Studies on tryptophan residues of *Abrus* agglutinin

Stopped-flow kinetics of modification and fluorescence-quenching studies

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The presence of two essential tryptophan residues/molecule was implicated in the binding site of *Abrus* agglutinin [Patanjali, Swamy, Anantharam, Khan & Surolia (1984) *Biochem. J.* **217**, 773–781]. A detailed study of the stopped-flow kinetics of the oxidation of tryptophan residues revealed three classes of tryptophan residues in the native protein. A discrete reorganization of tryptophan residues into two phases was observed upon ligand binding. The heterogeneity of tryptophan exposure was substantiated by quenching studies with acrylamide, succinimide and Cs^+ . Our study revealed the microenvironment of tryptophan residues to be hydrophobic, and also the presence of acidic amino acid residues in the vicinity of surface-localized tryptophan residues.

INTRODUCTION

The unique sugar-specific binding of phytohaem-agglutinins to cell-surface receptors is the first step in eliciting physiological responses such as mitogenesis, endocytosis, insulin-like hormonal action etc. (Novogrodsky & Katchalski, 1971; Moscona, 1971; Cuatrecasas & Tell, 1973; Goldstein & Hayes, 1978; Lis & Sharon, 1981, 1984). A clear understanding of the binding site, which mediates the process of binding, is essential to draw a structure–function correlation for lectins. Although many workers have reported sugar-specificities and worked out the association parameters for various sugars, a clear understanding of the binding site has remained largely elusive. It is therefore desirable to identify the essential amino acid residues required for sugar binding, to monitor the degree of their exposure to the solvent, and to probe the changes occurring, upon ligand binding, in their microenvironment and the overall disposition of similar residues on the surface and in the interior of the protein as an entity.

In previous work we have reported the involvement of tryptophan residues in the combining site of *Abrus* agglutinin (Patanjali *et al.*, 1984). This lectin, isolated from the seeds of the jequirity plant (*Abrus precatorius*), is a non-toxic but mitogenic and haemagglutinating protein (Olsnes *et al.*, 1974; Wei *et al.*, 1975). It binds to galactopyranosyl residues, and this binding is responsible for most of its biological activity. It has two binding sites/molecule (Olsnes *et al.*, 1974; Khan *et al.*, 1981) and requires one tryptophan residue/binding site for its binding activity (Patanjali *et al.*, 1984).

Although tryptophan residues were implicated in the binding activity of the agglutinin, relatively little is known about the environment of the tryptophan residues and their arrangement on the molecule as a whole, and the differential exposure of these residues before and after ligand binding. These aspects are investigated in the present work, which deals with the fast reaction kinetics of the oxidation of tryptophan residues by *N*-bromosuccinimide and the quenching of intrinsic fluorescence by neutral quenchers such as acrylamide and succinimide, as well as by a cationic quencher, Cs^+ .

MATERIALS AND METHODS

Agglutinin

Agglutinin was prepared from the seeds of the jequirity plant. A mixture of toxin and agglutinin was obtained by affinity chromatography on a cross-linked guar-gum column as described by Appukuttan *et al.* (1977). The toxin was separated from agglutinin on a Sephadex G-100 column. The agglutinin moved as a single band on acidic as well as basic polyacrylamide-gel electrophoresis (Davis, 1964).

Chemicals

All reagents used were of analytical grade. *N*-Bromosuccinimide and acrylamide, obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., were recrystallized from water and ethanol respectively. Optical-grade CsCl was a product of Sigma Chemical Co. Succinimide (Koch–Light Chemicals) was generously given by Professor N. Appaji Rao (Department of Biochemistry of the Institute). Lactose was obtained from BDH Chemicals, Poole, Dorset, U.K. Sephadex G-100 was a product of Pharmacia, Uppsala, Sweden.

Stopped-flow kinetic studies

These were performed on a Union Giken RA 401 stopped-flow spectrometer working in the absorbance mode. Changes in A_{280} were monitored by using 0.02 A_{280} unit as full scale and varying the sampling time from 1 to 10 s. The dead time of the instrument was determined to be 0.5 ms. The protein concentration employed was 1.05 μM . The resultant concentration of *N*-bromosuccinimide varied from 100 to 500 μM in the absence of sugar. The effect of the binding of sugar on the rate parameters was studied at a constant concentration of *N*-bromosuccinimide (250 μM) and different concentrations of lactose (100–500 μM). All the kinetic studies were performed in 0.1 M-sodium acetate buffer, pH 4.0, containing 0.15 M-NaCl at 25 °C. The sample compartments were maintained at 25 ± 0.1 °C by using a Lauda water bath. The kinetic data were recorded on a Union

Fig. 1. Typical stopped-flow trace of the modification of tryptophan residues of *Abrus* agglutinin by *N*-bromosuccinimide

Final concentrations of $1.05 \mu\text{M}$ -agglutinin and $250 \mu\text{M}$ -*N*-bromosuccinimide were employed. Details are given in the Materials and methods section. The logarithmic plots (insets a, b and c) show three classes of tryptophan residues greatly differing in reactivities. The continuous line adjoining the later points of inset (a) gives the rate constant for the slow-reacting component. Inset (b) is the logarithmic plot after the change in A_{280} corresponding to the slower component is subtracted from the total change. The slope obtained from this plot gives the rate for the medium-reacting component. The rate for the fast-reacting component (inset c) is obtained in a similar way by subtracting the contribution from the medium-reacting component.

Giken RA 452 X-Y recorder and analysed as described by Peterman & Laidler (1979).

Fluorescence measurements

These were taken on a Union Giken FS 501A fluorescence polarizer equipped with photon-counting photomultipliers. The instrument is controlled by a microprocessor, which typically averaged ten measurements to generate a single data point. Excitation was at 280 nm, and the emission was monitored by using a 320 nm-cut-off filter along with a 335 nm-band-pass filter ($\lambda_f = 6.5 \text{ nm}$).

The intrinsic fluorescence of *Abrus* agglutinin was quenched with two neutral quenchers, acrylamide and succinimide, and a cationic quencher, Cs^+ . Defined amounts of the quenchers from 1.0 M stock solution were added to 2.0 ml of protein solution with A_{280} in the range 0.05–0.10.

Quenching with acrylamide and Cs^+ was performed in the presence and in the absence of lactose. Lactose concentration was kept constant at 0.15 M. All the studies were done in 0.02 M-sodium phosphate buffer, pH 7.3, containing 0.15 M-NaCl. In order to observe the effect of pH, quenching studies were also performed with Cs^+ , in 0.1 M-sodium acetate buffer, pH 4.0, containing 0.15 M-NaCl in the presence and in the absence of lactose. The quenching data were plotted in accordance with the Stern-Volmer equation and analysed as described by Eftink & Ghiron (1981).

RESULTS AND DISCUSSION

In a previous paper (Patanjali *et al.*, 1984) we observed that the modification of tryptophan residues by *N*-bromosuccinimide and 2-hydroxy-5-nitrobenzyl bromide led to a complete loss of the haemagglutinating and saccharide-binding activities of *Abrus* agglutinin. We also ascertained by spectroscopic studies that *N*-bromosuccinimide specifically oxidized only indole side chains of tryptophan residues, and the modification does not impose any gross conformational change of the lectin. These studies, however, suggested a change in the exposure of tryptophan residues that were accessible for the oxidation before and after ligand binding. In order to understand this phenomenon in detail, we have performed stopped-flow kinetic studies on the modification of tryptophan residues of *Abrus* agglutinin.

Stopped-flow kinetic studies

The advantage of monitoring fast reaction kinetics lies in obviating the possibility of complicating factors of slow side reactions and secondary effect that may be caused by changes in protein folding patterns (Hiromi *et al.*, 1977).

A typical reaction curve in the absence of sugar is shown in Fig. 1. Semi-logarithmic plots of the second-order rate curves of *Abrus* agglutinin show a triphasic quenching pattern (Fig. 1 inset), indicating the presence of three different kinds of tryptophan residues. The rate

Fig. 2. Dependence of apparent rate constants for the modification of tryptophan residues of the *Abrus* agglutinin on the concentration of *N*-bromosuccinimide

Apparent rate constants ($k_{app.}$) are plotted as a function of the concentration of *N*-bromosuccinimide. The second-order rate constants were calculated from the slopes of these plots: (a) slow-reacting component; (b) medium-reacting component; (c) fast-reacting component. Insets represent the dependence of the second-order rate constants, calculated from each of the $k_{app.}$ values, on the concentration of *N*-bromosuccinimide.

Table 1. Second-order rate constants for oxidation of tryptophan residues of *Abrus* agglutinin in the presence and in the absence of lactose, and numbers of tryptophan residues/molecule modified in each kinetic phase

For full details see the text.

Condition of oxidation	k ($M^{-1} \cdot s^{-1}$)			No. of tryptophan residues/molecule modified in each kinetic phase		
	Phase 1	Phase 2	Phase 3	Phase 1	Phase 2	Phase 3
Lactose absent	$3.33(\pm 0.1) \times 10^3$	$3.63(\pm 0.25) \times 10^4$	$4.55(\pm 0.15) \times 10^5$	6	2	3
Lactose present (350 μM)	$0.8065(\pm 0.1) \times 10^4$	$1.4(\pm 0.15) \times 10^5$	—	7	—	4

constants of the reaction process obtained by a plot of the apparent rate constants ($k_{app.}$) versus the concentration of *N*-bromosuccinimide (Fig. 2) differed by approximately one order of magnitude from each other. These rate constants are $4.54 \times 10^5 M^{-1} \cdot s^{-1}$, $3.63 \times 10^4 M^{-1} \cdot s^{-1}$ and $3.33 \times 10^3 M^{-1} \cdot s^{-1}$, which represent the fast-, medium- and slow-reacting components respectively (Table 1). The amplitude of the signal change occurring in each phase is made use of in deducing

the number of residues existing in each of these phases. The numbers of residues/molecule modified were estimated by the method of Spande & Witkop (1967). It is observed that three, two and six residues are present in the fast-, medium- and slow-reacting components respectively (Table 1), when the reaction was monitored over a period of 10 s. Hence it appears that there are three tryptophan residues/molecule that are 'highly exposed', two 'partially exposed' and six 'buried'.

Fig. 3. Typical stopped-flow trace of the modification of tryptophan residues of *Abrus* agglutinin by *N*-bromosuccinimide in the presence of lactose

Final concentrations of $1.05 \mu\text{M}$ -lectin, $250 \mu\text{M}$ -*N*-bromosuccinimide and $250 \mu\text{M}$ -lactose were employed. Rates were evaluated as described in the legend to Fig. 1. Insets show logarithmic plots for (a) the slow component and (b) the fast component.

In our earlier paper (Patanjali *et al.*, 1984) a partial protection of tryptophan residues was inferred, as larger amounts of *N*-bromosuccinimide were required to achieve the same degree of modification in the presence of lactose. In order to investigate the effect of lactose on the reactivity of different classes of tryptophan residues, a study of kinetics of modification was undertaken in presence of various amounts of lactose (100 – $500 \mu\text{M}$) and a constant concentration of *N*-bromosuccinimide. Typical reaction curves are shown in Fig. 3. The semi-logarithmic plots (Fig. 3 inset) show only two phases, indicating that there is a definite alteration in the accessibility of tryptophan residues in the protein upon binding to lactose. The values of rate constants obtained from these plots are $1.4 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the fast component and $0.8065 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the slow component. The numbers of residues present in the fast and slow phases are four and seven respectively (Table 1).

Thus a partial protection of tryptophan residues is also evident in the fast-reaction-kinetics experiments. The number of residues modified after 10 s remains at 11 in the presence and in the absence of lactose. However, the accessibility of tryptophan residues to the reagent has changed from a triphasic to a biphasic nature. Binding of lactose principally causes relocation of tryptophan residues in the protein fabric: in effect a 3:2:6 separation of fast-, medium- and slow-reacting tryptophan residues of lectin in the absence of lactose redistributes to a 4:7 mode of fast- and slow-reacting species respectively. *N*-Bromosuccinimide thus primarily affects the 'partially exposed' residues. The fast component has decreased by

a factor of 1.5-fold in the presence of sugar. This component has a rate that lies between those of the fast and the medium components observed in the absence of sugar. That of the slow component in the presence of sugar lies between those of the slow and the medium components in the absence of sugar. These situations are represented schematically below:

where (S) and (A) represent the reaction components in the presence and in the absence of sugar respectively. The numbers in parentheses represent the corresponding numbers of tryptophan residues in each phase. This division of the reacting groups holds over the lactose range studied in Fig. 4.

The plots of k_{app} as a function of the concentration of *N*-bromosuccinimide for the slow- and the medium-reacting components do not go through the origin. However, the plot for the fastest-reacting residues does go through the origin (Fig. 2). For any reaction performed under pseudo-first-order conditions, the plot of the first-order rate constant (k_{app}) as a function of the excess reagent concentration (in our case *N*-bromosuccinimide) should extrapolate to the origin. The observed discrepancy may be due to the presence of some more components in the medium- and slow-reacting phases. As there are 11 tryptophan residues/molecule modified during the observed time scale of the reaction (10 s), theoretically it is possible to have 11 different components

in the reaction, assuming that all tryptophan residues are not identical with respect to their reactivity and environment. But under the observed experimental conditions these 11 residues group themselves grossly into three distinct sets. If individual elements (tryptophan residues) in each of these subsets do not differ in their reactivities, plots of the first-order rate constants versus the concentration of the reagent in excess pass through the origin, as observed in our fast step. Alternatively, if any of the subsets contain tryptophan residues that differ moderately but are indistinguishable, bearing in mind the sensitivity of observation, the aforementioned plots may not go through the origin, as observed in our medium and slow components. Fig. 2 inset shows the second-order rate constants (k , $\text{M}^{-1}\cdot\text{s}^{-1}$) calculated for each of the first-order rate coefficients ($k_{\text{app.}}$, s^{-1}) plotted as a function of the concentration of *N*-bromosuccinimide. Non-linearity of the plots of the slow and the medium components is suggestive of the presence of further differentiation in each of these components. The fast reaction rate constants are linear within a deviation limit (Table 1), indicating that this component may be homogeneous. Although in our previous study we did not observe any gross secondary-structural changes by c.d. measurements, a change in the exposure of the medium- and the slow-reacting tryptophan residues upon modification of the fast-reacting tryptophan residues may not be overruled. These changes need not be very large to divide the existing phases further into more subsets, although they may be influencing the rate constants. Two other possibilities, namely the pre-equilibrium and post-equilibrium, may also cause altered accessibility of the tryptophan residues. To sum up, keeping these limitations in mind, our studies suggest that there are at least three kinds of tryptophan residues in the native protein, of which two are appreciably altered upon sugar binding.

Fig. 4. Effect of lactose concentration on the apparent rate constants for the modification of tryptophan residues of *Abrus* agglutinin by *N*-bromosuccinimide

The apparent rate constants ($k_{\text{app.}}$) are plotted as a function of the concentration of lactose: (a) slow component; (b) fast component.

A complete protection of the tryptophan residues shows a pronounced dependence of $k_{\text{app.}}$ on the concentration of the ligand added, as observed in lysozyme (Hiromi *et al.*, 1977). However, in the present study the apparent rate constants do not show an appreciable dependence on the concentration of sugar added (Fig. 4). The association constants for the binding of lactose to *Abrus* agglutinin in 0.1 M-sodium acetate buffer, pH 4.0, at 25 °C was determined to be $7.24 \times 10^3 \text{ M}^{-1}$ by intrinsic-fluorescence titrations by the method of Lotan & Sharon (1973). On the basis of the above value, we employed a range of lactose concentrations that gave 35% to complete saturation of the ligand-binding sites of the agglutinin. It is generally expected that, if ligand binding leads to a protection of active-site residues, the saturation of the binding sites of the protein with the ligand could be reflected in the rate constants, i.e. at different degrees of saturation different rate constants would be observed. If the rate constant for a modification reaction is decreased in the presence of ligand, the extent of modification will inevitably be decreased. However, it may not be experimentally detectable, because the reaction may be essentially complete at the sampling time (10 s in this case), even in the presence of ligand. As a result the number of residues modified will be identical in the presence and in the absence of ligand, although a decrease in rate constants may occur. Nonetheless, these results strongly indicate altered accessibility of the indole side chains of the *Abrus* agglutinin on saccharide binding.

Quenching studies

Quenching of the intrinsic fluorescence of proteins by acrylamide, succinimide and Cs^+ has been used to probe the tryptophan environment in proteins and peptides. The data were analysed by the Stern–Volmer equation. The slopes obtained in Stern–Volmer plots are indicative of exposure of tryptophan residues, expressed as K_Q (Eftink & Ghiron, 1981).

Quenching with acrylamide and succinimide. Plots of F_0/F versus $[Q]$, where F_0 and F are the initial and final fluorescence intensities respectively and $[Q]$ is the concentration of the quencher, are essentially biphasic (Fig. 5a). Acrylamide, being a neutral quencher, can quench the fluorescence of partially as well as completely buried residues by diffusing into the protein core. Hence the data obtained with acrylamide give a comprehensive picture of the distribution of tryptophan residues as a whole. Quenching in the presence of lactose was also biphasic, exhibiting a decrease in the quenching constants, indicating protection of some residues in the presence of ligand. However, a redistribution of tryptophan residues upon binding to lactose, leading to differential exposure, cannot be ruled out. The various K_Q values are listed in Table 2.

Succinimide, being a bulkier molecule than acrylamide, is less effective as a quencher. The quenching pattern showed a slight upward curvature (Fig. 5b). The effective values of K_Q are listed in Table 2. Eftink & Ghiron (1984) suggest a method for calculating the microenvironment of tryptophan residues by analysing the ratio of the effective quenching constant of succinimide (K_S) to that of acrylamide (K_A). The present data suggest a K_S/K_A ratio of 0.2. On the basis of data of Eftink & Ghiron (1984), we conclude that the microenvironment of

Fig. 5. Quenching of the intrinsic fluorescence of *Abrus* agglutinin with neutral quenchers

Stern-Volmer plots are shown of the quenching of intrinsic fluorescence of *Abrus* agglutinin by (a) acrylamide [○, in the absence of lactose; △, in the presence of lactose (0.15 M)] and (b) succinimide in the absence of lactose. All titrations were performed with 1.0 M stock solutions of the quenchers. [The quenching plots are curvilinear. However, in calculating the microenvironment of tryptophan residues, effective K_Q values obtained from plots such as those reported by Eftink & Ghiron (1984) are employed.]

Table 2. Quenching constants for various quenchers of the fluorescence of tryptophan residues of *Abrus* agglutinin in the presence and in the absence of lactose

Titration were performed with 1.0 M stock solutions. For full details see the text.

Quencher	pH	K_Q (M^{-1})			
		Lactose absent		Lactose (0.15 M) present	
		Phase 1	Phase 2	Phase 1	Phase 2
Acrylamide	7.3	19.18(± 0.19)	27.61(± 0.22)	16.27(± 0.25)	24.00(± 0.28)
Succinimide	7.3	3.68(± 0.13)	—	—	—
CsCl	7.3	4.10(± 0.28)	1.88(± 0.18)	1.16(± 0.12)	0.714(± 0.085)
CsCl	4.0	← No quenching →		← No quenching →	

tryptophan residues available to these quenchers is essentially hydrophobic.

Quenching by Cs^+ . Quenching of the fluorescence by Cs^+ and other cations has two advantages. Since they do not permeate proteins, it is possible to determine the fraction of total fluorescence that is due to surface-localized tryptophan residues. Additionally, the quenching by cations is influenced by the presence of a negative charge in the vicinity of tryptophan residues. This effect can be monitored in terms of quenching as a function of pH. In our study we observed downward-sloping biphasic Stern-Volmer plots for *Abrus* agglutinin in the presence and in the absence of lactose (Fig. 6). There is a considerable decrease in the values of K_Q upon binding to lactose, suggesting that the residues involved in binding are not buried in the absence of lactose.

Fig. 6. Stern-Volmer plots of the quenching of the fluorescence of *Abrus* agglutinin with CsCl

○, In the absence of lactose; △, in the presence of lactose (0.15 M).

Fig. 7. Estimation of the fraction of tryptophan residues exposed in *Abrus agglutinin*

Data for quenching by CsCl are replotted according to the modified Stern–Volmer equation. The fraction of tryptophan residues exposed is calculated from the ordinate intercept (see the Results and discussion section for details): (a) in the absence of sugar; (b) in the presence of sugar (0.15 M); (c) loss of fluorescence of the lectin as a function of tryptophan modification by *N*-bromosuccinimide (data taken from Patanjali *et al.*, 1984).

The quenching data were analysed by the modified Stern–Volmer equation (Lehrer, 1971; Lacowicz, 1983):

$$\frac{F_0}{\Delta F} = \frac{1}{f_a K [Q]} + \frac{1}{f_a}$$

where f_a is the fraction of the initial fluorescence that is accessible to the quencher, F_0 is the initial fluorescence intensity, K is the Stern–Volmer quenching constant, $[Q]$ is the concentration of the quencher and ΔF is the change in fluorescence intensity upon addition of quencher. This modified form of the Stern–Volmer equation allows f_a to be determined graphically. A plot of $f_0/\Delta F$ versus $1/[Q]$ yields f_a^{-1} as the intercept. We observed that 40% of the initial fluorescence was available for Cs^+ quenching (Fig. 7a) in the absence of ligand, but only 20% in the presence of sugar (Fig. 7b). Our previous data suggest that 82% of the fluorescence is contributed by 16 tryptophan residues (Fig. 7c). As the quenching patterns are linear, it is possible to interpret that eight tryptophan residues are present on the surface, accessible for quenching by Cs^+ . Upon lactose binding four out of eight residues were available for quenching. This observation is also consistent with the acrylamide quenching patterns, suggesting that buried residues are not involved in the binding. When the pH of the buffer was changed from 7.3 to 4.0, there was no quenching by Cs^+ , in the presence as well as in the absence of lactose. This is indicative of a negatively charged residue in the vicinity of the binding locus.

A relatively high propensity for occurrence of tryptophan residues in the binding site of numerous lectins compelled our study on the tryptophan environment and distribution in *Abrus agglutinin*, modelled as a representative system (Ceramakova *et al.*, 1976; Privat *et al.*, 1976; Majumder *et al.*, 1981; Ashford *et al.*, 1981;

Patanjali *et al.*, 1984; Higuchi *et al.*, 1985; Khan *et al.*, 1986). We therefore believe that our present study, work hitherto not attempted in the field of lectins, will help to improve our understanding of the tryptophan environment and its relation to the structure and function of lectins.

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