Chapter 6

Intermediate States Of Lectin Under Alkaline Conditions

Work from this chapter (for revision) in:

6.1 Introduction

Among the earliest known agents affecting protein stability and aggregation were salts [209] and acids and bases [210]. The earliest understood electrostatic effect on proteins was the pH titration behavior of native molecules [211]. The pH of the solution is crucial for the proteins because their net charge depends not only on the amino acids constituting them but also on the acidity or basicity of the buffer environment. Behavior of a given protein at low or high pH is a complex interplay between a variety of stabilizing and destabilizing forces. Various interactions get altered due to the change in pH. Hydrogen bonds play a very crucial role in stabilizing the structure. Hydrogen bond occurs between two electronegative atoms for the same hydrogen atom, D-H—A. In protein major hydrogen bond donors are Arg with five hydrogens and lysine with three hydrogens. The strongest acceptors are Asp and Glu with 4 acceptor sites each. Upon reducing the pH of the solution the most of the acidic residues get protonated. It is very likely that extremely low pH would break salt bridge interactions. On the other hand increasing the pH would lead to the deprotonation of the basic residues Arg and Lys, probably involved in many hydrogen bonds in native state. Deprotonation would disrupt the stability of many residues involved, which may lead to unfolding of the protein. Also salt-bridges between ionizing groups may be weakened or disrupted at extreme pH, at which one of the interacting groups is no longer ionized.
6.2 Results and Discussion

6.2.1 Pea lectin in alkaline conditions: formation of MG-like intermediate and its structural and thermal studies under the influence of HFIP:

6.2.1.1 Alkaline denaturation of pea lectin

Pea lectin was subjected to alkaline denaturation in the range of pH 7 to 13. It was observed that pea lectin showed resistance to alkaline pH up to pH 10.5 but at pH 13 both the secondary as well as tertiary structure underwent denaturation. Fig. 50, inset, shows the change in secondary structure of the protein as a function of pH as detected by far-UV CD.

![Far-UV CD spectrum of pea lectin at pH 7 (- - -); pH 11 (----); pH 13 (--- --), and in presence of 6M GndHCl (.......). (Inset: pH transition curve in the far-UV region, monitored at 217 nm).](image)

As already mentioned, it can be seen from the figure that the lectin resisted any change in its structure up to pH 10.5. From pH 11 a slight change becomes evident, further increase in pH resulted in continuous loss of structure and at pH 13 the protein is almost completely denatured. Fig. 50 depicts the spectra of pea lectin under varying conditions,
spectrum of the lectin at pH 11 is only slightly different from that of native and the spectrum corresponding to pH 13 state closely resembles the spectrum of the completely denatured state at 6 M GdnHCl. Interestingly near-UV CD studies show that like secondary structure, tertiary structure of the protein also resisted any change upto pH 10.5. A slight loss in structure occurred at pH 11, denaturation in tertiary structure went on increasing with increase in pH (Fig. 51) and at pH 13 protein lost the signal at 275 nm, which was prominent in native state spectrum and also in the spectrum of the protein at pH 11, and approached the completely unfolded structure in presence of 6M GdnHCl.

Fig. 51  Near-UV CD spectrum of pea lectin at pH 7 (—–); pH 11 (——); pH 13 (—— • ——), and in presence of 6M GdnHCl (……...).  
(Inset: pH transition curve in the Near-UV region. Ellipticity measurements were carried out at 275 nm).

The known pea lectin amino acid sequence and knowledge of the secondary structure shows that it has 27 acidic residues and 20 basic amino acid most of them are approximately 50% exposed and few of these residues like Glu-119 and 223, Asp-81, 121, 140 and 195, Arg-213 and His-166 and 252 are buried in the native form.
It is probably the titration of the side chains of these charged amino acid residues that disrupted their stability at extreme alkaline pH, ultimately leading to the unfolding of protein. Basic residues Arg and Lys, probably involved in many hydrogen bonds in native state get deprotonated due to which the hydrogen bonds get disrupted. May be the salt-bridges between ionizing groups also got weakened, contributing to the alkaline unfolding of the protein.

Intrinsic fluorescence data, Fig. 52, shows a slight increase in fluorescence up to pH 8 (spectrum-2) as compared to pH 7 state (spectrum-1).

![Fluorescence intensity of pea lectin at 335 nm as a function of pH. Inset: Tryptophan fluorescence spectrum of pea lectin. Pea lectin at pH 7 (curve-1); pH 8 (curve-2); pH 9 (curve-3); pH 10 (curve-4); pH 11 (curve-5); pH 12 (curve-6); pH 13 (curve-7) and in presence of 6M GndHCl (curve-8)]
For this slight initial increase of intensity it could be hypothesized that up to pH 8 disturbance in the balance existing between the amino acids of native state due to the titration of side chains did not come into play and only resulted in some kind of rearrangements in the immediate environment of tryptophan residues that resulted in the initial increase. Further it was observed that with increase in pH, fluorescence intensity went on decreasing. This decrease of fluorescence intensity could be attributed to the exposure of tryptophan residues to polar environment as a consequence of alkaline unfolding. Out of the 5 tryptophan residues Trp-236 and 257 are predicted to be present buried in beta strands. Rest of them supposed to form coils are also buried to quite some extent in the native form. The 10 Tyrosine residues which also contribute to the intrinsic fluorescence are shown to be a part of coil and beta structures and are also present almost buried in native state. When the protein opens up in presence of alkaline conditions, these aromatic amino acid residues that are a part of native beta structure get exposed to the polar environment resulting in the decrease of fluorescence intensity.

As can be seen in Fig. 52 (inset), spectra from pH 9 show a continuous decrease in intensities and at pH 13 (spectrum-7) although there is an increase in intensity the spectrum is considerably red shifted and corresponds to the completely denatured state (spectrum-8) reflecting the loss of structure at pH 13, this result is in accordance with that observed by near-UV CD studies which also shows complete unfolding of pea lectin at pH 13. The extent of hydrophobic patches exposed in the entire alkaline range was monitored by extrinsic fluorescence experiments. Fig. 53 clearly shows maximum ANS binding at pH 11 state (spectrum-5).

On the basis of observations stated above we can say that pea lectin at pH 11 exists as Molten Globule like intermediate with native like secondary structure, retained tertiary structure and has maximally exposed hydrophobic sites. Increasing evidences support the idea that MG state besides having secondary structure may posses well defined tertiary contacts as well [73-76]. In the insets of figure-52 and figure-53 only selected spectra have been shown for the sake of clarity.
6.2.1.2 Effect of 1,1,3,3,3-hexafluoropropanol on MG like state of pea lectin

The stability of the MG-like folding intermediate obtained at alkaline pH 11 was further studied in presence of HFIP. Far-UV CD studies showed that on addition of the fluoroalcohol the native like structure retained by the intermediate underwent a sudden change in structure (CD values of pea lectin under different conditions have been summarized in table- IX).
Table IX:
Summary of different spectral properties of Pea lectin.

<table>
<thead>
<tr>
<th>Pea lectin under different conditions</th>
<th>MRE</th>
<th>Intrinsic Fluorescence</th>
<th>ANS Fluorescence Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>217 nm</td>
<td>275 nm</td>
<td>Relative Fluorescence intensity</td>
</tr>
<tr>
<td>Native at pH 7</td>
<td>-14.21</td>
<td>0.5</td>
<td>100</td>
</tr>
<tr>
<td>MG-like Intermediate at pH 11</td>
<td>-13.97</td>
<td>0.38</td>
<td>35.16</td>
</tr>
<tr>
<td>MG-like Intermediate in presence of 8% HFIP</td>
<td>-11.11</td>
<td>0.17</td>
<td>93.16</td>
</tr>
<tr>
<td>GdnHCl</td>
<td>-3.86</td>
<td>-0.74</td>
<td>30</td>
</tr>
</tbody>
</table>

Fig. 54 shows a continuous decrease in CD values with increasing HFIP concentration, inset shows, as expected, that under the influence of HFIP the residual beta structure of the protein in presence of 2% HFIP (curve-1) was transformed to typical alpha helical structure at 30% HFIP (curve-4) further addition of HFIP had no effect on the secondary structure. Spectrum-3 corresponding to the pH 11 intermediate in presence of 8% HFIP represents a state where the protein begins the structural transformation i.e. the transformation from beta to alpha. It is now an established fact that alcohol destabilizes hydrophobic cores because of its non-polar character while it enhances secondary structure formation of protein by minimizing exposure of peptide back bone [165-167]. They weaken nonlocal hydrophobic interactions while promoting local polar interactions (i.e., hydrogen bonds). In many cases alcohol induced unfolding of the protein is accompanied by stabilization of the extended helical rods in which the hydrophobic side chains are exposed but the polar amide groups are shielded from the solvent [179, 166].
It has also been observed that alcohols induce significantly higher helical structures in partially or completely unfolded proteins as compared to folded proteins [212].

Fig. 54 pH transition curve of pea lectin at pH 11 in presence of increasing concentrations of HFIP in the far-UV region, monitored at 217 nm. (Inset: Far-UV CD spectrum of pea lectin at pH 11 showing the effect of increasing concentrations of HFIP, 2% HFIP (curve-1); 4% HFIP (curve-2); 8% HFIP (curve-3); 30% HFIP (curve-4).

Near-UV CD was used as probe to monitor the changes occurring in the tertiary structure of the protein under the influence of HFIP. The results obtained showed disruption of the residual tertiary structure (Fig. 55).
Intrinsic fluorescence studies were also used to determine the tertiary structure of the intermediate in presence of HFIP. Fig. 56 shows an initial increase in fluorescence intensity at 2%, 4% and 6% HFIP concentrations as compared to the native and alkaline unfolded intermediate but as shown in inset the increase in intensity was accompanied by a considerable red shift in each case. Hence it can be concluded that the residual tertiary structure of the pH 11 induced intermediate underwent further disruption on addition of HFIP and the increase in intensity could be attributed to separation due to unfolding, of the chromophoric groups, originally present in vicinity of some quencher group in the native state.
Extrinsic fluorescence studies were done in order to find out the HFIP induced intermediate with maximally exposed hydrophobic patches. It was observed that ANS showed maximum binding to the pH 11 intermediate in presence of 8% HFIP (figure-57). It can be seen from the figure that ANS binding to the HFIP induced state is even more than that of pH 11 intermediate itself. Thus addition of 8% HFIP to the alkaline unfolded state resulted in formation of another intermediate having non-native secondary structure, disrupted tertiary structure and having maximally exposed hydrophobic patches.
6.2.2 Thermal studies

Comparative thermal studies were done on the above discussed intermediates of pea lectin, one obtained at pH 11, the alkaline denatured MG-like intermediate and the other obtained on treating the pH 11 intermediate with 8% HFIP. The thermal unfolding experiments of the intermediate states were performed with reference to the thermal unfolding of the native state of the protein. Figure-58 shows the thermal profiles of pea lectin under three different conditions monitored by far-UVCD at 217 nm. Series-1 (represented by filled circles) corresponds to pea lectin in the native state. It was observed that there was no considerable change in structure up to 80°C, protein was found to undergo thermal denaturation at 85°C as reflected by the decrease in negative CD values at 217 nm. Series-2 (represented by empty circles) corresponds to the
changes that occurred in the MG-like intermediate when subjected to thermal treatment, the unfolding temperature of the lectin under alkaline conditions was reduced to 75°C while the same intermediate in presence of 8% HFIP (series-3), represented by triangles, was discovered to be the most labile form which showed changes in its secondary structure at temperature as low as 65°C.

![Thermal transition curve as monitored by far-UV CD at 217 nm. Protein concentration was 0.5 mg ml⁻¹ in pH 7 (—•—), pH 11 (—○—) and pH 11 in presence of 8% HFIP (—▲—).](image)

Tertiary structure of the lectin reacted in a pattern very similar to that of secondary structure when exposed to increasing temperatures (figure-59). It is known that tertiary structure is more susceptible to heat than secondary structure. In our case also the native protein resisted change in structure upto 70°C and unfolded at 75°C, the MG-like state at pH 11 melted at 70°C and in presence of HFIP tertiary structure that had already suffered denaturation under alkaline conditions, disrupted at 55 °C. Alkaline pH producing a decrease of denaturation temperature has been reported [213]. Here we also report a decrease of denaturation temperature under alkaline conditions and the further weakening of the structure under the influence of 8% HFIP resulting in thermal denaturation at even lower temperature. CD and fluorescence values of the lectin under varying conditions have been summarized in Table-1.
Alkaline pH (pH 11) resulted in the formation of a MG-like intermediate of pea lectin with native like secondary structure and quite stable tertiary structure and showed maximum ANS binding. Addition of HFIP to the beta rich intermediate resulted in its transition to alpha helix dominated structure and in between the pathway of transition another intermediate state was formed in which the native like secondary structure was replaced by non-native structure induced by HFIP and the tertiary structure, which was retained in the MG-like state, was disrupted. Thus HFIP induced state was also found to have maximally exposed hydrophobic patches. Thermal studies showed that alkaline pH reduced the denaturing temperature of pea lectin and it became even more susceptible in presence of 8% HFIP.