Vol. 92, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1980 Pages 319-326

DIFFERENTIAL FLUORESCENCE ENHANCEMENT OF 8-ANILINO-1-NAPTHALENE SULFONIC ACID BY RICIN A AND B CHAINS

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Received December 3,1979

Incubation of 8-anilino-1-naphthalene sulfonic acid with ricin and its isolated A and B polypeptide chains showed an increase in fluorescence at 470 nm. The A chain induced more fluorescence enhancement than either ricin or ricin B chain. The addition of B chain to A chain resulted in decreased fluorescence enhancement which was pH dependent. Sephadex gel filtration showed that A and B chain efficiently reassociated and the reassociation was not dependent on formation of the interchain disulfide bond and could not be prevented by high salt concentration.

Ricin is an extremely potent inhibitor of protein synthesis of some, but not all, eucaryotic ribosomes (1). The toxin has a subunit structure in which two specialized polypeptides are covalently held together by a disulfide bond (2). Only the A chain is inhibitory to protein synthesis in cell free extracts. In order for the A chain to gain access to the ribosomes within a cell, the B chain must be present to bind the toxin to cell surface receptors. It has been suggested that the B chain may not be transported across the membrane with the A chain (3). However, prolonged incubation in high concentrations of mercaptoethanol is necessary to break the disulfide bond and to separate the chains. Little information is available about the liklihood of easily separating the two chains at the membrane surface or about forces which may stabilize the interaction of the two chains.

MATERIALS AND METHODS

Ricin, Ricinus communis agglutinin (RCA), and ricin A and B chains were purified as previously described (4) from castor beans. ANS (8-anilino-1-naphthalene sulfonic acid) was from Pierce Chemical Co. PBS is 5 mM sodium phosphate, pH 6.5, containing 200 mM NaCl. ANS fluorescence spectra were recorded using a Aminco-Bowman 4-8940SP spectrofluorometer. For ANS titration experiments, an excitation wavelength of 360 nm and an emission wavelength of 470 nm was used. A carefully measured volume (4 to 10 µl)

This work was supported by a Research Career Development Award from the National Cancer Institute to L.L.H. and grant CA16106 from the National Cancer Institute, National Institutes of Health, DHEW.

0006-291X/80/010319-08$01.00/0

of protein was added, quickly mixed by gentle shaking, and the fluorescence intensity recorded. Additional volumes were added such that the final increase in volume was less then 7%. No significant difference was found when the protein was added in one volume compared to adding it in increments.

RESULTS

When ricin$_1$, RCA, ricin$_1$ A chain, or ricin$_1$ B chain were added to ANS there was an increase in the fluorescence as determined by scanning the emission spectrum. The spectral change was typical of that observed with many other proteins when ANS was bound. The change in fluorescence enhancement as a function of pH is shown in Figure 1. Little change in the level of intensity was observed between pH 6 and 9.4 for ricin$_1$ A and B chains. The values immediately reached for all the proteins between pH 5.15 and 9.4 did not change for at least one hour at room temperature. There was a time dependent increase for all proteins at pH 4.3 which lasted for about one hour. A much larger increase occurred at pH 3.1 and was probably due to acid denaturation.

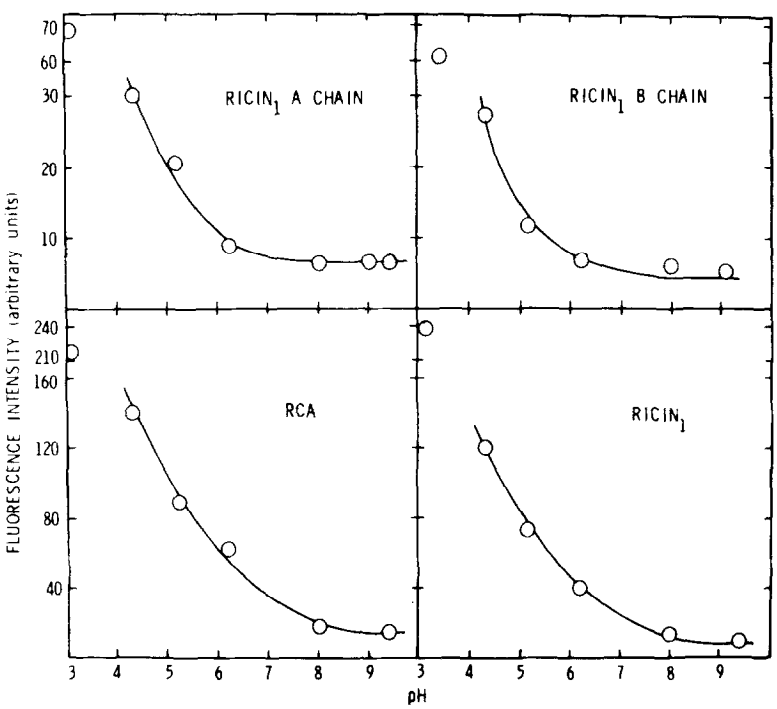

FIG. 1. Dependence of ANS fluorescence enhancement on pH: Small volumes (4 to 10 µl) of individual proteins were added to a solution containing 900 µl water, 100 µl 1 M buffer adjusted to the desired pH and 50 µl of 1 mM ANS. The pH was determined after the final addition of protein and was not changed from the initial value. The fluorescence intensity (λ excitation = 360 nm) was measured at 470 nm and the initial intensity of ANS was subtracted.

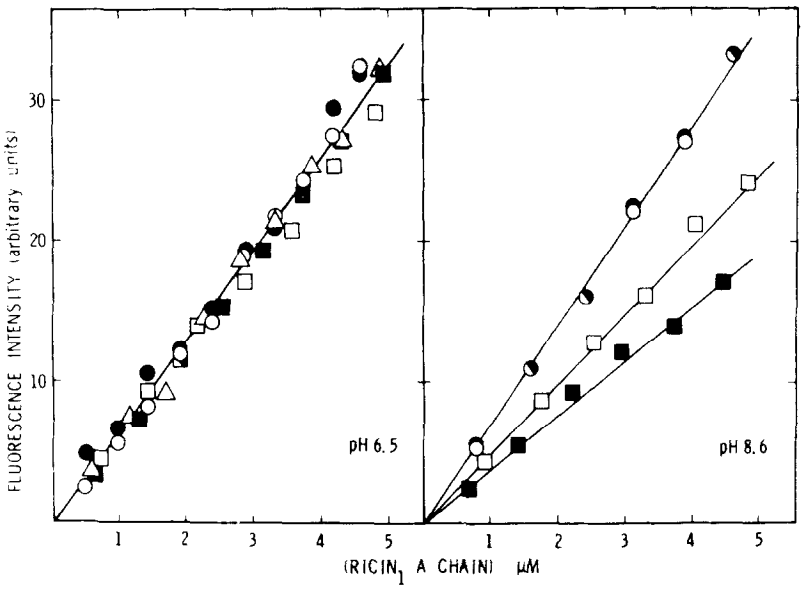

FIG. 2. Binding of ANS to A chain in the presence of B chain. Left: Four microliter volumes of ricin$_1$ A chain were sequentially added to 1.0 ml of pH 6.5 PBS to which 50 μl of 1 mM ANS had been added. The A chain was either taken directly from ice (○), thawed and frozen (△), or incubated at room temperature (22°C) for 60 minutes (●). Titration was carried out in 11.6 μM (■), or 29.1 μM (□) ricin$_1$ B chain. The fluorescence intensity due to ANS and B chain was substracted. Right: ANS was titrated with ricin$_1$ A chain taken from ice (○), A chain incubated for 60 minutes at room temperature (●), or added in the presence of 5.8 μM B chain (□) and 29.1 μM B chain (■) using pH 8.6 buffer.

Titration of a constant amount of ANS with ricin$_1$ A chain at pH 6.5 resulted in a linear increase in fluorescence intensity (Figure 2). The same dependence was obtained if the A chain was added directly to ANS from ice, was added after incubation for one hour at 22°, or was added after freezing and thawing. When the titration was carried out at pH 6.5 in increasing concentrations of B chain, little change in slope of the line was seen. In contrast, the presence of B chain during the titration considerably decreased the amount of fluorescence at pH 8.6. The amount of decrease was dependent of the concentration of B chain. The pH dependence of the fluorescence enhancement induced by the presence of the B chain is further illustrated in Figure 3. As the pH was increased, the B chain became more effective in decreasing the slope of the line. A plot of the slope of the straight lines against the pH (Figure 3 insert) closely parallels the shape of the curve obtained with ricin$_1$ shown in Figure 1. Galactose (14.8 mM) had no effect on the fluorescence enhancement of any of these proteins.

The converse experiment, the titration of ANS with ricin$_1$ B chain in the presence of a constant amount of A chain, also showed less fluorescence enhancement at pH 8.6

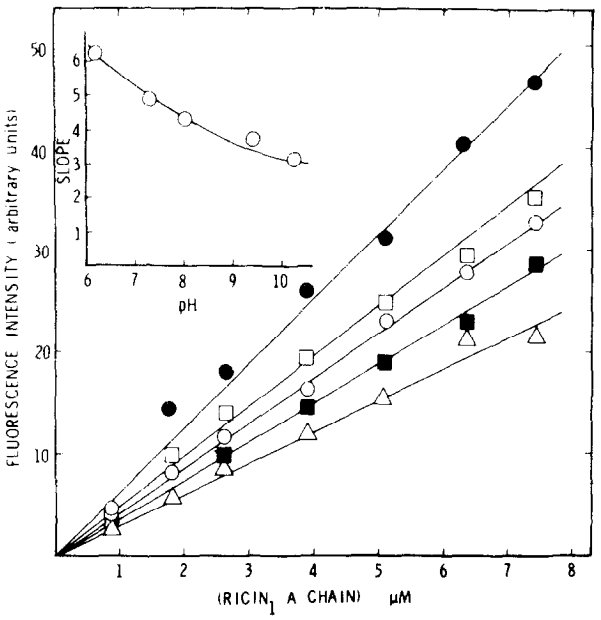

FIG. 3. ANS titration by A chain in the presence of B chain as a function of pH: Fifty microliters of 1 mM ANS was added to a solution containing 900 μl of water, 100 μl of 1 M buffer at pH 6.2 (●), pH 7.3 (□), pH 8.0 (○), pH 9.1 (■), or pH 10.3 (△), and 12.2 μM ricin$_1$ B chain. The intensity of the B chain alone in ANS buffer was subtracted.

compared to pH 6.5 (Figure 4). Instead of an increase in fluorescence, a decrease of about 65% was observed at pH 8.6. About a 2-fold excess of B chain was required to reach the maximal effect. No significant difference was observed if the A chain was first incubated with ANS and then the B chain added or if the A chain were incubated together and then ASN added.

The apparent interaction of the A chain and the B chain as measured by the decreased ANS fluorescence leads to the prediction that ricin should produce less enhancement of fluorescence than the theoretical combination of the individual chains. This is born out as shown in Figure 5. Ricin$_1$ A chain produced a much greater yield of fluorescence enhancement than ricin$_1$ or RCA (plotted as equivalents of A-B dimer to correct for the A_2B_2 structure of RCA). Somewhat surprisingly, the B chain was much less effective than the A chain in binding to ANS as measured by fluorescence enhancement. The B chain showed only 5.4% and 3.3% of the A chain intensity at pH 6.5 and 8.6, respectively. Ricin has only 40% of the theoretical amount of fluorescence enhancement at pH 6.5 and only half of that (23%) at pH 8.6. There was little difference

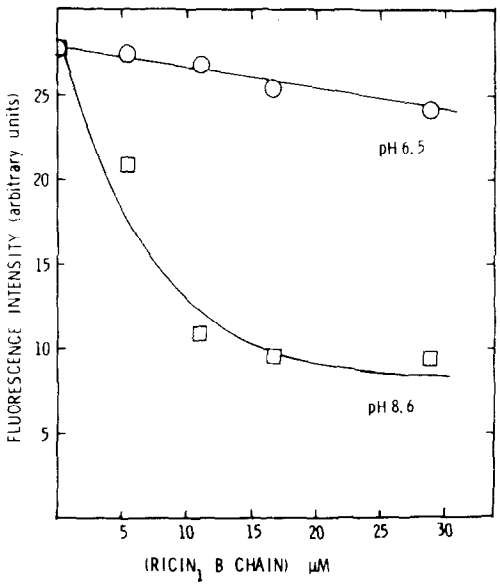

FIG. 4. ANS titration by B chain in the presence of A chain: Fifty microliters of 76.3 μM ricin$_1$ A chain was incubated with 100 μl of 1 M Tris chloride, pH 8.6, or 1 M sodium acetate, pH 6.5, and 50, 150 or 250 μl of 122 μM B chain for 20 minutes at 22°C. Water was added to make a volume of 1.0 ml, 50 μl of 1 mM ANS was added and the fluorescence determined.

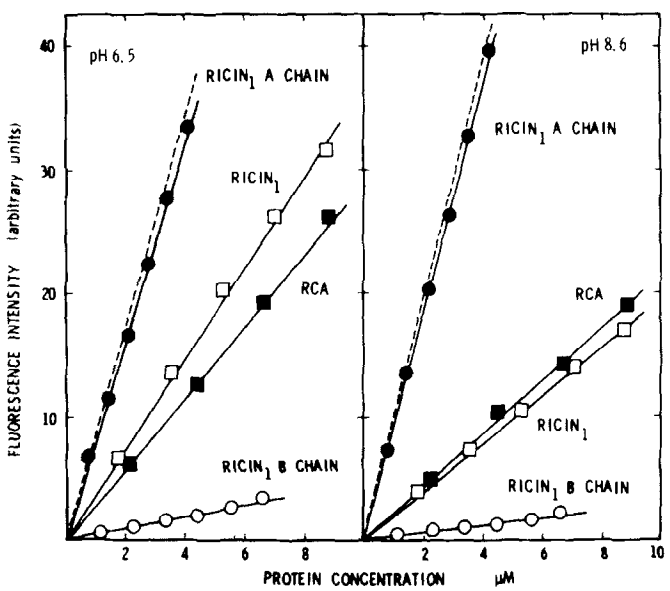

FIG. 5. Comparison of ANS binding to ricin and its subunits: The proteins were sequentially added to 1.0 ml of pH 6.5 PBS or 1.0 ml of 0.1 M Tris adjusted to pH 8.6 with HCl to which 50 μl of 1 mM ANS had been added. The dotted line is the theoretical fluorescence enhancement obtained by adding the slopes of the individual A and B chains. The line for RCA is plotted in equivalents of ricin A-B dimer.

in fluorescence enhancement of RCA and $ricin_1$ and many preparations were closer than shown in Figure 5.

The number of sites to which ANS binds and the strength of the binding could not be measured. Even by using very small ANS concentrations (0.5-1 μM), it was impossible to saturate ANS and evalute the maximal fluorescence enhancement using protein concentrations up to 1 mg/ml.

The ability of ANS to quench tryptophan fluorescence in $ricin_1$ and the A and B chain at pH 6.5 was determined by using 290 nm exciting light and monitoring tryptophan fluorescence by scanning the emission. The intensity of the emission peak decreased when ANS was added in small increments up to 100 mM. There were no significant differences in the plots of the percent decrease in intensity versus the ANS concentration for $ricin_1$, $ricin_1$ A and B chains, and for tryptophan which indicated that all quenching by ANS was due to the inner filter effect and there was no detectable energy transfer. Therefore, it appeared that ANS binding was not to tryptophan containing sites.

One interpretation of these data is that the fluorescence enhancement changes which are affected by the titration of one chain with the other is following the formation of the intact toxin. That there was indeed a strong interaction between the A and B chains is illustrated in Figure 6. The isolated A and B chains when chromatographed separately eluted at fraction number 38 on Sephadex G-100 in comparison to the appearance of ricin at fraction number 31. These data clearly show that the A and B chains formed a dimer at pH 6.5 when they were added together either in ice or incubated for a longer time at 22°. Prior modification of the thiol groups which participate in the interchain disulfide bond did not modify the ability of the A and B chains to dimerize. The effect of high ionic strength was evaluated by adding solid NaCl to separate samples of A and B chains to a final concentration of 2 M NaCl. After incubation at 22° for 10 minutes, the chains were mixed, incubated for 35 minutes at 22° and chromatographed at 4° in 5 mM sodium phosphate, pH 6.5, containing 50 mM galactose and 2 M NaCl. The protein peak was eluted at fraction number 30 which demonstrated that high ionic strength had no effect on the ability of A and B to dimerize. When a mixture of 100 μl of A chain added to 40 μl of 1 M Tris chloride, pH 8.6, followed by 250 μl B chain was incubated 5 minutes

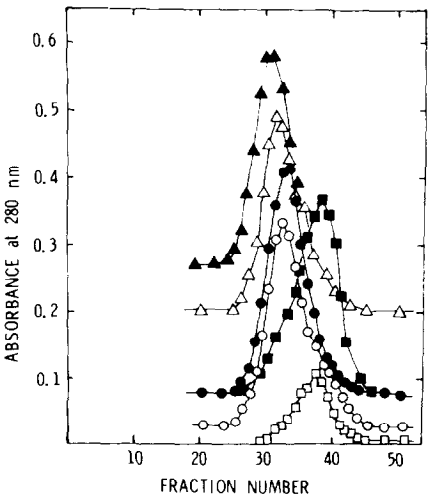

FIG. 6. Gel filtration of ricin and its peptides: Ricin (△), A chain (□) and B chain (■) were chromatographed at 4°C on a 0.9 x 55 cm column of Sephadex G-100 in PBS made 50 mM in galactose. Ricin A chain (100 µl containing 7.63 nmoles) was mixed on ice with 250 µl of B chain (30.5 nmoles) and immediatley chromatographed (○) or first incubated for 65 minutes at room temperature (●). Ricin A chain and B chain were separately labeled with a 20-fold excess of NEM at pH 7.6, dialyzed against PBS, mixed together and chromatographed (▲). Solid galactose was added (50 mM final concentration) to the proteins before placing on the column. The curves were separated by adding a constant absorbance to each fraction in an experiment.

at 22°C and chromatographed over the Sephadex G-100 column equilibrated with 0.1 M Tris chloride, pH 8.6, the peak of the protein eluted in the same fraction as untreated ricin. Fractions on the leading edge of the peak were reduced and analyzed by sodium dodecyl sulfate gel electrophoresis and compared to ricin. The relative intensity of Coomassie Blue staining of the A and B chain was identical in the chromatographed sample of ricin. The chromatographed sample showed identical concentration dependence as ricin in ANS titration shown in Figure 5.

DISCUSSION

Ricin A chain is much more efficient than B chain in interacting with ANS. It is unfortunate that the binding was not strong enough to quantitate the binding equilibrium of ANS to the two chains. The decreased ANS fluorescence enhancement observed upon the addition of one polypeptide to the other could result from change in the number of binding sites, change in the affinity of ANS for the polypeptides or change in the ability of the binding site to stimulate the fluorescence enhancement. The most likely explanation for the decreased enhancement when A and B chain are added together is that ANS

binding sites are lost due to the reassocation of the polypeptides to form intact toxin. The reassociated polypeptides showed the same ability as ricin to enhance ANS fluorescence. Because the A chain was better able to stimulate ANS enhancement than either ricin or B chain, it would appear that most of the effect of A-B association is to modify sites on the A chain, either by physically covering them or by modifying the strength of ANS association. The pH dependency of this modification of ANS fluorescence enhancement is not due to the lack of interaction of the A and B chain at pH 6.5, where the decrease in fluorescence enhancement was minimal (Figure 3 and 4). Sephadex gel filtration showed that association did occur at pH 6.5. Therefore, it would appear that the physical state of the ANS binding sites are different depending on the pH. The level of reduction in A chain fluorescence when titrated with B chain (Figure 4) approximated the predicted reduction from the data in Figure 5.

The changes in fluorescence intensity occurred within a time period that would preclude formation of a disulfide bond between the A and B chains. Because of this and the fact that NEM treatment did not prevent reassociation, it is likely that the formation of the interchain disulfide is not important at least in the initial association of A and B chains. The lack of an inhibitory effect on A and B chain association by high concentrations of salt suggests that ionic bonding does not play a primary role in the interaction.

ACKNOWLEDGEMENT

I thank Ms. Sophie Boguslawski for preparing the proteins used in this work and Dr. Richard Givens for the use of the spectrofluorometer.

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