Calorimetric studies on the stability of the ribosome-inactivating protein abrin II: effects of pH and ligand binding

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INTRODUCTION

Abrin II, a type II ribosome-inactivating protein (RIP) from Abrus precatorius seeds, consists of two subunits, the A-subunit (M, 30000) and the B-subunit (M, 33000), which are connected via a single disulphide bond [1]. The A-subunit is an N-glycosidase, and inactivates eukaryotic protein synthesis by cleaving the base adenine-4324 from the 28 S rRNA [2]. The B-subunit is a lectin, which binds to the cell-surface receptor that contains terminal galactose residues, thus facilitating the entry of the toxic A-subunit into the cell [3–6]. Reduction of the inter-subunit disulphide bond is necessary for the toxin to initiate its action [7]. The late endosomes and Golgi apparatus are implicated for the transmembrane transfer of the toxic A-subunit to the cytosol [8,9]. Recently it has been argued that the endoplasmic reticulum is the probable site of such a translocation [10,11]. Although the mechanism underlying the transmembrane transport of RIPs is not fully understood, these proteins have been used extensively for the construction of immunotoxins for therapeutic purposes. and have been useful as models for studies of the mechanisms by which soluble proteins are able to cross the membrane.

With regard to the action of toxins, the process of cell intoxication by diphtheria toxin (DT) is best understood. Subsequent to endocytosis, the endosomal pH induces conformational changes in DT, resulting in the transport of its A-subunit into the cytosol through the endosomal membrane [12]. In vitro, at low pH, DT undergoes partial unfolding. As a result it opens up the buried hydrophobic patches, which enable it to be translocated [13,14]. Abrin II, like DT, is a water-soluble protein, but in order to reach the ribosomes it has to cross the membrane. Earlier studies have shown that, at low pH, ricin (a type II RIP) and its subunits penetrate membranes [15,16]. Abrin II is expected to follow a similar itinerary within the cell.

A detailed thermodynamic study of abrin II will aid in the understanding of the mechanism of its transfer to the cytosol. Unfolding experiments using guanidine hydrochloride have demonstrated that, at low pH, ricin, mistletoe lectin and their A-subunits show an increase in stability, whereas their B-subunits show decreased stability [17,18]. In the present study, we investigate the effects of pH and ligand binding on the stability of abrin II by means of high-sensitivity differential scanning calorimetry (DSC). In these studies DSC has been used to determine the transition temperature (T) for the unfolding of abrin II, the calorimetric and van’t Hoff enthalpies (AH and AH, respectively), and the change in excess heat capacity (AC). Taken together, these data provide information on the conformational stability of the protein.

MATERIALS AND METHODS

Materials

DEAE-Sephacel, Sepharose-4B and Sephadex G-100 used for protein purification were products of Pharmacia (Uppsala, Sweden). Ultrapure lactose and dithiothreitol were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals used were of analytical grade and were obtained locally. Abrin II was isolated from seeds of Abrus precatorius using the procedure in [19]. The A-subunit of abrin II was

The effects of pH and ligand binding on the stability of abrin II, a heterodimeric ribosome-inactivating protein, and its subunits have been studied using high-sensitivity differential scanning calorimetry. At pH 7.2, the calorimetric scan consists of two transitions, which correspond to the B-subunit (transition temperature (T) 319.2K) and the A-subunit (T 324.6K) of abrin II, as also confirmed by studies on the isolated A-subunit. The calorimetric enthalpy of the isolated A-subunit of abrin II is similar to that of the higher-temperature transition. However, its T is 2.4K lower than that of the higher-temperature peak of intact abrin II. This indicates that there is some interaction between the two subunits. Abrin II displays increased stability as the pH is decreased to 4.5. Lactose increases the enthalpy between the two subunits. Abrin II displays increased stability as intact abrin II. This indicates that there is some interaction subsequent to endocytosis, the endosomal pH induces conformational changes in DT, resulting in the transport of its A-subunit into the cytosol through the endosomal membrane [12]. In vitro, at low pH, DT undergoes partial unfolding. As a result it opens up the buried hydrophobic patches, which enable it to be translocated [13,14]. Abrin II, like DT, is a water-soluble protein, but in order to reach the ribosomes it has to cross the membrane. Earlier studies have shown that, at low pH, ricin (a type II RIP) and its subunits penetrate membranes [15,16]. Abrin II is expected to follow a similar itinerary within the cell.

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Abbreviations used: DSC, differential scanning calorimetry; ITC, isothermal titration calorimetry; RIP, ribosome-inactivating protein; DT, diphtheria toxin; C, excess heat capacity; ΔC, change in excess heat capacity; T, temperature of the peak maximum; ΔH, molar transition enthalpy at T; ΔH, calorimetric enthalpy; ΔH, van’t Hoff enthalpy; n, number of binding sites.

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purified as described in [20]. The concentrations of abrin II and of its A-subunit were determined from their respective molar absorption coefficients at 280 nm of 100170 M$^{-1}$·cm$^{-1}$ and 23610 M$^{-1}$·cm$^{-1}$ [19,20].

**DSC**

All calorimetric measurements were carried out with a Microcal MC-2 ultrasonic differential scanning calorimeter. DSC measurements were carried out as a function of scan rate, pH, and protein and ligand concentrations. The data were analysed using Origin® software supplied by the DSC manufacturer. The ratio $\Delta H_p/\Delta H_m$ was utilized to infer the co-operativity of the transition. $\Delta H_m$ is calculated from the equation:

$$\Delta H_m = 4RT_b C_{\text{prmax}}/\Delta H_i$$  \hspace{1cm} (1)

where $R$ is the gas constant, $C_{\text{prmax}}$ is the maximal value of the excess heat capacity and $T_m$ is the temperature at $C_{\text{prmax}}$. The binding constant of lactose to the protein at the denaturation temperature [$K_b(T)$] is obtained from the following equation [21,22]:

$$K_b(T) = \frac{\exp[T_m - T_m \Delta H_p/nRT_m]}{[L]}$$  \hspace{1cm} (2)

where $T_m$ and $T_m$ are the denaturation temperatures in the presence and absence of lactose respectively, $\Delta H_p$ is the calorimetric enthalpy of the lactose-protein complex, $[L]$ is the ligand concentration and $n$ is the number of binding sites in the protein.

The protein samples were dialysed extensively with large volumes of the desired buffer and degassed prior to loading into the calorimetric cell. The sample pH was checked both before and after the scan. The buffers used were 50 mM sodium acetate for the pH range 4.5–5.8, 50 mM sodium phosphate for the pH range 6.4–7.9, 50 mM sodium borate for the pH range 8.3–9.2, and 50 mM sodium carbonate for pH 10.16. β-Mercaptoethanol (5 mM) was added to the buffer systems for experiments with the A-subunit of abrin II.

**Isothermal titration calorimetry (ITC)**

Isothermal calorimetric titrations were performed with an Omega microcalorimeter (Microcal), as described in [23,24]. Defined aliquots (5 μl) of lactose solution (10.5 mM) were added from the computer-controlled 250 μl rotating syringe (395 rev./min) to the protein solution (0.351 mM) kept in a 1.347 ml cell, and heat changes accompanying the additions were recorded. The time period between two consecutive injections was 2.5 min, to allow the exothermic peak, which appeared after each addition, to return to the baseline. The area under each peak represented the amount of heat accompanying binding of the added lactose to the protein. The total heat, $Q_i$, was then fitted via a non-linear least-squares minimization method to the total lactose concentration, $X_i$, using the following equation:

$$Q_i = n M_i \Delta H_v V_i [1 + X_i/nM_i + 1/nK_b M_i^{-1}] - [1 + X_i/nM_i + 1/nK_b M_i^{-1}]^2 - 4X_i/nM_i [1 + X_i/nM_i]$$  \hspace{1cm} (3)

where $n$ is the number of binding sites, $M_i$ is the total protein concentration, $V_i$ is the cell volume, $K_b$ is the binding constant and $\Delta H_v$ is the binding enthalpy. The heat released for the $i$th injection, $\Delta Q_i$, is given by the following equation:

$$\Delta Q_i = Q_i + dV_i/2V_i [Q_i + Q_{i-1}] - Q_{i-1}$$  \hspace{1cm} (4)

where $dV_i$ is the volume of the titrant added to the protein solution.

**RESULTS**

**Thermal unfolding of abrin II**

A plot of the baseline-subtracted excess heat capacity ($C_p$) against temperature for abrin II in 50 mM sodium phosphate buffer, pH 7.2, at a scan rate of 10 K·h$^{-1}$ is shown in Figure 1 (upper curve). The respective baseline-subtracted data (where the baseline was obtained with buffer in both the sample and the reference cuvettes of the calorimeter) were used for all analyses. The pre- and post-transition baselines were connected by the progress baseline option of the Origin® program and subtracted from the data, as shown in Figure 2. The free energy of unfolding of a protein can be obtained as a function of temperature, in principle, from a single DSC experiment by Gibbs–Helmholtz analysis of the DSC data. In practice, however, it is difficult to determine $\Delta C_p^\circ$ of unfolding accurately from a single scan. The measurement of $\Delta H_m$ as a function of $T_m$, obtained by carrying out DSC scans at different pH values, allows the estimation of $\Delta C_p^\circ$ from the slope of a plot of $\Delta H_m$ against $T_m$. The value of $\Delta C_p^\circ$ obtained by this method is considered a better estimate than that obtained directly as the difference between the pre- and post-transition baselines in a single DSC experiment, because the latter method is fraught with errors due to arbitrariness in baseline determination [25–28].

In the abrin II system, we have found that the $\Delta H_m$ against $T_m$ plot is fitted well by a straight line (correlation coefficients are 0.97 and 0.98 for the lower- and higher-temperature transitions respectively) over a range of $T_m$ values. In Figure 1, the dashed and the dotted–dashed lines are the deconvoluted least-squares fits of the calorimetric data to a two-stage transition model for each of the two transition peaks. The sum of these two transition peaks is represented by the continuous line, which follows closely the observed curve, represented by open circles. The lower-temperature transition peak has a $T_m$ of 319.2 K with $\Delta H_m =$
Figure 2  DSC scan of abrin II (92.8 μM) in the presence of 20 mM lactose, pH 7.2, at 10 K·h⁻¹

Table 1  Thermodynamic quantities for the thermal transition of abrin II and its isolated A-subunit as a function of pH

The DSC scan rate was 10 K·h⁻¹, except where indicated (*60 K·h⁻¹). \( T_m, \Delta H_c \) and \( \Delta H_v \) are the respective values for the lower-temperature transition, while \( T_m, \Delta H_c \) and \( \Delta H_v \) are the same quantities for the higher-temperature transition. The errors in the determination of \( T_m, \Delta H_c \) and \( \Delta H_v \) values were less than 0.05%, 3.0% and 3.5% respectively. The concentration of intact abrin II and of its isolated A-subunit was 92.8 μM.

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<th>( \Delta H_v ) (kJ·mol⁻¹)</th>
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| A-subunit
| 4.50| –            | –                        | –                        | –                        | 322.9        | 494                      | 502                      | 0.98                     |
| 7.20| –            | –                        | –                        | –                        | 322.2        | 506                      | 519                      | 0.98                     |

854 kJ·mol⁻¹, while the higher-temperature transition peak has a \( T_m \) of 324.6 K with \( \Delta H_m = 573 \) kJ·mol⁻¹ (Table 1). If abrin II is scanned up to a temperature at which the lower-temperature transition peak mostly appears, and is then cooled and scanned again, then only the higher-temperature transition peak is observed, indicating that the lower-temperature transition peak is irreversible. If the sample is scanned yet again past the second transition, no endotherm appears, suggesting that the higher-temperature transition peak is also irreversible. To rule out any kinetic effects on the unfolding of abrin II, data were also collected at several scan rates (10–60 K·h⁻¹). There is a slight increase in the transition temperature with increase in scan rate, but the thermodynamic parameters, \( \Delta H_c \) and \( \Delta H_v \), are independent of the scan rate. Therefore the two-state equilibrium model, and not the irreversible model [29], was applied to these transitions. The basis for this assumption is that the transition may be treated as a sequence of two processes; the first process is the reversible unfolding of the protein, described by thermodynamic parameters \( T_m, \Delta H_c \) and \( \Delta H_v \), which is followed by a slower irreversible process, e.g. aggregation. This treatment,
first demonstrated by Sturtevant and co-workers [30], yields results for the overall process that are the same as for the reversible process. This less conservative view is supported by others [31,32] and justifies treating the unfolding transition of abrin II from the standpoint of a thermodynamic model.

A plot of \( C_p \) against temperature for the isolated A-subunit of abrin II is also shown in Figure 1 (lower curve). This transition, which is symmetrical, is characterized by a \( T_m \) of 322.2 K and a \( \Delta H_m \) value of 506 kJ mol\(^{-1}\). These data are thus similar to those for the deconvoluted higher-temperature transition peak of abrin II. These data apparently indicate that, in intact abrin II, the higher-temperature transition peak at 324.6 K corresponds to its A-subunit. The values of \( \Delta H_m \) for these transitions are comparable with those reported for other globular proteins [33]. A lower value (by 2.4 K) for the \( T_m \) of the isolated A-subunit than for the higher-temperature transition peak of intact abrin II implies some intersubunit interaction within the intact toxin which contributes to the increased stability of the A-subunit. The B-subunit becomes completely insoluble when separated from the A-subunit. Consequently, the thermal unfolding behaviour of the isolated B-subunit could not be characterized. It may be noted that the \( \Delta H_m / \Delta H_p \) ratio for the low-temperature transition is nearly 2, while that for the high-temperature transition is close to 1. This indicates that, at their respective denaturation temperatures, two domains are involved in the unfolding of the first transition, while a single domain is implicated for the second one.

### Stabilization by ligand binding

In the presence of saturating amounts of lactose, the values of \( T_m \), \( \Delta H_m \) and \( \Delta H_p \) increased, indicating that lactose stabilizes the native conformation of abrin II (Figure 2). An increase in thermal stability due to an added ligand has been reported for a large number of proteins [34,35]. The denaturation transition for the liganded form can be written as follows [35]:

\[
\ln[L] = -\frac{\Delta H_v}{nRT_v} + \text{constant}
\]

where \( m \) is the number of native (N) or denatured (D) protein molecules and \( n \) is the number of ligand (L) molecules. At constant protein concentration this can be transformed to:

\[
\ln[L] = -\frac{\Delta H_v}{nRT_v} + \text{constant}
\]

where \( T_v \) is the temperature at which the transition peak is a maximum. The \( \Delta H_v \) value (595 kJ mol\(^{-1}\)) calculated from a plot of \( \ln[lactose] \) against \( 1/T \) (with \( n = 2 \)) for the lower-temperature transition is similar to the value obtained by the two-state fit of the transition data in the presence of lactose (Figure 3 and Table 2). Thus two lactose molecules bind to one molecule of abrin II. At equivalent concentrations of lactose, \( T_m \) for the first transition increases by 8.8 K at pH 7.2, while at pH 4.5 it increases by 5.5 K (Table 2). Although the second transition also shifts to higher temperatures in the presence of lactose, a strict correlation between ligand concentration and \( T_v \) is not discernible. A control experiment using isolated A-subunit saturated with 50 mM lactose gave identical results as with the A-subunit alone, indicating a lack of interaction of lactose with this subunit.

### Effect of salt

The denaturation temperature of abrin II increases with increasing NaCl concentration, without an appreciable change in the co-operativity of the lower- and higher-temperature

### Table 2 Thermodynamic quantities for the thermal transition of abrin II in the presence of lactose

<table>
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* The ratio \( K_{DSC}/K_{ITC} \) (471/508) i.e., the binding constant at the denaturation temperature determined by DSC to the extrapolated value of binding constant determined by ITC is 0.93.
agreement with the value obtained from ITC measurements.

$K$ denaturation temp.

$D$ and $m$ molecule of abrin II, with values for identical-site model shows that two lactose molecules bind to one site model, is given in Figure 4. The fit of the data to the identical-site model, along with a least-squares fit of the data to the identical-site model, is shown as a solid line. The scan rate was 10 K-°C h⁻¹; the buffers used were 50 mM sodium phosphate, pH 7.2.

The DSC scan rate was 10 K-°C h⁻¹. $T_m$, $\Delta H_m$ and $\Delta H_g$ are the respective values for the lower-temperature transition, while $T_m$, $\Delta H_m$ and $\Delta H_g$ are the same quantities for the higher-temperature transition. The errors in the determination of $T_m$, $\Delta H_m$ and $\Delta H_g$ values were less than 0.05%, 2.7% and 3.0% respectively. The concentration of abrin II was 92.8 $\mu$M.

<table>
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</table>

Figure 4 Calorimetric titration of abrin II with lactose

Upper panel: raw data obtained for 66 automatic injections, each of 5 $\mu$l of 10.5 mM lactose into 351 $\mu$l abrin II solution at 292.1 K. Lower panel: total heat released as a function of the molar ratio of lactose to abrin II for the titration shown in the upper panel. The solid line is the result of the least-squares fit of the data to eqn. (3). The buffer used was 50 mM sodium phosphate, pH 7.2.

Transitions. This implies increased stability of the protein with increasing salt concentration (Table 3).

Binding of lactose to abrin II

A typical isothermal measurement of the binding of lactose to abrin II, along with a least-squares fit of the data to the identical-site model, is given in Figure 4. The fit of the data to the identical-site model shows that two lactose molecules bind to one molecule of abrin II, with values for $K_b$ (the binding constant) and $\Delta H_b$ (the binding enthalpy) of 2980 ± 56 M⁻¹ and 33472 ± 408 KJ mol⁻¹ respectively. The binding constant at denaturation temp, $K_b(T_m)$, calculated using eqn. (2), is in good agreement with the value obtained from ITC measurements, which is extrapolated to the denaturation temperature (Table 2).

Effect of pH

Calorimetric scans were also carried out as a function of pH in the pH range 4.5–10.2. The results of the fits of the data are given in Table 1. DSC scans at the two pH extremes, i.e. pH 4.5 and 10.2, are shown in Figure 5. A plot of $\Delta H_m$ against $T_m$ is shown in Figure 6. The data are fitted to a straight line using linear regression analysis (Origin program). The slope (d$\Delta H_m$/dT_m) gives the estimate of $\Delta C_p$. The values of $\Delta C_p$ obtained from Figure 6 are 27 ± 2 kJ mol⁻¹ K⁻¹ and 20 ± 1 kJ mol⁻¹ K⁻¹ for the lower- and higher-temperature transition peaks respectively.

The free energy changes for these transitions as a function of temperature, $AG^o(T)$, are calculated from the values of $T_m$, $\Delta H_m$ and $\Delta C_p$ using the Gibbs–Helmholtz equation:

$$\Delta G^o(T) = \Delta H_m(T_m - T) + \Delta C_p[T - T_m - T \ln(T/T_m)]$$

The values of $\Delta H_m$, $T_m$ and $\Delta C_p$ used for the low-temperature transition at pH 7.2 are 854 kJ mol⁻¹, 319.2 K and 27 kJ mol⁻¹ K⁻¹ respectively. For the higher-temperature transition at pH 7.2, they are 573 kJ mol⁻¹, 324.6 K and $\Delta C_p = 20$ kJ mol⁻¹ K⁻¹.

The temperature at which the lower-temperature transition (B-subunit) of abrin II is maximally stable is 289 ± 2 K, with a free energy of stabilization of 41 ± 3 kJ mol⁻¹. For the higher-temper-
The solid lines are least-squares fits for the lower- (○) and higher- (□) temperature transition peaks. The correlation coefficients are 0.97 and 0.98 for the lower- and higher-temperature transition peaks respectively. The slopes of these lines yield \( \Delta C_p \) values of 
\[ 27 \pm 2 \text{ kJ mol}^{-1} \text{ K}^{-1} \] 
and 
\[ 20 \pm 1 \text{ kJ mol}^{-1} \text{ K}^{-1} \] 
for the lower- and higher-temperature transition peaks respectively.

The thermal stability of intact abrin II increases at pH 4.5. However, no such effect was observed for the isolated A-subunit (Table 1).

**DISCUSSION**

Abrin II is a heterodimeric protein consisting of A- and B-subunits connected by a single disulphide bond. The DSC scan of abrin II shows two peaks, indicating that the protein consists of two entities unfolding at different temperatures. The values of \( T_m \) and \( \Delta H_m \) for the higher-temperature transition peak are similar to those for the isolated A-subunit. Hence the higher-temperature transition is identified with the unfolding of the A-subunit at the denaturation temperature. The lower-temperature transition exhibits a value of 2 for the \( \Delta H_m / \Delta C_p \) ratio, suggesting that it represents the unfolding of two domains at the denaturation temperature. The data also indicate that these domains display identical thermal unfolding properties. Moreover, these domains appear to unfold, to a large extent, completely independently under a variety of conditions employed in these studies. These results also imply that the unfolding of the first transition is less co-operative than would be expected if the two domains were interacting with each other to a significant extent. This is not surprising if one examines the three-dimensional structure of abrin II. In the abrin II (a abrin II of these studies) structure, the two carbohydrate-binding domains of the B-subunit appear to interact very little with each other. The B-subunit is insoluble in aqueous solution when separated from the A-subunit. This could be due to the B-subunit being more hydrophobic, a view consistent with the higher value of \( \Delta C_p \) associated with its thermal unfolding [36]. Unlike the other isoforms of abrin, we were unable to solubilize the B-subunit with ionic and non-ionic detergents or by carboxymethylation of its thiol groups either with or without detergents [37]. These results indicate that the toxin is mostly stabilized by intrasubunit interactions, with very little intersubunit interaction, unlike DT, which is stabilized by a combination of intra- and inter-subunit interactions.

In the absence of similar studies on other RIPs, it is instructive to compare the thermal unfolding behaviour of DT and cholera toxin with that of abrin II. Intact DT, like abrin II, displays two thermal unfolding transitions [14]. The denaturation temperature of the isolated A-subunit of DT is 6.5 K lower than that of the lower-temperature transition (A-subunit) of intact DT. The B-subunit of DT is hydrophobic and is precipitated when separated from the A-subunit. Thus the thermal unfolding behaviours of DT and abrin II appear quite similar. In contrast, the A- and B-subunits of cholera toxin unfold independently. This observation was confirmed by the fact that the \( T_m \) and \( \Delta H_m \) values of isolated B-subunits were similar to those of the higher-temperature transition peak (B-subunit) of cholera toxin [38]. Similar observations have also been made in the case of several multidomain proteins, which are stabilized by intradomain interactions and not by interdomain interactions [39].

The \( T_m \) and \( \Delta H_m \) values of abrin II increase with decreases in pH, while the stability of the isolated A-subunit remains unchanged at various pH values (Table 1). There is an upward shift of approx. 22 K in the \( T_m \) between pH 7.2 and pH 4.5. Thus the stability of intact abrin II increases as the pH is lowered from neutral to pH 4.5. The stabilization at pH 4.5 could be due to a potentiation of interactions between the A- and B-subunits. Similar results are also observed for the guaainidine hydrochloride-induced unfolding of abrin II and its A-subunit (J. Krupakar, P. K. Das and S. K. Podder, unpublished work). Unfolding experiments using guainidine hydrochloride show that the stabilities of ricin and mistletoe lectin and their A-subunits are greater at lower pH values [17,18]. The \( \Delta C_p \) values observed in the case of abrin II, especially for its B-subunit, are higher than those for small globular proteins, and are consistent with the high proportion of hydrophobic residues in RIPs [36]. This is in turn consistent with the ability of these proteins to penetrate membranes [15,16].

The binding of lactose stabilizes the entire toxin, as indicated by the increased values of \( T_m \) and \( \Delta H_m \) for both transitions, suggesting that there is an interaction between the two subunits of the RIP. At equivalent concentrations of lactose and abrin II, the \( T_m \) shifts by approx. 8 K at pH 7.2 and approx. 4 K at pH 4.5, indicating that the ligand-induced stabilization is more pronounced at pH 7.2 than at pH 4.5. In the case of ricin, the values for the binding constant and binding stoichiometry with galactose at low pH are 10 and 25 %, respectively of those observed at neutral pH [40]. The lesser shift of \( T_m \) (by 4 K) in the case of abrin II ligated with lactose at pH 4.5 could be due either to weak binding of lactose to the native abrin II and/or to a decrease in the number of binding sites at acidic pH. The binding of two lactose molecules to one molecule of abrin II was surmised from the plot of ln[ lactose] against 1/\( T_m \) for the lower-temperature transition. Similar results were obtained from ITC measurements. Furthermore, \( K_M^{\text{DSC}} / K_M^{\text{ITC}} \) is close to 1, implying that lactose interacts with the folded form of abrin II preferentially. As the \( \Delta H_m / \Delta C_p \) ratio remains the same for the ligated form, it is apparent that the ligand stabilizes both domains to the same extent, and that one molecule of the carbohydrate ligand binds to each of these domains. This is consistent with the crystal structure of abrin-a, whose B-subunit consists of two similar subdomains and each subdomain contains one carbohydrate-binding site; this is presumed to have occurred by gene duplication [41]. Two binding sites have also been observed in the other isoforms of abrin [42,43].

Although the increase in thermal stability and poor binding to lactose at low pH appear contradictory and not conducive to the
release of the toxin into the cytosol, these data provide us with additional insight into the mechanism of transmembrane translocation of the toxic A-subunit of type II RIPs. The low pH in the endosomal compartment would allow dissociation of a significant proportion of the toxin from its receptor, thus facilitating recycling of the latter to the cell surface. The higher stability of the free toxin in the lumen of the endosome not only would enable it to resist degradation, but also would permit it to make its way to the Golgi apparatus and endoplasmic reticulum. On reaching the endoplasmic reticulum, the high pH of this compartment would destabilize the toxin, thus permitting reduction of the intersubunit disulphide bond and insertion of the highly hydrophobic B-subunit into the membrane. The A-subunit would then be able to enter the cytosol through the channel formed by the B-subunits or via retrotranslocation [44].

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REFERENCES


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