Guanidinium Chloride- and Urea-Induced Unfolding of the Dimeric Enzyme Glucose Oxidase†

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ABSTRACT: We have carried out a systematic study on the guanidinium chloride- and urea-induced unfolding of glucose oxidase from *Aspergillus niger*, an acidic dimeric enzyme, using various optical spectroscopic techniques, enzymatic activity measurements, glutaraldehyde cross-linking, and differential scanning calorimetry. The urea-induced unfolding of GOD was a two-state process with dissociation and unfolding of the native dimeric enzyme molecule occurring in a single step. On the contrary, the GdmCl-induced unfolding of GOD was a multiphasic process with stabilization of a conformation more compact than the native enzyme at low GdmCl concentrations and dissociation along with unfolding of enzyme at higher concentrations of GdmCl. The GdmCl-stabilized compact dimeric intermediate of GOD showed an enhanced stability against thermal and urea denaturation as compared to the native GOD dimer. Comparative studies on GOD using GdmCl and NaCl demonstrated that binding of the Gdm^+ cation to the enzyme results in stabilization of the compact dimeric intermediate of the enzyme at low GdmCl concentrations. An interesting observation was that a slight difference in the concentration of urea and GdmCl associated with the unfolding of GOD was observed, which is in violation of the 2-fold rule for urea and GdmCl denaturation of proteins. This is the first report where violation of the 2-fold rule has been observed for a multimeric protein.

The conformational stability of multimeric proteins can be measured by equilibrium unfolding studies using urea and GdmCl,[1] the two agents commonly employed as protein denaturants. Analysis of the solvent denaturant curves using these denaturants can provide a measure of the conformational stability of the protein (*1*, *2*). Protein unfolding/folding studies in GdmCl and urea solutions have focused on the identification of equilibrium and kinetic intermediates (*3*−*6*). The denaturant-induced unfolding of large or multimeric enzymes has mostly been found to be a multiphasic process with the stabilization of partially folded intermediates (*7*−*9*).

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 (*10*). Industrially it is being used in the removal of glucose or oxygen from food products and in production of gluconic acid (*11*). The most important application of glucose oxidase is as a molecular diagnostic tool as the enzyme is used in biosensors for the quantitative determination of D-glucose in samples such as body fluids, foodstuffs, beverages, and fermentation products (*12*, *13*). Since the discovery of the enzyme as an antibiotic, shown subsequently to be due to the peroxide formation (*14*), there has been an ever-increasing interest in glucose oxidase.

Glucose oxidase (GOD) from *Aspergillus niger*, a homodimer of molecular mass 160 kDa, is a glycoprotein with a carbohydrate content of 16% (w/w) (*15*, *16*). The carbohydrate moiety is of the high-mannose type, and the oligomeric polysaccharide is covalently attached to polypeptide chains via asparagine and serine or threonine residues (*17*). The enzyme contains two tightly bound but noncovalently linked flavin−adenine dinucleotides (FAD) per dimer (*18*, *19*). 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; however, at low pH (4.3 and below) it is susceptible to denaturation (*20*). 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 (*20*, *21*).

We have studied the structural and functional changes associated with GdmCl- and urea-induced unfolding of the dimeric enzyme GOD. Significantly different pathways of GOD unfolding were observed for the two denaturants. To understand the underlying mechanism of GdmCl-induced compaction of native GOD, comparative studies with NaCl were carried out.

EXPERIMENTAL PROCEDURES

Materials

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

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[1] Abbreviations: GOD, glucose oxidase; GdmCl, guanidinium chloride; FAD, flavin adenine dinucleotide; C_m, concentration of denaturant where 50% denaturation of protein is observed; T_m, midpoint of thermal denaturation; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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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 as described previously (*22*). The purity of the purified enzyme was evaluated on SDS−PAGE (*23*) followed by silver staining and was found to be about 99% pure.

Guanidinium Chloride and Urea Denaturation of GOD. GOD dissolved in sodium phosphate buffer (10 mM, pH 6.5) in the presence and absence of increasing concentrations of GdmCl or urea was incubated for 2 h at 25 °C before the measurements were made.

Assay of Enzymatic Activity. Glucose oxidase activity was determined by the colorimetric method using the coupled peroxide/*o*-dianisidine system as described previously (*22*).

Fluorescence Spectroscopy. Fluorescence spectra were recorded with a Perkin-Elmer LS 50B spectroluminescence meter in a 5 mm path-length quartz cell. GOD in 10 mM phosphate buffer, pH 6.5, was incubated in the presence of increasing GdmCl or urea 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 tryptophan and FAD fluorescence, excitation wavelengths of 290 and 365 nm, respectively, were used, and the spectra were recorded between 300 and 430 nm and between 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 denaturant under similar conditions.

Cross-Linking Using Glutaraldehyde. To native and GdmCl- or urea-treated (2 h at 25 °C) 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%) that 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 by 6% SDS−PAGE (*23*).

Differential Scanning Calorimetry. All calorimetric scans were performed with a Microcal MC-2 differential scanning calorimeter. The calorimetric unit was interfaced to 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 earlier (*24*). All the scans were found to be irreversible under the experimental conditions studied.

Size-Exclusion Chromatography. Gel filtration experiments were carried out on a Superdex 200HR 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 GdmCl or urea concentration at 25 °C. The GOD solution (4 μM) was incubated at the desired GdmCl or urea concentration for 12 h at 25 °C. Then 200 μL of this sample was loaded on the column and run at 25 °C; a flow rate of 0.3 mL/min with detection at 280 nm.

RESULTS

We have studied the effect of GdmCl- and urea-induced changes on the structural and functional properties of GOD.

Time-dependent changes in structural parameters and enzymatic activity of GOD at increasing GdmCl or urea concentrations (0.5, 2.5, and 5 M) were monitored to standardize the incubation time required for achieving equilibrium under these conditions. Under all of the conditions studied, the changes occurred within maximum of 1 h with no further alteration up to 12 h (data not shown). These observations demonstrate that an incubation time of 1 h is sufficient for achieving equilibrium under any condition of denaturant studied.

Changes in Molecular Properties of GOD Associated with GdmCl-Induced Unfolding. Enzyme activity can be regarded as the most sensitive probe to study 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 1A shows the effect of increasing concentrations of GdmCl on the enzymatic activity of GOD. A sigmoidal dependence of enzymatic activity on GdmCl concentration was observed. No significant effect of denaturant on enzymatic activity of native GOD was observed up to about 1.75 M GdmCl. However, between 2 and 4 M GdmCl, a steep decrease in enzymatic activity (from 100% to about 1%) and a complete loss of enzymatic activity above 5 M GdmCl were observed.

Optical spectroscopic studies on GOD in the presence of increasing GdmCl concentrations were performed to study the effect of denaturant on the structural properties of GOD.

The spectral parameters of tryptophan fluorescence emission such as position, shape, and intensity are dependent on the electronic and dynamic properties of the chromophore environment; hence, steady-state tryptophan fluorescence has been extensively used to obtain information on the structural and dynamical properties of the protein (*25*). The modification of the microenvironment of tryptophan residues of GOD due to denaturants has been monitored by studying changes in the intensity and wavelength of emission maxima (λ_{max})

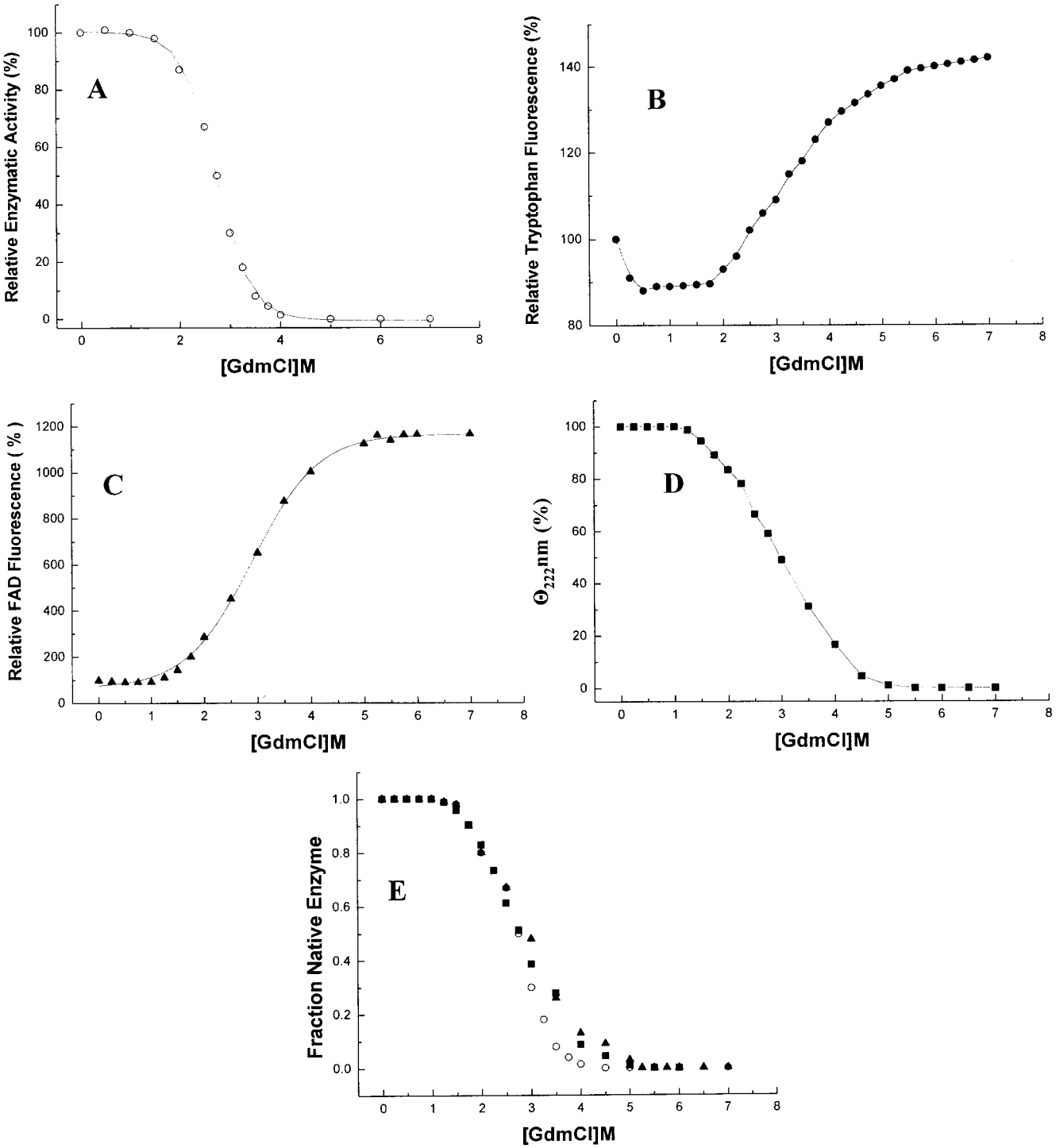

FIGURE 1: Changes in functional and structural properties of glucose oxidase in the presence of increasing concentrations of GdmCl at pH 6.5 and 25 °C. (A) Changes in enzymatic activity of GOD on treatment with increasing concentrations of GdmCl. GOD in 10 mM phosphate buffer, pH 6.5, was incubated with the desired concentration of GdmCl for 2 h at 25 °C, followed by measurement of enzymatic activity as described in Experimental Procedures. The data in the figure are expressed in terms of relative activity using the activity of the native enzyme as reference (100%). (B) Changes in tryptophan fluorescence of GOD on treatment with increasing GdmCl concentrations as monitored by fluorescence intensity at emission wavelength maxima under different conditions; excitation = 290 nm. The data are represented as the percentage of fluorescence, taking fluorescence of native GOD as 100%. (C) Changes in FAD fluorescence of GOD on treatment with increasing GdmCl concentrations as monitored by fluorescence emission at 524 nm; excitation = 365 nm. The data are represented as the percentage of fluorescence, taking fluorescence of native GOD as 100%. (D) GdmCl-induced changes in the secondary structure of GOD as monitored by following changes in ellipticity at 222 nm obtained from the far-UV CD curves of GOD at increasing concentrations of GdmCl. The data are represented as the percentage of ellipticity at 222 nm, taking the value observed for native GOD as 100%. (E) GdmCl-induced unfolding transition of GOD as obtained from enzymatic activity (panel A), FAD fluorescence (panel C), and ellipticity at 222 nm (panel D). 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 symbols are the same as in panels A, C, and D.

of tryptophan fluorescence as a function of denaturant concentration. Figure 1B illustrates changes in the tryptophan fluorescence intensity of GOD at increasing GdmCl concentrations. For native GOD, significant tryptophan fluorescence with emission λ_{max} at 329 nm was observed. The buried tryptophan residues in the folded protein show the fluorescence emission λ_{max} at 330−340 nm (*26*); hence, in native GOD the tryptophan moieties are buried in the hydrophobic core of the protein. Between 0 and 1.75 M GdmCl, quenching of tryptophan fluorescence intensity without any change in emission λ_{max} of the native enzyme was observed. The quenching of tryptophan fluorescence of native GOD has been reported to occur due to compaction of the native conformation of the enzyme (*22*); hence, treatment of GOD with low concentrations of GdmCl induces compaction of the native conformation of the enzyme. For increases in GdmCl concentration between 2 and 6 M, a significant enhancement in the tryptophan fluorescence along with a shift in emission λ_{max} to about 355 nm was observed. Normally, exposed tryptophan residues in the unfolded protein show emission maxima between 348 and 356 nm (*26*); hence, treatment of GOD with higher concentrations of GdmCl results in exposure of the buried tryptophan moieties present in native GOD to the solvent. Such a situation can happen only when the denaturant induces unfolding of GOD.

Studies on various flavanoproteins have reported that the fluorescent prosthetic groups 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 (*27*). For this reason the FAD group has been used as a natural marker to probe the dynamical microenvironment of the flavin fluorophore in flavanoproteins (*28*, *29*). GOD contains two tightly bound but noncovalently linked flavin−adenine dinucleotide (FAD) molecules. For the native enzyme, a significant fluorescence with the emission λ_{max} at 524 nm corresponding to a FAD molecule present in the enzyme was observed. The effect of addition of denaturants on the FAD microenvironment of GOD is summarized in Figure 1C where changes in FAD fluorescence intensity of GOD with increasing GdmCl concentrations are depicted. A sigmoidal dependence of FAD fluorescence intensity with GdmCl concentration was observed. No significant change in FAD fluorescence of the native enzyme was observed up to GdmCl concentrations of 1.5 M. However, between 2 and 6 M GdmCl a large enhancement (about 12 times) in FAD fluorescence intensity was observed. Enhancement of FAD fluorescence intensity of GOD has been associated with dissociation of the FAD molecule from the enzyme as a result of denaturation of the enzyme (*23*, *30*). For this reason studies were carried out to ensure that GdmCl-induced unfolding of GOD results in dissociation of the FAD molecule from the enzyme. The GdmCl (at increasing concentration) 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. Up to 1.5 M GdmCl, all of the FAD fluorescence was found (98% relative fluorescence) associated with the enzyme, and only background fluorescence (about 2% relative fluorescence) was observed in the filtrate. For 3.0 M GdmCl-treated GOD an almost equal amount of FAD fluorescence was observed both in the filtrate (49% relative fluorescence) and that associated with the enzyme (51% relative fluorescence). However, for increases in GdmCl concentrations to 6 M, all of the FAD fluorescence was found (98% relative fluorescence) in the filtrate. These observations demonstrate that denaturation of GOD with high GdmCl concentrations results in a complete dissociation of FAD from the enzyme.

Far-UV CD studies on GdmCl-induced unfolding of GOD were carried out to study the effect of GdmCl 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 (*22*). Figure 1D summarizes the effect of increasing GdmCl concentrations on the ellipticity at 222 nm for native GOD. Up to a GdmCl concentration of about 1.5 M, no significant change in ellipticity at 222 nm of native GOD was observed. However, between GdmCl concentrations of 2−5 M, a large gradual decrease in ellipticity at 222 nm from 100% to almost complete loss of signal was observed. These observations suggest that treatment of GOD with higher GdmCl concentrations results in complete unfolding of the enzyme.

Changes in the molecular properties of GOD such as enzymatic activity, CD ellipticity at 222 nm, and FAD fluorescence at increasing GdmCl concentrations however showed a sigmoidal dependence, but the profiles were not superimposable (Figure 1E), which suggests that GdmCl-induced unfolding of GOD is a multiphasic process with stabilization of intermediates. An experimental support for this suggestion comes from the tryptophan fluorescence studies (Figure 1B) where a biphasic profile, suggesting the stabilization of an intermediate, was observed for GdmCl denaturation of GOD.

Changes in Molecular Properties of GOD Associated with Urea-Induced Unfolding. Although urea and GdmCl are believed to have similar modes of action, GdmCl is a monovalent salt that has both ionic and chaotropic effects (*31*−*33*), whereas urea has only chaotropic effects. Thus urea is an ideal control agent for distinguishing between the ionic and chaotropic effects of GdmCl.

Figure 2 summarizes the urea-induced changes in structural and functional properties of GOD as studied by changes in enzymatic activity, FAD and tryptophan fluorescence, and CD ellipticity at 222 nm at increasing urea concentrations.

No significant change in enzymatic activity of native GOD was observed up to 2 M urea (Figure 2A). However, between 2 and 5 M urea, a sharp decrease in enzymatic activity from 100% to about 5% and above 6 M urea a complete loss of enzymatic activity were observed (Figure 2A).

Like enzymatic activity, a sigmoidal dependence of tryptophan fluorescence of GOD with increasing urea concentrations was observed (Figure 2B). A slight linear increase in tryptophan fluorescence of native GOD without any shift in emission λ_{max} (329 nm) was observed between 0 and 2 M urea concentration. A significant enhancement in tryptophan fluorescence with a shift in emission λ_{max} from 329 to 355 nm was observed between 2 and 6 M urea, which indicates that treatment of GOD with high concentrations of urea leads to unfolding of the enzyme (as discussed for GdmCl).

For FAD fluorescence also a sigmoidal dependence of fluorescence intensity with increasing urea concentration was

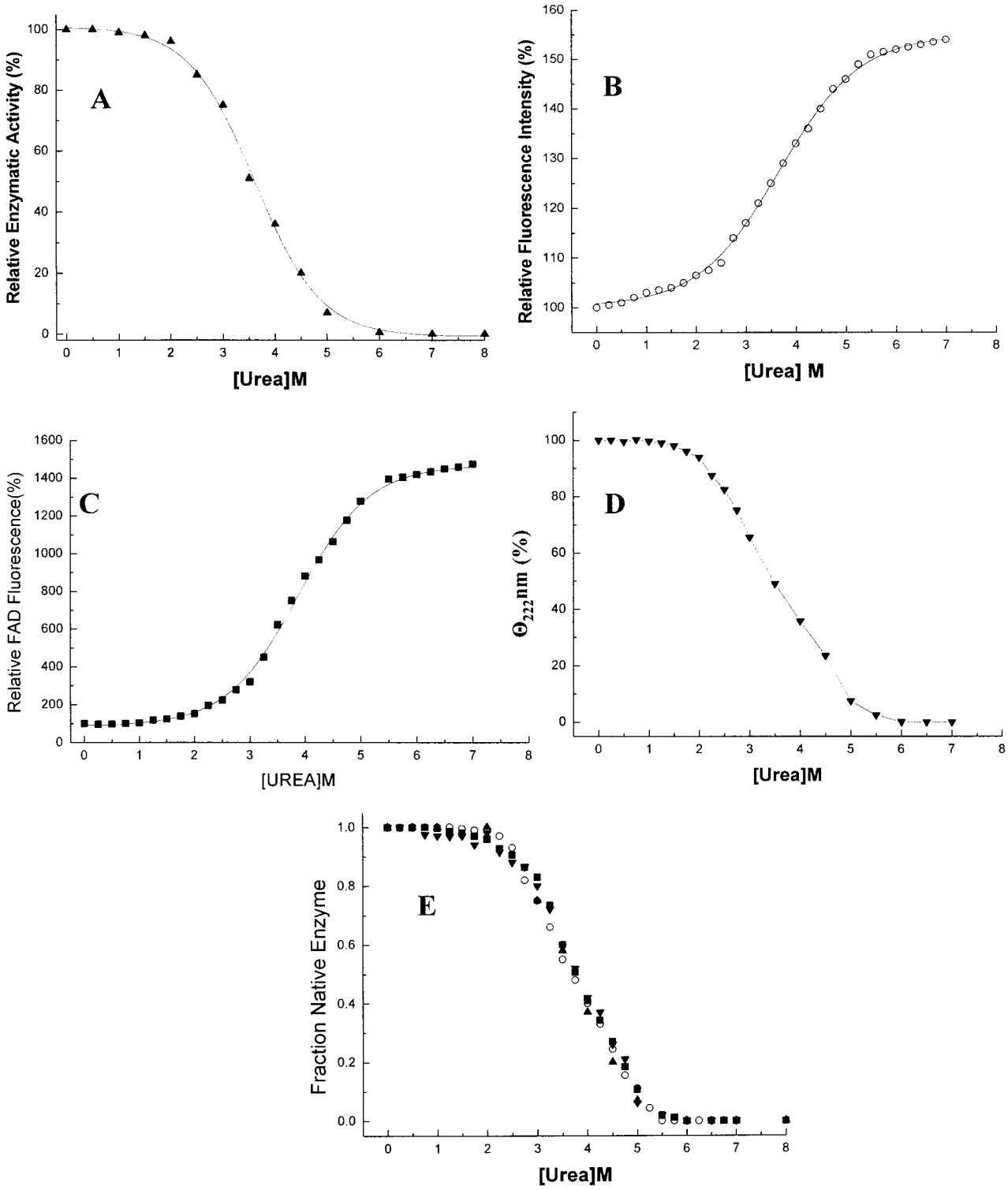

FIGURE 2: Changes in functional and structural properties of glucose oxidase in the presence of increasing concentrations of urea at pH 6.5 and 25 °C. (A) Changes in enzymatic activity of GOD on treatment with increasing concentrations of urea. GOD in 10 mM phosphate buffer, pH 6.5, was incubated with the desired concentration of urea for 2 h at 25 °C, followed by measurement of enzymatic activity as described in Experimental Procedures. The data in the figure are expressed in terms of relative activity using the activity of the native enzyme as reference (100%). (B) Changes in tryptophan fluorescence of GOD on treatment with increasing urea concentrations as monitored by fluorescence intensity at emission wavelength maxima under different conditions; excitation = 290 nm. The data are represented as the percentage of fluorescence, taking fluorescence of native GOD as 100%. (C) Changes in FAD fluorescence of GOD on treatment with increasing urea concentrations as monitored by fluorescence emission at 524 nm; excitation = 365 nm. The data are represented as the percentage of fluorescence, taking fluorescence of native GOD as 100%. (D) Urea-induced changes in the secondary structure of GOD as monitored by following changes in ellipticity at 222 nm from the far-UV CD curves of GOD at increasing concentrations of urea. The data are represented as the percentage of ellipticity at 222 nm, taking the value observed for native GOD as 100%. (E) Urea-induced unfolding transition of GOD as obtained from enzymatic activity (panel A), tryptophan fluorescence (panel B), FAD fluorescence (panel C), and ellipticity at 222 nm (panel D). The fraction of native enzyme was calculated as described in Figure 1D. The symbols are the same as in panels A−D.

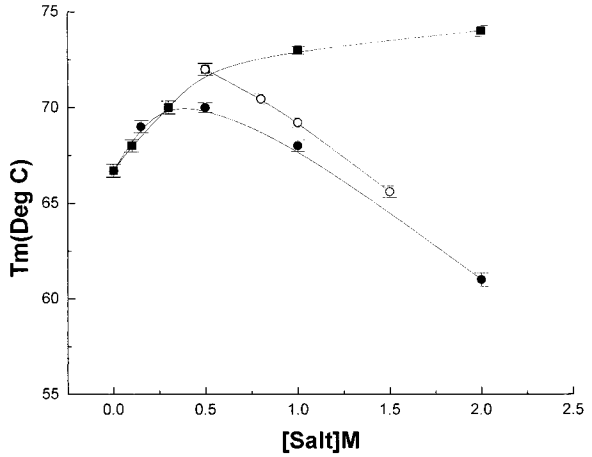

FIGURE 3: Dependence of GdmCl and NaCl of the thermal stabilities of GOD. The midpoints of thermal denaturation transitions are shown as a function of the concentration of added salts. The transitions are in the presence of NaCl (filled squares) and GdmCl (filled circles). In the experiment denoted by open circles 0.1, 0.3, and 0.5 M GdmCl was added to the 0.5 M NaCl-treated GOD sample. The values represent the mean ± SD of four different experiments.

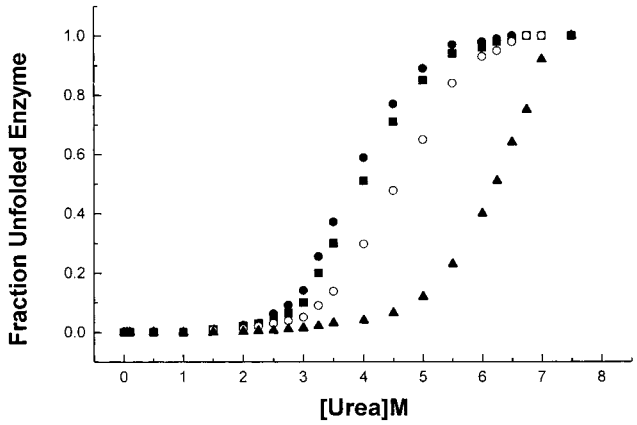

FIGURE 4: Urea-induced unfolding transition of native (filled squares), 0.5 M NaCl-treated GOD (filled triangles), and 0.5 M (open circles) and 2 M GdmCl-treated GOD (filled circles) at 25 °C. A linear extrapolation of the baselines in the pre- and posttransitional regions was used to determine the fraction of unfolded protein within the transition region by assuming a two-state mechanism of unfolding.

observed. No significant enhancement of FAD fluorescence intensity was observed up to about 1.75 M urea concentration. However, between urea concentrations of 2−6 M there was a large enhancement in FAD fluorescence intensity (about 14 times). Studies on FAD dissociation from GOD (as described for GdmCl above) on treatment with urea showed that up to 2 M urea the FAD molecule is associated with the enzyme (98% relative FAD fluorescence). However, treatment with 6 M urea resulted in complete dissociation of FAD (only 2% relative FAD fluorescence associated with the enzyme) from GOD.

The urea-induced changes in the secondary structure of GOD were studied by monitoring changes in CD ellipticity at 222 nm at increasing urea concentrations and are summarized in Figure 2D. A sigmoidal dependence of decrease in ellipticity at 222 nm with increasing concentrations of urea was observed with no change in value observed for the native enzyme up to 2 M urea. However, between 2 and 6 M urea a large decrease in ellipticity value at 222 nm and a complete loss of signal above 6 M urea were observed. These observations suggest that higher concentrations of urea (≥6 M) induce complete unfolding of GOD.

The urea denaturation profile of GOD as studied by monitoring the changes in all of the four techniques studied, i.e., enzymatic activity, FAD and tryptophan fluorescence, and CD ellipticity at 222 nm at increasing urea concentrations, showed a superimposable profile (Figure 2E). This suggests that the urea denaturation of GOD is a two-state process where the dissociation and unfolding of the native dimer occurs in a single step. A C_m of about 3.65 M urea was associated with urea unfolding of GOD.

Dependence on NaCl and GdmCl of the Thermal Stability of GOD. For demonstrating directly the unusual effects of low GdmCl concentrations on the stability of GOD, we compared thermal unfolding transitions of native GOD on treatment with GdmCl or NaCl. Figure 3 shows the T_m values obtained from DSC experiments on native GOD as a function of NaCl and GdmCl concentrations and 0.5 M NaCl-treated GOD as a function of GdmCl concentrations. NaCl stabilizes the enzyme at all concentrations, and a particularly strong increase in T_m was found on treatment of the enzyme with 0−0.5 M NaCl; however, between 0.5 and 2 M NaCl a significantly lesser increase in T_m was observed (Figure 3 and ref *22*). This reflects that the NaCl-induced stabilization of GOD probably has a contribution of two components: cation binding to the native enzyme as well as a general stabilizing effect of monovalent cations on the enzyme. For GdmCl at low concentrations (between 0 and 0.3 M) an enhancement in T_m similar to that for NaCl, under similar concentrations, was observed. Between 0.3 and 0.5 M GdmCl no further enhancement in T_m was observed. For 0.5 M GdmCl, a significantly lesser T_m than that for 0.5 M NaCl-treated GOD was observed, suggesting that NaCl treatment results in greater stabilization of enzyme against thermal denaturation as compared to GdmCl treatment. At 1 and 2 M GdmCl the denaturing effect of denaturant predominated, and a decrease in T_m with increasing GdmCl concentrations was observed. However, up to 1 M GdmCl concentration, the observed value for the T_m was higher (minimum of about 4 °C) than that of native GOD, but for 2 M GdmCl-treated GOD a slight decrease in T_m (about 5 °C) as compared to native GOD was observed.

For GdmCl denaturation of NaCl (0.5 M) stabilized GOD, between 0.3 and 1.5 M GdmCl, a linear decrease in T_m with increasing GdmCl concentrations was observed. These observations demonstrate that, unlike in the case of native enzyme where low concentrations of GdmCl (up to 0.5 M) induce stabilization of enzyme, for 0.5 M NaCl-treated enzyme similar concentrations of the GdmCl induced destabilization of the enzyme. These results provide strong support for the assumption that the stabilization of GOD by low concentrations of NaCl and GdmCl occurs by the same mechanism, probably by binding to the common cation-binding site.

Urea Denaturation Studies. The urea denaturation of native and 0.5 M NaCl- and 0.5 and 2 M GdmCl-treated GOD was carried out to study the effect of GdmCl and NaCl treatment on the stabilization of the enzyme. Figure 4 shows the enhancement in population of the unfolded enzyme with increasing urea concentrations as obtained from the changes

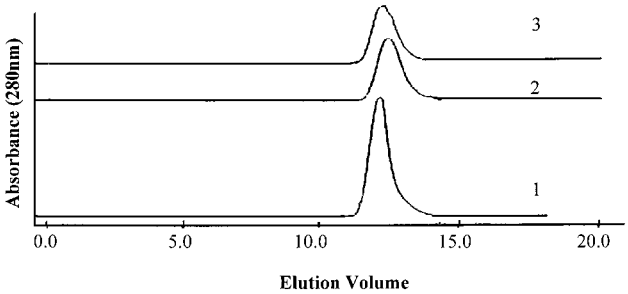

FIGURE 5: Size-exclusion chromatographic profiles on a Superdex 200HR column for native and 0.1 M GdmCl stabilized GOD at pH 6.5 and 25 °C. The experimental details are given in Experimental Procedures. Curves 1−3 represent samples of native, 0.1 M GdmCl-treated GOD, and aldolase (standard), respectively.

in FAD fluorescence intensity for these samples. For native GOD, a urea concentration of about 3.65 M was associated with 50% denaturation of the enzyme. However, for 0.5 M GdmCl- or NaCl-treated GOD an enhancement of about 0.5 M (4.25 M) and 2.7 M (6 M), respectively, in urea concentration as compared to native GOD was required for 50% denaturation of the enzyme. These observations demonstrate that treatment of GOD with GdmCl or NaCl (0.5 M) results in enhanced stability of the enzyme against urea denaturation. However, for same concentration of NaCl and GdmCl, a higher stabilization of the enzyme against urea denaturation was observed for NaCl treatment. These observations along with the results of thermal denaturation (reported above) demonstrate that as compared to GdmCl the NaCl treatment of GOD results in greater stabilization of the enzyme. For 2 M GdmCl-treated GOD, a profile similar to that for the native enzyme was observed. Similar results were observed when CD ellipticity changes at 222 nm under similar conditions were monitored.

Size-Exclusion Chromatography. For studying the effect of low concentrations of GdmCl on the molecular dimensions of native GOD, size-exclusion chromatographic studies on a S-200 superdex column in the presence and absence of GdmCl were carried out. Figure 5 summarizes the results of size-exclusion chromatographic experiments carried out on GOD in the presence and absence of GdmCl at 25 °C. For native GOD, a single peak at 12.15 mL was observed. Aldolase, M_r 158 kDa, on a S-200 column under identical conditions showed a single peak with a retention volume of 12.40 mL, which is slightly higher compared to the retention volume observed for native GOD. As the reported M_r of native dimeric GOD is 160 kDa (*15*, *16*), these observations indicate that native GOD under the conditions studied is in a dimeric configuration. However, when GOD treated with 0.1 M GdmCl was loaded on the same column and eluted, an enhancement in retention volume for the enzyme to 12.75 mL was observed. This increase in retention volume for the GdmCl-treated GOD is indicative of reduced hydrodynamic radii for the GdmCl-stabilized conformation of GOD as compared to the native enzyme. A similar reduction in hydrodynamic radii of native GOD on treatment with the monovalent cation has been reported earlier by us and was demonstrated to be due to compaction of the native conformation of the native enzyme (*22*). These observations suggest that GOD on treatment with low concentrations of GdmCl undergoes compaction of conformation.

Cross-Linking Studies. The effect of urea and GdmCl denaturation on the subunit structure of GOD was studied by carrying out glutaraldehyde cross-linking experiments. Figure 6 shows the results of cross-linking of native and denaturant-treated GOD. For native as well as GdmCl- or urea-treated GOD (up to 2 M denaturant concentration) the protein band of glutaraldehyde cross-linked samples corresponding to only dimers was observed. However, for GOD treated with higher concentrations of urea or GdmCl (5 or 6 M) only protein bands corresponding to monomers were observed.

The results of glutaraldehyde cross-linking along with the size-exclusion chromatography experiments suggest that

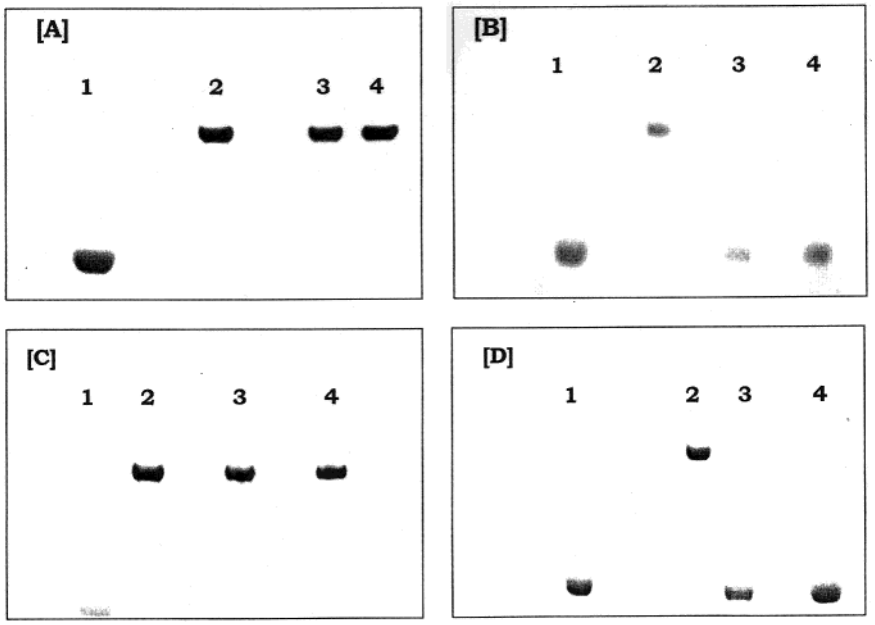

FIGURE 6: SDS−PAGE profile of glutaraldehyde cross-linked GdmCl-treated GOD (panels A and B) and urea-treated GOD (panels C and D). In all of the panels lanes 1 and 2 represent native and glutaraldehyde cross-linked native GOD samples, respectively. In panels A and C lanes 3 and 4 represent 1 and 2 M denaturant-treated GOD samples, respectively. In panels B and D lanes 3 and 4 represent 5 and 6 M denaturant-treated GOD samples, respectively.

Table 1: Comparison of Urea and Guanidinium Chloride C_m Values for Proteins

protein	oligomeric state	C_m (urea, M)	C_m (GdmCl, M)	C_m (urea)/ C_m (GdmCl)	ref
vitronectin	tetramer	6	2.8	2.1	*35*
phosphofructokinase	tetramer	2.8	0.6	4.6	*36*
triosephosphate isomerase	dimer	>6.0	1.4	>4.3	*8*
ceratinase	dimer	5.2	2.0	2.6	*37*
glucose oxidase	dimer	3.6	3	1.2	this paper
bovine growth hormone	dimer	8.3	3.8	2.1	*38*
invertase	dimer	5.25	1.6	3.28	*39*
glutathione transferase	dimer	5.5	1.5	3.66	*40*
RNase T1	monomer	4.6	2.9	1.59	*31*
RNase A	monomer	6.6	3.0	2.2	*31*
maltose binding protein	monomer	3.5	1.05	3.33	*41*
ubiquitin	monomer	3	3.9	0.77	*32*

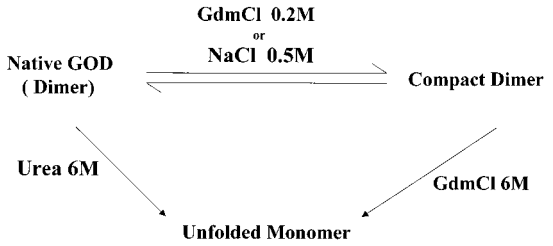

FIGURE 7: Schematic diagram of GdmCl- and urea-induced unfolding of GOD.

treatment of native GOD with low concentrations (up to 0.5 M) of GdmCl induces compaction of the native conformation of the enzyme only. However, treatment with higher concentrations of GdmCl or urea results in dissociation of GOD.

DISCUSSION

Guanidine hydrochloride and urea are the most common chemical denaturants that are used for protein denaturation. The conformational stability of multimeric proteins can be measured by equilibrium unfolding studies in urea or GdmCl solutions. The unfolding of GOD in urea and GdmCl suggests dramatically different unfolding pathways and mechanisms for the two denaturants. The two possible unfolding pathways in urea and GdmCl are represented schematically in Figure 7. The urea unfolding of GOD was found to occur by a two-state mechanism with dimer dissociation and enzyme unfolding occurring in a single step.

GdmCl displayed two opposing functions on the folding and stability of GOD. At low concentrations (0−2 M), GdmCl acted as a structure-stabilizing additive. It induced compaction of the native conformation of the enzyme and enhanced the stability of the enzyme against thermal and urea denaturation. However, at higher denaturant concentrations, the strong destabilization character of GdmCl was observed, and it behaved like a classical denaturant inducing extensive unfolding of the enzyme. There are several reports of GdmCl and urea providing different estimates for the conformational stability of a protein (*31*, *32*). In some cases these differences have been attributed to the ionic nature of GdmCl. GdmCl is an electrolyte with a pK_a of about 11, which means that at pH values below this the GdmCl molecule will be present in a fully dissociated form, i.e., as Gdm^+ and Cl^-. The presence of these ions would influence the stabilizing properties of proteins/enzymes. Mayr and Schmid (*31*) studied the effect of GdmCl and NaCl on the thermostabilities of RNase T1. Addition of 0.1−1 M NaCl or 0.1 M GdmCl resulted in an increase in the T_m of RNase T1; however, the enhancement in thermostability for GdmCl was significantly smaller than that for NaCl. These observations were interpreted in terms of stabilization by cation (Na^+ and Gdm^+) binding to the negatively charged moieties of the RNase T1 molecule.

We have earlier demonstrated that binding of monovalent cations to GOD induces a compaction in the native conformation of the enzyme with an enhanced stability against thermal and urea denaturation (*22*). The comparative studies with GdmCl and NaCl on GOD, as reported above, helped to clarify the mechanism of stabilization of GOD by low concentrations of GdmCl. NaCl stabilizes GOD against thermal denaturation presumably in a 2-fold manner. Binding to one or few sites with fairly high affinity leads to a strong increase in stability of the enzyme between 0 and 0.5 M salt. Additional weak interactions and/or indirect, solvent-mediated effects lead to a continuing but smaller increase in stability at higher salt concentrations (between 0.5 and 2 M) (*22*). Denaturation studies (both thermal and urea, Figures 3 and 4) indicate that GOD is indeed stabilized by NaCl or low concentrations of GdmCl, and hence the mechanism of stabilization of GOD by these molecules is closely related and mutually exclusive, probably due to cation binding. The suppression of the stabilizing effect of GdmCl on thermal denaturation of GOD by addition of about 0.5 M NaCl (Figure 3) provides strong support for this suggestion. Hence, it seems that at low GdmCl concentrations the strong stabilization by Gdm^+ cation binding to negatively charged sites in GOD dominates and compensates the general denaturation effect of GdmCl. A stabilization of proteins by ionic denaturants could be a fairly common process, because proteins frequently contain ion binding sites of varying affinity and specificity. However, such additional stabilization is not easily detected in single GdmCl-induced unfolding experiments, since the denaturing effect clearly dominates in the region of the cooperative transition. It can, however, be detected easily by a simple direct experimental approach where the thermal transition of the protein is measured in the presence of small concentrations of denaturants. The denaturing effect of GdmCl on folded proteins increases linearly with concentration as observed for GOD, at higher GdmCl concentrations (this paper), and various other proteins (*31*). These different stabilizing and destabilizing effects of GdmCl on GOD are apparently additive and lead to the observed complex dependence of the GdmCl concentration on the stability.

GdmCl is believed to be a much better denaturant than urea, implying that lower concentrations are required to unfold a protein by GdmCl than urea. For most monomeric proteins, GdmCl has been found to be approximately 2.3 times as effective a denaturant as urea, since for these proteins unfolding in urea occurs at a concentration twice that required in the case of GdmCl; this is suggestive of a "2-fold rule" (*33*, *34*) (Table 1). Multimeric proteins, on the other hand, have a higher ratio [C_m(urea)/ C_m(GdmCl)] (Table 1), which suggests that multimeric proteins are much more susceptible to GdmCl denaturation. Although for proteins with non-two-state transitions the C_m value may not be an accurate measure of the stability to the chaotrope, however, it does provide some indications for the differences in the interactions of the denaturants with different classes of proteins. Interestingly, the results presented above show that GOD is certainly an exception to the 2-fold rule of urea and GdmCl denaturation mentioned above. Figures 1D and 2D present the results of denaturation of GOD at pH 6.5 as monitored by the change in enzymatic activity and FAD fluorescence. The midpoint of the transition for urea unfolding was 3.65 M, and that for GdmCl was 3 M (as observed by FAD fluorescence spectroscopy). For small monomeric proteins violation of the 2-fold rule of urea and GdmCl denaturation has been observed in some cases, such as RNase T1 and ubiquitin (Table 1), but GOD seems to be the first multimeric protein for which such an observation is being reported.

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