Effect of Chemical Modification on the Hemolytic Activity of Asp-Hemolysin from *Aspergillus fumigatus*

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Amino acid residues involved in the hemolytic active site of Asp-hemolysin from *Aspergillus fumigatus* were analyzed. The hemolytic activity of the toxin for human erythrocytes was found to be inactivated by the following modification procedures: arginine residue with 1,2-cyclohexadione (CHD), arginine and tyrosine residues with 2,3-butanedione (BDO), and reductive methylation (RM) of lysine residue with formalin. The results indicate that arginine and/or lysine residues in toxin play an important role as a binding site to erythrocyte ghost membranes. Based on these results, a possibility of amino acid residues involved in the binding and the hemolytic active sites of Asp-hemolysin molecule was therefore studied.

Key word: *Aspergillus fumigatus*, Asp-hemolysin, hemolytic active site, binding site, chemical modification

Asp-hemolysin, a thermolabile protein toxin produced by *Aspergillus fumigatus* Fresenius-Muramatsu strain, has been purified and the biological properties of the toxin have been reported^{1,2)}. This toxin has been shown to promote the establishment and development of an experimental *Aspergillus* infection in mice³⁾. We have further observed multiple biological functions of Asp-hemolysin as a virulence factor⁴⁾. Amino acid residues in the hemolytic active site of Asp-hemolysin have been studied by photochemical oxidation⁵⁾. On the other hand, chemical modification of Asp-hemolysin with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), 2,2'-dithiodipyridine (2-PDS), 4,4'-dithiodipyridine (4-PDS), β -naphthoquinone-4-sulfonic acid (NQS), 2,4,6-trinitrobenzene sulfonic acid (TNBS) or glyoxal also suggested that one cysteine residue and/or or more arginine residue are involved in the hemolytic activity⁶⁾. We have reported that arginine residues

in Asp-hemolysin play an important role as a binding site on human erythrocyte ghosts⁷⁾. We recently reported that the toxin produces morphological changes in human erythrocyte membranes in which patches- or cap-forms are observed by scanning electron microscopy⁸⁾. A similar result was also found in chicken erythrocyte membranes⁹⁾.

In this study, we will extend our investigations on binding site and hemolytic active site in the Asp-hemolysin molecule. To investigate the presence of the two active sites, chemical modifications with 1,2-cyclohexadione (CHD) and 2,3-butanedione (BDO), and the reductive methylation (RM) of the toxin were carried out.

Materials and Methods

Materials: *Aspergillus fumigatus* was incubated under stationary condition at 30°C for 14 days in Sabouraud broth medium containing 0.5% heart infusion broth (Difco Lab., Detroit, Mich., U.S.A.). Highly purified Asp-hemolysin was prepared by the method using various column chromatographies described previously²⁾, and was proved to

be homogeneous by disc electrophoretic analysis and immunodiffusion plate test. CHD and BDO were purchased from Nakarai Chemicals Ltd., Kyoto. All other chemicals were commercial products of reagent grade.

Modification of the toxin: To determine the hemolytic active site and binding site of Asp-hemolysin, chemical modifications with CHD, BDO and RM were made under various conditions. The CHD modification of Asp-hemolysin was done according to the method of Patthy and Smith^{10,12)}. Various amounts of CHD were dissolved in 0.5 ml each of 0.2 M borate buffer (pH 8.1), and added to the toxin (1 mg). The mixture solution was incubated for 1 hr at 37°C. Chemical modification of the toxin (1 mg) with BDO was carried out by incubating the toxin with various concentrations of BDO in 0.2 M borate buffer (pH 8.1) for 2 hr at 25°C^{11,13)}. RM modification of the toxin was achieved by the method of Weisgraber et al.¹⁴⁾. The methylation of the toxin (1 mg) was performed in 0.3 M borate buffer (pH 9.0) at 0°C by addition of sodium borohydride (1 mg) followed by additions of 1 to 7 μ l of 37% formaldehyde solution (formalin) five times.

Assay of remaining hemolytic activity: After treatment with CHD, BDO and RM, excess chemical reagent in each modification was removed by gel filtration on a Sephadex G-25 column, and then the remaining hemolytic activity was measured according to the method reported in our previous paper¹⁾. The remaining hemolytic activities were expressed as percentages of that of the sample before preincubation (zero time; 100%).

Determination of binding rate: The rate of binding of the chemically modified Asp-hemolysin to human erythrocyte ghost membranes was determined according to the method of Mancini et al.¹⁵⁾. The mixture solution was incubated for 1 hr at 21°C, and centrifuged for 20 min at 15,000 rpm. The supernatant fluid was isolated and then the binding rate in supernatant fluid was determined by the single radial immunodiffusion technique using anti-Asp-hemolysin serum. The binding

rate was estimated by comparing the curves of the diameters of halos produced in the presence of various amounts of the toxin with the standard curve on semilogarithmic paper. When the diameter of halo was 0 mm, the binding rate was considered to be 100%, and when halos had the same diameter as that of toxin alone, the binding rate was 0%.

Amino acid analysis: Amino acid analysis was performed with a Hitachi Model 835-50 amino acid analyzer on samples hydrolyzed with 6N HCl in a sealed tube for 24 and 48 hr at 105°C.

Results and Discussion

Hemolytic activity of modified toxin

As shown in Fig. 1, hemolytic activity of Asp-hemolysin was rapidly inactivated by CHD- and BDO-modification. Both CHD and BDO have been widely used as a reagent for specific chemical modification of arginine residue in proteins^{10,11)}, and RM selectively modified lysine residue of lipoproteins¹⁴⁾. In CHD and BDO-modifications, the inactivation was achieved after the modification of 4 to 6 arginine residues per mole of the toxin. The hemolytic activity was inactivated completely by RM of 4 to 5 lysine residues in an Asp-hemolysin molecule. Considering the results of RM together with the complete inactivation of the toxin by CDH and BDO-modification, it is possible that lysine and arginine residues may contribute to in the hemolytic function of the toxin.

Binding ability of modified toxin

Figure 2 shows the binding rate of chemically modified Asp-hemolysin to human erythrocyte ghost membranes compared with that of the native toxin. In the control, to which chemical reagents were not added, binding of the toxin to the ghosts was observed, at an approximate rate of 100%. Both RM and CHD-modification strongly inhibited the binding. Similar results have been observed previously⁷⁾ in the binding function of the toxin modified with glyoxal, TNBS or DTNB. However, a significant decrease in the antigenic reactivity was observed when the toxin was

Fig. 1. Decrease in the content of specific amino acid residue and loss of hemolytic activity during modification of Asp-hemolysin by modification with 1,2-cyclohexanedione (A) and 2,3-butanedione (B), and by reductive methylation with formalin (C). The reaction conditions of chemical modification were determined as described in the text. After remove of excess chemical reagents by gel filtration the remaining hemolytic activity was assayed under standard conditions; Two milliliters of 2% human erythrocyte suspension were added to tubes containing 3 ml of the sample solution, and the mixture solution was incubated for 30 min at 21 ± 2°C. After the incubation, the mixture was centrifuged for 5 min at 1,500 rpm. Hemolysis was monitored by absorbancy at 541 nm.

Table 1. Amino acid composition of chemically modified Asp-hemolysin

Amino acid	Number ^{a)} of amino acid residue per mole of			
	Asp-hemolysin (Control)	Modified Asp-hemolysin		
		CHD	BDO	RM
Lys ^{b)}	21	20.5	21.4	16.8
His ^{b)}	3	3.8	3.0	2.7
Arg ^{b)}	9	5.3	3.0	9.2
Asp ^{b)}	36	36.0	36.3	36.0
Thr ^{c)}	18	17.2	17.8	18.2
Ser ^{c)}	22	21.0	21.0	21.0
Glu ^{b)}	18	18.0	18.4	17.6
Pro ^{b)}	6	6.3	6.0	6.3
Gly ^{b)}	30	29.7	30.0	29.0
Ala ^{b)}	14	13.8	14.2	14.0
Half-Cys ^{b)}	3	3.2	3.1	3.4
Val ^{d)}	20	19.9	20.1	20.4
Met ^{b)}	1	0.9	1.0	1.0
Ile ^{b)}	11	10.7	11.1	11.1
Leu ^{b)}	8	7.7	8.0	8.0
Tyr ^{b)}	8	7.8	5.2	8.1
Phe ^{b)}	8	8.3	8.2	8.1
Trp ^{e)}	4	3.8	3.9	4.0

Fig. 2. Rates of binding of chemically modified Asp-hemolysin to human erythrocyte ghost membranes. The binding rates were obtained by the single radial immunodiffusion technique of Mancini et al¹⁵⁾. Each bar indicates the mean ± S.E. (n=5). Binding ratio (%) was expressed as described in the text.

modified with BDO, and the BDO-modified toxin almost completely lost its capacity to bind to anti-Asp-hemolysin serum. Therefore, it was impossible to determine the binding rate for the toxin modified with BDO (data not shown).

Amino acid composition of modified toxin

The amino acid compositions of the native toxin

Amino acid content was analyzed at the point indicated by each arrow in Fig. 1.

- a) Based on a molecular weight of 30,000.
- b) Average value from 24 and 48 hr hydrolysis.
- c) Extrapolated to zero time.
- d) Analyzed as cysteic acid in the formic acid-oxidized Asp-hemolysin.
- e) Determined from the UV-spectrum.

and the modified toxin are shown in Table 1. The significant changes occurred only in the arginine, lysine and tyrosine residues. The other amino acid residues remained essentially unchanged during the chemical modification with CHD, BDO or by RM. In modification of the toxin with BDO, changes were detected in two amino acid residues, arginine and tyrosine. This result is not incompatible with our previous result obtained by chemical modification of the toxin with glyoxal⁶⁾.

In previous papers^{6,7)}, we reported individually the existence of binding and hemolytic active sites in Asp-hemolysin molecule using modification reagents. The present data proved simultaneously that Asp-hemolysin contain shemolytic active and binding sites based on findings by using chemical modification with CHD and RM. Further study of the hemolytic mechanism this toxin is needed to elucidate the role of the specific amino acid residues.

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