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THE EFFECT OF pH ON THE UNFOLDING PATHWAY AND STABILITY OF RIBOSOME-INACTIVATING PROTEIN ABRIN-II

Jayarapu Krupakar[1], Puspendu K. Das[1]* and Sunil K. Podder[2]†
[1]Department of Inorganic and Physical Chemistry and [2]Department of Biochemistry, Indian Institute of Science, Bangalore 560012, INDIA.

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Summary: The effect of pH on the unfolding pathway and the stability of the toxic protein abrin-II have been studied by increasing denaturant concentrations of guanidine hydrochloride and by monitoring the change in 8,1-anilino naphthalene sulfonic acid (ANS) fluorescence upon binding to the hydrophobic sites of the protein. Intrinsic protein fluorescence, far and near UV-circular dichroism (CD) spectroscopy and ANS binding studies reveal that the unfolding of abrin-II occurs through two intermediates at pH 7.2 and one intermediate at pH 4.5. At pH 7.2, the two subunits A and B of abrin-II unfold sequentially. The native protein is more stable at pH 4.5 than at pH 7.2. However, the stability of the abrin-II A-subunit is not affected by a change in pH. These observations may assist in an understanding of the physiologically relevant transmembrane translocation of the toxin.

Keywords: ribosome-inactivating protein, abrin-II, pH, stability, unfolding, guanidine hydrochloride, fluorescence, circular dichroism, 8,1-anilino naphthalene sulfonic acid, lactose.

Introduction

Ribosome-inactivating protein (RIP) toxins from plants are classified into two groups: Type I RIPs are single subunit proteins, e.g., saporin, gelonin, momordin. Type II RIPs are heterodimeric proteins, e.g., abrin, ricin, mistletoe lectin (1). The two subunits of type II RIPs are designated A- and B- subunits which are connected by a single disulfide bond (2). Type I RIPs and the A-subunit of type-II RIPs are N-glycosidases and are potent inhibitors of protein biosynthesis acting by the cleavage of the adenine 4324 residue from the 28S rRNA of cell free systems i.e., rabbit reticulocyte lysate (3). Type II RIPs are highly cytotoxic. They bind to cell surface receptors containing terminal galactose via its B-subunit (4) which facilitates the entry of the toxin into the cytosol (5). Both type I and type II RIPs have been used for the

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† CSIR emeritus scientist

*corresponding author,

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preparation of immunotoxins with potential uses in therapy against cancer and infectious diseases including AIDS (6). RIPs which in their native state are soluble globular proteins. But in order to reach their target they have to translocate across the membrane of an intracellular organelle. It is shown that the toxin enters the cell through receptor-mediated endocytosis (7), and that the cleavage of the disulfide bond connecting the A- and B- subunits is necessary for the toxin to exercise its action (8). Earlier it was thought that the late endosomes (9) and golgi (10) were probable sites for transmembrane transfer of toxin. Recently it was suggested that the endoplasmic reticulum is a probable site (11) for the transfer. However, the mechanism underlying the transmembrane transport by RIPs is not understood.

Unlike the case of RIPs from plants, the process of cell intoxication by diphtheria toxin (DT) is well understood. Low pH encountered in the endosomes induces conformational change in DT resulting in the transport of the A-subunit to the cytosol through the endosomal membrane (12). In vitro, DT undergoes a partial unfolding at low pH and this state is capable of translocation. Thus from studies on DT it was made clear that transient association of RIPs with the membrane is essential for translocation (13). Type-II RIPs are simple proteins which can serve as a model system to investigate the molecular mechanism of transmembrane translocation of cellular proteins. The unravelled mechanism will also assist in the design of therapeutically useful immunotoxins. In this study, detailed unfolding studies were carried out on abrin-II. The effects of pH on the unfolding pathway and stability of abrin-II after addition of the denaturant, guanidine hydrochloride, GdnHCl were investigated by employing fluorescence and circular dichroism, CD spectroscopic techniques.

Materials and methods

Materials: Abrin-II was purified from seeds of *Abrus precatorius* as described in (14). The A-subunit of abrin-II was purified using known procedures (15). The concentrations of abrin-II and its A-subunit were determined using extinction coefficients of 100170 $M^{-1}cm^{-1}$ and 23610 $M^{-1}cm^{-1}$ at 280 nm respectively. All column materials were obtained from Pharmacia (Uppsala, Sweden). Ammonium salt of 8,1-anilino naphthalene sulfonic acid (ANS), Guanidine hydrochloride (GdnHCl) and lactose were obtained from Sigma Chemicals Co., (St. Louis, MO) USA. The concentration of ANS was determined using an extinction of 4.91×10^3 $M^{-1}cm^{-1}$ at 363 nm. The concentration of GdnHCl was determined by the refractive index method (16). All other chemicals used were of analytical grade and obtained locally.

Denaturation / renaturation of protein: Denaturation of abrin-II and its A-subunit was monitored by measuring changes in fluorescence and circular dichroism, CD spectra. The protein was denatured in various concentrations of GdnHCl either in 50 mM sodium phosphate buffer (pH 7.2) or 50 mM sodium acetate buffer (pH 4.5). The protein-GdnHCl mixtures were

incubated overnight to achieve equilibrium denaturation at 25 °C. The apparent fraction of unfolded protein, F_{app} was calculated according to the equation(1)

$$F_{app} = (Y_{obs} - Y_n)/(Y_u - Y_n) \quad (1)$$

where Y_{obs} is the observed value of fluorescence/CD signal at any given denaturant concentration and Y_n and Y_u are the values of Y characteristic of native and unfolded forms of protein, respectively.

For renaturation experiments, stock solutions of denatured protein were diluted to the working concentration of the protein in buffer containing the indicated final concentration of GdnHCl. The mixtures were incubated overnight to achieve equilibrium renaturation at 25° C. All the spectroscopic measurements were made at 25° C. All the baseline corrections were made by subtracting the buffer spectrum from the sample spectrum.

Protein fluorescence: All fluorescence measurements were carried out using a Shimadzu RF-5000 Spectrofluorimeter. Intrinsic protein fluorescence spectra were recorded by setting the excitation wavelength at 280 nm and the emission was scanned between 300 and 400 nm. The slitwidths were 3 nm and 5 nm for excitation and emission respectively.

ANS fluorescence: The fluorescence spectra of ANS were collected on excitation at 420 nm with slitwidth of 5 nm and emission spectra were recorded between 440 nm and 530 nm with a slitwidth of 5 nm.

Circular dichroism(CD): CD spectra were recorded using a Jasco J-720 spectropolarimeter. Secondary structure was monitored by measuring ellipticity at 222 nm using a cell path length of 1 mm and tertiary structure was monitored in the wave length range 250-320 nm using a cell of path length 5 mm.

Results

Denaturation by Guanidine hydrochloride: Protein fluorescence and far UV-and near UV-CD were used to monitor changes in secondary and tertiary structure of the protein. The unfolding of the abrin-II induced by GdnHCl was assessed by monitoring the change in fluorescence at 330 nm and by following its far UV-CD at 222 nm. The apparent fraction of unfolded protein, F_{app} was calculated using equation (1). F_{app} was plotted against GdnHCl concentration in figure 1. At pH 7.2, the fluorescence-unfolding curve indicates the presence of two transitions caused by the increase in GdnHCl concentration. The midpoints of the two transitions were 1.4 M and 2.8 M GdnHCl. The midpoint of the far UV-CD curve was 2.8 M GdnHCl at pH 7.2. The midpoints of the fluorescence and far UV-CD curves were 2.5 M and 2.9 M GdnHCl, respectively at pH 4.5. Non-superimposable fluorescence and far-UV CD suggest the possible presence of intermediate states in the unfolding pathway at both pH 7.2 and 4.5. Abrin-II and its A-subunit completely unfold in 5 M GdnHCl at both pH 7.2 and 4.5.

ANS fluorescence: The unfolding exposes hydrophobic patches of the protein to the bulk water. This may result in excess binding of extrinsic hydrophobic probes like ANS to the protein (17). Figure 2 shows the binding of ANS to abrin-II and its A-subunit as a function of GdnHCl

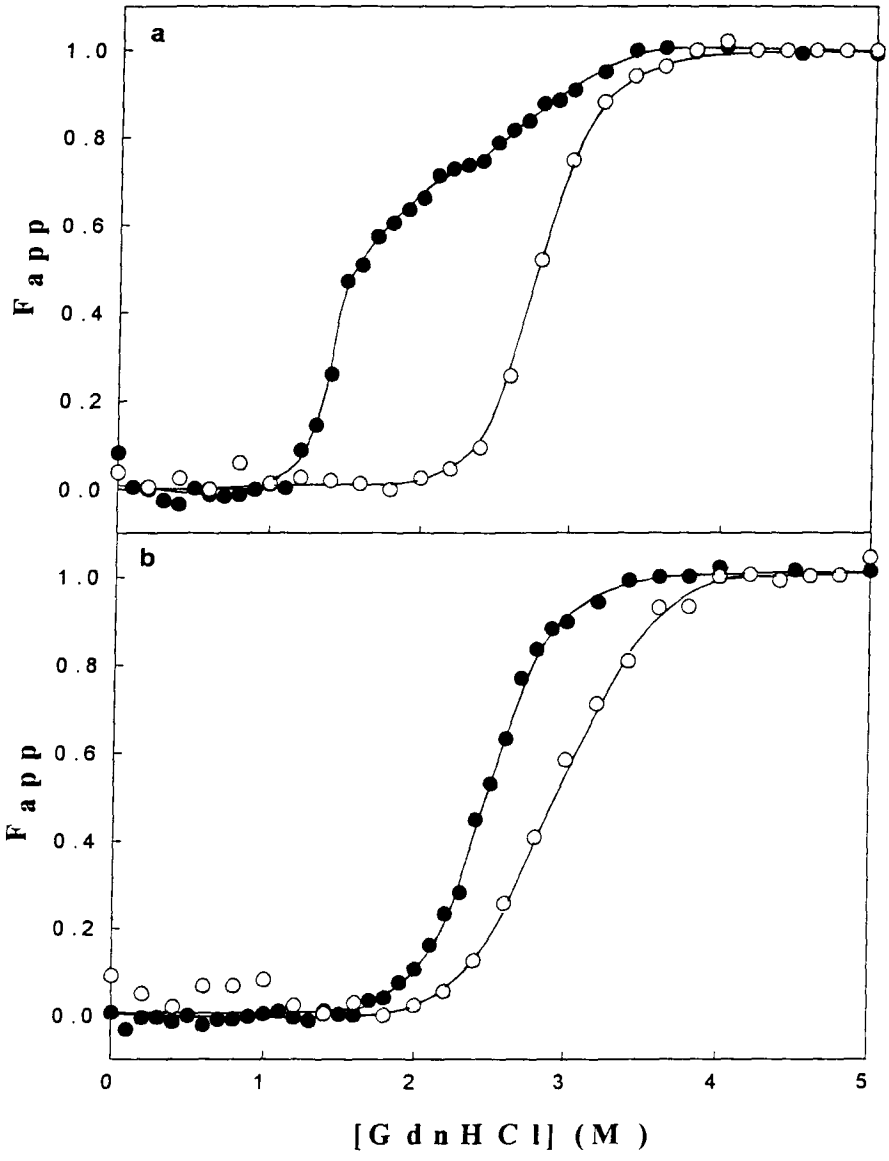

Figure 1: GdnHCl induced unfolding transition of Abrin-II. Unfolding was followed using fluorescence intensity at 330 nm (-•-) and for UV-CD (-o-) at 222 nm. Protein concentrations were 0.5 μM and 3.7 μM for fluorescence and CD, respectively a) pH 7.2 b) pH 4.5.

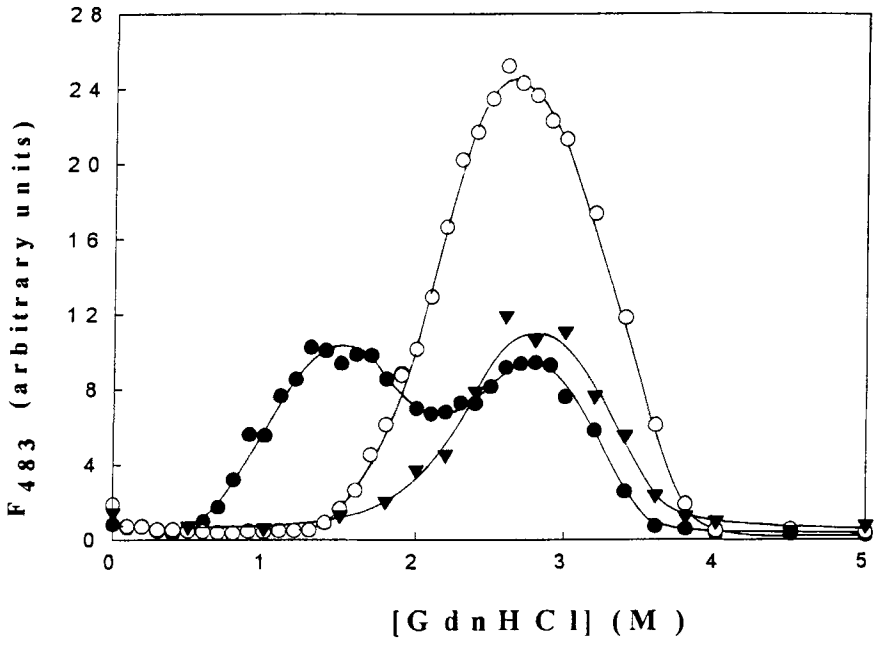

Figure 2: ANS binding as function of GdnHCl concentration. abrin-II pH 7.2 (-•-), abrin-II pH 4.5 (-o-), and abrin-II A-subunit pH 7.2 (-▼-). ANS concentration was 100 μM. Protein concentration was 2.9 μM for abrin-II and its A-subunit.

concentrations. For abrin-II two peaks were observed for bound-ANS fluorescence at pH 7.2 while a single peak was observed for both abrin-II at pH 4.5 and the A-subunit at pH 7.2.

Figure 3 shows the near-UV CD spectra of abrin-II in the absence and presence of 1.4 M GdnHCl at pH 7.2 and 2.2 M GdnHCl at pH 4.5. The changes in the relative fluorescence of abrin-II in the presence of lactose and the abrin-II A-subunit with increasing the concentration of GdnHCl alongwith refolded abrin-II A-subunit are shown in figure 4. The midpoints of the curves were 2.1 M and 2.7 M GdnHCl at pH 7.2 for abrin-II in presence of lactose and abrin-II A-subunit, respectively. The corresponding values of these at pH 4.5 are 2.55 M, 2.7 M.

Discussion

The non-coincidence of the unfolding curves shown in fig. 1 suggests the presence of intermediates along the unfolding pathways of the abrin-II at both pH 7.2 and 4.5. This suggestion is supported by results of the ANS binding (fig. 2) and the near UV-CD (fig. 3) studies. The transition midpoints at 1.4 M and 2.8 M GdnHCl obtained from the fluorescence-unfolding curve of abrin-II at pH 7.2 (fig. 1) match well with the two maxima in the ANS

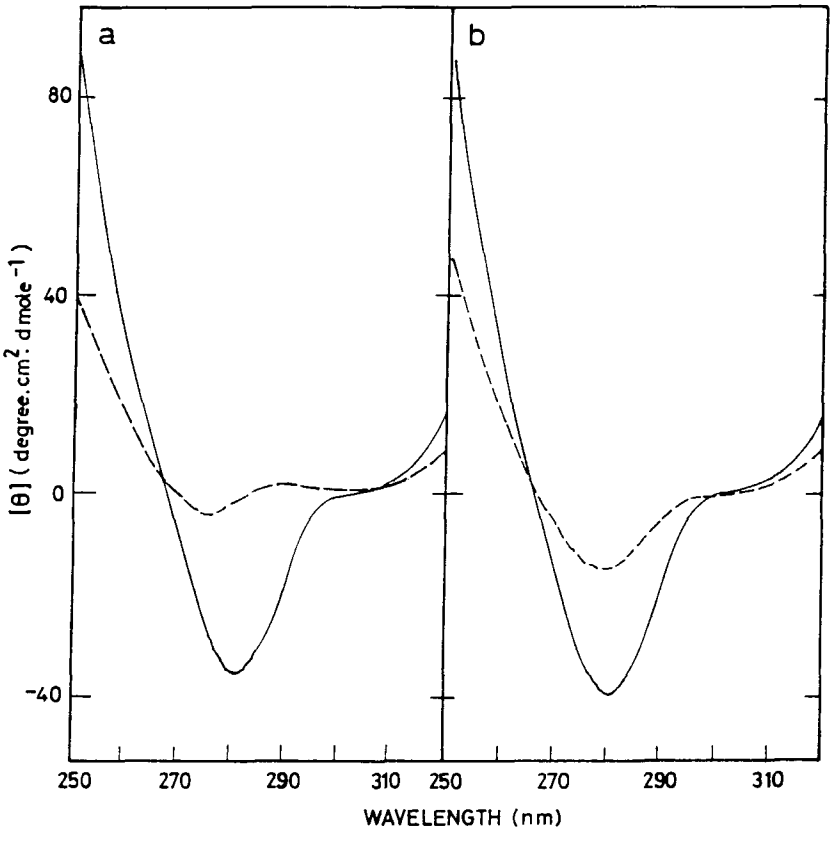

Figure 3: Dependence of mean residue ellipticity on wavelength in the near UV-CD region of abrin-II in presence of 0 M(—), 1.4 M(– –) at a) pH 7.2 and 0 M(—), 2.2 M(– –) at b) pH 4.5 GdnHCl. Protein concentration was 35 μM.

binding curve (fig. 2). At 1.4 M GdnHCl, the loss of tertiary structure (fig. 3) without change in secondary structure and ANS peak maximum indicate the presence of an intermediate. The transition midpoint at 2.8 M GdnHCl in the fluorescence unfolding curve of abrin-II matches with the second peak maximum of the ANS binding curve indicating another intermediate at pH 7.2. It also matches with the peak maximum of ANS binding curve and the midpoint of the isolated abrin-II A-subunit at pH 7.2. Therefore, the second transition in fig. 1 for the intact toxin may be attributed to the A-subunit. Abrin-II binds to carbohydrates such as lactose and galactose via the B-subunit. At pH 7.2, abrin-II in presence of lactose starts unfolding at 1.8 M of GdnHCl which is higher than that at 1.2 M in absence of lactose. At pH 4.5, the corresponding concentrations of GdnHCl are 2.0 M and 1.7 M, respectively. The first transition

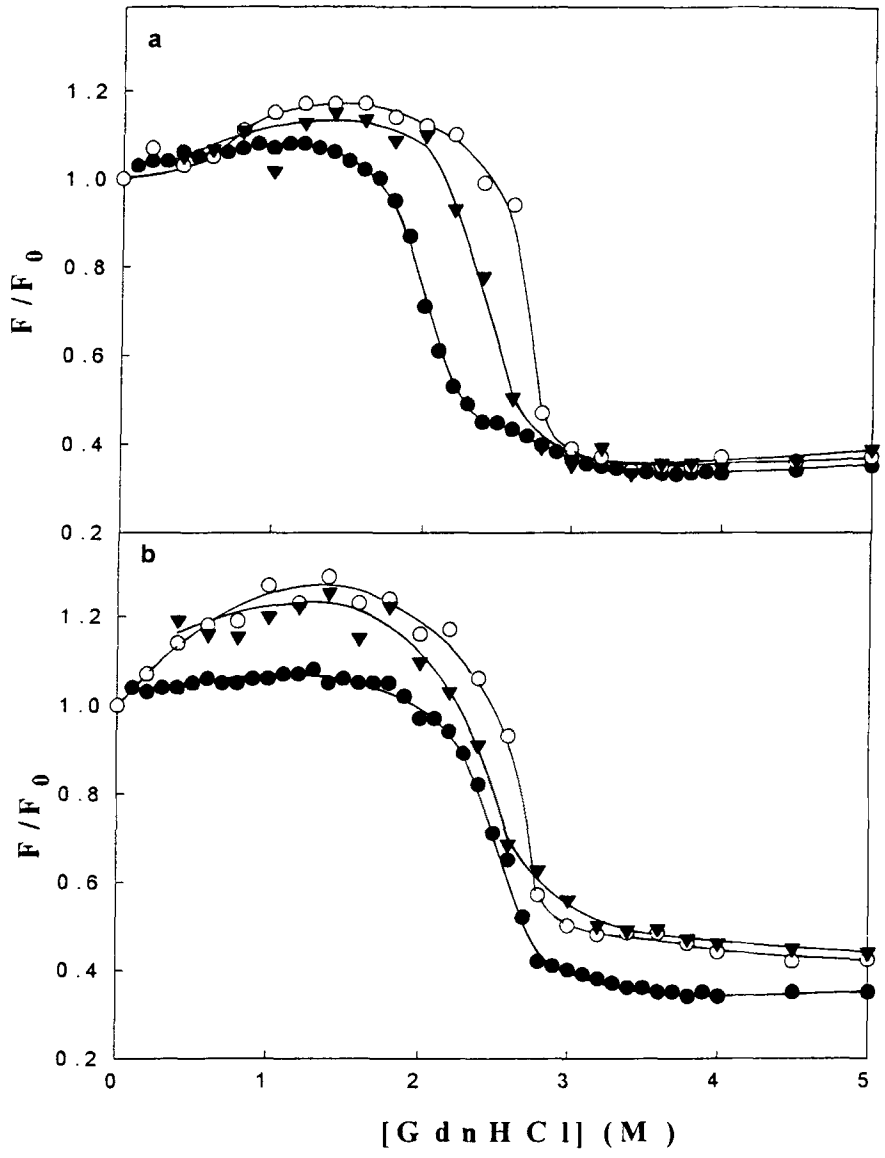

Figure 4: Relative fluorescence (F/F_0) as a function of GdnHCl. A) pH 7.2 b) pH 4.5 abrin-II + 50 mM lactose (-•-), its A-subunit (-o-) and refolded abrin-II A-subunit(-▼-). Protein concentration was 0.5 μM for abrin-II in presence of lactose, its A-subunit and refolded abrin-II A-subunit.

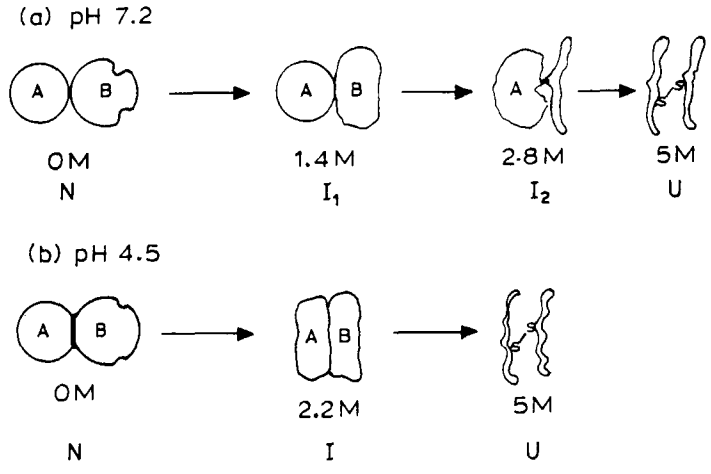

Figure 5: Schematic representation of abrin-II unfolding pathway. a) pH 7.2 b) 4.5 native protein (N), intermediates (I_1, I_2, I), unfolded protein (U).

midpoint of abrin-II at 1.4 M shifts to 2 M in the presence of lactose. There was no significant change in the midpoint of transition of abrin-II A-subunit in presence of lactose at both pH 7.2 and 4.5. This agrees well with the absence of binding sites for lactose in the A-subunit (18). Thus the first transition in fig. 1 may be due to the galactose binding B-subunit of intact abrin-II. The B-subunit is insoluble in aqueous solutions when separated from A-subunit. These results at pH 7.2, show that the B-subunit unfolds earlier than A-subunit in intact abrin-II, suggesting there is little interaction between the subunits. There were also two-intermediates along the unfolding pathway of intact abrin-II. At pH 4.5, non-coincidence of the fluorescence and far UV-CD unfolding curves, the presence of a single peak in ANS binding and reduced tertiary structure at 2.2 M GdnHCl, suggest the presence of an intermediary form along the unfolding path of the abrin-II. At pH 7.2 the onset of unfolding is at 1.2 M GdnHCl whereas it shifts to higher concentration of GdnHCl (1.7 M) at pH 4.5. This implies that the intact protein is more stable at the acidic pH. Such an increase in stability was also observed in ricin, mistletoe lectin and their A-subunits whereas their B-subunits exhibited show a decreased stability at low pH (19, 20). In contrast, there was no such effect on the A-subunit of abrin-II. Moreover, saporin, gelonin and abrin-II A-subunit (table 1) are more stable than the A-subunits of ricin and mistletoe lectin (21, 22). The midpoints of GdnHCl unfolding curves of these proteins are shown in table 1. The B-subunits of ricin, and mistletoe lectin undergo structural transitions at low pH (23). The GdnHCl induced unfolding pathway of ricin B-subunit occurs through a transition intermediate (24).

Table 1: GdnHCl concentrations corresponding to the midpoint of the unfolding transitions

Protein	[GdnHCl], M			
	neutral pH		low pH	
	Fluorescence	CD	Fluorescence	CD
[1]abrin-II	1.4, 2.8(7.2)	2.8(7.2)	2.5(4.5)	2.9(4.5)
+50 mM lactose	2.1,2.8(7.2)		2.55(4.5)	
A-subunit	2.7(7.2)		2.7(4.5)	
[2]ricin	4.1(7.5)		5.3(5.0)	
+50 mM lactose	5.2(7.5)		5.5(5.0)	
A-subunit	1.5(7.5)		2.0(5.0)	
B-subunit	1.3(7.5)		0.5(5.0)	
+50 mM lactose	2.7(7.5)		0.6(5.0)	
[3]mistletoe lectin	4.4(7.0)		5.1(5.0)	
+50 mM lactose	5.4(7.0)		5.6(5.0)	
A-subunit	1.7(7.0)		2.1(5.0)	
B-subunit	1.3(7.0)		1.0(5.0)	
+50 mM lactose	2.1(7.0)		2.2(5.0)	
[4]gelonin	3.0(7.4)		3.5(5.0)	

[1] this study

[2,3,4] Values are taken from (19), (20) and (22), respectively.

Figs in brackets are the pH values of the solutions.

At pH 4.5, the effect of lactose binding on the protein unfolding curve is less pronounced than that at pH 7.2. It is either due to weak binding of lactose to the protein and/or decrease in the number of binding sites at pH 4.5. The binding constant and binding stiochiometry of ricin to galactose at low pH is $1/10^{th}$ and $1/4^{th}$, respectively, of those at neutral pH (25). The reversibility of Abrin-II and its A-subunit unfolding has been checked by fluorescence change at both pH 7.2 and 4.5. The recovery of fluorescence is over 80% of the initial emission at pH 7.2 and 60% at pH 4.5(data not shown) for the intact protein. On the other hand, fluorescence is fully reversible at both pH values (fig. 4) for the A-subunit. The proposed unfolding pathway of abrin-II is shown in figure 5.

In summary the following three observations are linked to a proposition for transmembrane translocation: a) the stabilization induced by low pH b) weaker binding to lactose at low pH and c) intermediates at both pH 7.2 and 4.5. The low pH in the endosomal compartment would cause the dissociation of a significant proportion of the toxin from its receptor, allowing the recycling of the latter to the cell-surface. The free toxin in the lumen of the endosome, being stable, is not only able to resist degradation but is also able to make its way to the trans golgi network and the endoplasmic reticulum. The high lumenal pH destabilizes the toxin, facilitating

the reduction of inter-subunit disulfide bond. The A-subunit is now ready to enter the cytosol through retrotranslocation. Further characterization is necessary to understand the role of the intermediates in the transmembrane translocation of the toxin.

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