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Fluorescence studies on the interaction of adenine with ricin A-chain

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Ricin A-chain, an *N*-glycosidase that attacks 28S rRNA at a highly conserved adenine residue, has a unique tryptophan (Trp-211) in the putative active site cleft. Fluorescence spectroscopy revealed that specific binding of adenine to the A-chain caused a large enhancement of Trp-211 fluorescence (70%) and a concomitant red shift of the emission spectrum (8 nm). A Scatchard plot of the fluorescence enhancement data was not linear, indicating that the environment of Trp-211 was altered by heterogeneous binding of adenines. These results, taken together with the protective effect of adenine on the ribosome-inactivation by ricin A-chain, suggest that at least two adenines bind to the active site cleft.

Ricin A-chain; Adenine-protein interaction; Active site; Tryptophan fluorescence; Ribosome inactivation

1. INTRODUCTION

Ricin, a toxic protein from castor bean (*Ricinus communis*) seeds, consists of an A-chain (267 amino acid residues) linked by a disulfide bond to a B-chain (262 residues) [1]. The B-chain binds to galactose-containing receptors on the cell surface. The A-chain (RTA), after entering the cytosol, inactivates ribosomes by hydrolyzing the *N*-glycosidic bond of a single adenine residue in a highly conserved loop of the 28S rRNA (A4324 in rat liver 28S rRNA) [2,3]. This loop appears to be involved in the interaction of elongation factors [4].

X-Ray crystallography has resolved the three-dimensional structure of ricin that contains a putative active-site cleft in RTA [5,6]. Comparison of amino acid sequences of eleven ribosome-inactivating proteins has shown that this cleft contains several perfectly conserved amino acids [7]. Mutational analyses have suggested a role for RTA cleft amino acids in enzymic action [8–11]. The unique tryptophan at position 211 is one of such amino acid in the cleft: the substitution of phenylalanine for Trp-211 resulted in a nine-fold decrease in activity, suggesting an additive contribution to the RNA-binding [10].

Since adenine is a product of the *N*-glycosidase action of RTA, it is expected that adenine interacts with the active site. Binding of adenine to RTA, however, has not been detected by equilibrium dialysis [12]. Here, we report that adenine-binding induces a large enhancement of Trp-211 fluorescence, allowing us to characterize the adenine–RTA interaction. At least two adenines appear to bind to the active site cleft.

Abbreviations: RTA, ricin toxin A-chain; Ac-Trp-Et, *N*-acetyl-L-tryptophan ethyl ester.

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2. MATERIALS AND METHODS

Ricin was purified from seeds of *Ricinus communis* according to [13]. RTA was isolated from the reduced ricin as described in [14]. Rat liver 80S ribosomes were prepared as in [15]. Bases were purchased from Sigma Chemical Co. and *N*-acetyl-L-tryptophan ethyl ester (Ac-Trp-Et) was from Aldrich Chemical Co.

Poly(U)-directed polyphenylalanine synthesis by rat liver 80S ribosomes was assayed as described in [16].

Fluorescence measurements were made with a Hitachi 850 spectrofluorometer equipped with a data processor, at 25°C, using 5-nm excitation and emission band passes. An excitation wavelength of 295 nm was chosen to obtain the tryptophan fluorescence. The emission spectra of RTA were recorded after subtraction of a background spectrum, to remove the small Raman band of buffer. In the presence of higher concentrations of adenine, the fluorescence intensity was corrected for the inner filter effect due to excitation light absorbance by adenine. To assess the inner filter effect, increasing concentrations of L-tryptophan (up to A_{280} of 0.2) were excited at 280 nm and the observed fluorescence intensity was plotted against the absorbance at 280 nm.

The data of change in RTA fluorescence induced by adenine were plotted according to the method of Scatchard [17], using the following equation:

$$(\Delta F_{337}/F_{329})/[A_1] = -K_a \cdot \Delta F_{337}/F_{329} + K_a \cdot \Delta F_{max}/F_{329}$$

where ΔF_{337} and ΔF_{max} are the difference in fluorescence intensity at 337 nm between the free RTA and the complex, at a given adenine concentration and at saturation, respectively. F_{329} is the fluorescence intensity of free RTA at 329 nm. $[A_1]$ is the total adenine concentration and K_a is the association constant.

3. RESULTS

Fig. 1 shows the fluorescence spectra of RTA excited

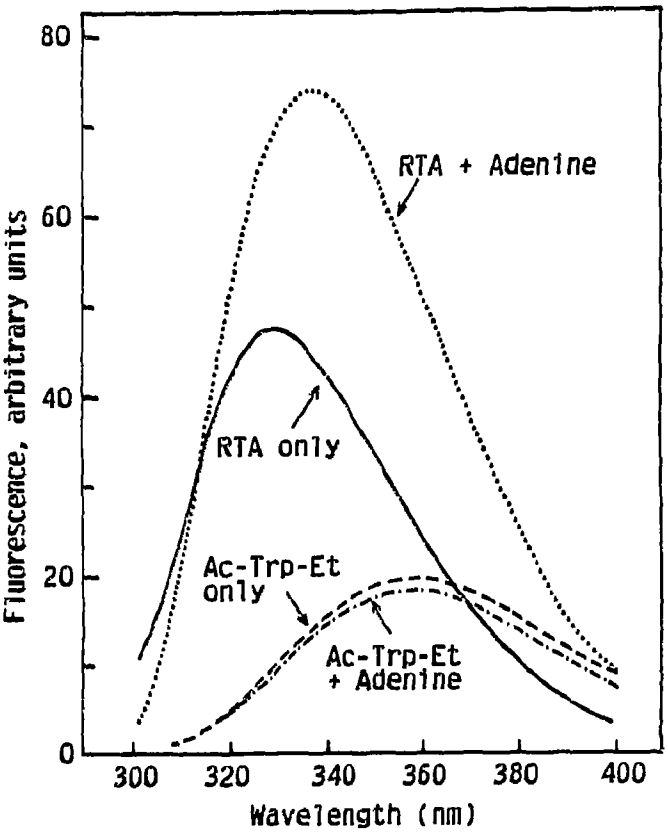

Fig. 1. Fluorescence emission spectra of RTA and Ac-Trp-Et in the absence and presence of adenine. (—) 3.2 μM RTA only; (.....) 3.2 μM RTA with 12.5 mM adenine; (----) 3.0 μM Ac-Trp-Et only; (-·-·-) 3.0 μM Ac-Trp-Et with 12.5 mM adenine. The samples in 20 mM potassium phosphate buffer (pH 7.0)/0.1 M KCl/0.1 mM dithiothreitol were excited at 295 nm, at 25°C. The spectra with adenine were corrected for inner filter effect.

at 295 nm in the presence and absence of adenine, in comparison with the spectra of the fully solvated model fluorophore *N*-acetyl-L-tryptophan ethyl ester. The spectrum of RTA in the absence of adenine had a maximum at 329 nm, indicating the nonpolar environment of tryptophan-211. Addition of adenine caused a marked increase in the intensity of the total emission and a concomitant red shift in the spectrum. Both the fluorescence intensity and the shift in the position of maximum emission increased with increasing adenine concentrations (inset in Fig. 2). The limiting values of the fluorescence enhancement and the red shift were 70% and 8 nm, respectively. In contrast, such a change in fluorescence spectrum was not observed when the same concentrations of adenine were added to Ac-Trp-Et (Fig. 1). Furthermore, neither uracil nor cytosine caused a significant change in the fluorescence spectrum of RTA; guanine was not able to be tested because of its low solubility in the neutral buffer. These results led us to conclude that the changes in the fluorescence spectrum induced by adenine reflect the specific binding of

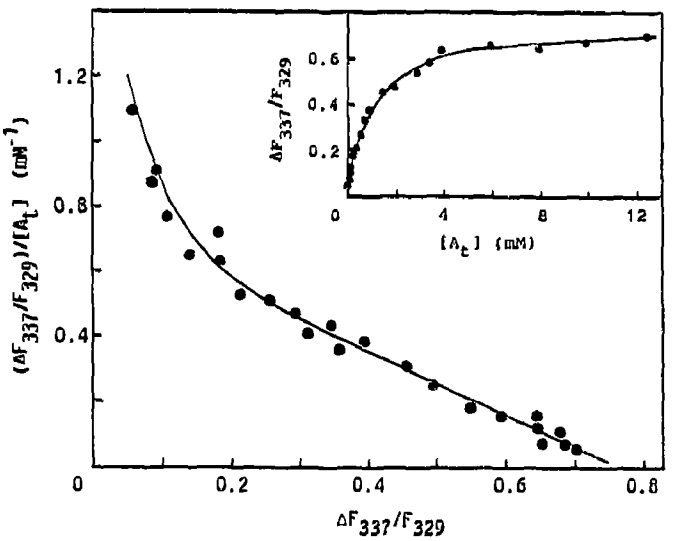

Fig. 2. Scatchard plots for binding of adenine to RTA. Measurements of RTA fluorescence were made in the presence of various concentrations of adenine under the same conditions as in Fig. 1. The inset shows the enhancement of fluorescence intensity as a function of adenine concentration.

adenine to RTA and are not due to the perturbing effect of the solvent on tryptophan.

From the double-reciprocal plot of ΔF_{337} versus adenine concentration (not shown), the association constant for adenine–RTA binding was estimated to be 1.0 $\times$ 10^3 M^{-1}. As shown in Fig. 2, however, the nonlinear

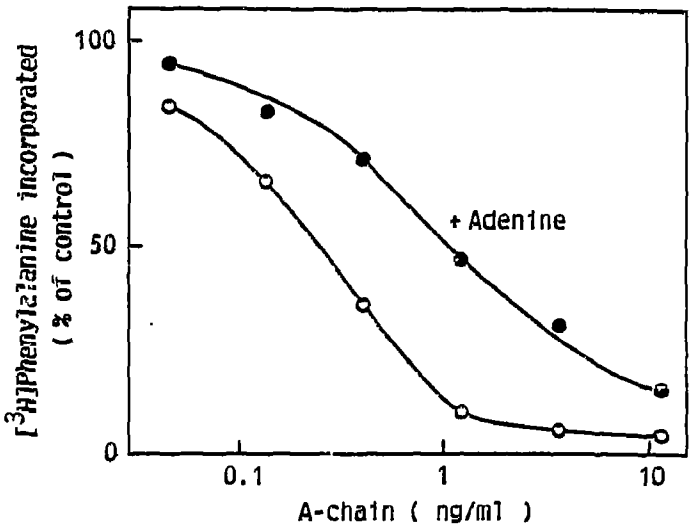

Fig. 3. Effect of adenine on inactivation of ribosomes by RTA. Rat liver 80S ribosomes (3.4 pmol) were incubated with various concentrations of RTA in the absence (○) or presence (●) of 10 mM adenine at 37°C for 10 min in 20 μl of 20 mM Tris-HCl buffer (pH 7.5)/5 mM magnesium acetate/100 mM NH_4Cl/1 mM dithiothreitol. The ribosomes (1.7 pmol in 10 μl) were then assayed for their ability to synthesize polyphenylalanine as in [16]. Control value of [^{3}H]phenylalanine incorporated, obtained with untreated ribosomes, was 6024 cpm.

Scatchard plot of the fluorescence enhancement data demonstrates that the fluorescence of Trp-211 is influenced by heterogeneous binding of adenines. This suggest that at least two binding sites are located in close proximity to each other and that binding of adenine to each site probably influences the binding to the other site. Therefore, further calculation for an association constant by assuming two independent binding sites [18] was not made.

The ability of RTA to inactivate ribosome was significantly reduced by addition of adenine. In the presence of 9 mM adenine, about 4-times the concentration of RTA was required to give 50% inhibition of polyphenylalanine synthesis (Fig. 3). The same concentration of cytosine or uracil did not show any effect on the ribosome-inactivation. Adenine is a product of *N*-glycosidase reaction by RTA. The protective effect of adenine demonstrates the specific binding of adenine to the active site of RTA.

4. DISCUSSION

The specific binding of adenine to RTA resulted in a large enhancement of Trp-211 fluorescence (70%) and a concomitant red shift in the emission spectrum (8 nm). This is the first direct observation of adenine–RTA interaction in the state of solution. A combination of the curvature in the Scatchard plots of the fluorescence enhancement data and the protective effect of adenine on the ribosome-inactivation suggests that at least two adenine-binding sites exist at the active site of RTA. This is consistent with the X-ray crystallographic study of ricin: Katzin et al. [6] tentatively stated that diffusion of adenine into ricin crystals gave a positive peak of difference electron density at the putative active site cleft, and that the difference density peak was quite large, being able to accommodate at least two adenines. The cleft contains several perfectly conserved amino acids including Trp-211 [7]. The present results further support the idea that this cleft containing Trp-211 is, in fact, the active site.

Stacking interaction of tryptophan with nucleic acid bases has been well established by investigators using oligopeptides containing tryptophan [19,20]. Since the stacking interaction has been shown to lead to a quenching of tryptophan fluorescence, the present result of fluorescence enhancement suggests that stacking of Trp-211 with adenine does not take place.

The adenine-induced change in the fluorescence probably reflects a conformational change in the active site cleft that alters the environment of Trp-211. The emission maximum at 329 nm, observed without adenine, indicates the nonpolar environment of Trp-211, in agreement with the previous reports of fluorescence measurement [21] and X-ray crystallography [6]. The red shift in the emission spectrum upon adenine-binding suggests that the environment of the tryptophan is made somewhat polar. The enhanced fluorescence can be accounted for by adenine-induced alleviation of quenching due to an increase in the distance between the indole ring of Trp-211 and a possible quenching residue.

It is interesting to consider that the specific recognition of the target RNA structure by RTA might involve two adenine-binding sites in the active cleft. This appears consistent with the proposal that the sequence GAGA in a loop with a stem is crucial for recognition by RTA [22].

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REFERENCES

[1] Olsnes, S. and Pihl, A. (1982) in: Molecular Action of Toxins and Viruses, vol. 2 (Cohen, P. and Van Heyningen, S. eds.) pp. 51–105, Elsevier, Amsterdam.
[2] Endo, Y., Mitsui, K., Motizuki, M. and Tsurugi, K. (1987) J. Biol. Chem. 262, 5908–5912.
[3] Endo, Y. and Tsurugi, K. (1987) J. Biol. Chem. 262, 8128–8130.
[4] Moazed, D., Robertson, J. and Noller, H. (1989) Nature 334, 362–364.
[5] Montfort, W., Villafranca, J.E., Monzingo, A.F., Ernst, S., Katzin, B., Rutenber, E., Xuong, N.H., Hamlin, R. and Robertus, J.D. (1987) J. Biol. Chem. 262, 5398–5403.
[6] Katzin, B.J., Collins, E.J. and Robertus, J.D. (1991) Proteins 10, 251–259.
[7] Funatsu, G., Islam, M.R., Minami, Y., Sung-Sil, K. and Kimura, M. (1991) Biochimie 73, 1157–1161.
[8] Frankel, A., Schlossman, D., Welsh, P., Hertler, A., Withers, D. and Johnston, S. (1989) Mol. Cell. Biol. 9, 415–420.
[9] Frankel, A., Welsh, P., Richardson, J. and Robertus, J.D. (1990) Mol. Cell. Biol. 10, 6257–6263.
[10] Bradley, J.L. and McGuire, P.M. (1990) Int. J. Peptide Protein Res. 35, 365–366.
[11] Ready, M.P., Kim, Y. and Robertus, J.D. (1991) Proteins 10, 270–278.
[12] Zamboni, M., Brigotti, M., Rambell, F., Montanaro, L. and Sperti, S. (1989) Biochem. J. 259, 639–643.
[13] Hara, K., Ishiguro, M., Funatsu, G. and Funatsu, M. (1974) Agric. Biol. Chem. 38, 65–70.
[14] Ono, M., Kuwano, M., Watanabe, K. and Funatsu, G. (1982) Mol. Cell. Biol. 2, 599–606.
[15] Blobel, G. and Sabatini, D. (1971) Proc. Natl. Acad. Sci. USA 68, 390–394.
[16] Watanabe, K. and Funatsu, G. (1987) Biochim. Biophys. Acta 914, 177–184.
[17] Scatchard, G. (1949) Ann. NY Acad. Sci. 51, 660–672.
[18] Halfman, C.J. and Nishida, T. (1972) Biochemistry 11, 3493–3498.
[19] Brun, F., Toulmé, J.J. and Hélène, C. (1975) Biochemistry 14, 558–563.
[20] Toulmé, J.J. and Hélène, C. (1977) J. Biol. Chem. 252, 244–249.
[21] Bushueva, T.L. and Tonevitsky, A.G. (1987) FEBS Lett. 215, 155–159.
[22] Endo, Y. and Tsurugi, K. (1988) J. Biol. Chem. 263, 8735–8739.