J. Biochem. **141,** 573–583 (2007)
doi:10.1093/jb/mvm057

Different Molten Globule-like Folding Intermediates of Hen Egg White Lysozyme Induced by High pH and Tertiary Butanol

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Received December 9, 2006; accepted February 7, 2007; published online February 16, 2007

We have provided evidence that hen egg white lysozyme (HEWL) existed in α helical and β structure dominated molten globule (MG) states at high pH and in the presence of tertiary butanol, respectively. Circular dichroism (CD), intrinsic fluorescence, ANS binding and acrylamide-induced fluorescence quenching techniques have been used to investigate alkali-induced unfolding of HEWL and the effect of tertiary butanol on the alkaline-induced state. At pH 12.75, HEWL existed as molten globule like intermediate. The observed MG-like intermediate was characterized by (i) retention of 77% of the native secondary structure, (ii) enhanced binding of ANS (∼5 times) compared to native and completely unfolded state, (iii) loss of the tertiary structure as indicated by the tertiary structural probes (near-UV, CD and Intrinsic fluorescence) and (iv) acrylamide quenching studies showed that MG state has compactness intermediate between native and completely unfolded states. Moreover, structural properties of the protein at isoelectric point (pI) and denatured states have also been described. We have also shown that in the presence of 45% tertiary butanol (*t*-butanol), HEWL at pH 7.0 and 11.0 (pI 11.0) existed in helical structure without much affecting tertiary structure. Interestingly, MG state of HEWL at pH 12.7 transformed into another MG state (MG2) at 20% *t*-butanol (v/v), in which secondary structure is mainly β sheets. On further increasing the *t*-butanol concentration α helix was found to reform. We have proposed that formation of both α helical and β sheet dominated intermediate may be possible in the folding pathway of α + β protein.

Key words: alkali induced unfolding, circular dichroism, fluorescence quenching, lysozyme, molten globule.

Abbreviations: ANS, 8-anilinonaphthalene-1-sulphonic acid; CD, circular dichroism; GnHCl, guanidinium hydrochloride; HEWL, hen egg white lysozyme; MG, molten globule; MRE, mean residual ellipticity; PFI, partially folded intermediate; pI, isoelectric point; RFI, relative fluorescence intensity; *t*-butanol, tertiary butanol; TFE, tri-fluoro ethanol; U_{alk}, alkali unfolded state; UV, ultra violet.

Proteins are known to unfold/refold through different denatured states. It is crucial to know the differences in residual structure between different denatured states along the pathway of folding. It is thought that such denatured states will provide useful information in understanding the mechanism of protein folding reaction. One such intermediate state know as 'molten globule' (MG) has attracted much attention in recent years because it is believed to be identical to the partially folded conformation transiently accumulated in the early stage of folding (*1*) and in *in vivo* folding (*2*, *3*). The MG is a state of the protein possessing native like 'format' with no global tertiary structure. The common structural characteristics of MG include: (i) the presence of pronounced amount of secondary structure, (ii) the absence of most of the specific tertiary structure produced by the tight packing of side chains and (iii) the presence of loosely packed hydrophobic core that increases the hydrophobic surface accessible to solvent (*4–6*). Recent evidence, however, suggests that MG may also possess well-defined tertiary contacts (*7–9*).

The hen egg white lysozyme (HEWL) belongs to the α + β class of proteins. It consists of two domains, an alpha domain, comprised of residues 1–36 and 87–129 and a beta domain consisting of residues 37–86 (*10*, *11*). Previous studies with many proteins have revealed the existence of intermediates with MG like characteristics at low pH. In acid denaturation intermolecular charge repulsion is a driving force for unfolding. As discussed earlier (*12*), protonation of all ionizable side chains below pH 3.0 leads to charge–charge repulsion and consequent unfolding of the protein. Further decrease in pH has no effect on the ionization state of the protein. On the other hand, increase in anion concentration leads to refolding to an A-state (*13*). On the basis of conformational states of proteins under condition of acid induced denaturation, Fink *et al*. (*12*) have classified the proteins into three major types. Type I proteins initially unfold in the vicinity of pH 3–4 and when the pH is further decreased they refold to a MG like conformation. Type II proteins do not fully unfold but directly transform to the MG states.

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Type III proteins do not unfold even at pH as low as 1. Lysozyme belongs to type III class of proteins which also include T4 lysozyme, ubiquitin, chicken lysozyme, chymotrypsinogen, protein A, β-lactoglobulin and concanavalin A.

One of the best studied cosolvents that modify protein structure is alcohol. Alcohols are known to weaken nonlocal hydrophobic interactions while promoting local polar interactions (*14*, *15*). Therefore, in many cases, alcohol induced denaturation is accompanied by stabilization of the extended helices in which hydrophobic side chains are exposed but the polar amide groups are shielded from the solvent (*16*, *17*). A recent report describes the existence of a native-like β structure in the partially folded state of tendamistat induced by TFE (*18*). Moreover, alcohols induce significantly higher helical structure in a partially or completely unfolded protein as compared to folded protein (*19*).

Based on the above information on structural behaviour of the proteins in low/high pH and alcohols, we here report the conformational behaviour of HEWL in alkaline pH region. A detailed investigation on the effect of tertiary butanol (*t*-butanol) on HEWL at pH 7.0, 11.0 [isoelectric point (pI)] and 12.75 (MG) state has been performed. We have identified and characterized the partially folded states of HEWL to examine the generality of the existence of intermediate conformational states of lysozyme, which can provide significant insight into the nature of protein folding pathway and the stability of the protein in alkaline and organic conditions.

MATERIALS AND METHODS

Materials—HEWL and guanidinium hydrochloride (GnHCl) were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Acrylamide was purchased from Qualigens Fine Chemicals (India). All other reagents and buffer components were of analytical grade.

Methods—Protein concentration determination

Protein concentrations were determined spectrophotometrically on a Hitachi U-1500 spectrophotometer using an extinction coefficient $E_{278}^{1\%} = 26.4$ (11) or alternatively by the method of Lowry *et al*. (*20*).

pH measurements

pH measurements were carried out on an Elico digital pH meter (model LI610).

Denaturation studies

HEWL solutions were prepared in buffers 20 mM each of different pH values ranging from pH 5 to 13.4. (pH 7–8, sodium phosphate buffer; pH 8–12.0, glycine–NaOH buffer, above 12 pH was monitored by NaOH). Before making measurements the solutions were incubated for 24 h at room temperature.

CD measurements

Circular dichroism (CD) measurements were carried out on a Jasco spectropolarimeter, model J-720, equipped with a microcomputer. The instrument was calibrated with D-10-camphorsulphonic acid. All the CD measurements were carried out at 25°C with a thermostatically controlled cell holder attached to a Neslab RTE-110 water bath with an accuracy of ± 0.1°C. Far-ultra violet (UV) CD spectra measurements were carried at a protein concentration of 3.0 μM and near-UV CD spectra were recorded at protein concentration of 30 μM. The path length was 1 mm and 1 cm, respectively. The results are expressed as mean residual ellipticity (MRE) in deg cm^2dmol^{-1} defined as

$$\mathrm{MRE} = \frac{\theta_{\mathrm{obs}}(\mathrm{mdeg})}{(10 \times \mathrm{n} \times \mathrm{Cp} \times \mathrm{l})}.$$

where θ_{obs} is the CD in millidegree; $n = 129$ (number of amino acid residues); l is the path length of the cell in centimeter and Cp is the molar fraction. The α-helical content of HEWL was calculated from the MRE values at 222 nm using the following equation as described by Chen *et al*. (*21*).

$$\%\mathrm{helix} = \left(\frac{\mathrm{MRE}_{222\,\mathrm{nm}} - 340}{30300}\right) \times 100$$

Fluorescence measurements

Fluorescence measurements were performed on a Shimadzu spectrofluorometer, model RF-540. For intrinsic fluorescence measurements, the protein solution was excited at 295 nm and the fluorescence emission spectra were recorded in the range of 300–400 nm. The protein concentration in all cases was 0.25 mg/ml

8-Anilinonaphthalene-1-sulphonic acid (ANS), a hydrophobic fluorescent dye is popularly used to monitor the exposure and/or disruption of hydrophobic patches of proteins during its unfolding/folding process (*22*). For ANS fluorescence in the ANS binding experiments, the excitation wavelength was set at 380 nm, and the emission spectra were recorded in the range of 400–600 nm.

Quenching Experiments

In the fluorescence quenching experiments, the concentration of the protein was taken as 0.25 mg/ml and quencher concentration was 0.1–1M. Excitation was set at 295 nm and the emission spectra were recorded in the range 300–400 nm. The fluorescence quenching data were analysed by monitoring the fluorescence intensity at 340 nm (λ_{max}) using the Stern–Volmer equation (*23*).

$$\frac{F_0}{F} = 1 + K_{\mathrm{sv}}(\mathrm{Q})$$

where F_0 and F are the fluorescence intensities at 340 nm in the absence and presence of quencher respectively, K_{sv} is the Stern–Volmer constant, and (Q) is concentration of the quencher.

RESULTS

Alkali Induced Unfolding of Hen Egg White Lysozyme: CD measurements—Alkali induced unfolding of HEWL was followed by far and near UV CD, ANS binding and acrylamide quenching studies. Far UV–CD spectra were

recorded at different pH values in the range of 7–13.2. The spectra are omitted for brevity. The spectra were analysed for secondary structural elements by analysing the signal obtained at 222 nm. The MRE at 222 nm showed no apparent change between pH 7.0 and 11.0, but when pH was increased above 11.0, MRE_{222} decreased markedly to a minimum value at pH 13.2 (Fig. 1). Thus, the pH-induced transition in the alkaline region, as monitored by ellipticity measurements at 222 nm was found to follow a single-step two-state transition. The results of secondary structure resolved analysis are presented in Table 1.

The near UV–CD spectra were recorded for the protein in the pH range 7–13.2 (the data not shown for brevity). Figure 2A and B show the alkali-induced unfolding of lysozyme as monitored by MRE measurements in the near-UV CD region at 255 nm and 291 nm. The near-UV CD signals in HEWL, one at 291 nm and another at 255 nm arise primarily from tryptophan and phenylalanine residues, respectively (*24–27*). As can be seen from the figure, MRE at 255 nm and 291 nm remained nearly constant between pH 7.0 and 10.5. However, further increase in pH leads to a decrease in MRE at 291 nm and an increase in MRE at 255 nm indicating loss of tertiary structure of the protein. Alkali induced transition curves as monitored by MRE measurements in the near-UV CD region were also found to be monophasic-like transition curve measured by far-UV CD (Fig. 1), a probe for secondary structure.

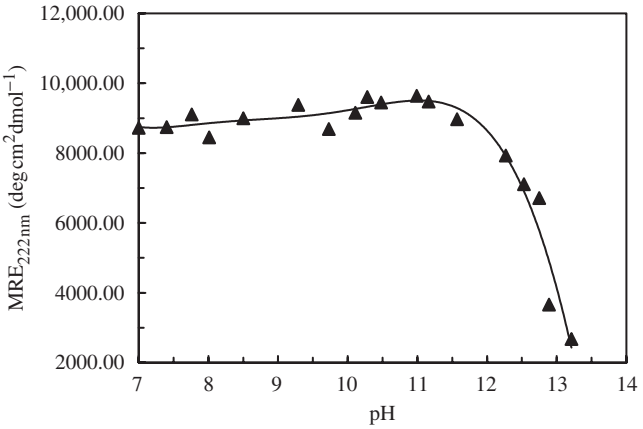
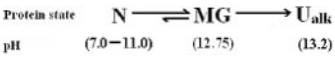


Fig. 1. **Mean residue ellipticity measurements at 222 nm.** Alkaline pH-induced unfolding profile of HEWL as monitored by MRE measurements at 222 nm. Each data point is the mean of three independent observations.

Intrinsic Fluorescence—The fluorescence emission spectrum of HEWL at excitation wavelength 295 nm is dominated by tryptophan fluorescence and shows maximal emission at 340 nm. Figure 3A and B show the alkaline pH induced unfolding of hen egg white lysozyme as observed by measurements of relative fluorescence intensity (RFI) at 340 nm and change in λ_{max} (maximum wavelength of emission), respectively. The fluorescence intensity and λ_{max} of HEWL in the basic pH region also showed a single step transition from pH 10 to 13.2. The λ_{max} of emission shifted from 340 at pH 7.0 to 350 nm at pH 13.2. The observed decrease in fluorescence intensity and increase of λ_{max} in the pH region 10–13.2 can be attributed to the loss of tertiary structure resulting from unfolding of the protein.

ANS Binding Studies—The solvent exposure of the hydrophobic surface in lysozyme at alkaline pH was studied by ANS binding. Binding of ANS to hydrophobic regions results in an increase in ANS–protein complex fluorescence intensity, which has been widely used to study the MG state of different proteins as reported in our earlier communications (*28*, *29*). As can be seen from Fig. 4, an increase in pH causes increased binding of ANS, with maximum binding occurring at pH 12.75. This suggests that HEWL at pH 12.75 has enhanced solvent accessible clusters of hydrophobic regions, which were initially buried in the interior of the protein.

Taken together, alkali-induced unfolding transition curves monitored by various spectroscopic techniques suggested that HEWL existed in a MG state at pH 12.75 and alkali unfolded state at pH 13.2. When the protein denatured at pH 12.75 was dialysed against a buffer of pH 5.0, the process was found to be partially reversible, however, increase in pH up to 13.2 makes the process nearly irreversible. Thus alkali-induced denaturation of HEWL may be approximated to a two state process and may be represented as



where N is native state, MG is alkali induced molten globule state and U_{alk} is alkali unfolded state of HEWL.

It is believed that structure of the intermediate and unfolded states of the protein can provide significant insight into nature of protein folding pathway, relationship between protein sequence, three-dimensional structure and stability of protein. Therefore, we aimed to characterize the non-native states of lysozyme observed during alkali denaturation in detail. Various structural properties of native state, state at pI, MG state and

Table 1. **Spectral properties of different alkali induced states of HEWL.**

Variables	N state (pH 7.0)	State at pI (pH 11.0)	MG state (pH 12.75)	U_{alk} (pH 13.21)	U_{GnHCl} (6.0 M)
$MRE^{a}_{222\,nm}$	−8725	−9474	−6712	−2676	−1546
$MRE^{a}_{255\,nm}$	4.6	210	337	246	139
$MRE^{a}_{291\,nm}$	364.7	374	292	264	232
$RFI^{b}_{340\,nm}$	100	97	48	43	145
λ_{max} (nm)[b]	340	342	350	350	352
$RFI^{c}_{480\,nm}$	100	127	523	202	117

[a]MRE, (deg cm^2 dmol^{-1}).
[b]Protein was excited at 295 nm, fluorescence intensity of native state at pH 7 was assumed to be 100.
[c]ANS– protein complexes were excited at 380 nm.

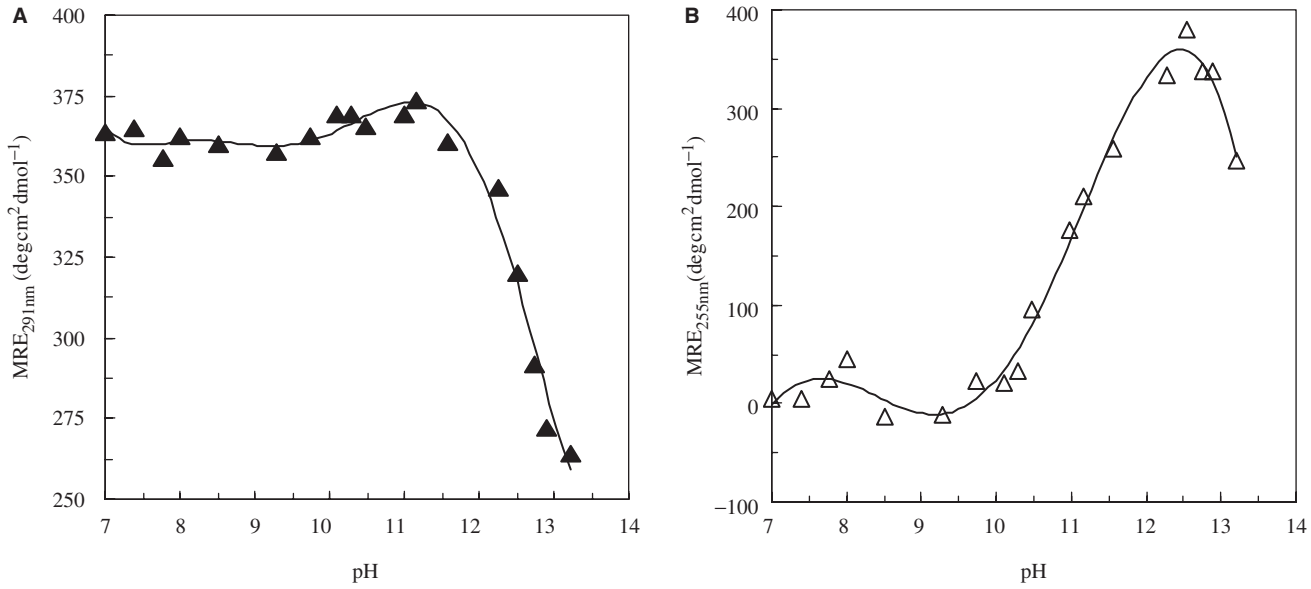


Fig. 2. **Mean residue ellipticity measurements at 291 nm and 255 nm**. Alkaline pH-induced unfolding of HEWL as monitored by MRE measurements at 291 nm (A) and 255 nm (B). Each data point is the mean of three independent observations.

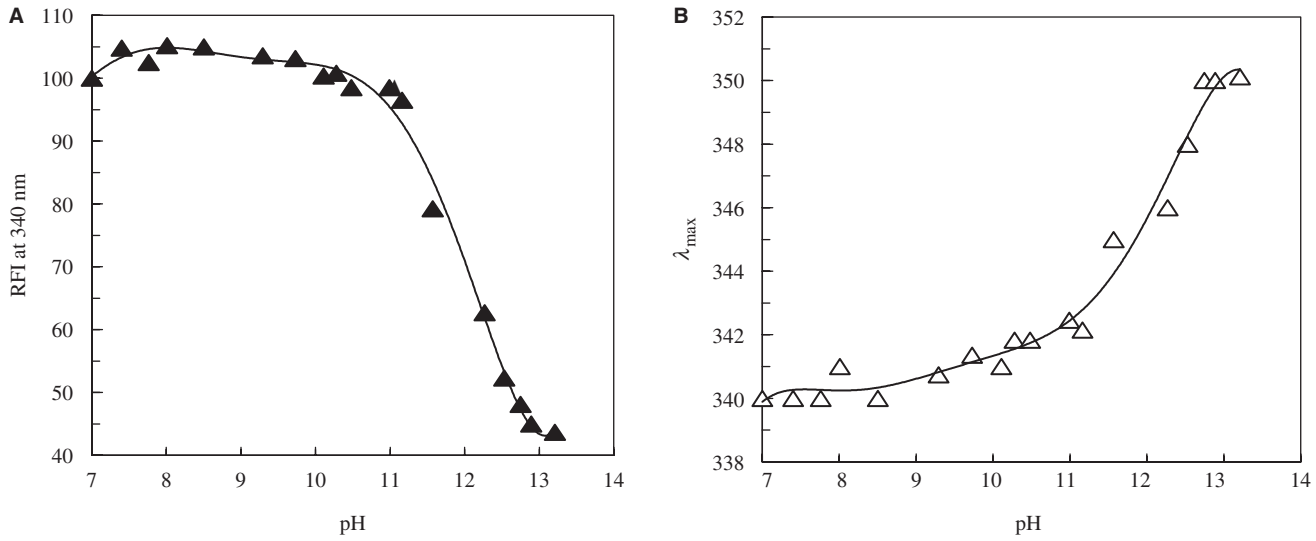


Fig. 3. **Intrinsic Fluorescence measurements**. Alkaline pH-induced unfolding of HEWL as monitored by tryptophanyl RFI (A) and maximum wavelength of emission (λ_{max}) (B). The protein was excited at 295 nm.

alkali unfolded state have been described subsequently and summarized in Table 1.

Structural Characteristics of HEWL in Native State, State at pI, MG State, Alkali Unfolded State and Completely Unfolded State: CD Measurements—Far-UV CD: Figure 5 shows the far-UV CD spectra of lysozyme at pH 7.0, 11.0 (pI), 12.75 (MG) and in presence of 6 M GnHCl (completely denatured state). HEWL at pH 7.0 revealed two negative peaks, one at 222 nm and another at 208 nm with the signal pronounced in magnitude at the 208 nm, a feature typical of $\alpha+\beta$ proteins. (*10*, *11*, *30*). The spectra of the protein at pH 11.0 (pI) retained all the features of secondary structure, an increase in the MRE values at 222 nm and a slight decrease at 208 nm (Table 1) was observed, indicating a 9% increment in α helical structure content. The spectrum of MG state was characterized by CD bands at 222 nm and 208 nm, indicating that it retained all the elements of secondary structure found in the native protein. There was however a decrease in the ellipticity value suggesting loss of secondary structure without affecting the basic format. Lysozyme in presence of 6 M GnHCl lost all the features of secondary structure and represented the completely unfolded state of the HEWL structure.

Near-UV CD: Near-UV CD spectra in the region 320–250 nm were used to probe the asymmetry of aromatic amino acids and disulphide bridge environment. The main contributions to the ellipticity of proteins come from tryptophan, tyrosine and phenylalanine with peaks at 291, 277 and 256 nm, respectively (*28–30*). Near-UV CD spectra of HEWL at pH 7.0 (native state), 11.0 (pI), 12.75 (MG state), 13.2 (alkali unfolded state) and in presence of 6 M GnHCl (completely denatured state) are shown in Fig. 5B. At pH 7.0, a broad and large maximum at 291 nm was observed which indicated major contribution from tryptophanyl residues, as HEWL contains 6 tryptophan, 3 tyrosine and 3 phenylalanines. By raising the pH of HEWL solution from 9.0 to 11.0 (pI), we observed a gain in the CD signal in the region between 250 and 270 nm with a maximal value obtained at 255 nm. It may be ascribed to changes in the phenylalanine environment. In addition, a shift of 291 nm peak towards longer wavelength was observed with a small change in the ellipticity in the region between 290 and 320 nm. The spectra of native state and state at pI are almost overlapping between regions 270 and 290 nm. On the other hand, spectrum of the MG state resembled neither the spectrum of the native state nor completely unfolded state. The CD band of native state at 291 nm shifted toward longer wavelength in the MG state and a significant increase in ellipticity was observed at 255 nm. This indicated a significant perturbation of aromatic amino acid and disulphide bond environment in the MG state compared to native state. As can be seen from the figure, alkali unfolded state lost nearly all of its tertiary structure and resembled more to the GnHCl denatured state. From these observations it appears that while some of the tryptophan residues of HEWL existed in a different environment at pI, others and some tyrosine residues were in the same environment as that of the native protein. Upon increasing the pH of the protein to 12.75 the spectrum showed two maxima, one at 255 nm and another at 300 nm, with a significant increase in ellipticity between 295 and 310 nm and a decrease between 273 and 295 nm. The signal at 255 nm became more pronounced at the MG state. This indicated that MG state possesses well-defined tertiary contacts, which were non-native.

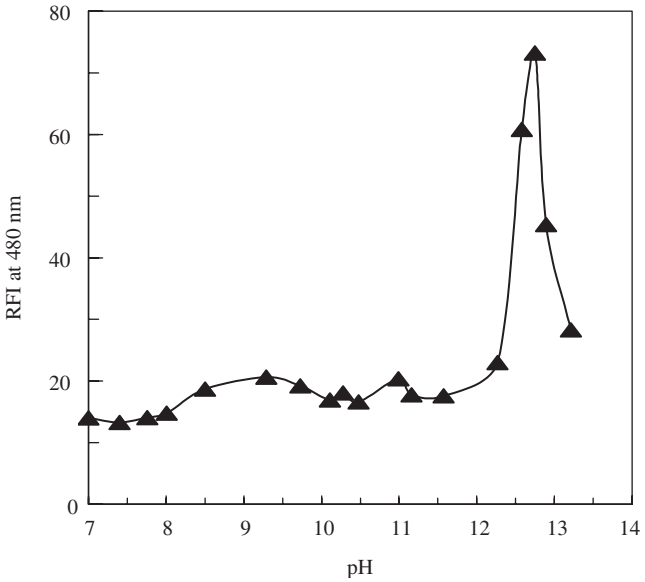


Fig. 4. **ANS–protein complex fluorescence at 480 nm.** Alkaline pH-induced unfolding of HEWL as monitored by ANS fluorescence at 480 nm after exciting the protein–ANS complex at 380 nm.

Intrinsic Fluorescence—The intrinsic fluorescence emission spectra of HEWL at pH 7.0 (native state), 11.0 (pI), 12.75 CMG state), 13.2 (alkali induced unfolded state) and in 6 M GnHCl (completely denatured state) are depicted in Fig. 6. The emission spectrum of HEWL at pH 7.0 was dominated by tryptophanyl fluorescence with emission maximum occurring at 340 nm. At pH 11.0 (pI) the relative fluorescence intensity (RFI) of the protein was almost same as that observed for the native state,

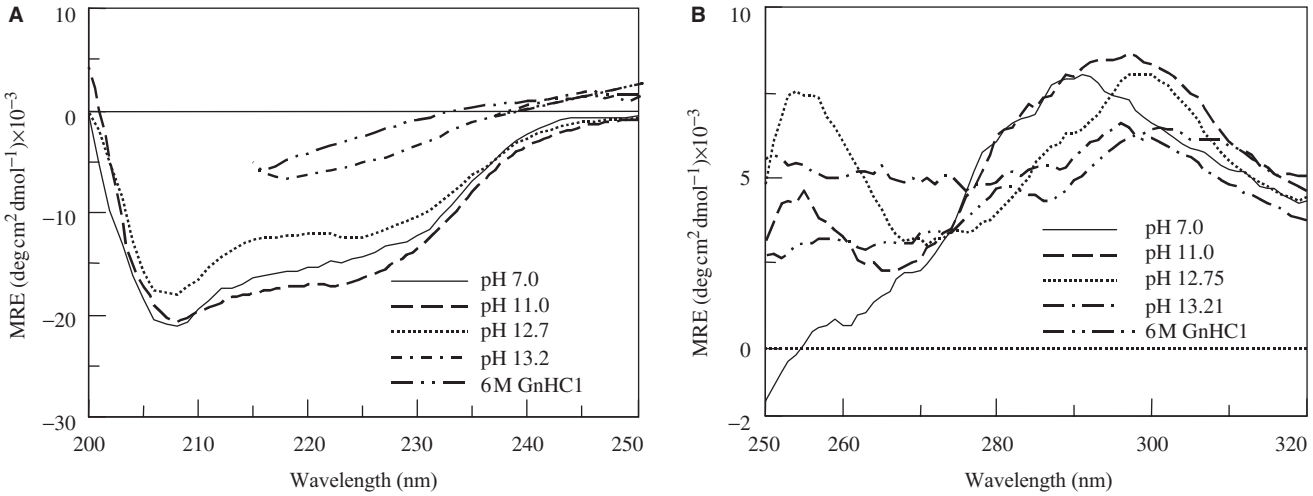


Fig. 5. **Circular dichroism studies.** Far-UV CD (A) and near -UV CD (B) spectra of HEWL at pH 7.0, 11.0, MG state at pH 12.7, alkali denatured state at pH 13.21 and guanidine hydrochloride (6 M) denatured state.

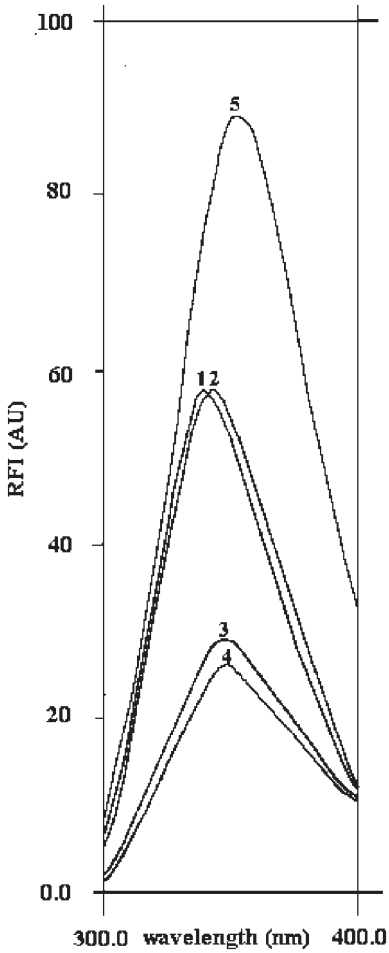


Fig. 6. **Intrinsic Fluorescence studies.** Tryptophanyl fluorescence spectra of native (curve 1), pH 11.0 (curve 2), MG state at pH 12.75 (curve 3), alkali denatured state at pH 13.2(curve 4) and guanidine hydrochloride (6 M, curve 5) denatured state of HEWL.

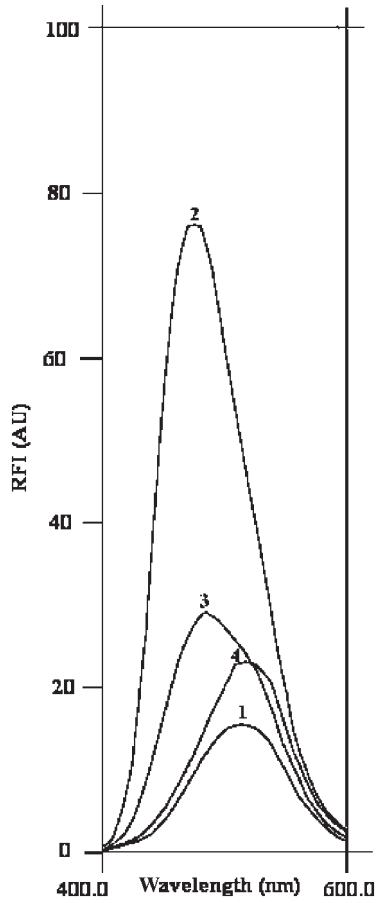


Fig. 7. **ANS fluorescence studies.** Fluorescence emission spectra of ANS bound to native HEWL (curve 1), MG state at pH 12.75 (curve 2), alkali denatured state at pH 13.2 (curve 3) and guanidine hydrochloride (6 M) denatured state (curve 4).

however, the emission maximum shifted from 340 nm to 342 nm. As the pH of the HEWL solution was increased to 12.75, the emission maximum showed a red shift of 10 nm from 340 nm to 350 nm with a significant decrease in RFI. On increasing the pH to 13.2 no further change in shape or intensity of the fluorescence spectrum was observed. Six molar GnHCl treated protein showed a marked increase in RFI and a red shift of 12 nm compared to the native protein. This indicated that MG state and U_{alk} state have similar tryptophanyl environment and resembled more to the denatured protein.

ANS Binding Studies—Figure 7 shows the fluorescence spectra of ANS–protein complex in 400–600 nm wavelength range at pH 7.0, (curve 1), 12.75 (curve 2), 13.2 (curve 3) and in 6 MGnHCl (curve 4). As can be seen, binding of ANS to the MG state at pH 12.75 produced a large increase in fluorescence intensity compared to native state and unfolded states. This shows that a sizeable amount of hydrophobic clusters are exposed in the MG state relative to native state where in they may be buried and the completely unfolded state where they may be disrupted. The protein at pI showed native like ANS binding property suggesting that buried region of the protein is not affected at this pH. Thus, retention of some amount of secondary structure with complete loss of tertiary structure along with maximum ANS binding at pH 12.75 is indicative of the presence of a MG state at this pH.

Acrylamide Induced Fluorescence Quenching—To confirm the environment of tryptophan residues, we compare the exposure of tryptophanyl residues in MG state with that in the native state and GnHCl induced state by a fluorescence quenching experiment, using uncharged molecules of acrylamide as described by Eftink and Ghiron (*23*). Figure 8 shows Stern–Volmer plots of quenching of fluorescence of lysozyme by acrylamide in the native state, MG state, state at pI, alkali denatured state and GnHCl denatured state. Table 2 shows the Stern–Volmer plot constant (K_{sv}) fitted to the linear parts of the curves in Fig. 8. K_{sv} for the MG state was found to be higher (6.47 M^{-1}) than native state and was accompanied by a red shift in λ_{max} from 340 to 350 nm. But K_{sv} value for GnHCl denatured

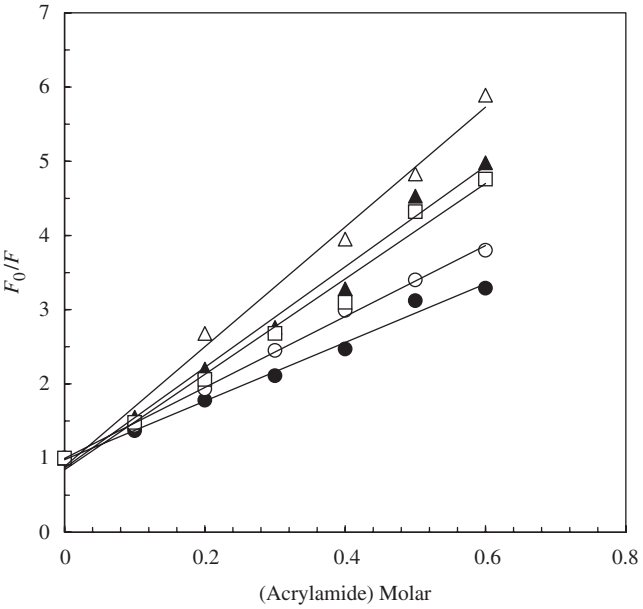


Fig. 8. **Acrylamide induced fluorescence quenching studies.** Stern–Volmer plots of acrylamide quenching for native HEWL at pH 7.0(closed circle), pH 11 (open circle), MG state at pH 12.75 (closed triangle), pH, 13.2 (open square) and GnHCl (6 M) denatured state. (open triangle)

Table 2. **Acrylamide quenching of different states of HEWL.**

States	K_{sv} (M^{-1})	$*R^2$
Native	3.9	0.989
At pH 11.0	4.8	0.997
MG (pH 12.75)	6.5	0.981
pH 13.2	6.8	0.982
GnHCl	7.8	0.99

$*R^2$ Correlation coefficient obtained by linear regression.

state was higher than MG state. These results show that MG state involves intermediate exposure of tryptophanyl residues relative to native and completely unfolded states.

t-Butanol Induced Conformational Changes in HEWL States—*t*-Butanol induced structural transitions of HEWL were monitored by far-UV CD, intrinsic and extrinsic fluorescence spectroscopic techniques at pH 7.0, 11.0 and 12.75. These pHs were selected because HEWL existed as native state, state at pI and MG states as discussed earlier.

CD Measurements—Figure 9A, B and C showed far-UV CD spectra in absence and presence of different concentrations of *t*-butanol at pH 7.0, 11.0 and 12.75, respectively. The spectra were recorded for *t*-butanol concentration in the range 5–60% *t*-butanol. However for the sake of brevity, the data are represented for 30 and 45% *t*-butanol concentration only, which showed significant changes. As is clear from these spectra, the CD signal shows significant changes at 222 nm on addition of *t*-butanol at all the pH values. The data are

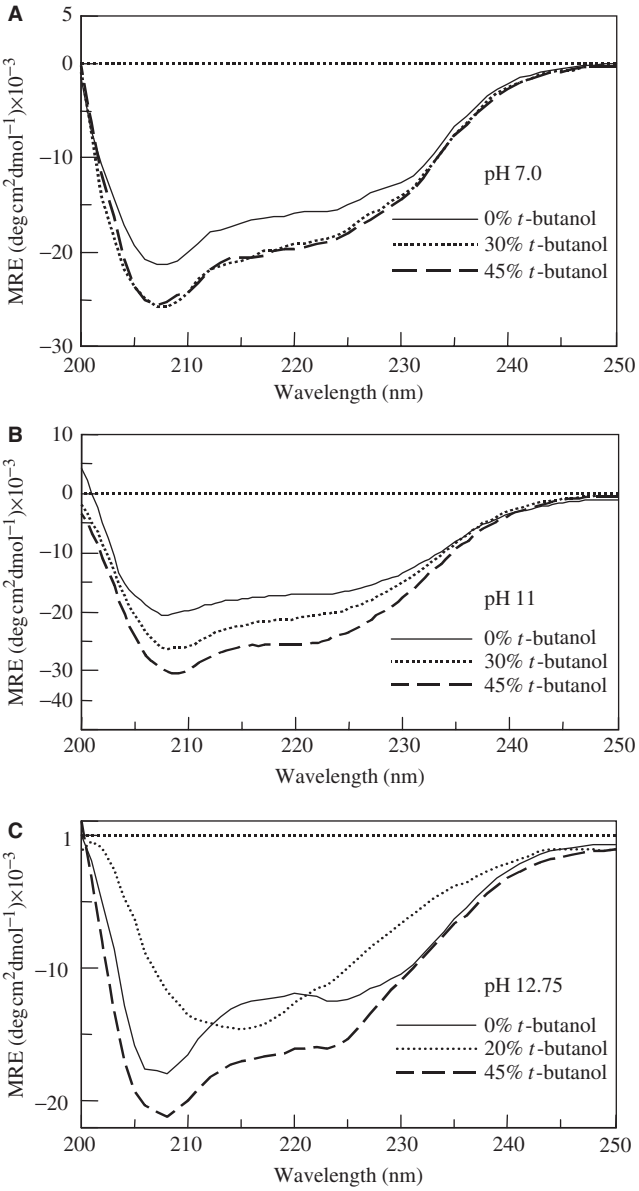


Fig. 9. **Effect of *t*-butanol on different states of HEWL far-UV CD spectra.** Far-UV CD spectra of native (A), pH 11 (B) and pH 12.75 (C) in the absence and presence of different concentrations of *t*-butanol.

represented in Fig. 10 as a plot between alcohol concentration and MRE at 222 nm.

For the native protein (at pH 7.0) the addition of *t*-butanol up to a concentration of 10% showed no effect, however, a linear increase in the MRE at 222 nm was observed when the concentration of *t*-butanol was between 10 and 20%. For the protein at pH 11.0 (pI), the addition of *t*-butanol showed a linear increase in MRE at 222 nm when the concentration of the alcohol was between 20 and 30%. These results suggest that the transition is highly cooperative. When the *t*-butanol concentration was increased to 45% the MRE at 222 nm showed no further significant change in the case of native protein. However, in the case of protein at

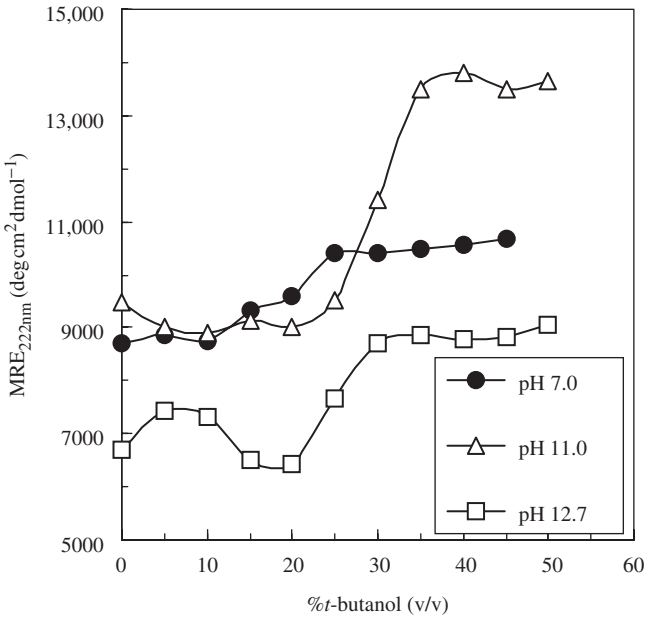


Fig. 10. **Effect of increasing concentration of *t*-butanol on MRE at 222 nm on native, pH 11.0 and pH 12.75 states of HEWL.**

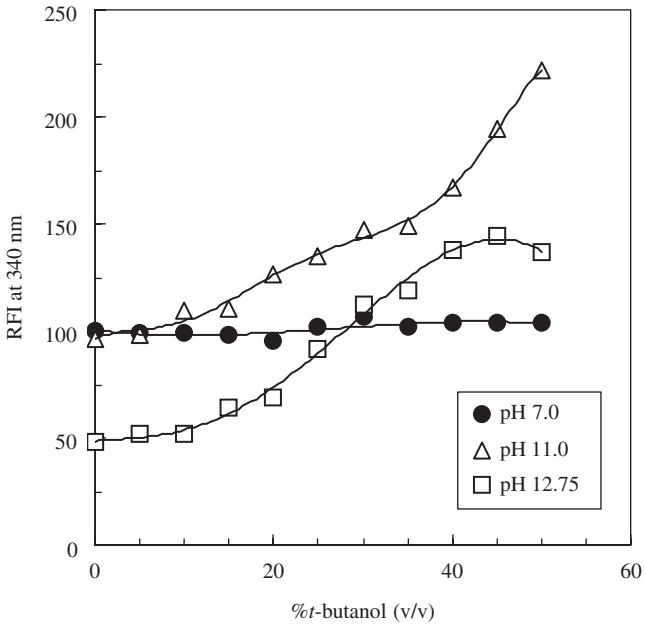


Fig. 11. **Effect of *t*-butanol on different states of HEWL intrinsic fluorescence.** Tryptophanyl fluorescence spectra of native, pH 11.0 and pH 12.7 states of HEWL in the presence of increasing concentrations of *t*-butanol.

Table 3. **Spectral characteristics of *t*-butanol-induced states of HEWL.**

Conditions	$MRE_{222\,nm}$	$RFI_{340\,nm}$	λ_{max}	$RFI_{480\,nm}$
pH 7.0, 30% *t*-butanol	−10,388.9	107	340	6
pH 7.0, 45% *t*-butanol	−10,577.55	104	340	−1
pH 11.0, 30% *t*-butanol	−11,415.1	148	340	1
pH 11.0, 45% *t*-butanol	−13,938.8	195	338	4
pH 12.75, 20% *t*-butanol	−6434.17	113	348	33
pH 12.75, 45% *t*-butanol	−8841.4	145	350	−2

pH 11.0 (pI), a significant change in the MRE values was observed when *t*-butanol concentration was increased from 30% to 45% (Table 3). This suggests the appearance of an increased amount of α-helical conformation in this state, being induced by *t*-butanol treatment. Moreover, spectral features of the HEWL at pH 7.0 and pH 11.0 in the presence of *t*-butanol remained almost unchanged.

At pH 12.75, the behaviour of HEWL in the presence of *t*-butanol was completely different from that of the protein at pH 7.0 and 11.0 (Fig. 9C). At this pH, the addition of 10–20% *t*-butanol-induced alterations in the CD spectrum with a negative band appearing at 215 nm with near disappearance of the bands observed at 222 and 208 nm. Although the change in the MRE value at 222 nm was insignificant, however, the spectral features point towards a shift in the conformational state of the protein from α-helix to β sheet. When the concentration of *t*-butanol was increased beyond 20%, the spectral features were indicative of another transformation with the negative CD band at 215 nm dipping gradually with concomitant reappearance of bands at 222 and 208 nm, till the bands at these wavelengths were prominent with the complete disappearance of 215 nm band obtained at 45% *t*-butanol concentration, indicating reformation of α-helical conformation. The various structural characteristics of native and unfolded states and that of the intermediates existing on alkali-induced unfolding pathway as revealed by various secondary and tertiary structural probes are summarized in Table 2. Whereas the protein at pH 11.0 is more or less native like, the intermediate state obtained at pH 12.75 is characterized by retention of secondary structure, enhanced ANS binding and signifying partial disruption of tertiary structure, which are characteristics of an MG state.

Intrinsic Fluorescence—Figure 11 shows the effect of *t*-butanol (0–50%) on the HEWL at pH 7.0, 11.0 (pI) and 12.75 (MG). The λ_{max} for emission remained unaltered for all the alcohol concentrations for the native protein (data not shown). Fluorescence intensity also remained unchanged for the protein at pH 7.0. The addition of *t*-butanol up to 45% to the protein at pH 11.0 (pI) caused increase in tryptophanyl fluorescence with a blue shift of 2 nm (Fig. 11). These changes may be ascribed to the formation of slightly hydrophobic environment around a few tryptophanyl residues. This is supported by the induced conformational changes in the peptide backbone as discussed earlier. A sigmoidal increase in the fluorescence intensity of MG state was observed upon addition of up to 45% (v/v) *t*-butanol. A 2 nm decrease in λ_{max} from 350 to 348 nm was noted at around 20% *t*-butanol, which however got restored back on addition of alcohol up to 45%. The decrease in λ_{max} at 20% *t*-butanol concentration may be due to formation of β sheet structure at this concentration of *t*-butanol (Fig. 9C). Further addition of *t*-butanol reverses the

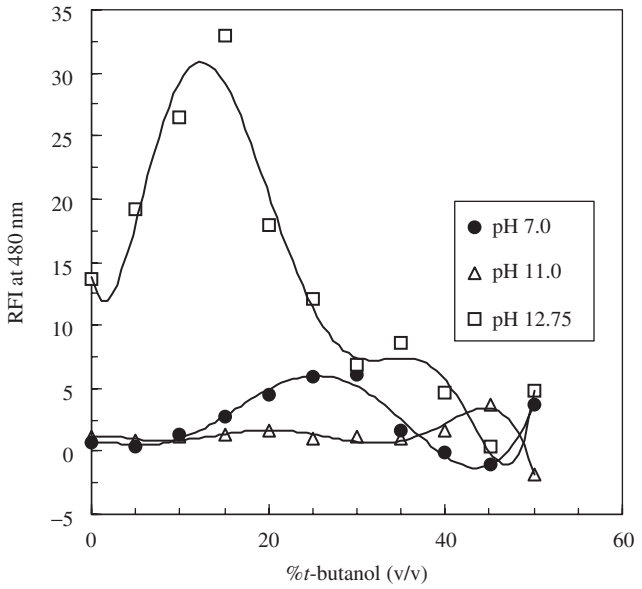


Fig. 12. **Effect of *t*-butanol on different states of HEWL ANS fluorescence.** ANS–protein complex fluorescence intensity at 480 nm in the presence of increasing concentrations of *t*-butanol.

β-sheet conformation and stabilizes the α-helical environment that may be responsible for restoration of the λ_{max} to its original position. In all these measurements, the contributions of *t*-buatanol to the emission spectra were corrected by using respective blanks.

ANS Binding Studies—ANS has higher affinity for the intermediate state of protein than the protein in the native state or completely unfolded state. This is because the intermediate conformations of the protein have exposed hydrophobic pockets that are easily accessible to ANS, than the native state of the protein where the hydrophobic groups are generally buried and thus inaccessible or accessible, but partially. In the completely unfolded states of the protein the hydrophobic patches are actually disrupted and thus possess reduced affinity for ANS. (*31*). We therefore attempted to identify the intermediate and denatured states of HEWL by following the ANS binding in the presence of various concentrations of *t*-butanol.

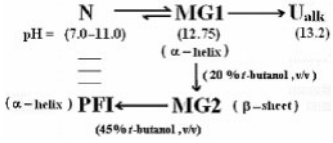
Figure 12 shows *t*-butanol-induced conformational changes of HEWL at pH 7.0, 11.0 and 12.75 as monitored by ANS–protein fluorescence. The fluorescence of the complex was monitored by exciting the complex at 380 nm and recording the emission at 480 nm. The *t*-butanol-buffer mixture was found to show significant amount of ANS binding. To exclude the contribution of the alcohol to the emission spectra, the fluorescence emission corrections for respective blanks were made.

As is shown in Fig. 12, the concentration of *t*-butanol that caused maximal binding of ANS was 30% for the native protein, 45% for the protein at pH 11.0 and 20% for the MG state of the protein. Since ANS binds more effectively to hydrophobic patches on the protein, these observations corroborate with the changes in the conformational state of the protein as observed with CD and fluorescence measurements and discussed earlier.

DISCUSSION

It has been previously shown that acid-and alkali-induced denaturation of proteins leads to the formation of partially folded intermediate, which resembled a MG state (*32–40*). The treatment of proteins with different alcohols has been shown to induce MG state in different proteins as reported earlier from our lab (*36–40*). Characterization of such intermediate states is important and can give significant clues leading to an understanding of the protein-folding phenomenon. The structural properties of such non-native states aid in determining the major factors involved in guiding a protein on the pathway of folding. We followed the alkali-induced unfolding of HEWL between pH 7.0 and 14, and found that pH-induced unfolding of lysozyme went through at least two partially folded intermediate states (PFI), one stabilized at pH 11.0 (the isoelectric pH) and another at pH 12.75 and it has been reported that unlike many other proteins as reported from our lab including α + β class (*33–37*) protein, lysozyme did not become MG like even at very low pH (*12*). Our report on MG state of HEWL at high pH (12.75), although specific forp lysozyme, a highly stable basic protein, may provide evidence to the generality of MG state on the folding pathway of a protein.

We extended our studies by studying the effect of alcohol (*t*-butanol) on native state, state at pI and MG state of HEWL in order to get more structural information about these states. The preliminary studies on HEWL with methanol, ethanol, propanol and butanol, and our previous results with other proteins (unpublished data) have shown that the changes were more prominent with butanol, a relatively more non-polar alcohol. The protein in native state and at pI was found to become more helical without significantly affecting tertiary contacts with the addition of up to 45% *t*-butanol. It was however interesting to note that MG state of lysozyme behaved uniquely with increasing concentration of *t*-butanol. We found that the MG state of lysozyme went through an α → β transition in its structure in the presence of low concentration of *t*-butanol [≤20% (v/v)]. The α → β transition of the MG state was also accompanied by enhanced binding of ANS indicating the availability of more hydrophobic surfaces. However, when the concentration of *t*-butanol was increased up to 45% (v/v), it resulted in the reversal of α → β transition and produced an α-helix dominated PFI. On the basis of earlier discussions the conformational behaviour of HEWL in alkali and alcohol can be summarized as follows



A model showing the behaviour of HEWL in alkaline conditions and in the presence of butanol. N represents native state, MG1 is alkali-induced MG state, MG2 is *t*-butanol-induced MG state, U_{alk} is alkali unfolded state, PFI is partially folded intermediate.

The marked β sheet and α-helical propensity of the MG states, respectively in low and high *t*-butanol concentrations, which is determined mainly by local polar interactions leads us to suggest that this protein may assume a β and/or α-helical structure in the intermediate stage of protein folding. As HEWL is an α + β protein, formation of both β sheet and α-helical dominated structures in the folding pathway of lysozyme is also a possibility. This also provides evidence that HEWL may follow alternative pathway of folding under different environmental conditions. The reversal of the backbone conformation on increasing the concentration of *t*-butanol to 45% leads to another conformation that is predominantly α- helical and quite similar to the native state, and may proceed through reversal to the MG1 state. The thioflavin T assay indicated that prior to the treatment of protein with butanol, the protein does not undergo any sort of aggregation at any of the pH values.

The financial assistance to M. H from university of Kashmir, and to B. A. from the Council of Scientific and Industrial Research (CSIR), Government of India, is acknowledged. Facilities provided by A.M.U are gratefully acknowledged. The authors are also thankful to DST (FIST) for providing lab facilities.

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