Monovalent Cation-Induced Conformational Change in Glucose Oxidase Leading to Stabilization of the Enzyme[†,‡]

Atta Ahmad, Md. Sohail Akhtar, and Vinod Bhakuni*

Division of Membrane Biology, Central Drug Research Institute, Lucknow 226 001, India

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ABSTRACT: Glucose oxidase (GOD) from *Aspergillus niger* is an acidic dimeric enzyme having a high degree of localization of negative charges on the enzyme surface and dimer interface. We have studied the effect of monovalent cations on the structure and stability of GOD using various optical spectroscopic techniques, limited proteolysis, size exclusion chromatography, differential scanning calorimetry, and enzymic activity measurements. The monovalent cations were found to influence the enzymic activity and tertiary structure of GOD, but no effect on the secondary structure of the enzyme was observed. The monovalent cation-stabilized GOD was found to have a more compact dimeric structure but lower enzymic activity than the native enzyme. The enzyme's K_m for D-glucose was found to be slightly enhanced for the monovalent cation-stabilized enzyme (maximum enhancement of about 35% for LiCl) as compared to native GOD. Comparative denaturation studies on the native and monovalent cation-stabilized enzyme demonstrated a significant resistance of cation-stabilized GOD to urea (about 50% residual activity at 6.5 M urea) and thermal denaturation (ΔT_m maximum of 10 °C compared to native enzyme). However, pH-induced denaturation showed a destabilization of monovalent cation-stabilized GOD as compared to the native enzyme. The effectiveness of monovalent cations in stabilizing GOD structure against urea and thermal denaturation was found to follow the Hofmeister series: $K^+ > Na^+ > Li^+$.

The complex three-dimensional structures of proteins are stabilized by a number of noncovalent interactions such as hydrogen bonds, van der Waals interactions, electrostatic interactions and the hydrophobic effect. One of the components that is significantly important for stability and function of a protein is its ionic composition. Both short- and long-range interactions contribute to the stability of proteins (*1*−*6*). For most of the proteins, the charges are arranged on the surface such that there are more attractive rather than repulsive electrostatic interactions (*7*). However, many proteins contain clusters of positively or negatively charged residues, due to which there are more repulsive than attractive electrostatic interactions in these molecules. Recently reported studies have demonstrated that alleviating unfavorable surface interactions in proteins results in stabilization of structure (*8*, *9*). The most common methods used for neutralization of surface charge interactions in a protein molecule are site-directed mutations (selectively) (*6*, *8*, *9*) and by either chemical modification of amino acids or ion binding (nonselectively) (*10*−*12*).

Glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) is a flavoprotein which catalyzes the oxidation of β-D-glucose by molecular oxygen to δ-gluconolactone, which subsequently hydrolyzes spontaneously to gluconic acid and hydrogen peroxide. The enzyme is of considerable commercial importance (*13*). Industrially, it is being used in the removal of glucose or oxygen from food products and in production of gluconic acid (*14*). The most important application of glucose oxidase is as a molecular diagnostic tool. The enzyme is used in biosensors for the quantitative determination of D-glucose in samples such as body fluids, foodstuffs, beverages, and fermentation products (*15*, *16*). Since the discovery that the enzyme is an antibiotic, shown subsequently to be due to the peroxide formation (*17*), there has been an ever increasing interest in glucose oxidase.

Glucose oxidase (GOD)[1] from *Aspergillus niger*, a homodimer with a molecular mass of 160 kDa, is a glycoprotein with a carbohydrate content of 16% (w/w) (*18*, *19*). The carbohydrate moiety is of high-mannose type, and the oligomeric polysaccharide is covalently attached to polypeptide chains via asparagine and serine or threonine residues (*20*). The enzyme contains two tightly bound, but noncovalently linked, flavin adenine dinucleotide (FAD) molecules per dimer (*21*, *22*). These flavin cofactors are responsible for the oxidation−reduction properties of the enzyme. GOD is an acidic protein and shows resistance to SDS denaturation at pH 6.0 even after prolonged incubation; however, at low pH (≤4.3), it is susceptible to denaturation (*23*). Dissociation of the subunits of GOD has been reported to be possible only under denaturing conditions and is accompanied by the loss of cofactor FAD (*23*, *24*).

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[‡] CDRI Communication 6050.

* To whom correspondence should be addressed: Division of Membrane Biology, Central Drug Research Institute, Lucknow 226 001, India. Fax: 91-522-223405. E-mail: root@cscdri.ren.nic.in.

[1] Abbreviations: GOD, glucose oxidase; NaCl, sodium chloride; KCl, potassium chloride; LiCl, lithium chloride; FAD, flavin adenine dinucleotide; NBS, *N*-bromosuccinimide; CD, circular dichroism; T_m, midpoint of thermal denaturation; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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GOD is an acidic protein having a net negative charge at neutral pH (*23*). We have carried out a systematic investigation on the monovalent cation-induced changes in conformational and functional parameters of GOD. To determine the effect of monovalent cation-induced structural changes on the enzyme stability, comparative studies on the thermal-, urea-, and pH-induced denaturation of native and monovalent cation-stabilized GOD were carried out.

EXPERIMENTAL PROCEDURES

Materials

All the chemicals, including GOD, were purchased from Sigma Chemical Co. (St. Louis, MO) and were of the highest available purity.

Methods

Purification of GOD. Commercial glucose oxidase (type X-S, Sigma Chemical Co.) was purified to homogeneity by ion-exchange chromatography followed by ammonium sulfate precipitation. Briefly, GOD (1−2 mg/mL) was dissolved in 10 mM phosphate (pH 6.0) and applied to a Hi-Trap Q column (5 mL) (Amersham Pharmecia Biotech, Uppsala, Sweden) pre-equilibrated with 10 mM phosphate (pH 6.0). The column was washed with 10 mM phosphate containing 100 mM NaCl (pH 6.0). The enzyme was eluted from the column at a flow rate of 2.5 mL/min with a linear gradient of 100 to 300 mM NaCl [in 10 mM phosphate buffer (pH 6.0)]. Fractions containing GOD activity were pooled and desalted. This partially purified enzyme was subjected to 47% ammonium sulfate precipitation. The precipitate was resuspended in 10 mM phosphate buffer (pH 6.5) and dialyzed overnight against the same buffer at 4 °C with three changes of buffer. The purity of the enzyme thus obtained was evaluated on SDS−PAGE followed by silver staining and was found to be about 99% pure.

Assay of Enzymic Activity. Glucose oxidase activity was determined by the colorimetric method using the coupled peroxide/*O*-dianisidine system (*25*). Briefly, 2.5 mL of the *O*-dianisidine buffer mixture [0.1 mL of 1% *O*-dianisidine in 12 mL of 0.1 M potassium phosphate buffer (pH 6.5) saturated with oxygen within 30 min of use], 0.3 mL of 18% glucose (mutarotated for 6 h), and 0.1 mL of peroxidase (0.2 mg/mL) were taken. The mixture was thoroughly mixed, and finally, 0.24 mL of GOD (0.27 μM diluted 100 times just before taking measurements or mentioned otherwise) was added. The reaction mixture was incubated for 4 min, during which the measurements were carried out by monitoring the absorption at 460 nm and 25 °C.

Fluorescence Spectroscopy. Fluorescence spectra were recorded with a Perkin-Elmer LS 5B spectroluminescencemeter in a 5 mm path length quartz cell. GOD in 10 mM phosphate buffer (pH 6.5) was incubated in the presence of increasing salt concentrations for 2 h at 25 °C before the spectra were recorded. The protein concentration was 3.0 μM for all experiments, and the measurements were carried out at 25 °C. For monitoring FAD fluorescence, the excitation wavelength was 365 nm, and the spectra were recorded between 450 and 550 nm.

Circular Dichroism Measurements. CD measurements were made with a Jasco J500 spectropolarimeter at 25 °C. The 0.2 and 1 cm path length cuvettes were used for 190−250 and 250−500 nm, respectively. The results are expressed as the mean residual ellipticity [θ], which is defined as $100\theta_{obs}/(lc)$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in moles of residue per liter, and l is the length of the light path in centimeters. The enzyme samples in increasing salt concentrations were prepared as described above. The CD spectra were measured at enzyme concentrations of 0.75 and 6.0 μM for far- and near-UV measurements, respectively. The values that were obtained were normalized by subtracting the baseline recorded for the buffer having same concentration of salts under similar conditions.

Size Exclusion Chromatography. Gel filtration experiments were carried out on a Superdex 200HR 10/30 column (manufacturer's exclusion limit of 600 kDa for proteins) on AKTA FPLC (Amersham Pharmacia Biotech). The column was equilibrated and run with sodium phosphate buffer [10 mM phosphate (pH 6.5)] containing the desired salt concentration at 25 °C. The GOD solution (3.0 μM) was incubated at the desired salt concentration for 2 h at 25 °C. Two hundred microliters of this sample was loaded on the column and run at a flow rate of 0.3 mL/min, with detection at 280 nm.

Limited Proteolysis. The native and 2 M NaCl-treated GOD samples were subjected to limited proteolysis with subtilisin. GOD (3 μM) dissolved in sodium phosphate buffer [10 mM phosphate (pH 6.5)] in the presence and absence of 2 M NaCl was incubated for 2 h at 25 °C followed by incubation with subtilisin (protease:enzyme weight ratio of 1:3) for 6 h at 25 °C. Adding 2 mM PMSF to the reaction mixture stopped the protease reaction. The samples were loaded on a Superdex 200HR 10/30 column on AKTA FPLC. The column was pre-equilibrated and run with 10 mM sodium phosphate (pH 6.5) at a flow rate of 0.3 mL/min, with detection at 280 nm for both the native and monovalent cation-stabilized GOD samples obtained after proteolysis.

Chemical Modification of Tryptophyl Residues by N-Bromosuccinimide (NBS) and Studies Using the Modified Enzyme. The NBS titration of native and 2 M NaCl-treated GOD was carried out as described previously (*26*). Briefly, GOD was dialyzed against pH 5.6 buffer (0.1 M sodium acetate). The enzyme (3.0 μM) at pH 5.6 was incubated in the presence and absence of 2 M NaCl for 2 h. One milliliter of this enzyme solution was taken in a cuvette, and its absorption at 280 nm and FAD fluorescence (as described in the fluorescence measurement section) were measured. For NBS modification, to this solution was added a desired volume of 7 mM NBS to obtain a final NBS concentration varying from 0 to 160 μM with a 2 mm cell at 25 °C. After each addition, the sample was incubated for 3 min with intermittent shaking before measurements were taken. The values observed for FAD fluorescence were corrected by subtracting the effect of dilution due to NBS addition.

Cross-Linking Using Glutaraldehyde. To native and NaCl (0.5 and 2 M)-treated GOD (0.208 μM) was added an aliquot of 25% (w/v) glutaraldehyde to achieve a final glutaraldehyde concentration of 1%. This sample was incubated at 25 °C for 5 min followed by quenching the cross-linking reaction by adding 2 μL of β-mercaptoethanol. After incubation for 20 min, 3 μL of 10% aqueous sodium deoxycholate was added. The pH of the reaction mixture was lowered to 2−2.5

by addition of orthophosphoric acid that resulted in precipitation of the cross-linked protein. After centrifugation (13237*g* at 4 °C), the obtained precipitate was redissolved in 0.1 M Tris-HCl (pH 8.0), 1% SDS, and 50 mM dithioerythritol and heated at 90−100 °C. SDS−PAGE was carried out on 8% gels.

Differential Scanning Calorimetry. All calorimetric scans were performed with a Microcal MC-2 differential scanning calorimeter. The calorimetric unit was interfaced with an IBM PC microcomputer using an A/D converter board (Data Translator DT-2801) for automatic data collection and analysis. The protein concentration used for these studies was 4.5 μM; a 1.3 mL sample was introduced into the sample cell, and a similar amount of buffer was introduced into the reference cell. Samples were scanned at a rate of 60 °C/h. The samples were degassed for 15 min at room temperature before being scanned in the calorimeter. Data reduction and analysis were performed as described previously (*27*). All the scans were found to be irreversible under the experimental conditions that were studied.

Urea Denaturation Studies. GOD (0.012 μM) in 10 mM phosphate buffer (pH 6.5) was incubated in the presence and absence of 2 M salt at 25 °C for 2 h. Fifty microliters of this solution was added to 250 μL of the buffer solution containing the desired urea concentration and 2 M salt and incubated for 2 h at 25 °C. The pH of solution was maintained at 7.0. The final enzyme concentration was kept constant at 0.002 μM and enzymic activity measured as mentioned above.

Thermal Denaturation Studies. GOD (0.002 μM) in 10 mM phosphate buffer (pH 6.5) was incubated in the presence and absence of 2 M salt at 25 °C for 2 h. These samples were then incubated at the desired temperature for 30 min followed by quenching in ice for 4 min, and finally, the enzymic activity was measured.

pH Studies. Ionic strength buffers (0.1 M) of varying pH (3−10) [HCl/KCl (pH 1 and 2), citrate (pH 3−5), sodium phosphate (pH 6 and 7), and glycine-KOH (pH 9−12)] were prepared. GOD (0.012 μM) was incubated in the presence and absence of 2 M salt for 2 h. Fifty microliters of this enzyme solution was added to 250 μL of buffer solution of varying pH (pH of the solution maintained) for a further 2 h. Enzymic activity of the resulting 0.002 μM enzyme solution was measured by the previously described method.

RESULTS

We have studied the effect of NaCl, KCl, and LiCl on the structural and functional properties of GOD. As these salts contain the same anion (i.e., Cl^-) but different monovalent cations such as K^+, Na^+, and Li^+, the various changes that are observed in the comparative study using these salts will mainly be due to the different monovalent cations that are present in these salts.

Time-dependent changes in structural parameters and enzymic activity of GOD at increasing salt concentrations were monitored to standardize the incubation time required for achieving equilibrium under these conditions. At 0.05, 0.5, and 2 M NaCl, both the FAD fluorescence and enzymic activity were found to decrease up to 15 min only, and no further change in the values was observed for increases in time up to 6 h (data not shown). Similar results were also observed for GOD treated with other salts used in the study. These results suggest that a minimum incubation time of 30 min is sufficient for achieving equilibrium under any salt condition that was studied.

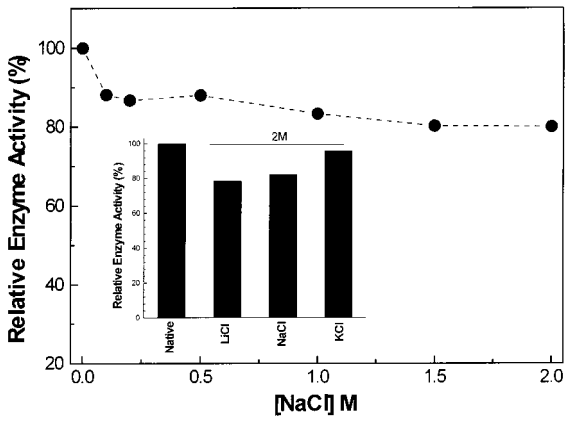

FIGURE 1: Effect of monovalent cations on the enzymic activity of glucose oxidase at pH 6.5 and 25 °C. Changes in enzymic activity of GOD on treatment with increasing concentrations of NaCl. The inset shows the relative enzymic activity of the native and NaCl-, KCl-, and LiCl-stabilized enzyme (salt concentration kept constant at 2 M). GOD in 10 mM phosphate buffer (pH 6.5) was incubated with the desired concentration of salt for 2 h at 25 °C followed by measurement of enzymic activity as mentioned in Experimental Procedures. The data are expressed in terms of relative activity using the activity of the native enzyme as a reference (100%).

Effect of Monovalent Cations on the Functional and Structural Properties of GOD. Enzymic Activity. Enzyme activity can be regarded as the most sensitive probe for studying the changes in the enzyme conformation during various treatments as it reflects subtle readjustments at the active site, allowing very small conformational variations of an enzyme structure to be detected. Figure 1 shows the effect of increasing concentrations of NaCl on the enzymic activity of GOD. Addition of NaCl (even 0.02 M) was found to reduce the enzymic activity (82%) of GOD. Furthermore, no significant effect on this decrease in enzymic activity of GOD by NaCl was observed with an increase in NaCl concentration up to 2 M. However, a distinct effect of treatment of GOD with different monovalent cations on the loss of enzymatic activity was observed. For GOD treated with 2 M KCl, NaCl, and LiCl, relative enzymic activities of about 95, 82, and 78%, respectively, compared to that of the native enzyme were observed (Figure 1 inset), indicating that the decrease in enzymic activity of GOD on treatment with various monovalent cations follows the order $K^+ > Na^+ > Li^+$.

As a decrease in enzymic activity of GOD was observed on treatment with monovalent cation, studies were carried out to determine whether this was a consequence of a monovalent cation-induced conformational change in the enzyme under these conditions or deactivation of the enzyme due to the presence of these cations. It was observed that the presence of 0.2−2 M NaCl, KCl, or LiCl in the assay reaction mixture did not result in any change in the enzymic activity of native GOD; however, for the same salt concentration in preincubations of the enzyme, a decrease in enzymic activity was observed. These observations demonstrate that NaCl, KCl, and LiCl induce conformational changes in GOD, leading to stabilization of a conformation

which has reduced enzymic activity compared to the native enzyme.

Changes in Molecular Properties. Optical spectroscopic studies on GOD in the presence of increasing monovalent cation concentrations were performed to study their effect on the structural properties of GOD.

Studies on various flavanoproteins have shown that fluorescent prosthetic groups of FAD or FMN exhibit different spectral characteristics in different proteins, reflecting the specific environmental property of isoalloxazine, which is the chromophore present in the molecule (*28*). For this reason, the fluorescent FAD prosthetic group has been used as a natural marker to probe the dynamical microenvironment of the flavin fluorophore in flavanoproteins (*29*, *30*). GOD contains two tightly bound, but noncovalently linked, flavin adenine dinucleotide (FAD) molecules. For native GOD, a significant fluorescence with an emission λ_{max} at 524 nm corresponding to the FAD molecule present in the enzyme was observed (Figure 2A inset). The effect of NaCl addition to the FAD micorenvironment of GOD is summarized in Figure 2A where changes in the FAD fluorescence intensity of GOD with increasing NaCl concentrations are depicted. Addition of increasing concentrations of NaCl (in the range of 0−2 M) to native GOD resulted in quenching of FAD fluorescence with increases in NaCl concentration. The quenching of FAD fluorescence observed in the presence of NaCl indicates that the FAD cofactor present in the native enzyme exhibits a movement to a more nonpolar environment in monovalent cation-stabilized GOD than in the native enzyme.

The effect of monovalent cation-induced changes in GOD conformation on the FAD microenvironment was further studied by FAD fluorescence quenching using KI. Figure 2B shows the fractional fluorescence of FAD in native and 2 M NaCl-stabilized GOD as a function of KI concentration. A significant quenching of FAD fluorescence was observed for both conformations of the enzyme; however, a difference in the relationship between the decrease in FAD fluorescence intensity and the increase in the KI concentration was observed for the two conformations. For native GOD, a nonlinear curve with a marked downward curvature was observed as the KI concentration was increased, indicating the existence of more than one class of FAD residues having quenching constants differing by a factor of 4 or more, accessible to the quencher (*31*). On the contrary, for 2 M NaCl-stabilized GOD, under similar conditions a linear curve with increasing KI concentrations was observed, indicating the existence of a single class of FAD residues accessible to the quencher. These observations indicate that the FAD molecules are present in a significantly different microenvironment in the native and monovalent cation-stabilized conformations of GOD.

To study the effect of NaCl on the secondary structure of GOD, far-UV CD studies on GOD in the presence and absence of NaCl were carried out. In the far-UV region, native GOD exhibited a CD spectrum indicating the presence of substantial α-helical conformation (Figure 2C). No significant change in ellipticity at 222 nm of native GOD was observed on treatment with increasing concentrations of NaCl (Figure 2C inset), and for GOD in the presence of 2 M NaCl, a far-UV CD spectrum similar to that of native GOD was observed.

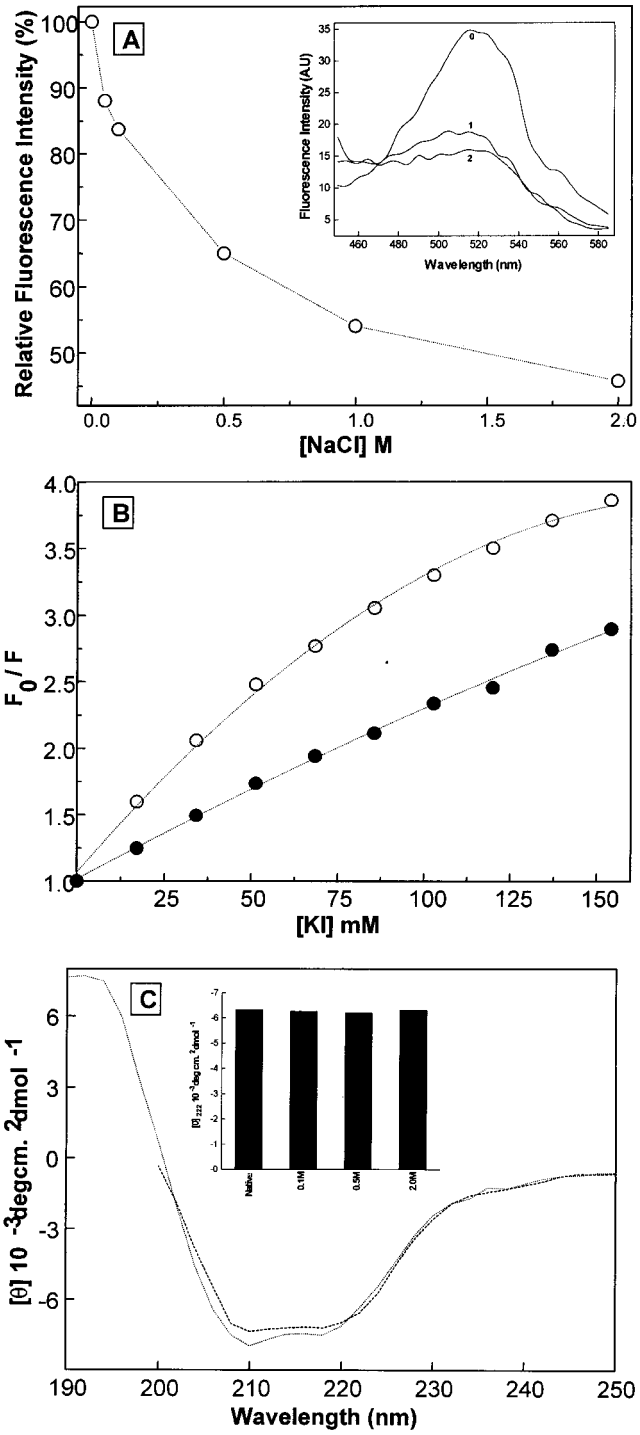

FIGURE 2: Changes in the structural properties of glucose oxidase in the presence of increasing concentrations of NaCl at pH 6.5 and 25 °C. (A) Changes in FAD fluorescence of GOD on treatment with increasing NaCl concentrations as monitored by fluorescence emission at 524 nm and excitation at 365 nm. The data are represented as a percentage of fluorescence taking the fluorescence of native GOD to be 100%. The inset figure shows the FAD fluorescence emission spectra of (0) native GOD and GOD in the presence of (1) 1 M NaCl and (2) 2 M NaCl. (B) Stern−Volmer plot of FAD fluorescence quenching by KI of native (○) and 2 M NaCl-stabilized (●) GOD. Quenching with KI was performed at pH 6.5 and 25 °C by addition of 3 μL of KI (4 M stock solution) to 700 μL of sample, to obtain a final KI concentration between 0 and 200 mM, and monitoring the decrease in fluorescence emission at 524 nm. An enzyme concentration of 3 μM was used in the study. (C) Far-UV CD spectra of native (···) and 2 M NaCl-stabilized (- - -) GOD. The inset shows the ellipticity at 222 nm observed for GOD in the presence of increasing concentrations of NaCl.

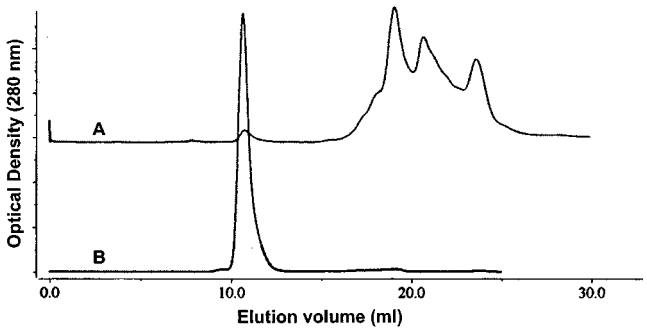

FIGURE 3: Size exclusion chromatographic profiles on a Superdex 200HR column for subtilisin-treated (A) native and (B) 2 M NaCl-stabilized GOD at pH 6.5 and 25 °C. The experimental details are given in Experimental Procedures.

The comparative structural studies on native and monovalent cation-stabilized GOD as reported above suggest that monovalent cations induce significant modification in the tertiary structure of native enzyme, whereas the secondary structure of the enzyme is not much affected.

Affinity of D*-Glucose for the Enzyme.* As the enzymic activity and the structural parameters of GOD were found to be significantly affected by monovalent cations, the affinity of D-glucose for native and 2 M NaCl-, KCl-, and LiCl-treated GOD was determined. The dissociation constant for dissociation of D-glucose from the native enzyme was found to be 31.25 mM, which is in accordance with the previously reported value (*32*, *33*). For 2 M NaCl-, KCl-, and LiCl-treated GOD, the dissociation constants of D-glucose were found to be 36.36, 38.46, and 43.47 mM, respectively. The slight enhancement in the K_m values for D-glucose observed for monovalent cation-stabilized GOD suggests that the modification of enzyme conformation on treatment with monovalent cation results in the alteration of functional properties of the enzyme.

Effect of Monovalent Cations on Molecular Dimensions. Limited Proteolysis. The factors determining the vulnerability for proteolysis of a protein by protease depend on the conformational parameters such as accessibility, segmental motion, and protrusions. For this reason, limited proteolysis has been effectively used to monitor protein surface regions, ligand-induced conformational changes, and protein folding and unfolding (*34*). When the protein is stabilized and destabilized as a result of folding and unfolding, the resulting conformational change must decrease and increase the inherent susceptibility of the protein to proteolytic attack, respectively.

Figure 3 shows the proteolytic susceptibility of native and 2 M NaCl-stabilized GOD to protease, subtilisin, as studied by analysis of the fragmentation profile by size exclusion chromatography on the S-200 Superdex column. Size exclusion column chromatography of native GOD treated with subtilisin at pH 7.0 showed the presence of several peaks with significantly large retention volumes of >19 mL compared to the native GOD (10.64 mL), indicating extensive fragmentation of the enzyme by protease into small polypeptide fragments under these conditions. However, size exclusion chromatography using buffer having no salt (mentioned in Experimental Procedures) after subtilisin treatment of NaCl-stabilized GOD showed the presence of only a single peak having a retention time of 10.64 mL corresponding to that of native GOD (as discussed in the size exclusion chromatography section) with complete absence of any fragmented polypeptide peak. A similar result was observed for subtilisin treatment of 2 M KCl and LiCl-stabilized GOD (data not shown).

As no significant proteolysis of monovalent cation-stabilized GOD was observed with subtilisin, the effect of monovalent cations on the protease, subtilisin, was studied to ensure that the loss of proteolysis of monovalent cation-stabilized GOD by subtilisin is due to a change in the conformation of native GOD under these conditions and not due to the loss of proteolytic activity of subtilisin under high-salt conditions. The proteolytic activity of subtilisin incubated in the presence and absence of 2 M NaCl was studied by following the proteolysis of a synthetic substrate Boc-Phe-OMe with respect to time and monitoring the product formation by thin layer chromatography. The rate and extent of proteolysis of Boc-Phe-OMe by native and 2 M NaCl-treated subtilisin were found to be similar, suggesting that subtilisin treated with 2 M NaCl has a proteolytic activity similar to that of native protease. However, treatment of subtilisin with a very high concentration of NaCl (4 M) was found to produce significantly decreased proteolytic activity as only about 50% proteolysis of Boc-Phe-OMe as compared to that of the native protease was observed under these conditions (data not shown).

The observations presented above indicate that interaction of monovalent cation with GOD leads to stabilization into a different conformation in which the sites for protease attack present in the native enzyme are hindered probably as a result of compaction of the enzyme conformation under these conditions.

Effect of Tryptophan Modification by NBS on the Structural Properties of the Native and Monovalent Cation-Stabilized Enzyme. The interaction between the flavin coenzyme and the aromatic amino acid in GOD has been extensively studied by fluorescence quenching and ultraviolet spectroscopy (*26*, *35*, *36*). Studies on NBS modification of trytophan residues present in native GOD have demonstrated that in the native conformation of the enzyme the tryptophan residue and FAD cofactor are in proximity, because of which there is quenching of FAD fluorescence as a result of resonance energy transfer between these two moieties (*26*). Hence, a comparative study of the changes in FAD fluorescence intensity on NBS modification of tryptophan residues in native and monovalent cation-stabilized GOD under similar conditions would provide useful information about the proximity of the two moieties in the two conformations.

Figure 4 summarizes the NBS titration for tryptophan modification of the native and 2 M NaCl-stabilized GOD at pH 5.4 and its effect on FAD fluorescence. With increases in NBS concentration, a decrease in absorbance at 280 nm as a result of modification of tryptophyl residues and an increase in FAD fluorescence were observed under both conditions, indicating that in both conformations the FAD fluorescence is quenched by tryptophan residues. Furthermore, a similar pattern of decrease in tryptophan absorbance at 280 nm (from 100 to 90%) was observed for both the native and monovalent cation-stabilized GOD with increasing NBS concentrations, indicating a similar accessibility of tryptophan residues to NBS in both conformations. However, a significantly better enhancement of FAD fluorescence in

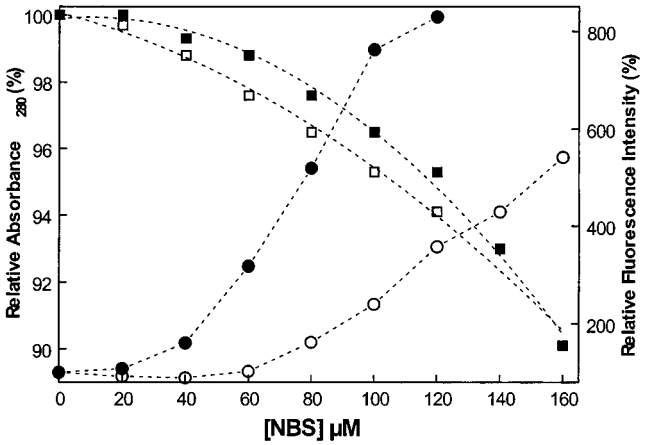

FIGURE 4: NBS titration of native and 2 M NaCl-stabilized GOD at pH 5.6 at 25 °C. The decrease in optical density at 280 nm (squares) and the increase in FAD fluorescence (circles) at 524 nm were measured after mechanical stirring for 3 min at room temperature. The white symbols represent data for native GOD and the black symbols for 2 M NaCl-stabilized GOD. The enzyme concentration used in the study was 3 μM. Data are expressed as the percent relative absorbance or fluorescence using optical density at 280 nm and fluorescence intensity at 524 nm for native and 2 M NaCl-stabilized GOD individually as references (100%).

2 M NaCl-stabilized GOD compared to that of the native enzyme under similar conditions was observed at all NBS concentrations and was much more prominent at higher NBS concentrations (above >60 μM). These observations collectively suggest that the reactive tryptophan residues in the native and 2 M NaCl-stabilized conformations of GOD are in a similar environment but there is a significantly better quenching of FAD fluorescence by the tryptophyl moiety in the NaCl-stabilized conformation than in the native enzyme. As the efficiency of resonance energy transfer between the donor and acceptor depends on their distance in space and has an inverse relationship (*37*), the better quenching of FAD fluorescence by tryptophan observed for the NaCl-stabilized conformation of GOD indicates that the tryptophan and FAD moieties in the NaCl-stabilized conformation are in closer proximity than in the native enzyme. Such a situation can presumably be a result of a more compact structure of the cation-stabilized enzyme compared to native enzyme.

Size Exclusion Chromatography. For studying the effect of monovalent cations on the molecular dimensions of native GOD, size exclusion chromatographic studies on the S-200 Superdex column in the presence and absence of NaCl were carried out. Figure 5 summarizes the results of size exclusion chromatographic experiments carried out on GOD in the presence and absence of NaCl at 25 °C. For native GOD, a single peak at 10.64 mL was observed. Aldolase (M_r = 158 kDa) on the S-200 column under identical conditions exhibited a single peak with a retention volume of 10.9 mL which is slightly higher than the retention volume observed for native GOD. As the reported M_r of native dimeric GOD is 160 kDa (*18*, *19*), these observations indicate that native GOD under the conditions studied is in a dimeric configuration. However, when GOD treated with either 0.5 or 2 M NaCl was loaded on the same column and eluted, a significant enhancement in retention volume for the enzyme to 12.10 mL was observed. This increase in retention volume for the NaCl-treated GOD is indicative of significantly

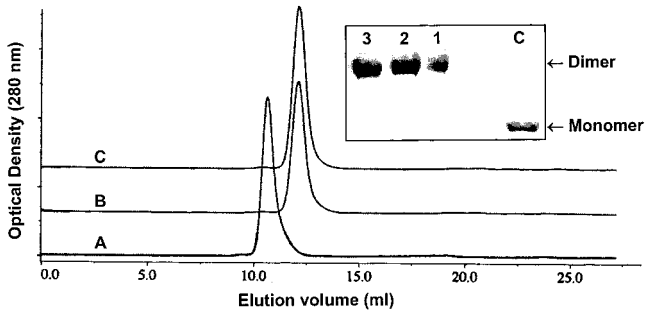

FIGURE 5: Size exclusion chromatographic profiles of native GOD on a Superdex 200HR column at various NaCl concentrations at pH 6.5 and 25 °C. The various curves represent (A) native GOD, (B) GOD treated with 0.5 M NaCl, and (C) GOD treated with 2.0 M NaCl. The inset shows the SDS−PAGE profile of glutaraldehyde cross-linked samples: (1) native, (2) GOD treated with 0.5 M NaCl, and (3) GOD treated with 2.0 M NaCl along with (C) a non-cross-linked native GOD sample as a control.

reduced hydrodynamic radii for the NaCl-stabilized conformation of GOD as compared to the native enzyme.

Cross-Linking Studies. A significant reduction in the hydrodynamic radii of monovalent cation-stabilized GOD as compared to native enzyme was observed by size exclusion chromatography; two possible explanations arise for this observation: (i) the enzyme is present in a monomeric configuration in monovalent cation-stabilized GOD and (ii) due to neutralization of negative charges present in native enzyme by monovalent cations the enzyme undergoes compaction, resulting in a decrease in the hydrodynamic radii, as has been pointed out in the above discussion. To differentiate between these possibilities, cross-linking studies using glutaraldehyde were carried out. The Figure 5 inset shows the results of cross-linking studies. For native as well as monovalent cation-stabilized GOD, the Coommassie-stained protein band corresponding to only dimers was observed, indicating that the monovalent cation treatment of GOD does not result in dissociation of native GOD.

The results of glutaraldehyde cross-linking along with the size exclusion chromatography experiments reported above suggest that the monovalent cation treatment of native GOD does not induce dissociation of the native dimeric enzyme but results in stabilization of the dimeric structure which has a significantly more compact conformation than the native enzyme.

Comparative Denaturation Studies on Native and Monovalent Cation-Stabilized GOD. Thermal Denaturation. Thermal denaturation studies on native and salt-stabilized conformations of GOD have been conducted by incubating the enzyme at variable temperatures for 30 min and evaluating the denaturation of the enzyme by monitoring the loss in enzymic activity associated with the increase in temperature. Figure 6 shows the effect of increasing temperature on the loss of enzymatic activity in the temperature range of 20−90 °C. For both native and 2 M NaCl-stabilized GOD, a sigmoidal decrease in enzymic activity with increasing temperature was observed. However, a significant difference in the temperature corresponding to half-denaturation (T_m) of the enzyme under these conditions was observed for the two conformations. For the native enzyme, a T_m of about 57.5 °C was observed under these conditions, whereas for the 2 M NaCl-stabilized enzyme, an enhancement of about 10 °C in T_m (65 °C) was observed.

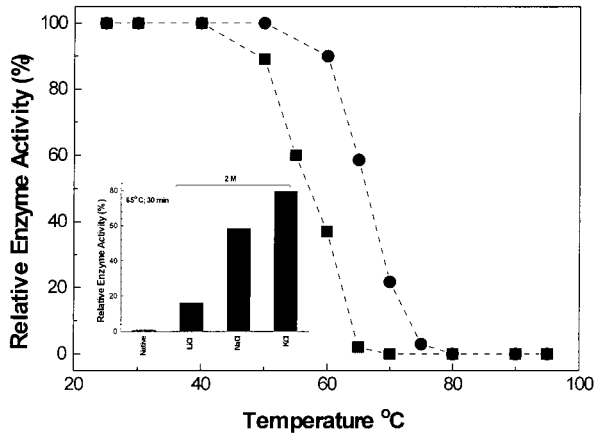

FIGURE 6: Thermal denaturation of native and monovalent cation-stabilized GOD at pH 6.5. The thermal unfolding transition of native (■) and 2 M NaCl-stabilized (●) GOD. At each given temperature, the samples were incubated for 30 min and then the reactions quenched for 4 min at 4 °C before the activity measurements were carried out. The inset shows the effect of thermal denaturation, at 65 °C for 30 min, on native and 2 M NaCl-, KCl-, and LiCl-stabilized GOD as observed by loss of enzymic activity. The enzyme concentration used for the study was 0.33 μg/mL. Data are expressed in terms of relative activity using the activity of native and 2 M NaCl-stabilized GOD individually as references (100%). The activity of 2 M NaCl-stabilized GOD corresponds to 80% of that of native GOD.

The thermal stability of various monovalent cation-stabilized GOD was also studied. The Figure 6 inset summarizes the percent residual activity of LiCl-, NaCl-, and KCl-stabilized GOD on heating at 65 °C for 30 min. For LiCl-, NaCl-, and KCl-stabilized GOD, residual enzymatic activities of 16, 58, and 79%, respectively, were observed. On the basis of these observations, the effectiveness of monovalent cations in stabilizing GOD against thermal denaturation at 65 °C was found to follow the order $K^+ > Na^+ > Li^+$.

Differential Scanning Calorimetry. Differential scanning calorimetric studies on GOD in the presence and absence of increasing concentrations of NaCl were carried out. Figure 7A shows the DSC curves for native GOD and that in the presence of increasing NaCl concentrations. For native GOD, a single transition curve with a T_m (temperature of the midpoint of transition) of 66.82 °C was observed. In the presence of NaCl, the T_m of GOD was found to increase with increases in NaCl concentration (Figure 7B), and at 2 M NaCl, a T_m of 74.07 °C was observed. These observations demonstrate that NaCl-induced changes in GOD conformation result in stabilization of the enzyme against thermal denaturation.

DSC studies were also carried out for the LiCl- and KCl-stabilized GOD (salt concentration of 2 M). Figure 7C summarizes the T_m values obtained for all these samples under identical conditions. For LiCl-stabilized GOD, no significant shift in T_m (67.76 °C) as compared to that of the native enzyme (66.82 °C) was observed. A maximum shift of 10 °C in T_m was observed for KCl-stabilized GOD (76.19 °C). On the basis of these observations, the effect of monovalent cation on stabilization of GOD against thermal denaturation was found to follow the order $K^+ > Na^+ > Li^+$.

Urea Denaturation. The stability of native and monovalent cation-stabilized GOD against urea denaturation was studied by monitoring the changes in enzymic activity at increasing urea concentrations. Figure 8 shows the effect of increasing concentration of urea, in the concentration range of 0−7 M, on the enzymic activity of native and 2 M NaCl-stabilized GOD. For the native enzyme, an initial slight enhancement in enzymic activity (maximum of 106%) at low urea concentrations (0−2 M) followed by a steep decrease (between 2 and 4 M urea) and finally total loss of enzymic activity at urea concentrations of >4.5 M was observed. The $C_{1/2}$ (urea concentration required to bring about 50% denaturation) for the native enzyme was found to be about 3.25 M. However, for NaCl-stabilized GOD, a large enhancement in enzymic activity (maximum to about 153%) was observed at low (1−3 M) urea concentrations, and at even 6.5 M urea, an enzymic activity of about 53% was observed. These observations indicate that the NaCl-stabilized GOD exhibits higher resistance to urea denaturation than the native enzyme.

The stability of various monovalent cation-stabilized GOD against urea denaturation was studied by carrying out denaturation of the enzyme at 6.5 M urea. The Figure 9 inset summarizes the percent residual activity of LiCl-, NaCl-, and KCl-stabilized GOD on denaturation in 6.5 M urea for 2 h. For LiCl-stabilized GOD, a complete loss of enzymic activity similar to that of the native enzyme was observed under these conditions. A maximum of about 73% residual activity was observed for KCl-stabilized GOD. Like thermal denaturation, the effectiveness of various monovalent cations in stabilizing the GOD against urea denaturation was found to follow the order $K^+ > Na^+ > Li^+$.

pH Denaturation Studies. The pH−activity profile of the native and 2 M NaCl-stabilized enzyme was determined at 25 °C in the pH range of 3−10 and is summarized in Figure 9. The native enzyme exhibited considerable enzymic activity in the pH range of 4−9 with a maximum at pH 5.7 which is in agreement with the previous reports (*38*). However, for the 2 M NaCl-treated enzyme, a significant decrease in the pH range sensitivity to enzymic activity was observed. At pH <5.0 and >8.0, no enzymic activity was observed for these samples. Furthermore, the pH corresponding to the enzymic activity maximum was found to be shifted to 6.5, and under these conditions, an enhancement (maximum of 120% residual activity) in enzymic activity compared to that of the native enzyme was observed. These observations suggest that the NaCl-stabilized conformation of GOD is more susceptible to both acidic and alkaline pH denaturation than the native enzyme.

The stability of native and various monovalent cation-stabilized GOD against pH denaturation was studied by monitoring the loss of enzymic activity of these samples at pH 5.0 relative to that of native GOD at pH 6.0 and is summarized in the Figure 9 inset. For native GOD, no significant loss of enzymic activity at pH 5.0 was observed. However, for salt-stabilized GOD, a significant loss of enzymic activity (minimum of about 70%) at pH 5.0 was observed. Furthermore, the effectiveness of various cations in stabilizing GOD against pH denaturation was found to follow the sequence $Li^+ > Na^+ > K^+$.

DISCUSSION

Physiological environments typically contain monovalent cations such as Na^+ or K^+ (at a concentration of ∼0.1 M)

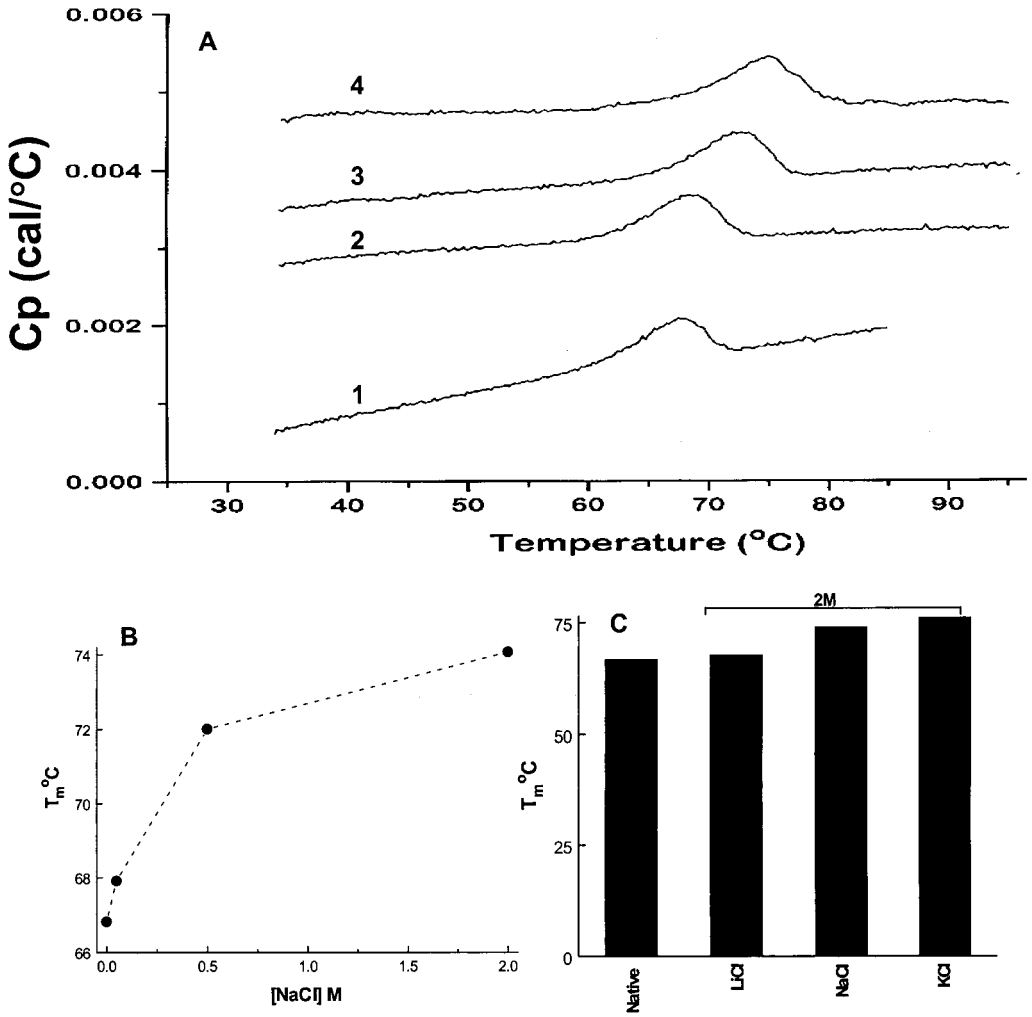

FIGURE 7: (A) Differential scanning calorimetric scans of native GOD in the presence of increasing concentrations of NaCl at pH 6.5. Curves 1−4 represent DSC scans for native GOD and GOD in the presence of 0.05, 0.5, and 2 M NaCl, respectively. The curves are displaced in the *Y*-axis for the purpose of clarity. (B) Dependence of T_m on NaCl concentration for GOD as obtained from the DSC curves under these conditions. The experimental details are mentioned in Experimental Procedures. (C) Comparative profile of T_m for native and 2 M NaCl-, KCl-, and LiCl-stabilized GOD at pH 6.5 as obtained from the DSC curves under these conditions.

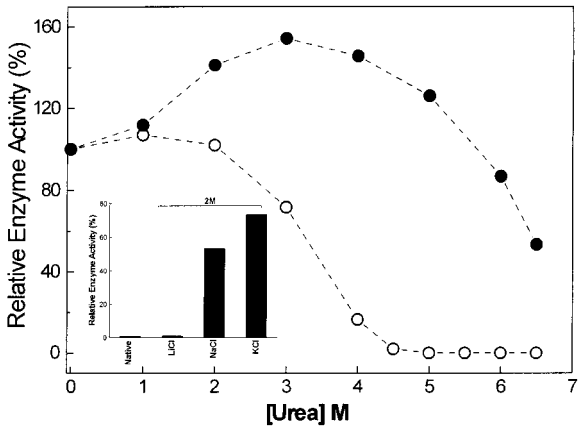

FIGURE 8: Urea denaturation of native and monovalent cation-stabilized GOD at pH 6.5. Urea unfolding transition of native (○) and 2 M NaCl-stabilized (●) GOD at 25 °C. At each given urea concentration, the samples were incubated for 2 h before the readings were taken. The inset shows the effect of urea denaturation at 6.5 M urea for 2 h, on native and 2 M NaCl-, KCl-, and LiCl-stabilized GOD as observed by loss of enzymic activity. The data are expressed in terms of relative activity as mentioned in the legend of Figure 5.

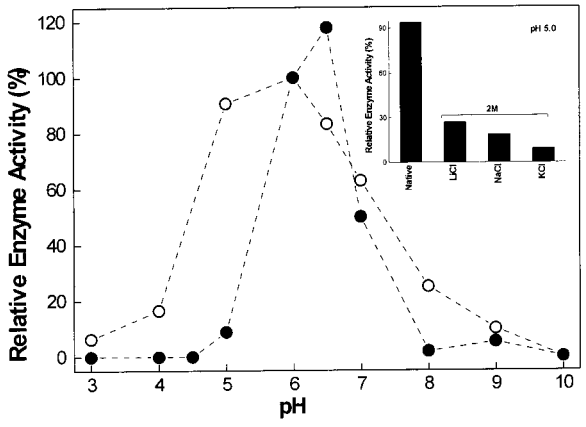

FIGURE 9: pH denaturation of native and monovalent cation-stabilized GOD. The pH−activity profile of native and 2 M NaCl-stabilized GOD at 25 °C. The inset shows the GOD denaturation at pH 5.0 (2 h) for native and 2 M NaCl-, KCl-, and LiCl-stabilized enzyme as observed by loss of enzymic activity. The data are expressed in terms of relative activity as mentioned in the legend of Figure 5.

which act as counterions for biomolecules such as proteins or enzymes. Monovalent cations have been shown to affect both the conformational and catalytic properties of several enzymes. Pyruvate kinase was the first enzyme for which a dependence of the enzymic activity on monovalent cations

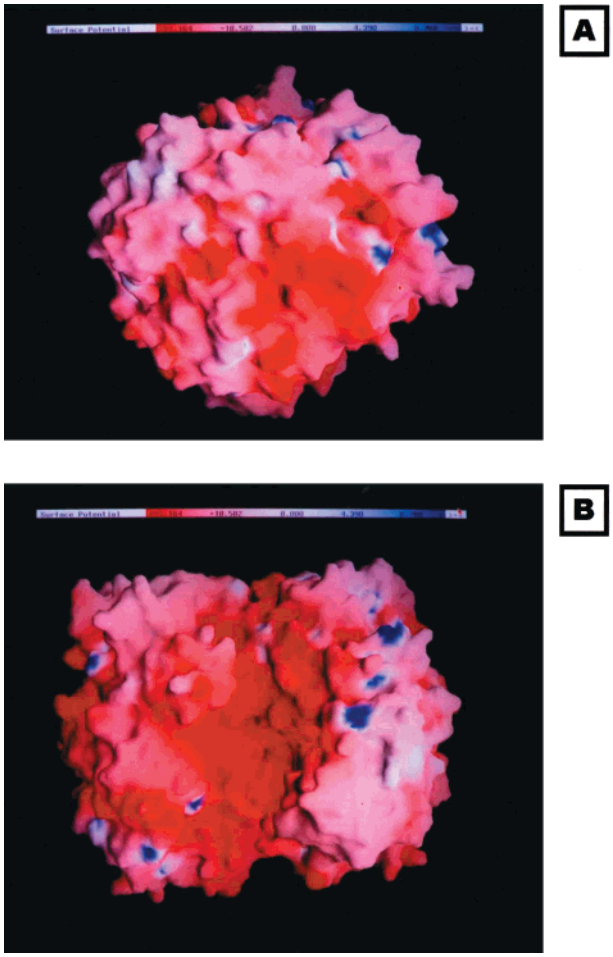

FIGURE 10: Molecular surface of the GOD dimer of *A. niger* colored according to electrostatic potential (blue for negative potential and red for positive potential). Panels A and B represent the GOD dimer molecule in two different orientations. The molecular surface was displayed using GRASP.

was documented (*39*). Since then, several enzymes have been shown to have an absolute requirement of alkali metal ions for activity (*40*). For some enzymes such as the tryptophan synthase $\alpha_2\beta_2$ complex (*41*), PLP-dependent dialkylglycine carboxylase (*42*), pyruvate kinase (*43*), and RNase T1 (*11*), monovalent cations have been shown to be affecting the conformational and functional properties. The thermal stability of proteins has also been reported to be influenced by cations. Acyl carrier protein (*44*), alkaline phosphotase (*45*), thermolysin (*46*), paravalbumin (*47*), and yeast alcohol dehydrogenase I (ADH) (*48*) are some of the proteins which have been shown to be much more stable in the presence of ions. Although there is no common understanding of the stabilization and destabilization of proteins by ions, a number of possible mechanisms such as specific and nonspecific binding of ions to the protein molecules, electrostatic shielding of charges, etc., have been proposed (*10*−*12*).

GOD is an acidic enzyme having an acidic to basic amino acid residue ratio of 3.4 (*49*). At pH 7.7, the enzyme has a charge of −77 (*50*). The crystal structure of deglycosylated GOD has recently been reported (*51*). The electrostatic surface potential of GOD from this crystal structure as obtained by GRASP (*52*) is shown in panels A and B of Figure 10. The enzyme has a predominant negative charge with a high concentration of negative charges localized in the dimeric interface and clusters of negative charges on the surface of the enzyme molecule. The ionized carboxyl groups present in GOD have been found to contribute significantly to these negative charges as observed from the crystal structure (*52*) and demonstrated experimentally by modification of these groups using glycine methyl ester (*23*).

Charge repulsion is one of the most important factors determining the conformation and stability of a protein (*53*). In a negatively charged protein like GOD, the enzyme molecule will have more repulsive than attractive electrostatic interactions at neutral pH. The repulsion between the similarly charged groups present in the protein will be the main force which would drive the protein to be stabilized in a relatively open conformation under physiological conditions. The stabilization of native GOD in an open conformation is supported by the extensive fragmentation of native GOD by subtilisin into small polypeptides on proteolytic digestion as reported in this paper. The interaction of monovalent cations with negatively charged moieties present in the native enzyme may help to compensate for the shortage of basic residues in the enzyme resulting in weakening of the repulsive electrostatic force present in the native enzyme molecule, thus permitting forces favoring compaction of the enzyme's conformation to become strengthened and manifest themselves. The cation-induced compaction of the GOD molecule is supported by the significant reduction in the hydrodynamic radii of native GOD on treatment with monovalent cations as observed by size exclusion chromatography. Furthermore, the limited proteolytic digestion studies using subtilisin showed that in the monovalent cation-stabilized enzyme the protease is inaccessible to the proteolytic sites present in the native enzyme which probably is a result of compaction of the structure of GOD in the presence of monovalent cations. A similar compaction of the protein conformation as a result of cation binding to protein has been reported for two other acidic proteins, RNase T1 and acyl carrier protein (*11*, *44*). These observations collectively suggest that cation-induced compaction of a polypeptide conformation is probably a universal phenomenon for acidic polypeptides and proteins.

Besides having a significant role in the maintenance of the quaternary structure of the enzyme, the negative charges present in native GOD also seem to have an important role in the modulation of enzymic activity. The enzymatic activity of monovalent cation-stabilized GOD was found to be significantly reduced (depending on the cation studied) as compared to that of the native enzyme. Comparative studies on the enzyme's K_m for glucose showed that a significantly enhanced K_m (16−35% depending on the monovalent cation that was studied) as compared to native GOD was observed for the monovalent cation-stabilized enzyme. However, a straightforward correlation between the enhancement in K_m and the decrease in enzymatic activity was not observed as K_m values of 38.46 and 36 mM and residual enzymatic activities of 95 and 82% were observed for 2 M KCl- and NaCl-stabilized GOD, respectively. These observations suggest that enhancement in K_m is not the sole reason for the loss of enzymatic activity observed for the monovalent cation-stabilized enzyme.

Previous studies have demonstrated that the enzymic activity of GOD can be modulated or completely lost on either protonation at low pH or chemically blocking of the carboxy groups present on the amino acid side chain by glycine methyl ester (*23*). These observations suggest that neutralization of negative charges present in the side chain carboxy groups in GOD leads to loss of enzymic activity of GOD. Hence, it seems that interaction of monovalent cations with carboxy groups present in native GOD leads to neutralization of negative charge, and as a consequence, there is a significant loss of enzymic activity.

The stabilizing or destabilizing effects of salts on proteins arise either by effects on water structure or by interaction with charged groups. The stabilizing effects of salts on protein follow the Hofmeister series. According to the lyotropic series of Hofmeister (*54*), it is possible to classify the ions following their ability to organize the water molecules. In the case of monovalent cations, this series is as follows: $Cs^+ > K^+ > Na^+ > Li^+$. If the degree of organization of water molecules influences enzyme stability in general and GOD in particular, the stabilizing effect induced using these ions ought to evolve in the same order as this series. The stability of GOD against thermal and urea denaturation as reported in this paper was found to increase in proportion to the ability of these monovalent cations to organize water, thus indicating that the stability of the GOD structure provided by monovalent cations depends on their ability to organize the water molecules. This is further supported by the comparative stability study of GOD in deuterium oxide (a solvent more structured than water) and water as reported earlier (*55*), where it was demonstrated that GOD in deuterium oxide is far more resistant to thermal denaturation than GOD in water.

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