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Effect of Trifluoroethanol on Native and Acid-Induced States of Glucose Oxidase from *Aspergillus niger*

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Abstract—We have studied the effect of trifluoroethanol (TFE) on the native (pH 7.0), acid unfolded (pH 2.6), and molten globule (pH 1.4) states of glucose oxidase (GOX) by circular dichroism and fluorescence spectroscopy. In the presence of 50% TFE, at pH 7.0 and 2.6, GOX exhibited a transition from native coiled-coil and acid unfolded state to non-associating α-helical state. Interestingly, at pH 1.4, 15% TFE induced the formation of β-structured intermediate by loss of 1-anilino-8-naphthalenesulfonate binding site and almost all tertiary contacts. The β-structured intermediate converted into open helical conformation on further addition of TFE.

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Key words: glucose oxidase, molten globule, coiled-coil state, acid unfolded state, trifluoroethanol

Glucose oxidase (GOX) (α-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) from *Aspergillus niger* is a dimeric enzyme of molecular mass 160 kDa [1]. The holoenzyme is made up of two identical 80 kDa subunits. The monomers are connected noncovalently via a long but narrow contact area. There are 120 contact points between the dimers centered on 11 residues, which form either salt bridges or hydrogen bonds. The monomeric molecule is a compact spheroid with approximate dimensions 60 × 52 × 37 Å. The monomer folds into two structural domains. One of the domains binds FAD and the other is involved in substrate binding. The subunit contains one disulfide bridge. The corresponding dimensions of the dimer are 70 × 55 × 80 Å. The minimum distance between the flavin and the surface of the monomer is 13 Å. The two isoalloxazine moieties are separated by about 40 Å, a distance which excludes any electrical communication between them [2]. It is an acidic protein having a net negative charge of –77 at neutral pH [3], which is mainly due to the presence of side chain carbohydrate groups present in the amino acids [2]. Under denaturing conditions, the subunits of GOX dissociate accompanied by release of FAD molecules [4, 5].

Glucose oxidase catalyses the oxidation of β-D-glucose to D-glucono-1,5-lactone and hydrogen peroxide, using molecular oxygen as the electron acceptor. The initial product of glucose oxidation is D-glucono-1,5-lactone. This is a weak competitive inhibitor of GOX, which hydrolyses spontaneously to gluconic acid. This has made it an important industrial enzyme. Industrially, it has found applications in the removal of glucose or oxygen from food products and in the production of gluconic acid. The most important application of GOX is as a molecular diagnostic and analytical tool as the enzyme is used in biosensors for the quantitative determination of D-glucose in body fluids, foodstuffs, beverages, and fermentation products. Discovery of antibiotic properties of GOX due to peroxide formation has increases the interest of biochemists in the enzyme [6-10].

Understanding structural and folding behavior of GOX in various solvent conditions might provide additional insight into the function of the protein. The effects of alcohols on proteins and peptides are useful for considering how specific structures of proteins are stabilized in aqueous environments. Alcohols are known to disrupt tertiary and quaternary contacts by weakening hydrophobic interactions and strengthening α-helical propensities

Abbreviations: ANS, 1-anilino-8-naphthalenesulfonate; CD, circular dichroism; GdnHCl, guanidine hydrochloride; GOX, glucose oxidase; MG, molten globule; MRE, mean residue ellipticity; TFE, 2,2,2-trifluoroethanol; U_A, acid-induced unfolded state.

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by promoting local polar (hydrogen bonding) interactions [11-14]. They are also found to disrupt coiled-coil interactions of helical proteins and thus stabilize non-interacting α-helices [15]. Thus, alcohols, mainly fluorinated alcohols such as 2,2,2-trifluoroethanol (TFE), have been used to transform native and denatured proteins into molten globule-like intermediates [11, 16, 17], which are believed to be identical to the partially folded conformations transiently accumulated in the early stage of folding and in *in vivo* folding [18]. Very little information about the folding/unfolding aspects of GOX is currently available. The process of thermal denaturation of GOX is irreversible and results in the loss of secondary and tertiary structure, leading to the unfolding and nonspecific aggregation of the enzyme molecules because of hydrophobic interactions of side chains [19]. The urea-induced unfolding of GOX is a two-state process with dissociation and unfolding of the native dimeric enzyme molecule occurring in a single step. In contrast, the guanidine hydrochloride (GdnHCl)-induced unfolding of GOX is a multiphasic process with stabilization of a conformation more compact than the native enzyme at low GdnHCl concentrations and dissociation along with unfolding of the enzyme at higher concentrations of GdnHCl [20, 21].

The present study describes the effect of increasing concentration of TFE on the structural parameters of GOX at pH 7.0, 2.6, and 1.4. These pH values were chosen for the study because GOX exists in native, acid unfolded, and molten globule states at these pH values as reported from our laboratory earlier [22].

MATERIALS AND METHODS

Materials. Glucose oxidase from *Aspergillus niger*, 1-anilinonaphthalene-8-sulfonate (ANS), and TFE were obtained from Sigma (USA). All other chemicals used in this study were of analytical grade.

Commercial preparations of glucose oxidase were further purified to homogeneity by gel filtration chromatography. Glucose oxidase (GOX) thus obtained was found to be homogeneous with respect to size and charge as judged by gel filtration chromatography and polyacrylamide gel electrophoresis. These preparations were used throughout the study.

Concentration of the protein was determined from the value of specific extinction coefficient ($A^{1\%}_{1cm}$ = 13.8) by measuring the absorbance of protein solution at 280 nm [22] on a Hitachi U-1500 spectrophotometer (Japan). ANS concentration was also determined spectrophotometrically using a molar absorption coefficient of 5000 $M^{-1}\cdot cm^{-1}$.

Preparation of different states of GOX. The native, acid unfolded, and molten globule states of GOX were produced by mixing 20 μl of GOX stock solution (250 μM) with 980 μl of 60 mM sodium phosphate (pH 7.0), 10 mM glycine-HCl (pH 2.6), 10 mM glycine-HCl (pH 1.4) buffers at room temperature as described earlier [22]. pH was measured using an Elico LI 610 digital pH meter using a PPC combined electrode, type CL-51, consisting of glass and reference electrodes in a single entity. The least count of the pH meter was 0.01 pH unit.

TFE-induced conformational transition of GOX states. Stock protein solutions of pH 7.0, 2.6, and 1.4 were prepared in the respective buffer solutions to be 10 times the required final protein concentration. Different volumes of buffers, TFE, and 20 μl of protein stock solution were mixed to give a final volume of 1 ml. This yielded final TFE concentration from 0 to 50% (v/v) and desired final protein concentration. Each sample was mixed by vortexing and was incubated overnight before spectroscopic measurements.

Fluorescence measurements. Fluorescence measurements were performed on a Shimadzu RF-540 spectrofluorimeter equipped with a DR-3 data recorder. The fluorescence spectra were measured at 25 ± 0.1°C with a 1 cm pathlength cell. The excitation and emission slits were set at 5 and 10 nm, respectively. Intrinsic fluorescence was measured by exciting the protein solution at 280 or 295 nm, and emission spectra were recorded in the range of 300-400 nm. For ANS fluorescence in the ANS binding experiments, the excitation wavelength was set at 380 nm and the emission spectra were taken in the range of 400-600 nm or at fixed wavelength of 480 nm. For FAD fluorescence, the excitation wavelength was set at 365 nm and the emission spectra were taken in the range of 500-600 nm or at fixed wavelength of 520 nm.

Circular dichroism measurements. Circular dichroism (CD) was measured with a Jasco J-720 spectropolarimeter equipped with a microcomputer. The instrument was calibrated with d-10-camphorsulfonic acid. All the CD measurements were made at 25°C with a thermostatically controlled cell holder attached to a Neslab RTE-110 water bath with an accuracy of ±0.1°C. Spectra were collected with scan speed of 20 nm/min and response time of 1 sec. Each spectrum was the average of 3-4 scans. Far and near-UV CD spectra were obtained with 1- and 10-mm pathlength cells, respectively. The results are expressed as MRE (mean residue ellipticity) in $deg\cdot cm^2\cdot dmol^{-1}$, which is defined as $MRE = \theta_{obs}/(10nlCp)$, where θ_{obs} is the CD in millidegree, n is the number of amino acid residues, l is the path length of the cell, and Cp is mole fraction. Helical content was calculated from the MRE values at 222 nm using the following equation:

$$\%\ \alpha\text{-helix} = [(MRE_{222} - MRE_{min})/MRE_{max}] \times 100,$$

where MRE_{222} is the molar ellipticity of the protein, MRE_{min} (2340) is the minimum value of molar ellipticity at 222 nm calculated for the unordered fraction of five proteins, and MRE_{max} (30,300) is the maximum value for

the ellipticity at 222 nm as measured for the helical fraction of the five proteins [23].

RESULTS

Far-UV CD. The GOX from *A. niger* is known to exist in native, acid unfolded, and molten globule states at pH 7.0, 2.6, and 1.4, respectively [22]. The effect of TFE on the far-UV CD spectra of native (pH 7.0), acid unfolded (pH 2.6), and molten globule (pH 1.4) states of GOX are shown in Fig. 1, demonstrating a pattern of formation of secondary structures with increasing concentration (0-50%) of TFE (v/v). Far-UV CD spectra of the each of the three states of GOX revealed troughs at 222 and 208 nm typical of a helical structure as reported earlier [22]. TFE is known to stabilize helical structure and disrupt tertiary and quaternary interactions that are stabilized by hydrophobic forces [11-14]. Changes in the ellipticity at 222 nm and the ratio of the ellipticities at 222 and 208 nm (MRE_{222}/MRE_{208}) are useful probes for visualizing varying helical content and the presence of non-interacting and interacting α-helixes, respectively [15]. For a non-interacting α-helix, the ratio MRE_{222}/MRE_{208} has been found to be between 0.8 and 0.9, while for two-stranded coiled-coils the ratio was calculated to be 1 ± 0.03 [24-29]. In accordance, the effect of increasing concentrations of TFE on MRE at 222 nm and the ratio MRE_{222}/MRE_{208} are presented in Fig. 2.

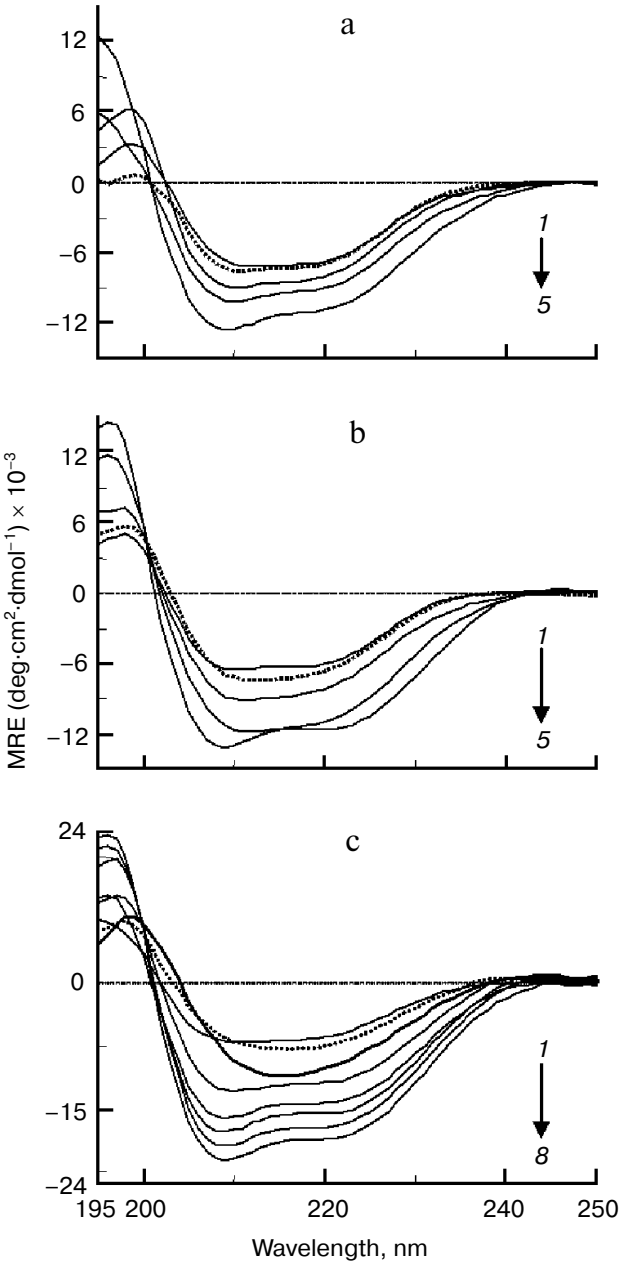


Fig. 1. Far-UV CD spectra of GOX (a) at pH 7.0 in the presence of 0, 20, 30, 40, and 50% TFE (*1-5*, respectively), (b) at pH 2.6 in the presence of 0, 25, 30, 40, and 50% TFE (*1-5*, respectively), and (c) at pH 1.4 in the presence of 0, 10, 15, 20, 25, 30, 40, and 50% TFE (*1-8*, respectively). Dashed lines correspond to curves *1*.

At pH 7.0, the MRE_{222} and MRE_{222}/MRE_{208} ratio of GOX were −6717 deg·cm^2·dmol^{-1} and 1.08, respectively, indicating around 15% interacting α-helices. A small decrease in the ellipticity at 222 nm (11.1%) and the ratio MRE_{222}/MRE_{208} (from 1.08 to 0.92) was noted upon titration of the native protein with TFE up to 15% (Fig. 2a, filled circles). However, above 20% TFE, an increase in the ellipticity (~41%) with decrease of the ratio MRE_{222}/MRE_{208} (to 0.82) was observed up to 50% TFE, indicating formation of non-interacting helices. As can be seen from Fig. 2a (open squares), an increase in the ellipticity of acid unfolded GOX by 119% was observed upon addition of the TFE up to 50%. The increase in MRE_{222} of acid-induced unfolded state (U_A) was noted only above 20% TFE concentration. MRE_{222} remained essentially constant between 0-20% TFE concentration. The ratio MRE_{222}/MRE_{208} at 0 to 30% TFE concentration increased from 0.89 to 0.96 indicative of increase in helix interaction. However, at 50% TFE the ratio decreased to 0.88 indicating re-dissociation of the helices.

Interestingly, addition of TFE up to 15% appears to have the opposite effect on the backbone conformation of the molten globule state of GOX that exists at pH 1.4. As can be seen from Fig. 1c, the far-UV CD spectrum of GOX at pH 1.4 in 15% TFE shows a 217 nm CD band indicative of formation of β-sheet conformation. Abrupt increase of the MRE_{222}/MRE_{208} ratio to 1.3 in 15% TFE (Fig. 2b) provided further evidence of α-helix to β-sheet conformational transition of the molten globule (MG) state. The increase in the MRE_{222}/MRE_{208} ratio was due to appearance of a more prominent minimum at 217 nm and loss of the minimum at 208 nm. However, increase in TFE concentration above 15% TFE re-induced the 208 and 222 nm bands characteristic of α-helical conformation. The intensities of these bands increased (Figs. 1c

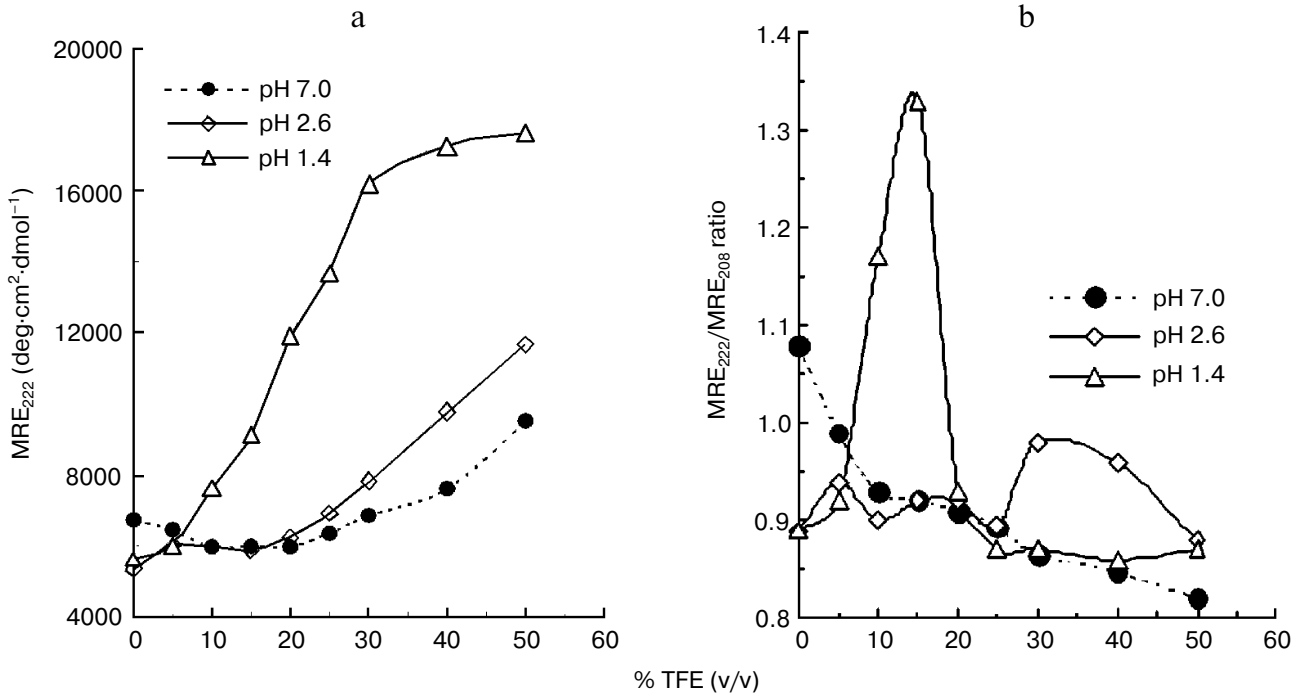


Fig. 2. Effect of increasing concentration of trifluoroethanol on native, acid unfolded, and molten globule states of GOX as monitored by changes in mean residue ellipticities at 222 nm (a) and MRE_{222}/MRE_{208} ratio (b).

and 2a, opened triangles) on further addition of TFE up to 30%; above 30% TFE no increase in ellipticity was observed, suggesting a saturable build-up of helical conformation in the molten globule state of GOX. Based on these results, it appears that TFE-induced backbone conformation transition of GOX is highly pH and/or structure dependent.

Near-UV circular dichroism. Near-UV CD spectra of the native state showed a prominent positive CD band at 273 nm (Fig. 3a). In the acid unfolded state, there was a decrease of signals in the entire region from 320 to 250 nm; nonetheless, the protein retained all the features of native proteins. Interestingly, at pH 1.4 the near-UV CD spectra approached the native state, suggesting regain of tertiary contacts. Solid lines of Fig. 3 (a-c) represent near-UV CD spectra of native, acid unfolded, and molten globule states of GOX. Since all the states retained the characteristic CD band at 273 nm, TFE-induced changes in tertiary contacts were monitored by measurement of MRE at 273 nm (Fig. 4). No change in the MRE at 273 nm was observed with native state up to 40% TFE. At 50% TFE, only an around 16% decrease in the MRE value was observed without any loss of characteristic spectral features. Acid-denatured GOX seems to regain ellipticity of native state at 5% TFE and it remained constant up to 25% TFE. Appearance of tertiary interaction might be due to mimicking of hydrophobic surfaces brought about by TFE, because TFE not only disrupts tertiary structure, but the hydrophobic surface areas brought about by it can mimic non-native or possibly even native-like tertiary interactions as well [30]. Above 25% TFE, a gradual loss of MRE values at 273 nm was noted suggesting loss of tertiary structure only above 25% TFE. On the other hand, the molten globule state at pH 1.4 showed a sigmoidal decrease in the MRE at 273 nm with increasing concentration of TFE. The transition was found to start at 10% TFE and was completed around 25% TFE. A comparison of near-UV CD spectra of the protein at pH 1.4 in the presence of 50% TFE and GdnHCl-denatured GOX showed that the MG state had lost almost all its tertiary contacts.

Intrinsic fluorescence. Figure 5 shows the dependence on TFE concentration of the fluorescence intensity at 340 nm and the wavelength of maximum emission (λ_{max}), respectively. With an increase in TFE concentration, a small red shift in λ_{max} (2 nm) with a slight decrease of relative fluorescence intensity of native GOX was observed. These findings evidently agree with the TFE-induced near-UV CD results of the native protein, which showed only small changes in the tertiary structure up to 50% TFE. The acid unfolded state of GOX showed essentially no change in λ_{max} of emission and relative fluorescence intensity at 340 nm in the range 0-20% TFE concentrations. At TFE concentrations above 20%, relative fluorescence intensity at 340 nm increased by ~43% with a red shift of 5 nm from 336 to 341 nm (Fig. 5, open rectangles) up to 50% TFE. On the other hand, the molten globule state at pH 1.4 showed a gradual red shift of 13 nm

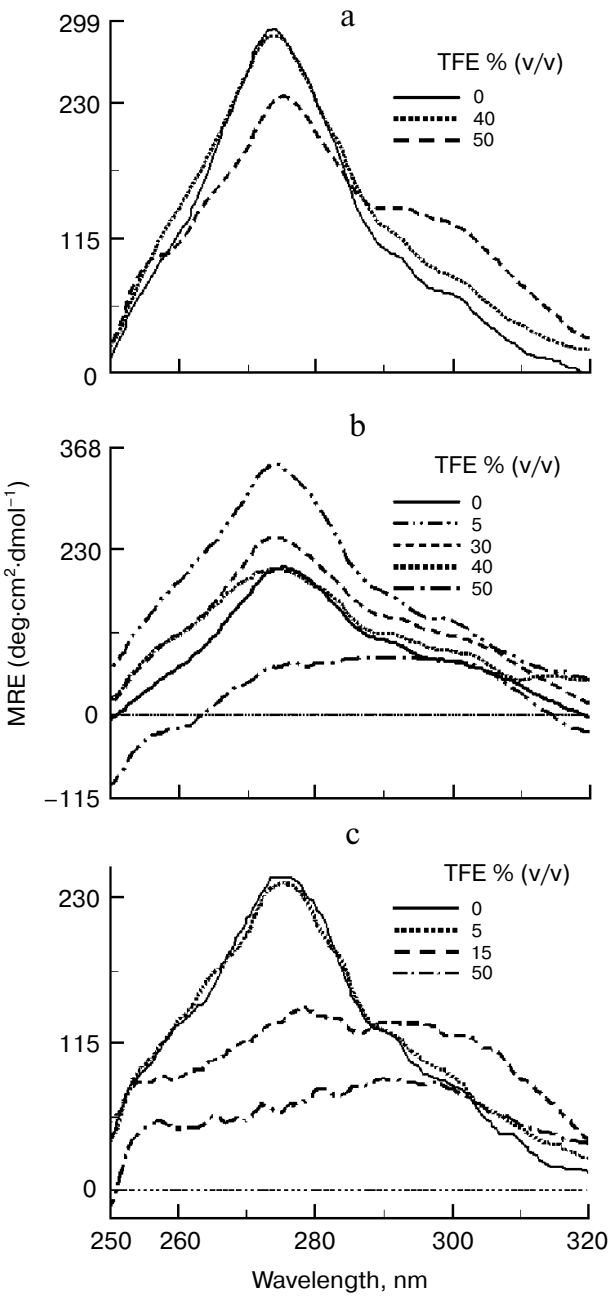


Fig. 3. Near-UV CD spectra of GOX (a) at pH 7.0 in the presence of 0, 40, and 50% TFE, (b) at pH 2.6 in the presence of 0, 5, 30, 40, and 50% TFE, and (c) at pH 1.4 in the presence of 0, 5, 15, and 50% TFE.

of the maximal wavelength (Fig. 5b, open triangles) in the TFE concentration range 0-30%. No change in the λ_{max} of emission was observed above 30% TFE, which is suggestive of maximal effect of TFE on the conformation of the protein at pH 1.4. At pH 1.4, the TFE-induced transition as measured by relative fluorescence intensity at 340 nm (Fig. 5b, open triangles) is clearly biphasic. As TFE was added, there was an increase (~80%) in relative fluorescence intensity between 0 to 15% TFE and then there was a decrease in intensity as the TFE concentration was further increased up to 50%.

ANS binding. Changes in ANS fluorescence are frequently used to detect intermediate conformations of globular proteins [31]. ANS fluorescence intensity at 480 nm increased markedly beyond 25% TFE and was maximal (~12 times compared to native protein) in the 40-50% TFE concentration range (Fig. 6, filled circles). This result suggests the presence of a large number of solvent accessible nonpolar clusters in the protein molecules in the 40-50% of TFE. On the other hand, a sigmoidal decrease of ANS fluorescence with the protein states at pH 2.6 and 1.4 was observed indicating cooperative loss of exposed hydrophobic clusters of these states in TFE. The decrease in ANS binding with the protein state at pH 1.4 in the presence of 40% TFE was comparable to that in 6 M GdnHCl unfolded state of GOX, while protein states at pH 2.6 retained a significant amount of ANS binding ability in the mentioned concentration range of TFE.

FAD fluorescence. Glucose oxidase contains one tightly, noncovalently bound FAD cofactor per monomer [2]. The FAD group has been used as a natural marker to probe the dynamic microenvironment of the flavin fluorophore in flavoproteins [32, 33]. Association/dissociation of FAD molecules to/from the protein can be used to probe the tertiary and/or quaternary state of the protein as the FAD fluorescence is significantly quenched for native dimeric GOX and a large enhancement in FAD fluorescence is observed on denaturation of the enzyme

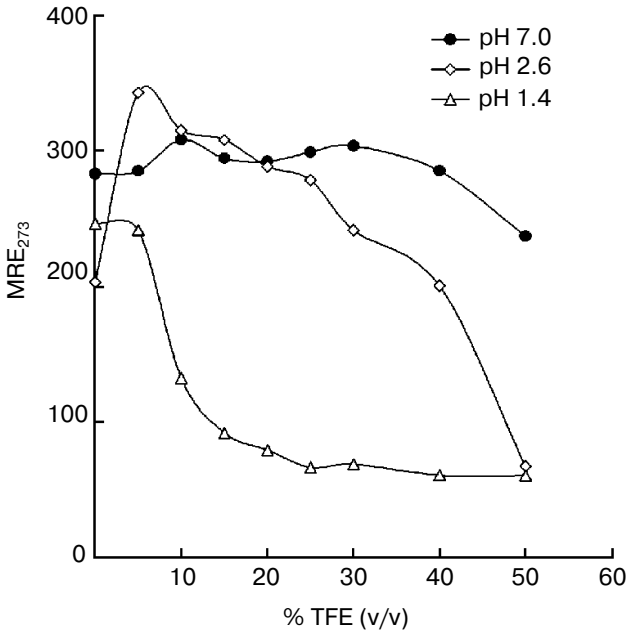


Fig. 4. Effect of increasing concentration of TFE on native, acid unfolded, and molten globule states of GOX as monitored by changes in MRE at 273 nm.

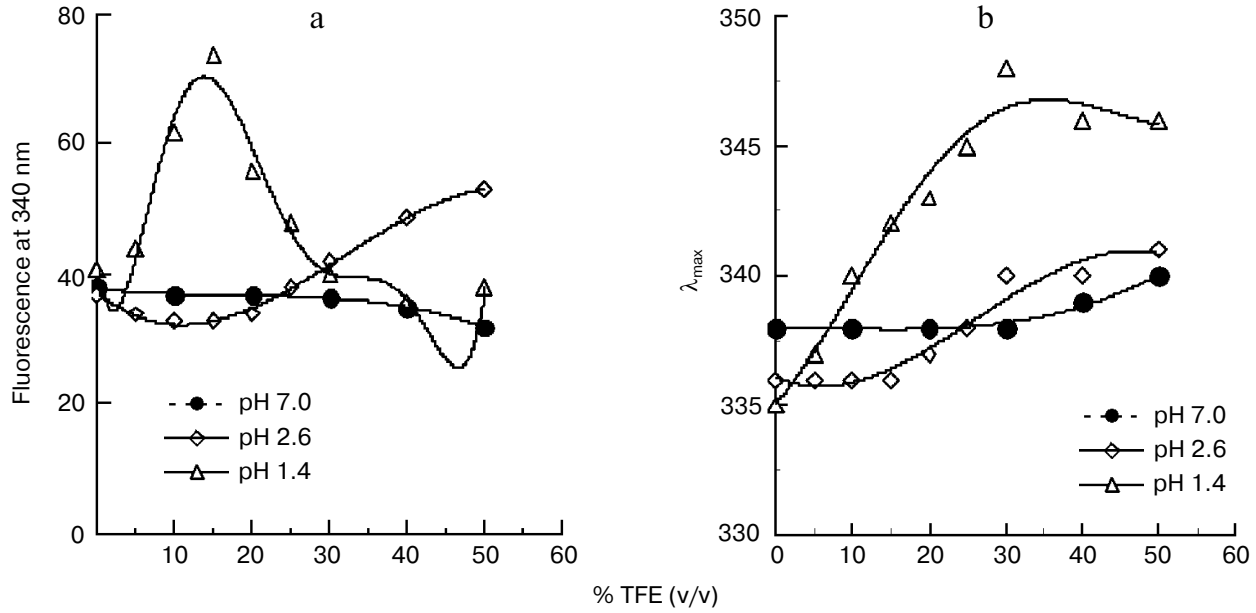


Fig. 5. Effect of increasing concentration of TFE on tryptophan fluorescence of GOX states. The protein was excited at 296 nm, and the relative fluorescence intensity at 340 nm (a) and maximum wavelength of emission (λ_{max}) (b) were measured.

[34, 35]. Figure 7 shows the changes in FAD fluorescence intensity at 520 nm of native, acid-denatured, and molten globule states of GOX on incubation with increasing concentrations of TFE. Incubation of native and acid-denatured GOX up to 30 and 20% TFE, respectively, did not show a significant change in the FAD fluorescence intensity. Above these concentrations of TFE, fluorescence intensity of both states increase gradually up to 50% TFE. However, a continuous increase of FAD fluorescence of molten globule state was noted in the TFE concentration range 0-30%, and no significant enhancement was observed thereafter. At 50% TFE the enhancements in the FAD fluorescence intensity of acid-denatured and molten globule states were found to be more as compared to the native state. It is known that under conditions where FAD is released from the enzyme, the dissociation

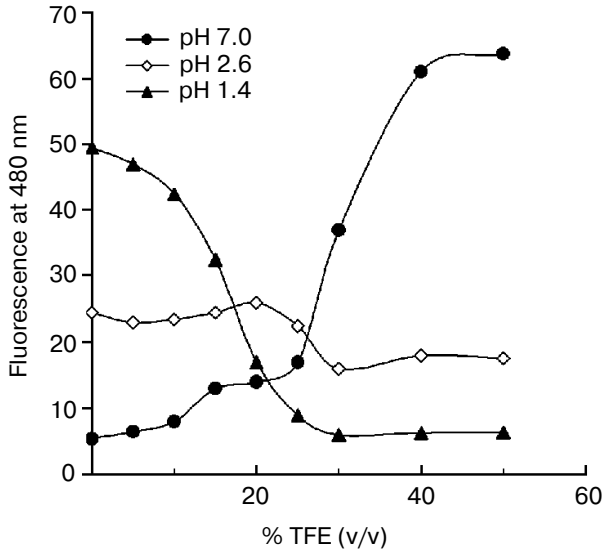


Fig. 6. Effect of increasing concentration of TFE on the ANS fluorescence intensity at 480 nm of GOX states with excitation of the ANS–protein complex at 380 nm.

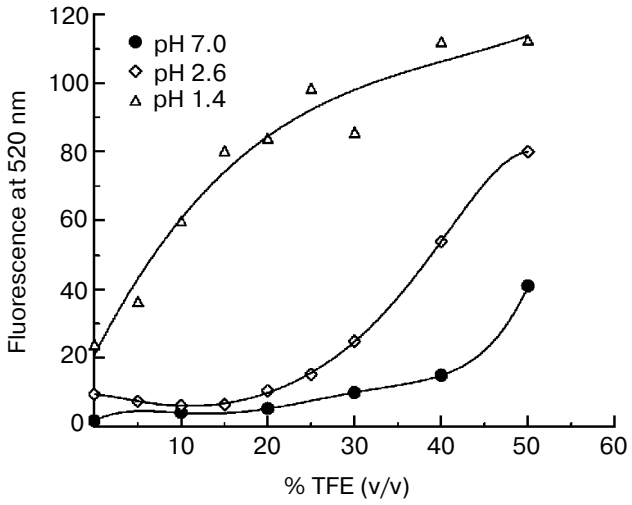


Fig. 7. Effect of increasing concentration of TFE on the FAD fluorescence intensity at 520 nm of GOX states with excitation of the FAD at 365 nm.

of dimer into monomer also takes place [4, 34]. Therefore, it appears from the above results that 50% TFE-induced state of GOX at pH 1.4 can exist in monomeric state, because maximum dissociation of FAD has been observed in this case.

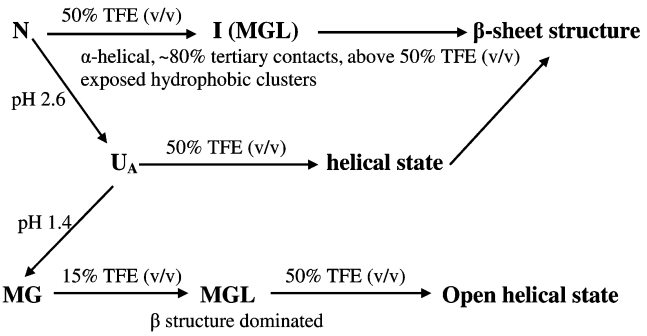
DISCUSSION

Effect of TFE on native GOX at pH 7.0. In the concentration region 0-30%, TFE was found to disrupt the coiled-coil state of native dimeric GOX without any significant change in the far-UV CD spectrum between 250 and 200 nm. Tertiary structure of the enzyme as probed by near-UV CD and intrinsic fluorescence measurement, binding of hydrophobic dye ANS, and bound status of cofactor FAD were also found to be unaffected in this TFE concentration region. Beyond 30% TFE, formation of non-interacting α-helix was observed and the maximum percentage of helix induced was 41%. A slight red shift (2 nm) with a small decrease in intensity of tryptophan fluorescence, about 16% decrease of MRE value at 273 nm, and significant dissociation of FAD were observed at 50% TFE, suggesting small but obvious loss of tertiary contacts. ANS, a hydrophobic dye with a greater affinity for the molten globule-like state shows maximum binding at 50% TFE. Taken together, it appears that native GOX exists as an α-helical intermediate around 50% TFE that has a significant amount of exposed hydrophobic clusters characteristic of a molten globule state. That ANS binds in a saturable manner together with high propensity of the state to aggregation on addition of more TFE provide clear evidence of its intermediate conformation.

Effect of TFE on the acid-denatured GOX at pH 2.6. Like native state, induction of α-helix in the acid-denatured state is cooperative and non-saturable, but slightly more helix is induced compared to the native state. Probes for tertiary structure (near-UV CD, intrinsic fluorescence, and FAD fluorescence) indicated a significant loss of tertiary contacts only at high concentration of TFE. Formation of non-native tertiary contacts were also observed at low concentration of TFE (5%) that remained unaffected up to 20% TFE. Binding of ANS was also found unchanged in the concentration range 0 to 20%. This suggests that formation of tertiary contacts as shown by the near-UV CD spectrum in the concentration range 0-20% might be due to local interactions of side chains, as TFE is known to induce local nonspecific interactions of side chains [34]. Decrease of ANS binding above 20% TFE might be due to disruption of hydrophobic clusters present in the acid-unfolded state of GOX. Thus, around 50% TFE, acid-unfolded state of GOX exists as an open helical coil.

Effect of TFE on the MG state of GOX obtained at pH 1.4. The far-UV CD spectrum of the MG state of GOX at pH 1.4 is representative of α-helical conformation. The far-UV CD spectrum of GOX at pH 1.4 in the presence of 15% TFE shows a 215 nm CD band, suggesting α-helix to β-sheet transition in the TFE concentration range 5-15%. Probes for tertiary structure (near-UV CD, intrinsic fluorescence) show almost complete loss of tertiary contacts. Loss of ANS binding ability and release of FAD molecules are also maximal at this concentration. Beyond 20% TFE concentration, the 208 and 222 nm CD bands characteristic of helical conformations are reinduced with further loss of tertiary structure. At 40-50% TFE concentration, maximal helix content was induced, which was almost twice that observed in either the native state or the acid-unfolded state in the presence of same concentration of TFE.

The effect of TFE on the different states of GOX can be summarized by the following scheme.



To understand the phenomenon of protein folding, all conformational states should be described with respect to their structure, because such conformational states might resemble intermediate states along the *in vivo* protein-folding pathway and thus play an important role in understanding the mechanism of protein folding [18]. Concerning the present case studies, formation of different intermediate states (molten globule like state in the presence of 50% TFE at pH 7.0, β-structured intermediate in the presence of 15% TFE at pH 1.4, and open helical intermediates in the presence of 50% TFE at pH 2.6 and 1.4) suggest that folding is not a unique process and might possibly involve more than one pathway, as postulated by the new view of protein folding. Concerning the process of protein folding in cells, the TFE-denatured state might be just as important as GdnHCl- or urea-induced states, because a similar hydrophobic environment as in the presence of TFE does exist for a protein during translocation through a membrane [36].

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