 4000 rpm, and the
supernatant was passed through a G-75 column (4 cm x 0.75 cm). The eluted sample was used to measure the residual activity of GOD. This procedure ensured that the half-life did not decrease after reaching the maximum. Increasing the lysozyme to GOD mole ratio above 110 gave no further improvement in the thermal stability of GOD, lysozyme concentration corresponding to this mole ratio was employed for thermal inactivation studies. The requirement of a relatively high mole ratio (110) of lysozyme to stabilize GOD suggested that the interaction between lysozyme and GOD was nonspecific. Increased stability of GOD, in the presence of lysozyme in acidic pH (pH 6.0), confirmed the ionic interactions between lysozyme and GOD. At pH 7.7, the net charge on GOD was reported as –77 (18). The net charge (Lys and Arg) of lysozyme at pH 6.0 is positive (19).

**Effect of lysozyme incorporation on the kinetic parameters of GOD**

To obtain a better understanding of the stabilization of GOD by lysozyme, kinetic parameters viz., Michaelis-Menten constant (Kₘ) and maximum reaction velocity (Vₘₐₓ) were determined in the presence as well as absence of lysozyme (Fig. 4B). Kₘ and Vₘₐₓ of GOD increased from 7.5 to 36.4 mM and 0.33 to 0.73 µM min⁻¹ respectively, when the lysozyme concentration was varied from 0 to 1.1 mM. There was a decrease in affinity of GOD for β-D-glucose in the presence of lysozyme. Interaction of GOD with lysozyme resulted in alteration of its functional properties.

**Thermal inactivation of GOD: Circular dichroism (CD) measurements**

The effect of thermal inactivation on the FAD environment, tertiary and secondary structures of GOD were studied by measuring CD spectra in the visible, near and far-UV regions respectively. GOD exhibited characteristic FAD band at 375 nm in the visible region, a strong CD band at 274 nm in the near-UV region and minima around 208 and 222 nm in the far-UV
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region. The analysis of secondary structure by the method of Yang et al., (17) indicated 14% α-helix and 64% β-structure in the molecule. Due to the thermal inactivation of the enzyme, there were changes in all the 3 regions of the spectra (Fig. 5A, B and C). The intensity at 208 nm and 222 nm decreased, suggesting a loss in α- helix structure. The secondary structure analysis also supported this. The thermally inactivated enzyme had 9% α- helix content and 65% β-structure. In the near-UV region, the 274 nm band caused by the asymmetric environment of aromatic amino acids completely disappeared, indicating disruption of the native tertiary structure. The intensity of the CD band at 375 nm decreased and the maxima was shifted to 335 nm.

Addition of either 0.2 M K₂SO₄ or 0.6 M NaCl to the native enzyme did not affect the CD bands at 274 nm. In the far-UV region too, there was no change in the intensity of 222 nm band. Addition of either NaCl or K₂SO₄ did not alter the backbone or side chain conformation. Spectra of thermally inactivated enzyme at 60°C in the presence of either 0.6 M NaCl (results not shown) or 0.2 M K₂SO₄ suggested (a) a small decrease in the intensity of both 274 nm band and 375 nm band indicating the partial prevention of loss in tertiary structure and protection of the environment around FAD; (b) no significant change in the far-UV region, pointing to the prevention of loss of α- helix, attributable to these salts. The comparison of CD spectra of thermally inactivated GOD in the region 300 – 450 nm with that of free FAD suggested possible dissociation of FAD from GOD (Fig. 5C).

Effect of lysozyme, K₂SO₄ and NaCl on FAD environment of GOD

To follow the conformational changes in GOD due to interaction with lysozyme, the changes in CD spectra in the region 300 – 450 nm, where lysozyme does not contribute (even at high protein concentrations) were measured. The addition of lysozyme and NaCl resulted in
small changes in the spectra indicating a change in the environment of FAD in GOD (Fig. 6). K$_2$SO$_4$ did not affect the FAD band indicating no significant changes in the environment of FAD.

**Thermal unfolding monitored by CD measurements**

The CD measurements of native and thermally inactivated GOD pointed to structural changes in the molecule. Secondary structural changes could be followed by changes in ellipticity values at 222 nm. Changes in tertiary structure were reflected at 274 nm while the dissociation of FAD could be followed by changes in the ellipticity values at 375 nm. Thermal unfolding transitions of GOD in the temperature range 25 - 90°C as followed at 222, 274 and 375 nm are shown (Fig. 7A – C). The loss of tertiary and secondary structure in the native GOD, evident from [θ]$_{274 \text{ nm}}$ and [θ]$_{222 \text{ nm}}$, occurred over a temperature range of 55 to 65°C with a $T_m$ of 62°C. It was found that the loss of FAD (starting at 50°C) was complete by 63°C with a $T_m$ of 59°C.

**Effect of NaCl, K$_2$SO$_4$ and lysozyme on thermal unfolding**

To understand the mechanism of stabilization by NaCl and K$_2$SO$_4$, thermal unfolding transitions of GOD in the presence of 0.6 M NaCl and 0.2 M K$_2$SO$_4$, were followed by CD measurements at 222 nm, 274 nm and 375 nm (Fig. 7A-C). $T_m$, followed at 274 nm, shifted from 62 to 68 and 72°C for native, 0.2 M K$_2$SO$_4$ and 0.6 M NaCl stabilized GOD, respectively (Fig. 7A and 7C). The $T_m$, followed at 375 nm, shifted from 59°C to 68 and 72°C for native, 0.2 M K$_2$SO$_4$ and 0.6 M NaCl, respectively. NaCl was seen to stabilize the tertiary structure and the environment around FAD better compared to K$_2$SO$_4$. The only contrasting difference observed was the transition at 222 nm. K$_2$SO$_4$ was a marginally better stabilizer of the secondary structure compared to NaCl (Fig 7B). Thus, it is evident that NaCl affected the side chain interactions (reflected by $T_m$ measurements at 274 and 375 nm) more favorably while K$_2$SO$_4$ primarily
affected the backbone interactions. Tm followed at 375 nm, shifted by 7°C in the presence of lysozyme (results not shown).

Steady – state fluorescence measurements

For the native GOD, fluorescence emission maximum was observed at 338 nm. The intrinsic fluorescence spectra of the holoenzyme in 20 mM phosphate buffer (pH 6.0), when excited at 285 nm, was significantly quenched compared to the heat inactivated enzyme (Fig 8A). For the heat inactivated enzyme, a significant enhancement of fluorescence intensity along with a small shift in the emission maximum was observed. The dissociation of FAD from the enzyme due to heat inactivation resulted in an increase in the quantum yield. Studies on the reduced and oxidized holoenzyme as well as the apoenzyme revealed that in the native conformation of the enzyme, seven tryptophan residues and FAD are in proximity. The quenching of fluorescence was the result of a Förster energy transfer from the tryptophan residues to the flavin group (20). Addition of salts to the native enzyme did not affect the tryptophan fluorescence emission significantly.

ANS has been shown to bind to hydrophobic regions of proteins. To assess the relative contributions of salts to hydrophobic interactions, the fluorescence of the ANS bound GOD was measured. The fluorescence emission of the ANS bound GOD in 20 mM phosphate buffer (pH 6.0) is shown in Fig 8B. The emission maximum of the ANS bound GOD was at 517 nm when excited at 375 nm. In the presence of 0.6 M NaCl and 0.2 M K₂SO₄, there was an enhancement in fluorescence intensity compared to GOD (control). This suggested that ANS was being displaced into a more apolar environment in salt solutions. Further, the fluorescence intensity was higher in presence of K₂SO₄ compared to NaCl suggesting ANS bound GOD being in a more apolar environment in presence of K₂SO₄.
Role of FAD in activity and structure

The normalized plot for the loss of activity, FAD, secondary and tertiary structure as a function of temperature is shown in Fig. 9. That the activity loss was due to dissociation of FAD from the holoenzyme can be made out from the fact that the loss of activity and loss of FAD followed the same curve. There was simultaneous loss of secondary and tertiary structure, after dissociation of FAD, confirming the role of FAD in activity and structure.

Size exclusion chromatography

The changes in the molecular dimensions of GOD due to the thermal unfolding and the effect of additives, were followed by size exclusion chromatography on HPLC. To follow the dissociation of FAD from GOD, the chromatograms were monitored both at 280 nm and 375 nm for protein and FAD respectively (Fig. 10A & B). In 20 mM phosphate buffer (pH 6.0), GOD eluted as a single peak at 8 ml, corresponding to a molecular weight of 160 kDa. Both protein peak and FAD peak were identical, indicating that under the conditions studied the protein was a dimeric molecule and FAD is associated with it. The thermally unfolded enzyme eluted at 6.5 ml suggesting that protein was aggregated. The aggregated protein peak was not associated with FAD. The dissociated FAD eluted at 11.8 ml. The FAD peak was positively identified by eluting free FAD on the column (Fig. 10B). In presence of 0.2 M K$_2$SO$_4$, thermally unfolded enzyme at 60°C, eluted at 5.77 ml, 8 ml and 11.8 ml. The 375 nm absorbance made it evident that the aggregate eluting at 5.77 ml was not associated with FAD. The enzyme eluting at 8 ml had FAD associated with it (corresponding to the native enzyme). The peak at 11.8 ml corresponded to free FAD. It was clear that after the dissociation of FAD from the holoenzyme, the apoenzyme was not in a monomeric state. Dissociation of FAD exposed many hydrophobic sites which lead to association of enzyme. This thermally inactivated apoenzyme could not bind FAD and regain...
its activity. In the presence of K$_2$SO$_4$, there was partial dissociation of FAD from the holoenzyme and the apoenzyme did not dissociate to monomers but formed different types of aggregates.

The dissociation of FAD from the holoenzyme was confirmed by CD and fluorescence measurements. Comparison of the CD band of free FAD in the region 300–450 nm with the thermally unfolded enzyme (Fig. 5C) revealed that FAD was dissociating from the holoenzyme, resulting in its inactivation. Fluorescence measurements revealed an increase in fluorescence intensity without change in emission maximum of the protein moiety. This indicated possible dissociation of FAD from the holoenzyme. Energy transfer from the tryptophan residues to the flavin cofactor appeared to influence quantum yields of tryptophan. As reported earlier, all the tryptophan residues of each of the subunits transfer energy to the flavin moiety (20).

**Effect of K$_2$SO$_4$ on Stokes’ radius**

To understand the mechanism of stabilization of GOD by sulfate, the Stokes’ radius of GOD was measured at different concentrations of K$_2$SO$_4$. The elution volume of the enzyme increased with increasing molarity of K$_2$SO$_4$, suggesting a compaction of the enzyme molecule (Fig. 11). The Stokes’ radius decreased from 51.4 to 48.5 Å (Fig. 11 inset). These measurements indicated that K$_2$SO$_4$ did not affect the association/ dissociation of GOD in the native state. GOD was more compact in sulfate solutions. There was a significant decrease in the hydrodynamic volume (2.9 Å) of the sulfate stabilized conformation.

**Exposure of cysteine residues during thermal unfolding**

GOD contains 2 free cysteine residues per dimer. The exposure of cysteine residues during thermal unfolding in 20 mM phosphate buffer (pH 6.0) was followed by change in absorbance at 412 nm in the presence of DTNB. The accessibility of thiols to DTNB did not
change during thermal unfolding of protein as reflected in change in absorbance at 412 nm from 30 to 70°C.

**DISCUSSION**

Interactions of proteins with perturbants like salts, temperature, pH and solvent delineate the relative role of these non covalent interactions in the structure and stability of proteins. A number of noncovalent interactions such as hydrogen bonds, van der Waals, ionic and hydrophobic interactions contribute to the structure and stability of proteins.

Glucose oxidase from *A. niger* is a homodimer with a carbohydrate content of 16% v/v (21). The enzyme, a homodimer of 160 kDa molecular weight, contains two tightly bound, noncovalently linked, FAD molecules (22). These flavin cofactors are responsible for the oxidation – reduction properties of the enzyme. Various biophysical approaches have been employed to understand the mechanism of thermal inactivation of GOD. The transition from active to inactive enzyme is found to be highly cooperative and occurred over a very narrow range of temperature. The activation parameters, in presence of lysozyme, NaCl and K₂SO₄, point to the role played by ionic and hydrophobic interactions in the stabilization of GOD. The *Eₐ* values are found to vary in the order K₂SO₄ > NaCl > lysozyme. This indicates that the hydrophobic interactions play a vital role in the stabilization of GOD compared to ionic interactions. The observed *Eₐ* values in 1 M NaCl and lysozyme are very similar suggesting the ionic nature of stabilizing forces in these solutions. Both lysozyme and NaCl affect stability of GOD by affecting the ionic interactions. However, in 0.2 M K₂SO₄, the *Eₐ* values are found to be higher compared to NaCl and lysozyme. Thus, K₂SO₄ primarily strengthens hydrophobic interactions. The fluorescence emission of ANS bound GOD being higher in the presence of K₂SO₄, compared to NaCl of similar ionic strength, supports this. Based on *Eₐ* values in different
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salt solutions and lysozyme, it is concluded that hydrophobic interactions contribute to greater stability compared to ionic interactions. The nature of interactions and the mechanism of stabilization by different salt solutions is also evident from CD measurements.

GOD exhibited CD bands at 375 nm, 274 nm and 222 nm which were used as probes for following the (a) environment around bound FAD, (b) the tertiary structure and (c) the secondary structure of the enzyme respectively. The intensity of the band at 375 nm decreased due to thermal unfolding. This decrease could be due either to the change in the environment around the FAD molecule or to the dissociation of FAD from GOD. Size exclusion chromatography and CD measurements clearly established the change in the state of association of apoenzyme following the dissociation of FAD (due to thermal unfolding). However, there are conflicting reports on the dissociation of FAD from GOD due to thermal unfolding (11, 23). The dissociation of FAD from GOD leads to considerable loss of secondary and tertiary structure (there was a loss of $\alpha$-helix-36% of the total helical content). The complete disappearance of the band at 274 nm, caused by the asymmetric environment of aromatic amino acids indicates the disruption of native tertiary structure. Intrinsic protein fluorescence measurements indicate a higher quantum yield for tryptophan without a change in the emission maximum. Changes in the fluorescence intensity are the result of FAD removal only (20). The overlapping melting curves for the loss of enzyme activity and FAD dissociation, secondary and tertiary structure loss emphasize the critical role of FAD in maintaining the active structure of the enzyme. GOD has one cysteine residue per monomer buried in the interior, which is not essential for enzyme activity. Reaction of GOD with DTNB during the course of thermal unfolding (results not shown) indicates that there was no further exposure of cysteine residues. The dissociation of FAD from GOD did not lead to any exposure of sulfhydryl groups.
The stabilizing effects of salts on proteins involve both ionic and hydrophobic effects. The effect of salts on the ionic interactions largely affects $\alpha$-helices, while the hydrophobic interactions mostly affect $\beta$-sheets (24). Sulfate is a known stabilizing (kosmotrope) agent for proteins while chloride is relatively neutral (25). Ion – induced effects on the water structure may in turn affect the hydrophobic interactions within the protein at high salt concentrations. The preferential exclusion of the salt from the vicinity of the surface of the protein may lead to increased compactness of the native states (26).

Charge repulsions contribute to the conformation and stability of proteins (27). Under physiological conditions, the repulsion between charged groups present in the protein is the main driving force for the protein to be stabilized in open conformation. GOD has a high content of acidic amino acids, which contribute to the net negative charge at neutral pH. The cations stabilize GOD by virtue of their ability to organize the water molecules leading to a compaction of the structure (6).

$K_2SO_4$ did not affect the secondary and tertiary structures or the environment around FAD, however, it prevented the loss of $\alpha$-helix during thermal unfolding of GOD (Fig. 7B). NaCl protected the tertiary structure and the environment around FAD better than $K_2SO_4$. However, $K_2SO_4$ exhibited better preservation of the $\alpha$-helix. Thus, NaCl stabilized the side chain interactions whereas $K_2SO_4$ was a better stabilizer of the backbone conformation.

A considerable reduction in the hydrodynamic volume (Fig. 11) of GOD in the presence of $K_2SO_4$ has been observed. Decrease in repulsive interactions in salt solutions may lead to GOD being more compact. Compaction of the enzyme molecule does result in stabilization, which has been evidenced in low concentrations of guanidine hydrochloride also (28). The
results of size exclusion chromatography have to be viewed with caution since increased hydrophobic interactions in high salt solutions could also, in turn, affect the elution volumes.

The results obtained in the present study could be represented as in Scheme I for the thermally induced unfolding and inactivation of GOD.

Scheme I

\[
\text{Acidified} \ (\text{NH}_4)_2\text{SO}_4 \quad [1] \\
\text{GOD (Dimer)} \quad \xrightarrow{\text{Thermal unfolding}} \quad \text{Apoenzyme (monomer) + FAD} \\
\text{Apoenzyme (monomers) + FAD} \quad \xrightarrow{\text{Aggregates + FAD}}
\]

Thermal perturbation of GOD led to the dissociation of FAD resulting in loss of activity followed by loss of secondary and tertiary structure. Unfolding led to the exposure of hydrophobic surfaces resulting in the formation of aggregates (Scheme 1). The aggregates could not bind FAD due to non-availability of interfacial area between the subunits. The thermostability of GOD was dependent on the microenvironment of the enzyme and on its subunit reorganisation. Protein stability changed in the presence of solvents or additives in the environment of the enzyme, but not independently of the molecular nature of the enzyme (11).

In conclusion, the thermal inactivation of GOD in 20 mM phosphate buffer (pH 6.0) followed first order kinetics both in the absence and presence of the additives. Addition of lysozyme, NaCl and K_2SO_4 enhanced the half-life at 60°C by 3.5, 33.4 and 23.7 fold.
respectively. The loss of activity and structure of GOD was due to the dissociation of FAD from GOD. The irreversible nature of the inactivation was due to the change in the state of association of the apoenzyme. CD and size exclusion chromatography indicated that the thermal inactivation of GOD involved the dissociation of FAD followed by conformational changes, unfolding and nonspecific aggregation of the enzyme molecule. Based on the thermodynamic activation parameters, in the presence of additives, hydrophobic interactions appeared to play a dominant role compared to ionic interactions in the stabilization of GOD. The stabilization of GOD by lysozyme and NaCl was due to charge effects. K₂SO₄ stabilized GOD against thermal inactivation by decreasing the hydrodynamic volume of the enzyme and strengthening the hydrophobic interactions.
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REFERENCES

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The abbreviations used are: GOD, glucose oxidase; NaCl, sodium chloride; K$_2$SO$_4$, potassium sulfate; $T_m$, midpoint of thermal transition; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid), FAD, flavin adenine dinucleotide; CD, circular dichroism; HPLC, high performance liquid chromatography, ANS, 8-Anilino-1-naphthalenesulfonic acid.
FIGURES LEGENDS

Fig. 1. HPLC and SDS-PAGE analysis of purified GOD.

Size exclusion chromatography on HPLC was carried out on a Shodex® Protein KW-803, column (300 x 8 mm i.d.) at 30°C and a flow rate of 0.5 ml/ min. Elution was isocratic using 20 mM phosphate buffer (pH 6.0). Detection was at 280 and 375 nm using a photodiode array detector. GOD was purified by gel filtration on Sephacryl S-200 HR (45 x 2.1 cm) column at a flow rate of 10 ml/ hour.

Inset: Gel electrophoresis profile of GOD on 17.5 % polyacrylamide gels according to Laemmli (16).

Fig. 2. Effect of additives on thermal inactivation of GOD

—◊—, Native enzyme; —▼—, GOD in the presence of lysozyme (7.1 x 10⁻⁵ M); —△—, GOD in the presence of 0.2 M K₂SO₄; —○—, GOD in the presence of 1 M NaCl. The enzyme in 20 mM phosphate buffer (pH 6.0) was assayed after incubation in a water bath at the desired temperature for 15 min. Samples were immediately cooled to 4°C and assayed. The enzyme assay was carried out at 30°C as described under “Experimental Procedures”. Activity of the unincubated sample was taken as 100% and the % remaining activity of heated samples were calculated.

Fig. 3. Effect of additives on kinetics of thermal inactivation of GOD

A: Native (in the absence of additives) B: GOD (7.1 x 10⁻⁵ M Lysozyme) C: GOD in the presence of 1 M NaCl and D: GOD in the presence of 0.2 M K₂SO₄ —●—, 56°C; —□—, 60°C; —▲—, 63°C; —○—, 67°C. Samples were incubated at required
temperatures in the absence or presence of different additives. Aliquots of the enzyme were drawn at different time intervals and assayed as given under “Experimental Procedures”.

Fig. 3D -Inset: Arrhenius plots of GOD inactivation

—♦—, GOD (native); —□—, GOD in the presence of Lysozyme(7.1 x 10^{-5} M);

—▲—, GOD in the presence of K_{2}SO_{4}(0.2 M); —○—, GOD in the presence of NaCl (1 M).

Fig. 4. Effect of lysozyme concentration on thermal inactivation and kinetic parameters

A. Thermal inactivation of GOD in presence of lysozyme

Enzyme was in 20mM phosphate buffer (pH 6.0). ■—■, without removal of the aggregated lysozyme and ◆—◆, aggregate removed by centrifugation.

B. Effect of lysozyme concentration on K_{m} and V_{max} of GOD

Enzyme was assayed in 20mM phosphate buffer (pH 6.0) as described under “Experimental Procedures”.

—□—, GOD without lysozyme; —▲—, GOD in presence in 0.69mM lysozyme;

—■—, GOD in presence of 0.87mM lysozyme; —◆—, GOD in presence of 1.05mM lysozyme.
Fig. 5. **Effect of thermal inactivation on the structure of GOD**

A. Far -UV CD spectra in the absence and presence of $K_2SO_4$.

   Enzyme concentration was 0.2 – 0.25mg/ ml. Cell path length used was 1mm. Scans are the average of 3 runs at a speed of 10nm/ min. Native enzyme (*Solid line*), enzyme in presence of 0.2 M $K_2SO_4$ (*dashed line*); enzyme heated at 60°C for 15min (*dashed and dotted line*) and enzyme heat inactivated at 60°C for 15 min in the presence of 0.2 M $K_2SO_4$ (*dotted line*)

B. Near –UV CD spectra in the absence and presence of $K_2SO_4$

   Enzyme concentration was 0.7 – 0.8 mg/ ml. Path length of cell used was 5 mm. Scans are the average of 3 runs at a speed of 10 nm/ min. Native enzyme (*Solid line*), enzyme in presence of 0.2 M $K_2SO_4$ (+ + +); enzyme heated at 60°C for 15 min (*dotted line*) and enzyme heated at 60°C for 15 min in the presence of 0.2 M $K_2SO_4$ (*dashed and dotted line*).

C. Visible CD spectra of native and heat inactivated GOD

   Native enzyme (*Solid line*), enzyme heated at 60°C for 15 min (*dotted line*). Enzyme concentration was 2.5 – 3.5 mg/ml. Cell path length used was 10 mm. Scans are the average of 3 runs at a speed of 10 nm/ min.

Fig. 5C - Inset: **CD signal of free FAD**
Fig. 6. **The effect of additives on FAD environment of GOD**

Native enzyme without additives (*solid line*); enzyme in the presence of K₂SO₄ (*dotted line*); enzyme in the presence of lysozyme (*dashed line*) and enzyme in the presence of NaCl (*short vertical line*).

The effect of lysozyme (7.1 x 10⁻⁵ M), 1 M NaCl and 0.2 M K₂SO₄ on the FAD environment was followed by CD spectra in the visible region (300 – 450 nm). The enzyme was incubated in the additive for 1 h and the spectra run at 30°C at a speed of 10 nm.min⁻¹ in a 10 mm path length cell. A mean residue weight of 115 was assumed for calculation of the molar ellipticity. The buffer used was 20 mM phosphate (pH 6.0).

Fig. 7. **Effect of salts on the thermal unfolding of GOD**

A. Ellipticity at 274 nm; B. Ellipticity at 222 nm; C. Ellipticity at 375 nm

Native enzyme in 20 mM phosphate, pH 6.0 (*solid line*), enzyme in the presence of 0.2 M K₂SO₄ (*dotted line*) enzyme in presence of 0.6 M NaCl (*dashed line*). Thermal inactivation of GOD was followed in the temperature range of 25 to 90°C. Temperature was increased by 1°C. min⁻¹. Protein concentrations were as given under “Experimental Procedures”. A mean residue weight of 115 was used for calculation of the molar ellipticity values.
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Fig. 8. Steady-state fluorescence measurements with GOD

A: Effect of thermal inactivation on the intrinsic fluorescence emission spectra of GOD

—○—, native enzyme in 20 mM phosphate buffer (pH 6.0), —■—, heat inactivated enzyme. Enzyme was inactivated by heating at 60°C for 15 min. Slit widths for excitation and emission were 5 nm. Protein concentrations of 1.5 µM were used. All measurements were made at 30°C.

B: ANS bound GOD fluorescence spectra in the presence of salts

—○—, native enzyme; —●—, GOD in presence of 0.6 M NaCl; —○—, in presence of 0.2 M K₂SO₄; —□—, free ANS in 20 mM phosphate buffer (pH 6.0).

Fig. 9. Thermal unfolding transition curves of GOD

Unfolding was followed by enzyme activity, ellipticity at 222 nm, ellipticity at 274 nm and dissociation of FAD (375 nm). All the measurements were performed in 20 mM phosphate buffer (pH 6.0).

—○—, % residual activity; —△—, dissociation of FAD from the holoenzyme;
—■—, loss of tertiary structure (274 nm) and —△—, loss of secondary structure (222 nm).

FIG. 10. Size exclusion chromatography of native and heat inactivated enzyme in the presence and absence of K₂SO₄

A. Peaks detected at 280nm, B. Peaks detected at 375nm
The enzyme was heat inactivated in the absence and presence of 0.2 M \( \text{K}_2\text{SO}_4 \) at 60\(^\circ\)C for 15 min. a: native enzyme; a’: Enzyme heated without \( \text{K}_2\text{SO}_4 \); b: Native enzyme in the presence of 0.2 M \( \text{K}_2\text{SO}_4 \); b’: Enzyme heated in the presence of 0.2 M \( \text{K}_2\text{SO}_4 \). The column (Shodex\textsuperscript{\textregistered} PROTEIN KW-803, exclusion limit \( 1.5 \times 10^5 \), 300 x 8 mm) was equilibrated in 20 mM phosphate buffer (pH 6.0). Elution was isocratic in the above buffer at a flow rate of 0.5 ml.min\(^{-1}\). 20 \( \mu \)l of 1 mg.ml\(^{-1}\) protein concentration was injected.

Fig.11. \textbf{The effect of K}_2\text{SO}_4\textbf{ concentration on the hydrodynamic volume of GOD}

The column (Shodex\textsuperscript{\textregistered} PROTEIN KW-803, exclusion limit \( 1.5 \times 10^5 \), 300 x 8mm) was equilibrated in at least 2 bed volumes of 20 mM phosphate buffer (pH 6.0) containing 0 – 0.4 M \( \text{K}_2\text{SO}_4 \). Samples were eluted isocratically in the same buffer. Flow rate was maintained at 0.5 ml.min\(^{-1}\). 20 \( \mu \)l of sample was injected and the peaks were detected at 280 and 375 nm.

Fig. 11-Inset : \textbf{Reduction of Stokes’ radius with increasing molarity of K}_2\text{SO}_4
### Table 1 Activation parameters of GOD in the presence of additives

<table>
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<th>Incubation temperature (°C)</th>
<th>Half – life (min)</th>
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<th>$\Delta G^*$ (kcal. mol$^{-1}$)</th>
<th>$\Delta H^*$ (kcal. mol$^{-1}$)</th>
<th>$\Delta S^*$ (cal.deg$^{-1}$. mol$^{-1}$)</th>
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![Graph showing elution volume vs. absorbance units with markers at 97.4, 68, 43, 26, 20, and 14.3](image)

Fig. 1
Mechanism of Thermal Inactivation of Glucose Oxidase

Fig. 2
Fig. 3
Mechanism of Thermal Inactivation of Glucose Oxidase

Fig. 4
Fig. 6
Temperature (°C)

Mechanism of Thermal Inactivation of Glucose Oxidase

Fig. 7
Mechanism of Thermal Inactivation of Glucose Oxidase

Fig. 8
Mechanism of Thermal Inactivation of Glucose Oxidase

Fig. 9

Temperature (°C) vs. % Normalized signal

- Temperature axis: 20 to 80
- % Normalized signal axis: 0 to 100

Graph showing the relationship between temperature and the % normalized signal.
Fig. 10
Mechanism of Thermal Inactivation of Glucose Oxidase

Fig. 10

Elution Volume (ml)

Free FAD

a

b

a'

b'

B

AU