Divalent Cation Induced Changes in Structural Properties of the Dimeric Enzyme Glucose Oxidase: Dual Effect of Dimer Stabilization and Dissociation with Loss of Cooperative Interactions in Enzyme Monomer†

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ABSTRACT: Glucose oxidase (GOD) from *Aspergillus niger* is a dimeric enzyme having high localization of negative charges on the enzyme surface and at the dimer interface. The monovalent cations induce compaction of the native conformation of GOD and enhance stability against thermal and urea denaturation [Ahmad et al. (2001) *Biochemistry 40*, 1947−1955]. In this paper we report the effect of the divalent cations Ca^{2+} and Mg^{2+} on the structural and stability properties of GOD. A divalent cation concentration dependent change in native conformation and subunit assembly of GOD was observed. Low concentration (up to 1 M) of $CaCl_2$ or $MgCl_2$ induced compaction of the native conformation of GOD, and the enzyme showed higher stability as compared to the native enzyme against urea denaturation. However, higher concentration (≥2.0 M) of $CaCl_2$ or $MgCl_2$ induced dissociation of the native dimeric enzyme, resulting in stabilization of the enzyme monomer. An interesting observation was that the 3 M $CaCl_2$-stabilized monomer of GOD retained about 70% secondary structure present in the native GOD dimer; however, there was a complete loss of cooperative interactions between these secondary structural elements present in the enzyme. Regarding the mechanism of divalent cation induced structural changes in GOD, the studies suggest that organization of water molecules by divalent cation results in stabilization of enzyme at low divalent cation concentration, whereas direct binding of these cations to the enzyme, at higher divalent cation concentration, results in dissociation and partial unfolding of the dimeric enzyme molecule.

A wide variety of different residue types, including acidic and basic groups, are present in the amino acid sequence of proteins. The dispersion of these charged residues on the protein surface certainly plays an important role on the solubility of proteins, but the effect that electrostatic interactions or, more so, the electrostatic potential, due to these charged residues, has on the stability of proteins is not very well understood. Electrostatic interactions between the charged groups in proteins play a major role in stabilizing the proteins (*1*−*3*). However, it has recently been demonstrated that stabilization of the protein can be achieved by alteration in the surface electrostatic potential only (*4*), indicating that the ion pair or the ion network is not an essential requirement for stabilization of proteins.

Glucose oxidase (GOD)[1] from *Aspergillus niger*, a homodimer of molecular mass 160 kDa, is a glycoprotein that catalyzes the oxidation of β-D-glucose by molecular oxygen to δ-gluconolactone, which subsequently hydrolyzes spontaneously to gluconic acid and hydrogen peroxide (*5*, *6*). GOD is an acidic protein having a net negative charge of −77 at neutral pH (*7*). The surface potential calculations from the X-ray crystal structure of the deglycosylated GOD showed that the dimer interface and the enzyme surface contain clusters of negative charges which are mainly due to the presence of side chain carboxylate groups present in amino acids (*8*). Interaction of monovalent cations with native GOD leads to compaction of the native enzyme conformation, resulting in stabilization of the enzyme (*9*).

In this paper we report the effect of divalent cations on the conformation and subunit structure of GOD. The divalent cation induced significant changes in both the native conformation and quaternary structure of GOD. The mechanism of divalent cation induced changes in the GOD structure has also been discussed.

EXPERIMENTAL PROCEDURES

Materials

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

Methods

Purification of GOD. Commercial glucose oxidase (type X-S, Sigma Chemical Co., St. Louis, MO) was purified to homogeneity by ion-exchange chromatography followed by ammonium sulfate precipitation as described previously (*9*). The purity of the purified enzyme was evaluated on SDS−PAGE followed by silver staining and was found to be about 99% pure (*9*).

Fluorescence Spectroscopy. Fluorescence spectra were recorded with a Perkin-Elmer LS 5B fluorometer in a 5 mm path length quartz cell. GOD in 10 mM phosphate buffer,

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[1] Abbreviations: GOD, glucose oxidase; FAD, flavin adenine dinucleotide; NBS, *N*-bromosuccinamide; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CD, circular dichroism.

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pH 6.5, was incubated in the presence of increasing salt concentrations for 2 h at 25 °C before the spectra were recorded. Protein concentration was 3.0 μM for all experiments, and the measurements were carried out at 25 °C. For monitoring tryptophan and FAD fluorescence the excitation wavelengths of 290 and 370 nm, respectively, were used, and the spectra were recorded between 300 and 430 nm and 450 and 550 nm, respectively.

Circular Dichroism Measurements. CD measurements were made with a Jasco J800 spectropolarimeter calibrated with ammonium (+)-10-camphorsulfonate. The results are expressed as the mean residual ellipticity [θ], which is defined as $[\theta] = 100\theta_{obs}/(lc)$, 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 CD spectra were measured at an enzyme concentration of 0.75 μM with a 1 mm cell at 25 °C. The values obtained were normalized by subtracting the baseline recorded for the buffer having the same concentration of salts under similar conditions.

Chemical Modification of Tryptophan Residues by N-Bromosuccinimide (NBS) and Study Using the Modified Enzyme. The NBS titration of native and 3 M $CaCl_2$-treated GOD was carried out as described previously (*9*). 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 3 M $CaCl_2$ for 2 h. Then 1.0 mL of this enzyme solution was added to 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 a desired volume of 7 mM NBS was added 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 made. The values observed for FAD fluorescence were corrected by subtracting the effect of dilution due to NBS addition.

Size Exclusion Chromatography. Gel filtration experiments were carried out on a Superdex 200 HR 10/30 column (manufacturer's exclusion limit 600 kDa for proteins) on AKTA FPLC (Amersham Pharmacia Biotech, Sweden). The column was equilibrated and run with sodium phosphate buffer (10 mM, pH 6.5) containing the desired $CaCl_2$ concentration at 25 °C. The GOD solution (4 μM) was incubated at the desired $CaCl_2$ concentration for 2 h at 25 °C. Then 200 μL of this sample was loaded on the column and run at 25 °C at a flow rate of 0.3 mL/min and detection at 280 nm.

Cross-Linking Using Glutaraldehyde. To native and $CaCl_2$- or $MgCl_2$-treated GOD (0.208 μM) was added an aliquot of 25% (m/v) glutaraldehyde so as to make a final concentration of 1% glutaraldehyde. This sample was incubated at 25 °C for 5 min followed by quenching the cross-linking reaction by addition of 97 mM glycine. For salt-treated samples buffer exchange was carried out for the removal of salts. After 20 min incubation 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 (85%), which resulted in precipitation of the cross-linked protein. After centrifugation (13237*g*, 4 °C, 20 min) the obtained precipitate was redissolved in 0.1 M Tris-HCl, pH 8.0, 1% SDS, and 50 mM dithiothreitol and heated at 90−100 °C. Samples were analyzed on 8% SDS−PAGE (*10*).

Limited Proteolysis. The native and 3 M $CaCl_2$-treated GOD samples were subjected to limited proteolysis with α-chymotrypsin. GOD (3 μM) dissolved in sodium phosphate buffer (10 mM, pH 6.5) in the presence and absence of 3 M $CaCl_2$ was incubated for 2 h at 25 °C followed by incubation with α-chymotrypsin (protease:enzyme weight ratio of 1:6) for 6 h at 25 °C. Adding 2 mM PMSF to the reaction mixture stopped the protease reaction. The samples were analyzed on 8% SDS−PAGE (*10*).

RESULTS

We have studied the effect of $CaCl_2$ and $MgCl_2$ on the structural and stability properties of GOD. Studies using monovalent cation containing chloride salts such as NaCl, KCl, and LiCl have demonstrated that Cl^- ions do not interact with GOD; however, the cations induce significant changes in structural and functional properties of the enzyme (*9*). As both $CaCl_2$ and $MgCl_2$ used in this study contain Cl^-, various changes that are observed in the structural and functional properties of GOD using these salts will mainly be due to the divalent cations Ca^{2+} and Mg^{2+} only.

Time-dependent changes in structural parameters of GOD at increasing salt concentrations were monitored for standardizing the incubation time required for achieving equilibrium under these conditions. For 1.0 and 3.0 M $CaCl_2$- or $MgCl_2$-treated GOD the changes in FAD and tryptophan fluorescence with time were monitored for studying the time required for achieving equilibrium under these conditions. The changes in both of the measurements were observed within 10 min with no further change in the observed values up to 5 h (data not shown). These results suggest that a minimum incubation time of 30 min is sufficient for achieving equilibrium under any condition of the salt studied.

Effect of Divalent Cations on the Structural and Functional Properties of GOD. (A) Changes in Molecular Properties. Optical spectroscopic studies on GOD in the presence of increasing concentrations of divalent cation were performed to study their effects on the structural properties of GOD.

(B) FAD Microenvironment. The spectral characteristics of the fluorescent prosthetic group FAD in proteins have been demonstrated to be sensitive to the microenvironment (*11*, *12*). GOD contains two tightly bound, but noncovalently linked, flavin adenine dinucleotide (FAD) molecules (*8*). For native dimeric GOD, the FAD fluorescence is significantly quenched, and a high fluorescence polarization is observed (*9*, *13*). However, a large enhancement in FAD fluorescence and decrease in fluorescence polarization are observed on denaturation of the enzyme (*13*, *14*). Due to these characteristics, the changes in FAD fluorescence can be effectively used for studying changes in native protein conformation on folding/unfolding of GOD.

Figure 1A summarizes the changes in the FAD fluorescence intensity and polarization of native GOD on incubation with increasing concentrations of $CaCl_2$ or $MgCl_2$. Addition of a low concentration (up to 1.5 M) of these salts resulted in slight quenching of FAD fluorescence intensity and slight linear enhancement in FAD fluorescence polarization (from 0.34 to 0.38) of native GOD. This indicates that the FAD cofactor present in the native enzyme shows a slight movement to a more nonpolar environment (*9*) due to which it will have a restricted motion. However, an increase in concentration of $CaCl_2$ or $MgCl_2$ between 1.5 and 3 M

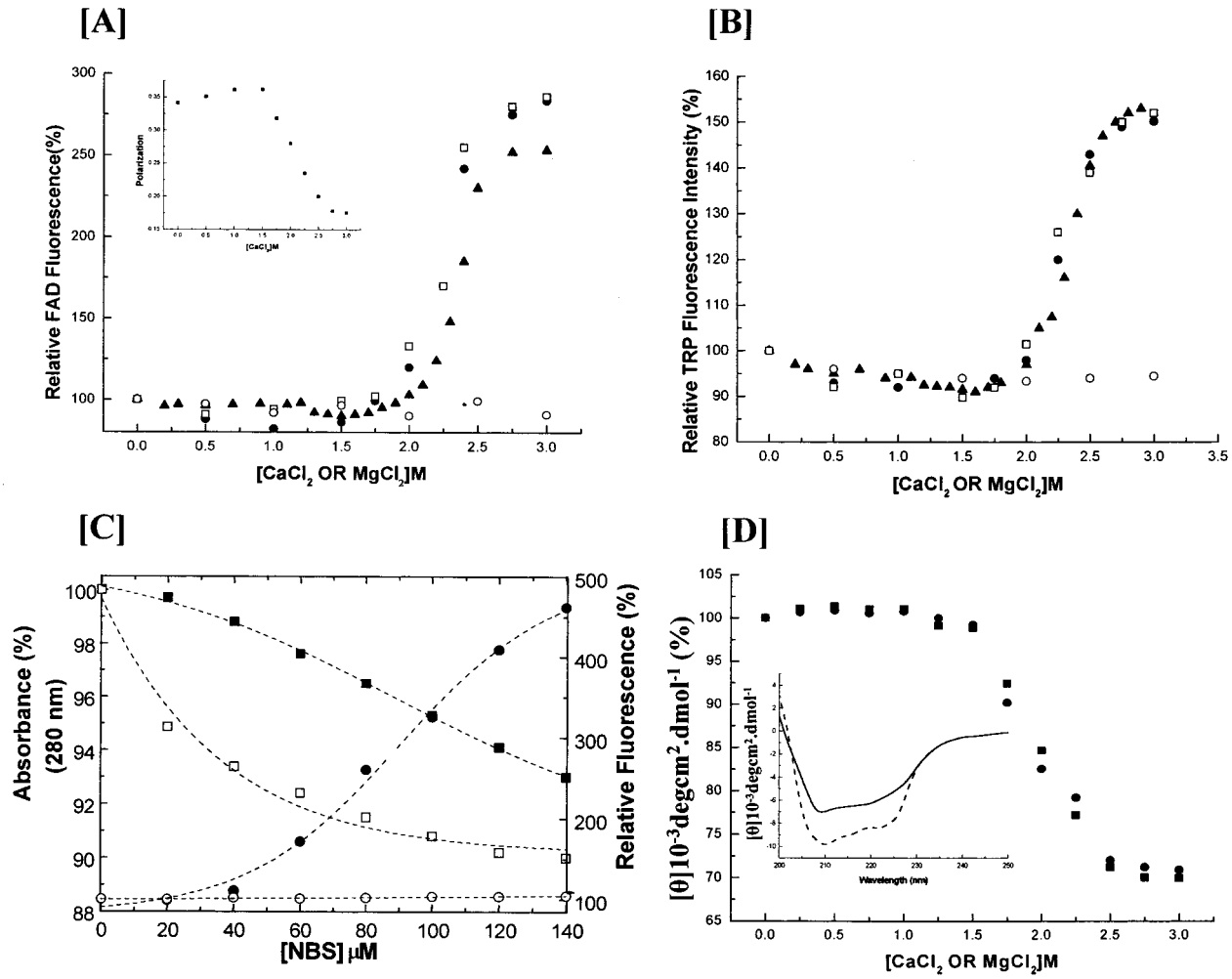

FIGURE 1: Divalent cation induced changes in structural properties of glucose oxidase at pH 6.5 and 25 °C. (A) Changes in FAD fluorescence intensity of GOD on incubation with increasing $CaCl_2$ or $MgCl_2$ concentration as monitored by fluorescence emission at 524 nm and excitation 370 nm. The squares represent data for $CaCl_2$, and the triangles represent data for $MgCl_2$. The closed circles represent data for changes in FAD fluorescence intensity of 0.5 M NaCl-stabilized GOD on incubation with increasing $CaCl_2$ concentration, and open circles represent data for free FAD in the presence of increasing $CaCl_2$ concentration (control). The data are represented as the percentage of fluorescence, taking fluorescence of native GOD as 100%. For free FAD the fluorescence in the absence of $CaCl_2$ is taken as 100%. The inset shows the changes in FAD fluorescence polarization of GOD on incubation with increasing $CaCl_2$ concentration as monitored by fluorescence emission at 524 nm and excitation 370 nm. (B) Changes in tryptophan fluorescence intensity of GOD on incubation with increasing $CaCl_2$ or $MgCl_2$ concentration as monitored by excitation at 290 nm and intensity at fluorescence emission maxima. The symbols are same as in panel A, except that the open circles represent data for tryptophanamide in the presence of increasing $CaCl_2$ concentration (control). The data are represented as the percentage of fluorescence, taking fluorescence of native GOD as 100%. For tryptophanamide the fluorescence in the absence of $CaCl_2$ is taken as 100%. (C) NBS titration of native and 3 M $CaCl_2$-treated 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 filled symbols represent data for native GOD, and the open symbols represent data for 3 M $CaCl_2$-treated GOD. The enzyme concentration used in the study was 3 μM. Data are expressed as percent relative absorbance or fluorescence using optical density at 280 nm and fluorescence intensity at 524 nm for native and 3 M $CaCl_2$-treated GOD individually as reference (100%). (D) $CaCl_2$- or $MgCl_2$-induced changes in the secondary structure of GOD on incubation as monitored by following changes in ellipticity at 222 nm obtained from the far-UV CD curves at increasing concentration of $CaCl_2$ (squares) or $MgCl_2$ (circles). The data are represented as the percentage of ellipticity at 222 nm, taking the value observed for native GOD as 100%. The inset shows the far-UV CD spectra of the native (dashed line) and 3 M $CaCl_2$-stabilized GOD (continuous line).

resulted in a significant enhancement (about three times) in FAD fluorescence intensity and a significant decrease in fluorescence polarization of GOD (Figure 1A). These observations indicate that treatment of GOD with a higher concentration of $CaCl_2$ or $MgCl_2$ results in alteration in the native GOD conformation such that the FAD molecules get either solvent exposed or dissociated from native molecule.

For GOD, the enhancement of FAD fluorescence intensity is associated with dissociation of the FAD molecule from the enzyme as a result of denaturation of the enzyme (*14*, *15*). As a large enhancement in FAD fluorescence intensity of GOD was observed on treatment with higher concentrations of both $CaCl_2$ and $MgCl_2$, the possibility of dissociation of FAD from the enzyme under these conditions was studied. The 2 and 3 M $CaCl_2$- or $MgCl_2$-treated GOD samples were concentrated on a Centricon of 3 kDa cutoff, and the presence of FAD in free form (in filtrate) and protein-bound form (in protein fraction) was monitored by fluorescence spectroscopy. For 2 M $CaCl_2$-treated GOD, an almost equal fraction of FAD fluorescence was found both in the filtrate (48% relative fluorescence) and in that associated with the enzyme (52% relative fluorescence). However, for increase in $CaCl_2$

concentration to 3 M, a major fraction of FAD was observed in the filtrate (about 77.7% relative fluorescence) with some fraction associated with the enzyme also (about 22% relative fluorescence). Similar results were observed for $MgCl_2$-treated GOD (data not shown). These observations suggest that treatment of GOD with higher concentrations of $CaCl_2$ and $MgCl_2$ leads to dissociation of the FAD molecule from the enzyme. However, a complete dissociation of FAD from the enzyme was not observed under any condition of salt studied.

(C) Tryptophan Microenvironment. Steady-state tryptophan fluorescence has been extensively used to obtain information on the structural and dynamic properties of proteins as the spectral parameters of fluorescence emission such as position; shape and intensity are dependent on the electronic and dynamic properties of the chromophore environment (*16*). For native GOD, significant tryptophan fluorescence with emission λ_{max} at 329 nm was observed (*9*). As buried tryptophan residues in the folded protein show the fluorescence emission λ_{max} at 330−340 nm (*17*), these observations indicate that the tryptophan molecules in native GOD are buried in the hydrophobic core of protein and not significantly exposed to the solvent. Figure 1B illustrates changes in the tryptophan fluorescence intensity of GOD in the presence of increasing $CaCl_2$ and $MgCl_2$ concentration. Up to $CaCl_2$ or $MgCl_2$ concentration of about 1.5 M, a quenching of tryptophan fluorescence without any shift in emission λ_{max} was observed. Between 2 and 3 M $CaCl_2$ or $MgCl_2$ concentration, a significant enhancement in tryptophan fluorescence intensity along with shift in emission λ_{max} to 355 nm was observed. Normally, exposed tryptophan residues in the unfolded protein show emission maxima between 348 and 356 nm (*17*). Hence, the above results indicate that the tryptophan moieties present in GOD get fully exposed on treatment of the enzyme with higher concentrations of $CaCl_2$ or $MgCl_2$, suggesting a divalent cation induced unfolding of GOD.

For $CaCl_2$ and $MgCl_2$ treatment of GOD, quenching of both FAD and tryptophan fluorescence was observed at low concentration of these salts. Quenching of FAD and tryptophan fluorescence of native GOD has been demonstrated to be a result of compaction of the native conformation of the enzyme in the case of interaction of GOD with monovalent cations (*9*). These observations indicate that low concentrations of divalent cations induce compaction of the GOD native conformation. At higher concentration of $CaCl_2$ or $MgCl_2$, a large enhancement in fluorescence of both FAD and tryptophan along with shift in emission λ_{max} of tryptophan fluorescence to 355 nm was observed. These observations suggest that treatment of GOD with a higher concentration of divalent cation results in exposure of buried tryptophan and FAD moieties present in the native enzyme to the solvent. Such a situation can happen only when the enzyme undergoes unfolding on treatment with divalent cations. The above-reported results suggest that $CaCl_2$ or $MgCl_2$ induces two opposing effects of compaction and unfolding on GOD, depending on the divalent cation concentration.

Effect of Tryptophan Modification by NBS on Structural Properties of Native and Divalent Cation Treated Enzyme. Each GOD monomer contains four tryptophan residues located in the flavin coenzyme activity domain out of which three residues are coupled with the flavin (*8*, *18*). The interaction between flavin coenzyme and aromatic amino acids (tryptophan and tyrosine) in GOD has been extensively studied using fluorescence quenching, resonance energy transfer, and ultraviolet spectroscopy (*14*, *18*). Chemical modification of tryptophan residues of the native enzyme has been demonstrated to influence the FAD fluorescence (*14*). The flavin coenzyme is embedded in the native GOD and hence does not generate significantly high fluorescence emission; however, modification of the native enzyme with NBS results in a large enhancement in FAD fluorescence probably due to oxidation of the binding site to FAD with NBS, thus resulting in alteration in the tryptophan−flavin interaction (*14*). The changes in the native conformation of the enzyme have been reported to influence both the NBS modification of the tryptophan residues and subsequent changes in the FAD fluorescence of the enzyme (*9*, *14*). For this reason, a comparative study on the changes in FAD fluorescence intensity on NBS modification of tryptophan residues in native and divalent cation treated GOD would provide useful information on the divalent cation induced changes in the enzyme.

Figure 1C summarizes the NBS titration for tryptophan modification of the native and 3 M $CaCl_2$-treated GOD at pH 5.6 and its effect on FAD fluorescence. For both samples, with increase in NBS concentration, a decrease in absorbance at 280 nm as a result of modification of tryptophyl residues was observed, indicating that tryptophan residues of the enzyme are being modified. However, for 3 M $CaCl_2$-treated GOD a much sharper decrease in absorbance at 280 nm was observed at low NBS concentration, indicating that the tryptophan residues in the 3 M $CaCl_2$-treated GOD are significantly more accessible to NBS for modification as compared to that of native GOD. For changes in FAD fluorescence on modification of tryptophyl residues with increasing concentration of NBS, a significant enhancement in FAD fluorescence intensity was observed for native GOD, which is similar to the earlier reports (*9*, *14*). However, for 3 M $CaCl_2$-treated GOD no change in FAD fluorescence intensity was observed on modification of tryptophan residues with increasing concentration of NBS. These observations suggest that the tryptophan and FAD residues, which are in close proximity in the native enzyme, have moved apart in the 3 M $CaCl_2$-treated GOD, indicating a significant unfolded conformation for 3 M $CaCl_2$-treated GOD.

Divalent Cation Induced Changes in the Secondary Structure of GOD. Far-UV CD studies on divalent-induced unfolding of GOD were carried out to study the effect of these cations on the secondary structure of the enzyme. In the far-UV region, the CD spectrum of native GOD shows the presence of substantial α-helical conformation (Figure 1D inset) (*9*). Figure 1D summarizes the effect of increasing $CaCl_2$ and $MgCl_2$ on the ellipticity at 222 nm for native GOD. Up to $CaCl_2$ or $MgCl_2$ concentration of about 1.5 M, no significant change in ellipticity at 222 nm of native GOD was observed. However, a gradual decrease in ellipticity at 222 nm from 100% to about 70% (Figure 1D inset) was observed between $CaCl_2$ and $MgCl_2$ concentration of 1.5−3 M. These observations demonstrate that even 3 M $CaCl_2$ or $MgCl_2$ induces only partial unfolding of GOD as only about 30% of the α-helical structure present the native enzyme is lost under these conditions.

The FAD and tryptophan fluorescence studies (Figure 1A,B) reported above suggested an extensive unfolding of GOD at high divalent cation concentration, which is in contrast to the far-UV CD observations according to which about 70% of the secondary structure present in the native enzyme is retained under these conditions. This discrepancy in the results obtained using different techniques is due to the fact that both the FAD and tryptophan fluorescence provide information only about the local environment (where these fluorophores are present), whereas far-UV CD provides information on the global structure of the enzyme. On treatment of GOD with higher concentrations of $CaCl_2$ or $MgCl_2$, the FAD cofactor gets dissociated from the enzyme (discussed in the text); hence, under these conditions no information about the structural changes in the enzyme can be obtained by monitoring FAD fluorescence.

Effect of Divalent Cations on the Subunit Assembly and Molecular Dimension of GOD. (A) Cross-Linking Studies. Glutaraldehyde cross-linking studies were carried to study the effect of $CaCl_2$ or $MgCl_2$ treatment on the subunit configuration of GOD and are summarized in Figure 2A. For GOD treatment with $CaCl_2$ up to 1.6 M, only dimers were observed. However, on increase in $CaCl_2$ concentration to 2 M the presence of both dimers and monomers was observed, indicating dissociation of the native dimeric enzyme molecule under these conditions. For further increase in $CaCl_2$ concentration to 3 M an increase in monomer population with concomitant decrease in dimer population was observed. For $MgCl_2$ treatment of GOD also a dissociation of the native dimer into monomers was observed at higher concentration of $MgCl_2$ (data not shown).

(B) Size Exclusion Chromatography. For studying the effect of low concentration of divalent cations on the molecular dimension of native GOD, size exclusion chromatographic studies on a Superdex S-200 column in the presence and absence of $CaCl_2$ were carried out and are summarized in Figure 2B. For native GOD dimer, a single peak with retention volume of 10.8 mL was observed. However, for 0.5 M $CaCl_2$-stabilized GOD an enhancement in retention volume to 12.2 mL was observed. The increase in retention volume compared to native enzyme for the 0.5 M $CaCl_2$-stabilized GOD is indicative of the significantly reduced hydrodynamic radii for the enzyme under these conditions.

The results of glutaraldehyde cross-linking along with the size exclusion chromatography experiments reported above suggest that treatment of GOD with 0.5 M $CaCl_2$ results in stabilization of a dimeric structure of GOD having more compact conformation than the native enzyme. A similar compaction of the native conformation of GOD has been reported for the monovalent cation treatment of GOD (*9*).

(C) Limited Proteolysis. The factor determining the vulnerability for proteolysis of a protein by protease depends on 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 (*19*).

Figure 2C shows the proteolytic susceptibility of native and 3 M $CaCl_2$-treated GOD to protease and α-chymotrypsin, as studied by analysis of the fragmentation profile by SDS−PAGE. No effect of proteolysis using α-chymotrypsin was observed for native GOD as the protein band corresponding to the enzyme only was observed on SDS−PAGE. However, for 3 M $CaCl_2$-treated GOD an extensive digestion of the enzyme by protease was observed.

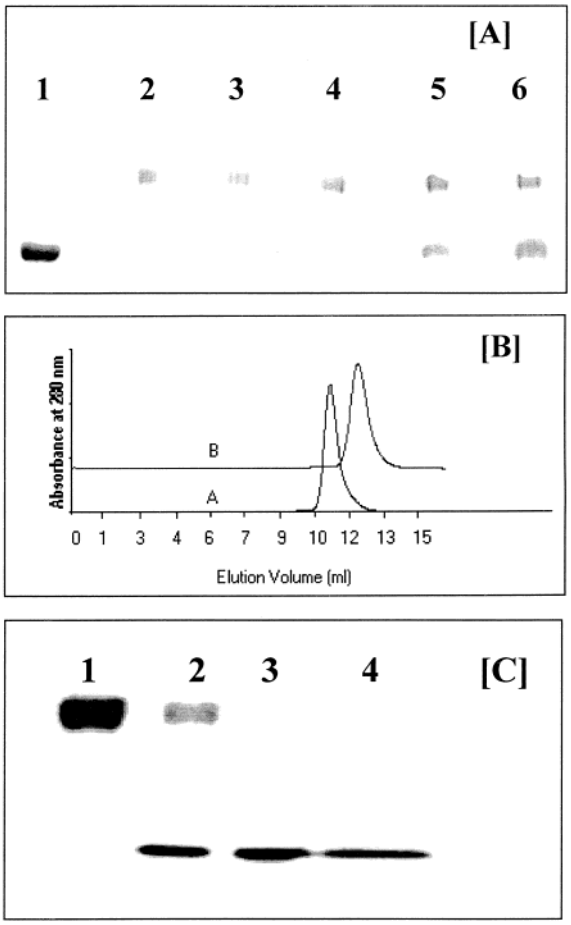

FIGURE 2: Effect of divalent cations on the subunit configuration and molecular dimension of GOD. (A) SDS−PAGE profile of glutaraldehyde cross-linked samples of GOD and on incubation with increasing concentration of $CaCl_2$. In the figure lanes 1 and 2 represent native and native cross-linked GOD. Lanes 3−6 represent cross-linked products of 1, 1.6, 2, and 3 M $CaCl_2$-treated GOD. (B) Size exclusion chromatographic profiles of native and 0.5 M $CaCl_2$-treated GOD on a Superdex 200 HR column at pH 6.5 and 25 °C. The various curves represent (A) native GOD and (B) 0.5 M $CaCl_2$-stabilized GOD. Curve B has been displaced in the *Y*-axis for presentation. (C) SDS−PAGE profile of α-chymotrypsin-treated native GOD and $CaCl_2$-treated GOD. In the figure lanes 1−4 represent native GOD, α-chymotrypsin-treated native GOD, α-chymotrypsin-treated 3 M $CaCl_2$-stabilized GOD, and α-chymotrypsin samples, respectively. The experimental details are given in Experimental Procedures.

The far-UV studies on 3 M $CaCl_2$-treated GOD have demonstrated that only partial unfolding (about 30% secondary structure loss) of the enzyme occurs under these conditions; however, for these samples an extensive proteolysis with protease was observed. This can happen only when the cooperative interactions between the secondary structural elements present in the enzyme have been disrupted and the enzyme has an open conformation, thus enabling the easy access of protease to various sites within the protein. This is further supported by the observation from DSC studies (data not shown) where no transition was observed for 3 M $CaCl_2$-treated GOD.

Stability of 0.5 M $CaCl_2$-Treated GOD against Urea Denaturation. The urea denaturation studies on native and

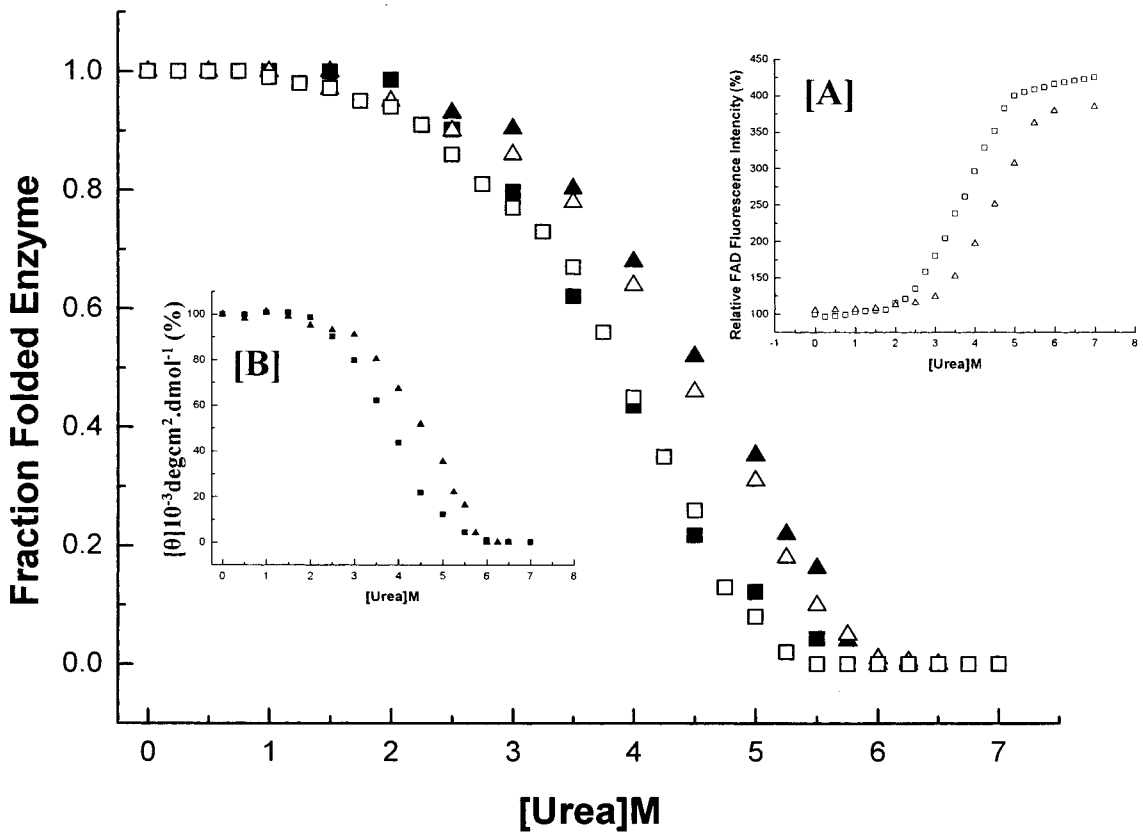

FIGURE 3: Urea denaturation of native and 0.5 M $CaCl_2$-stabilized GOD. Urea-induced unfolding transition of native and 0.5 M $CaCl_2$-stabilized GOD as obtained from FAD fluorescence (inset A) and CD ellipticity at 222 nm (inset B). A linear extrapolation of the baselines in the pre- and posttransitional regions was used to determine the fraction of folded protein within the transition region by assuming a two-state mechanism of unfolding. The squares represent data for native GOD, and the triangles represent data for 0.5 M $MCaCl_2$, respectively. The filled symbols represent data obtained from CD ellipticity at 222 nm, and the open symbols that from FAD fluorescence. Inset A shows changes in FAD fluorescence intensity of native and $CaCl_2$-treated GOD on incubation with increasing urea concentration as monitored by fluorescence emission at 524 nm and excitation at 370 nm. The symbols are same as in the main figure. The data are represented as the percentage of fluorescence, taking fluorescence at 0 M urea for each condition as 100%. Inset B represents changes in the secondary structure of native and $CaCl_2$-treated GOD on incubation with increasing urea concentration as monitored by CD ellipticity at 222 nm. The symbols are same as in the main figure. The data are represented as the percentage of CD ellipticity at 222 nm, taking ellipticity of 0 M urea for each condition as 100%.

0.5 M $CaCl_2$-treated GOD were carried out to see the effect of $CaCl_2$ treatment on the stability of the enzyme. Figure 3 shows the changes in FAD fluorescence CD ellipticity at 222 nm for native and 0.5 M $CaCl_2$-treated enzyme at increasing concentration of urea. For native GOD a urea concentration of about 3.8 M was found to be associated with 50% denaturation of the native enzyme. However, for 0.5 M $CaCl_2$-treated GOD an enhancement of about 0.8 M urea (4.6 M) as compared to native GOD was found to be associated with 50% denaturation of the enzyme. This observation suggests that treatment of GOD with 0.5 M $CaCl_2$ stabilizes such a conformation of enzyme that shows higher stability against urea denaturation. A similar enhancement in stability of native GOD against urea denaturation has been reported by us for monovalent cation treatment of GOD (*9*).

Divalent Cations Interact at the Same Site in GOD. To ascertain whether both divalent cations, Ca^{2+} and Mg^{2+}, interact at the same site in the enzyme, we carried out studies on 1.5 M $CaCl_2$-treated GOD at increasing $MgCl_2$ concentration and vice versa. The results of these studies are summarized in Figure 4. Treatment of native GOD with low concentrations (between 0.5 and 1.5 M) of either $CaCl_2$ or $MgCl_2$ does not induce any significant change in native enzyme structure/conformation as no significant change in FAD or tryptophan fluorescence of native GOD is observed on treatment of enzyme up to about 1.5 M concentration of these salts. However, for 1.5 M $MgCl_2$-treated GOD, addition of 0.5, 1, and 1.5 M $CaCl_2$ resulted in a large enhancement of both FAD (Figure 4A) and tryptophan fluorescence (Figure 4B). Similar results were observed for addition of 0.5, 1, and 1.5 M $MgCl_2$ to 1.5 M $CaCl_2$-treated GOD (Figure 4). These observations demonstrate that $CaCl_2$ and $MgCl_2$ have an additive effect on the changes induced by these salts in the enzyme structure and hence provide strong support for the assumption that both divalent cations interact at the same site in the enzyme.

DISCUSSION

The divalent cations were found to induce two opposing effects of stabilization and dissociation with partial unfolding of the GOD monomer, depending on the concentration of the cation. At low divalent cation concentration, a compaction of the native conformation of the enzyme with enhancement of enzyme stability against urea denaturation was observed. For monovalent cations a similar compaction of the native conformation of GOD and enhancement of enzyme stability has been reported earlier by us (*9*). However, higher

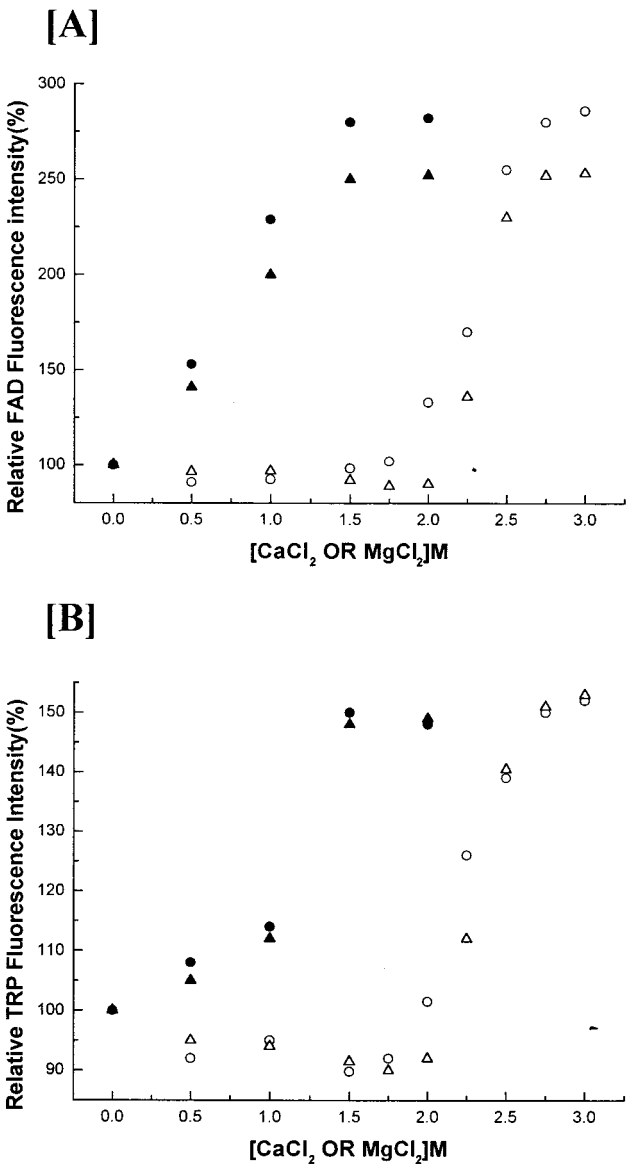

FIGURE 4: Effect of $MgCl_2$ on $CaCl_2$-treated GOD and vice versa. (A) Changes in FAD fluorescence intensity of native and 1.5 M $CaCl_2$- or $MgCl_2$-treated GOD on incubation with 0.5, 1, 1.5, and 2 M $MgCl_2$ or $CaCl_2$ (so that the effective concentration of salt becomes 2, 2.5, 3, and 3.5 M) as monitored by fluorescence emission at 524 nm and excitation at 370 nm. The circles represent data for $CaCl_2$, and triangles represent data for $MgCl_2$ treatment. The filled symbols represent data for changes in FAD fluorescence intensity of 1.5 M $CaCl_2$- or $MgCl_2$-treated GOD on incubation with 0.5, 1, and 1.5 M $CaCl_2$ or $MgCl_2$. The open symbols represent data for treatment of native GOD with increasing concentration of $CaCl_2$ or $MgCl_2$. (B) Changes in tryptophan fluorescence intensity of 1.5 M $CaCl_2$-treated GOD on incubation with increasing $MgCl_2$ concentration as monitored by fluorescence emission at 342 nm and excitation at 290 nm. The symbols are the same as in panel A.

concentrations of divalent cations induced dissociation of the native dimer into monomers along with dissociation of the FAD cofactor from the enzyme. Furthermore, the divalent cation-stabilized monomer of GOD however retained about 70% of α-helical structure present in the native enzyme, but a complete loss of cooperative interaction between these secondary structural elements was observed.

From the crystal structure of GOD it is observed that a short contiguous region formed by residues 75−98, known as the "FAD covering lid" is located in the dimer interface in GOD and effectively couples the dimer stabilization with FAD binding (*8*). It was hypothesized that detachment of FAD from the native GOD would lead to an open conformation of the FAD covering lid with detachment of the lid from the dimer surface. Due to elimination of major dimer contact in the native enzyme this would result in dissociation of the dimer into monomer. The experimental support for this hypothesis came from the analysis of the subunit structure of GOD under conditions where FAD was dissociated from the enzyme. For example, the apo-GOD was found to be present as monomers rather than the dimer (*20*). The FAD fluorescence studies presented in this paper demonstrate that incubation of GOD with higher concentrations of $CaCl_2$ and $MgCl_2$ leads to dissociation of FAD from the GOD molecule; hence, under these conditions it is expected that the native dimer of GOD should also undergo dissociation. Studies on the effect of divalent cations on subunit assembly of GOD demonstrate that only under conditions where FAD was dissociated from the enzyme (higher concentration of salts) is the dissociation of dimer into monomer also observed. Furthermore, under no concentration of divalent cation studied was a complete dissociation of both FAD molecules from the enzyme and dimer to monomer observed. These observations provide strong experimental support to the hypothesis that for GOD the dissociation of FAD from enzyme and dimer to monomer occurs in a concerted fashion.

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 (*21*), it is possible to classify the ions by following their ability to organize the water molecules. In the case of cations, this series is as follows: $Cs^+ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+}$. If the organization degree 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 stabilization of GOD by monovalent cation containing salts, against urea denaturation as reported earlier (*9*) and divalent cations (this paper), was found to increase in proportion to the ability of the various cations to organize water, thus indicating that the stability of GOD structure provided by low concentrations of cations depends on their ability to organize the water molecules.

High concentrations of divalent cations were found to induce dissociation and partial unfolding of the GOD monomer. To understand the underlying mechanism of divalent cation induced dissociation of GOD, comparative FAD and tryptophan fluorescence studies on the divalent cation induced changes in native and NaCl-stabilized GOD (*9*) were carried out. Figure 1 summarizes the effect of $CaCl_2$ on the native and NaCl-stabilized GOD as monitored by the changes in FAD (Figure 1A) and tryptophan fluorescence (Figure 1B) of 0.5 M NaCl-treated GOD at increasing concentration of $CaCl_2$. For both native and NaCl-stabilized GOD, a similar profile of changes in both FAD and tryptophan fluorescence at increasing concentration of $CaCl_2$ was observed, thus demonstrating that the NaCl stabilization of GOD has no effect on the $CaCl_2$-induced denaturation of GOD. These observations suggest that probably direct binding of divalent cations to negatively charged groups in GOD is responsible for the dissociation of the native dimer

of the enzyme. This suggestion is further supported by the earlier reported observation where at low pH, which would lead to protonation of negatively charged carboxylate moieties resulting in neutralization of charge, dissociation of GOD is reported (*15*).

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