Chapter 5

Formation And Stabilization Of Different Intermediate States Of Some Acid Denatured Lectins In Presence Of Polyethylene Glycols

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5.1 Introduction

5.1.1 Polyethylene Glycols

Polyethylene glycols (PEGs) are nontoxic water-soluble synthetic polymers widely used in chemical and biomedical industries. Different molecular weight PEGs exhibit different solubility, surface tensions, viscosities, freezing points, and melting points. All have sufficient solubility in water. In spite of their relatively inert chemical properties, PEGs have been found to have some interesting biological effects. For example, they cause profound changes in the conformation of DNA polymers [195] promote the fusion and hybridization of cells [196], and potentiate mitogen-induced stimulation of lymphocytes, presumably by enhancing the lateral aggregation of receptors in the membrane [197]. Polyethylene glycols are also extensively used for the precipitation and crystallization of proteins [198,199]. The mechanism by which PEG induces protein aggregation has been studied extensively, the PEG polymer adopts a random coil configuration in solution, acting as if it were a sphere with an effective radius. The center of mass of the PEG polymer cannot come closer to a protein than its effective radius. Thus the PEG is excluded from a volume around the protein defined by the surface area of the protein and the effective radius of the PEG. Removal of the protein surface area formed by protein-protein interactions effectively decreases the volume that is not available for solvating the PEG. This provides an entropic effect promoting protein-protein association. The greater the volume that becomes accessible for PEG solvation upon formation of the protein complex, the greater the effect of PEG [200]. For PEGs two possible mechanisms of stabilization have been suggested, steric exclusion and exclusion due to protein charge.
Exclusion of PEGs leads to preferential hydration that stabilizes the protein structure. But here it should be mentioned that studies have shown that PEGs can interact with proteins in a number of ways. They may have destabilizing effects on proteins also depending on the chemical nature of the protein [202]. Possible destabilizing effect of high molecular weight PEGs (PEG-6000 and 20,000) was found on the acid unfolded state of stem bromelain [203]. In coming section we have shown the effect of low, medium and high molecular weight PEGs on an intermediate state of pea lectin obtained at pH 2.4 [204].
5.2 Results and Discussion

5.2.1 Transition of a compact intermediate state of pea lectin under the influence of different molecular weight polyethylene glycols.

5.2.1.1 Effect of PEGs on the secondary structure of pH 2.4 intermediate

When different molecular weight PEGs were added to the previously characterized compact intermediate of pea lectin at pH 2.4 [204] some interesting observations were made. Fig. 42 shows the transition plot of pH 2.4 intermediate as a function of increasing concentrations of different molecular weight PEGs.

![Transition plot showing the changes in CD mdeg with increasing concentrations of different PEGs. PEG-400 (— — ), PEG-4000 (— — ), PEG-20,000 (— — ). CD changes were monitored at 217 nm.](image)

As can be seen from the figure increasing concentrations of PEG-400 from 10% to 80% (v/v) resulted in a continuous increase in CD values at 217 nm. Figure shows that non-
native secondary structure was induced by the low molecular weight PEG and causes the transition of pH 2.4 intermediate to a different state.

On addition of medium weight PEG i.e. PEG-4000 there was no significant change in CD values at 217nm up to 25% v/v. From 30% PEG-4000 a slight decrease in θ mdeg was observed along with a shift in trough from 217 to 219 that is again indicative of formation of some new conformation different from the pH 2.4 intermediate.

In case of PEG-20,000 the MRE goes on decreasing with increasing PEG concentration. This shows that the high molecular weight PEG caused destabilization of the pH 2.4 intermediate may be by reducing some of hydrogen bonds responsible for holding together the beta-structure of the intermediate form that closely resembles to that of native.

Similar studies on protein and polyethylene glycol interactions [205] have shown that low molecular weight PEG 600 at low concentration causes a decrease in the secondary structure of the protein and has no effect on its tertiary structure.

In another study [206] it was shown that PEG at low concentrations had no effect on the secondary structure while on increasing the concentration a considerable decrease in structure was observed.

Thus it has been found that PEGs tend to have different effects on different proteins depending upon the chemical nature of proteins. It has been earlier reported from our lab that high molecular weight PEG-6000 and PEG-20,000, which generally stabilize proteins by preferential hydration mechanism [207], were found to play a denaturing role on acid unfolded stem bromelain [203].

According to Arakawa and Timasheff [202] PEGs may have a destabilizing effect because they are essentially non-polar and can bind to the protein through hydrophobic interactions.
Fig. 43 Far-UV CD spectrum of pH 2.4 intermediate of pea lectin in presence of varying concentrations of different PEGs.
(a) 10% PEG-400, (curve-2); 50% PEG-400, (curve-3); 70% PEG-400, (curve-4); in presence of 6M GndHCl, (curve-5). (b) 5% PEG-4000, (curve-2); 40% PEG-4000, (curve-3); in presence of 6M GndHCl, (curve-4). (c) 4% PEG-20,000 (curve-2); 20% PEG-20,000 (curve-3); 32% PEG-20,000 (curve-4); in presence of 6M GndHCl, (curve-5).
In all the above three cases the intermediate state of pea lectin at pH 2.4 is represented by curve-1.

Due to greater nonideality, high molecular weight PEGs acquire more compact structure as compared to low molecular weight PEGs and the possibility of their steric exclusion is
reduced. This allows these molecules to penetrate the hydration layer of the protein and bind to the hydrophobic sites that leads to the unfolding of the protein.

Fig. 43 (a), (b) and (c) depict the CD spectra measured in the far-UV range and summarizes the observations discussed above. In all the figures curve-1 represents the pH 2.4 state of the lectin while all the other spectra correspond to pH 2.4 states in presence of different concentrations of PEGs. Only selected spectra are shown the for the sake of clarity.

5.2.1.2 Effect of PEGs on the tertiary structure of pH 2.4 intermediate

*Intrinsic fluorescence studies*

![Graph showing the fluorescence intensity of pea lectin at 340 nm as a function of varying concentrations of different molecular weight PEGs. PEG-400 (— ● —), PEG-4000 (— ■ —), PEG-20,000 (— ▲ —).]

In the previous study [204] it was observed that the pH 2.4 intermediate not only possessed substantial secondary structure but to some extent also native like tertiary contacts.
Fig. 44 shows the transition of the pH 2.4 intermediate as a function of increasing concentrations of the three different PEGs. Increasing concentrations of PEG-400 caused a continuous increase in fluorescence intensity of the acid unfolded intermediate at pH 2.4. While probing the secondary structure through far-UV CD we came across that PEG-400 caused an increase in the secondary structural elements, here the probe for tertiary structure indicates that it also caused an increase in the tertiary contacts.

As reported earlier, pea lectin has been shown to possess a total of 29 aromatic amino acid residues out of which 5 are tryptophan residues. Trp-53, Trp-128 and Trp-206 are a little exposed while Trp-52 and Trp-227 are buried in the native form. Rest of the aromatic residues are contributed by 10 tyrosine and 14 phenylalanine amino acids. All the phenylalanine residues are highly buried while in case of tyrosine Tyr-46, Tyr-100, Tyr-179 and Tyr-191 are more buried than the rest. When treated with the low molecular weight polyol the increased tertiary contacts possibly caused the burial or internalization of the exposed tryptophan residues, attributing to the increased fluorescence.

PEG-4000 induced a small decrease in the fluorescence intensity. The decrease in intensity could be due opening of the tertiary structure exposing the tryptophan residues to polar environment.

On addition of PEG-20,000 again a decrease in the fluorescence intensity was observed along with a slight red shift of about 2-3 nm (fig. 45(c)). The explanation to the decreased fluorescence is same as that for PEG-4000 i.e. the loss in tertiary structure leading to the exposure of tryptophan residues, red shift observed further supports the disruption of the tertiary structure.

Fig. 45 (a), (b) and (c) depict the selected fluorescence spectra of pH 2.4 intermediate treated with PEG-400, PEG-4000 and PEG-20,000 respectively. In all the three cases curves 1, 2 and 5 represent the native state, the pH 2.4 intermediate and 6M GdnHCl denatured state. Curves-3 and 4 represent the lowest and highest concentrations of the PEGs while all the other spectra corresponding to the other concentrations lie between curves 3 and 4 (not shown in fig).
Fig. 45 Intrinsic fluorescence spectra of pea lectin in presence of different molecular weight PEGs.
(a) 10% PEG-400 (curve-3), 80% PEG-400 (curve-4). (b) 5% PEG-4000 (curve-3), 45% PEG-4000 (curve-4). (c) 4% PEG-20,000 (curve-3), 32% PEG-20,000 (curve-4). In all the above cases pea lectin in Native state, curve-1 (—), Intermediate state of the lectin at pH 2.4, curve-2 (— — —) and the 6M GndHCl denatured state curve-5 (......).

ANS binding studies
It is a well-known fact that ANS, which is a fluorescent hydrophobic dye, binds to the hydrophobic patches present on the surface of the proteins [208]. It has a higher affinity for the intermediate states of the proteins as compared to the native state or completely denatured state. Intermediate states have such a conformation in which hydrophobic patches are exposed for ANS binding which are less accessible in the native and completely denatured forms [69]. Hence, ANS binding is extensively used to monitor the exposure of hydrophobic patches of proteins during their folding or unfolding process. Figure-46 shows the binding pattern of ANS to pH 2.4 intermediate treated with different molecular weight PEGs.
As is evident from the figure ANS maximally bound to the pH 2.4 intermediate. In presence of PEGs and with their increasing concentrations ANS fluorescence went on decreasing and at higher concentrations of the polyols, became negligible. The loss of ANS binding could be explained on the basis of CD studies that tell us about the secondary structure of the protein and in the light of intrinsic fluorescence studies that give an idea about the tryptophan environment in particular as the protein was excited at 280 nm. When the pH 2.4 intermediate was treated with PEG-400 there was induction of secondary (Fig.43 (a)) and non-native tertiary contacts were formed resulting in the internalization of tryptophan residues (Fig.45 (a)). We can speculate that due to the formation of the non-native tertiary structure the hydrophobic patches also got buried and became more and more inaccessible for ANS binding with increasing polyol concentration. In case of PEG-4000 and PEG-20,000 loss in secondary as well as tertiary structure was observed. But the CD and fluorescence spectra of the intermediate treated with PEG-4000 and 20,000 in all the observations were different from that of 6M.
GdnHCl treated preparation indicating that the intermediate did not undergo complete
denaturation. It can be hypothesized that PEG-4000 and 20,000 altered the conformation
of the pH 2.4 and resulted in its transition to another intermediate state in which the
hydrophobic patches were not as accessible as they were earlier. This explains the
decrease in ANS fluorescence of the intermediate state when exposed to medium and
high molecular weight PEGs. No Molten Globule state was observed in the protein
folding/unfolding pathway induced by polyethylene glycols.

The difference in the effect of low molecular weight PEG (PEG-400) and medium and
high molecular weight PEGs (PEGs-4000 and 20,000) on the pH 2.4 intermediate could
be due to the difference in the proposed mechanism of action of these polyols. PEG-400
due to greater exclusion size as compared to higher molecular weight PEGs leads to
protein preferential hydration, which stabilizes the structure. PEG-400 acts on the protein
remaining away from the main protein structure, while high molecular weight PEGs (in
our case PEG-4000 and 2,000) owing to their compact structure penetrate the hydration
layer and directly bind to the protein bringing about the alterations in the structure. In
earlier discussion it was observed that, when the compact pH 2.4 intermediate of pea
lectin was treated with fluoroalcohols it was converted to an MG-like intermediate at
80% TFE and 4% HFIP. Although the two fluoroalcohols differ in their helix inducing
capacity and have different modes of action on a particular protein leading to different
types of structure at respective concentrations but in case of pea lectin an intermediate
structure was obtained that was common to both TFE and HFIP. The MG-like
intermediate had non-native secondary structure and absence of tertiary structure and
showed maximum ANS binding. In our present study we observed that the pH 2.4
intermediate underwent transitions that led to the formation of three different
intermediates under the influence of low, medium and high molecular weight PEGs. The
difference in the intermediate states obtained was that the one formed by the action of
PEG-400 was due to the induction of non-native secondary as well as tertiary structure
and those formed by the action of PEG-4000 and PEG-20,000 were due to the loss of
secondary structure and rearrangement in the tertiary contacts. Formation of PEG-400
induced intermediate along with PEG-4000 and PEG-20,000 induced intermediate states
has been discussed in detail in the previous section. Formation of secondary structure on
addition of low molecular weight PEG-400 is supported by the continuous increase in the CD values corresponding to the increasing concentrations of the polyol, moreover the spectral features as shown in Fig-43 (a) indicate formation of non-native secondary structure. Formation of tertiary structure on addition of PEG-400 has been hypothesized considering the fact that tryptophan fluorescence was found to increase with the increasing polyol concentration, which shows that the chromophoric groups get internalized during the process of formation of tertiary contacts. On the other hand in the intermediates induced by medium and high molecular weight PEGs a loss in secondary structure was observed, loss was more significant with PEG-20,000 as compared to PEG-4000 probably due to reasons discussed earlier. The loss in structure can be seen in figures 43(b) and (c) where the spectra are seen approaching towards the guanidium hydrochloride denatured state. Our speculation of rearrangements occurring in the tertiary structure of the pH 2.4 intermediate is based on the results obtained by spectrofluorometry. Also the ANS binding studies showed the absence of any MG or MG-like structures formed in the folding/unfolding pathway induced by PEGs.
5.2.2 Structural Intermediates of acid unfolded Con-A in presence of low, medium and high molecular weight Polyethylene Glycols (PEGs)

5.2.2.1 MG-like state of Con-A under the influence of different molecular weight PEGs.

Stability and structural properties of the intermediate state of Con-A obtained at pH 2 were studied in presence of low molecular weight (PEG-400), medium molecular weight (PEG-4000) and high molecular weight (PEG-20,000) polyethylene glycols.

Generally it is known that high molecular weight PEGs stabilize proteins by preferential hydration mechanism [207]. In one study [205] it was shown that low molecular weight PEG 600 at low concentration causes a decrease in the secondary structure of the protein and has no effect on its tertiary structure. In another study [206] it was shown that PEG at low concentrations had no effect on the secondary structure while on increasing the concentration a considerable decrease in structure was observed.

It has been found that PEGs tend to have different effects on different proteins depending upon their chemical nature. Earlier studies from our lab on acid unfolded stem bromelain have reported the destabilizing effect of high molecular weight PEGs 6000 and 20,000 [203]. In our present work we have shown the different transitional states through which the acid unfolded Con-A go through on exposure to different molecular PEGs.

**Low molecular weight PEG-400**

On addition of PEG-400 to the intermediate state of Con-A it was found that the low molecular weight PEG resulted in gradual increase in secondary structure up to 60% v/v. Further increase in concentration of the PEG up to 70% and 80% led to an abrupt increase in far-UV CD values pointing out the possibility of more intense structural changes occurring in presence of high concentrations of the polyol (Fig. 47 (a), series-1).
Fig. 47 Transition plots showing the changes in (a) far-UV CD and (b) near-UV CD with increasing concentrations of different PEGs. PEG-400 (--- ● ---), PEG-4000 (--- ■ ---), PEG-20,000 (--- ▲ ---).

(In figures, circles represent series-1, squares represent series-2 and triangles represent series 3). The somewhat different behavior of the acid unfolded structure of Con-A in presence of high concentrations of low molecular weight PEG is also reflected in the tertiary structure changes as observed by near-UV CD (Fig. 47 (b), series-1). It can be seen in figure that addition of 10% PEG-400 significantly increased the CD value at 293 nm after which only a slight increase was observed upto 40% PEG-400 while from 50% the values decreased with increasing concentration. Fig. 48 shows tryptophan fluorescence in presence of PEG-400 (series-1).
Fig. 48 Transition curve showing the fluorescence intensity of pH 2 intermediate of Con-A at 340 nm as a function of varying concentrations of different molecular weight PEGs. PEG-400 (---•---), PEG-4000 (---■---), PEG-20,000 (---▲---).

Fluorescence intensity went on increasing with increasing concentrations of the polyol but it can be observed that upto 40% PEG the increase was gradual. At 50% the intensity increased significantly and at 70% and 80% PEG-400 intensity increased abruptly. The increase of both CD values and the fluorescence intensity upto 40% PEG-400 indicates that the tertiary structure of the pH 2 intermediate is stabilized. Decrease in CD values at higher concentrations (>40%) indicates alteration in tertiary structure. This change in tertiary confirmation might have occurred in such a way that it changed the position of one or more tryptophan residues putting them apart from some fluorescence quenching group accounting for the sudden increase in tryptophan fluorescence especially at 70% and 80% of the PEG. Bringing together the results obtained from far and near UV CD and tryptophan fluorescence it can be concluded that PEG-400 seems to induce non-native secondary structure in the pH 2 intermediate while it seems to stabilize the tertiary structure only at low concentrations.
Medium molecular weight PEG-4000

Fig. 47 (a), (series-2) shows that secondary structure was induced in the acid unfolded intermediate of Con-A with increasing concentration of PEG-4000 up to 35% but at 40% it resulted in decrease in CD values or we can say a sudden transition in structure occurred at the highest concentration. CD studies in the near UV range show that addition of 5% PEG-4000 considerably changed the tertiary structure of the Con-A intermediate (Fig. 47 (b), series-2). On further increasing the concentration no considerable changes were observed, only a slight loss of structure was seen up to 20% of the polyol while at still higher concentrations the effect became almost constant. In the light of intrinsic fluorescence, the change occurring in the tertiary structure of Con-A intermediate can be interpreted as overall loss of tertiary contacts, as can be seen in the figure- fluorescence intensity of the intermediate increases at 5% PEG-4000 then goes on decreasing with further increase in concentration (Fig. 48, series-2).

High molecular weight PEG-20,000

High molecular weight PEG (PEG-20,000) showed similar effects on the Con-A intermediate as the medium molecular weight PEG (PEG-4000). Low concentrations of PEG-20,000 like PEG-4000 showed induction of non-native secondary structure but the transition that occurred at 40% PEG-4000 (Fig. 47 (a), series-3) took place at a lower concentration in case of PEG-20,000. Figure shows a clear decrease in CD values at 28% PEG-20,000. On adding PEG-20,000 tertiary structure changed significantly and, unlike PEG-4000, increase in concentration resulted in considerable loss of structure (figure-47 (b), series-3). This loss of tertiary structure is also evident from decrease in fluorescence intensity with increasing polyol concentration (Fig. 48, series-3). Transition in tertiary structure mainly occurred at 16% PEG-20,000 while in case of PEG-4000 the transition started at around 20% of the polyol. So we can say PEG-20,000 altered the tertiary structure of the protein in a way similar to that of PEG-4000 but the effect of PEG-20,000 was found to be more intense than PEG-4000 and the changes in structure occurred at lower concentrations of PEG-20,000 as compared to PEG-4000.
**ANS binding studies**

![Graph showing ANS fluorescence transition curve of pH 2 intermediate Con-A as a function of varying concentrations of different molecular weight PEGs.](image)

**Fig. 49** ANS Fluorescence transition curve of pH 2 intermediate Con-A as a function of varying concentrations of different molecular weight PEGs. PEG-400 (— ■ —), PEG-4000 (—♦—), PEG-20,000 (—▲—).

In order to identify the induction of any intermediates extrinsic fluorescence studies were performed in presence of ANS, a fluorescent hydrophobic dye widely used to detect the intermediate states of proteins, as it has higher affinity for partially folded or unfolded states with exposed hydrophobic patches [69]. PEGs show a significant amount of ANS binding. To rule out the contribution of PEGs, their respective blanks were prepared and fluorescence emission corrections for possible contribution of PGs were made. Fig. 49 shows the binding of ANS to acid unfolded Con-A in presence of different molecular weight PEGs. A decrease in ANS binding with increasing concentrations of all the PEGs was observed. When the pH 2 intermediate was treated with PEG-400 tertiary structure was stabilized only at low concentrations, at higher concentrations alteration in structure was seen. Induction of non-native secondary structure was found with increasing concentrations of PEG-400. In case of PEG-4000 and PEG-20,000 loss of tertiary structure was observed with increasing polyol concentrations. There was induction of secondary structure initially but at higher concentrations loss in structure occurred. Loss of tertiary contacts bringing the pH 2 intermediate in a conformation where the hydrophobic patches became less accessible as compared to the pH 2 intermediate of Con-A explains the decrease in ANS fluorescence when exposed to PEGs. CD and
fluorescence spectra of the intermediate treated with the PEGs in all the observations were different from that of 6M GdnHCl treated preparation indicating that the intermediate did not undergo complete denaturation. It can be hypothesized that the polyethylene glycols altered the conformation of the pH 2 intermediate and resulted in its transition to another intermediate state in which the hydrophobic patches were not as accessible as they were earlier.

On the basis of above results obtained we can summarize that the folding intermediate of Con-A obtained at pH 2 underwent a series of conformational changes when exposed fluoroalcohols and to different molecular weight PEGs. In presence of fluoroalcohols the pH 2 intermediate was transformed to a state with predominant α-helical secondary and lost tertiary structures. In the pathway of these transformations MG-like intermediates were formed at 10% TFE and 6% HFIP. In presence of low molecular weight PEG, PEG-400, non-native secondary structure was induced and tertiary structure was stabilized at low concentrations while loss of tertiary structure was seen at higher concentrations. In presence of PEG-4000 and 20,000 there was induction of secondary structure initially but at higher concentrations (40% PEG-4000 and 28% PEG-20,000) transition in structure was observed while loss in tertiary structure was seen with both medium and high molecular weight PEGs. As shown by ANS binding studies, in the intermediate states induced by the PEGs the hydrophobic patches were not as much exposed as in the pH 2 intermediate itself. Hence ANS binding was found to be maximum with the pH 2 intermediate which decreased on exposure to PEGs and went on decreasing with their increasing concentrations.