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INACTIVATION OF STAPHYLOCOCCAL ENTEROTOXIN A BY CHEMICAL MODIFICATION

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Carboxymethylation of either five or six histidine residues of enterotoxin A caused a slight reduction in the ability of the enterotoxin to react with antibody specific for enterotoxin A and a significant reduction in toxicity. The loss of toxicity of the enterotoxin was not associated with a conformational change but appeared to be due to the modification of an amino acid residue within the toxic site of the molecule.

The staphylococcal enterotoxins are a closely related family of proteins that can cause emesis and diarrhea when ingested by humans. Antigenically distinct enterotoxins have been identified as enterotoxins A, B, C, etc. on the basis of their reactions with specific antibodies (1). The enterotoxins are single polypeptide chains with molecular weights of 26,000 to 30,000 daltons. They are resistant to proteolytic enzymes and relatively resistant to heat. The amino acid sequence of staphylococcal enterotoxin B has been determined (2). The half-cystine residues of enterotoxin B were found to create a "cystine loop" of twenty amino acid residues between positions 92 and 112. Although the entire amino acid sequence of staphylococcal enterotoxin A has not been resolved, a sequence adjacent to the cystine loop that is homologous to that of enterotoxin B has been determined (3). The first seven amino acid residues of this sequence are identical as are four of the next seven residues:

-Thr-Cys-Met-Tyr-Gly-Gly-Val-Thr-Leu-His-Asp-Asn-Asn-Arg-Leu-Thr-Glu- (underlined residues are common to enterotoxin B). This was a logical region to investigate in an attempt to locate the toxic site because the homologous

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sequence is adjacent to a structural feature, the cystine loop, that is common to all of the enterotoxins (4,5).

In this paper we present the results of the substitution with bromoacetic acid of the histidine residues in enterotoxin A on: (1) its activity when given orally to rhesus monkeys and (2) the effect of its reaction with the specific antibody to enterotoxin A.

Materials and Methods

Materials. Staphylococcal enterotoxin A was prepared by the method of Schantz et al. (6) and antiserum specific for enterotoxin A was prepared in rabbits as described by Bergdoll et al. (7). The bromoacetic acid was obtained from Aldrich Chemical Co. (Milwaukee, WI).

Chemical Modification and Analyses. The histidine residues were modified with bromoacetic acid at pH 7.0 by the method of Harris and Hill (8). Samples for amino acid analysis were hydrolyzed with 6 N HCl in vacuum-sealed tubes at 110°C for 48 h. Amino acid compositions were determined on a Beckman model 120B amino acid analyzer. Carboxylated histidine residues were determined by measuring the difference between histidine content of the untreated and the bromoacetic acid treated enterotoxin A.

The concentrations of solutions of chemically modified enterotoxin A were measured by the method of Lowry et al. (9) using enterotoxin A as the standard. The quantitiative precipitin test (10) and monkey feed (11) experiments were used for enterotxoin analysis and detection. The fluorescence spectra of native and modified enterotoxin A were determined in an Amino-Bowman spectrofluorimeter as described by Chu and Bergdoll (12).

Results

Modification of Histidine. The rate of carboxymethylation of enterotoxin A was similar to the rate observed by Harris and Hill (8) with myoglobin. The first two histidine residues of enterotoxin A were inactivated within 12 h, while the others were modified very slowly. The fifth and sixth histidine residues were not modified until eight and fourteen days, respectively. The reaction of bromoacetic acid with enterotoxin A was highly specific for histidine residues. Only one other residue, a lysine residue, was affected, even after 14 days.

A comparison of the fluorescence spectra of native enterotoxin A and enterotoxin with 5 and 6 residues modified showed no evidence of conformational change as a result of the modification reaction (Figure 1). The reaction of the carboxymethylated enterotoxin A molecules with antiserum specific for the native enterotoxin A (quantitative precipitin assay) indicated that the

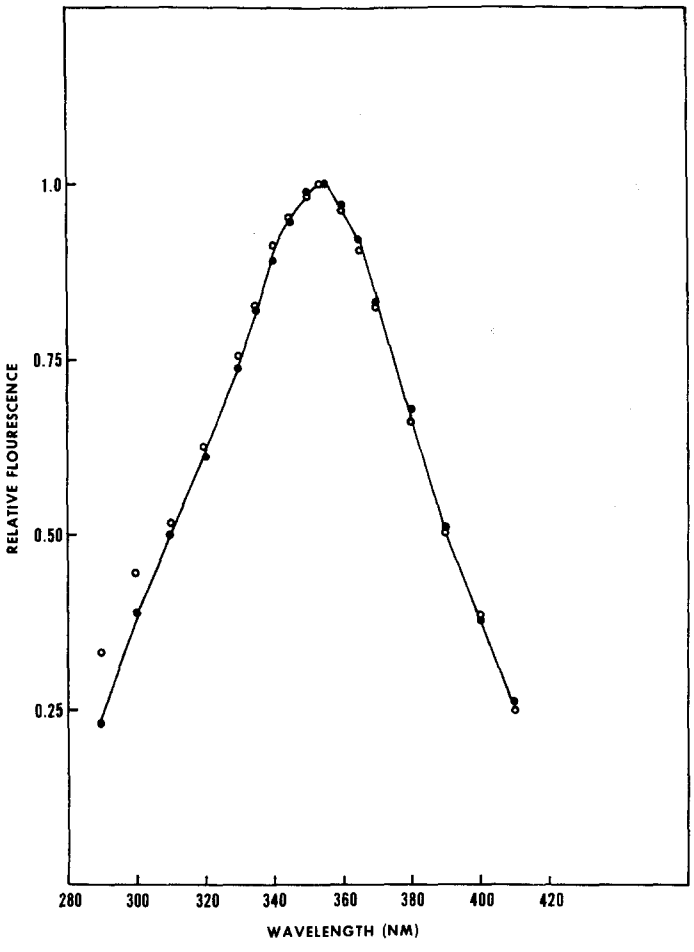

Figure 1. Fluorescence spectra of native and carboxymethylated enterotoxin A. Relative fluorescence of native enterotoxin, ●——●. Relative fluorescence of carboxymethylated enterotoxin, O——O.

modification of all six histidine residues of enterotoxin A resulted in only a slight reduction in the affinity of the toxin for the anti-enterotoxin A (Figure 2). Because no gross conformational change was measured in the modified enterotoxins, the slight change in affinity of the toxins for the antiserum could be due to the modification of an amino acid residue within one of the antigenic sites of the enterotoxin A molecule or to a localized conformational change near one of the antigenic sites.

Effect of Histidine Modification on the Biological Activity of Enterotoxin A. The results of testing the modified enterotoxin for biological activity by the monkey feeding test are given in Table 1. The results show

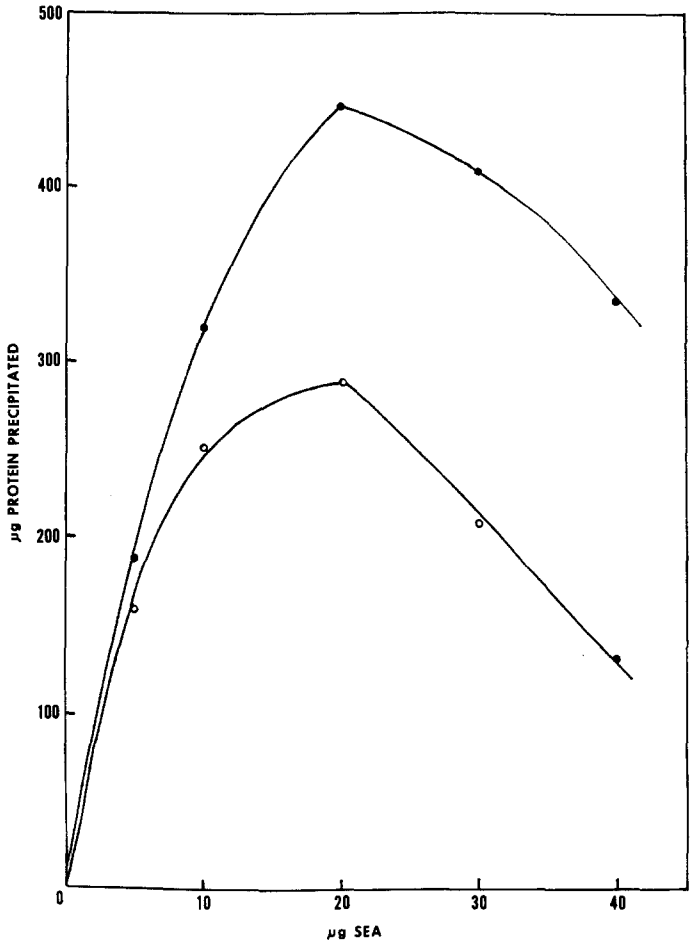

Figure 2. Precipitin analysis of native enterotoxin A and enterotoxin A modified by treatment with bromoacetate for 14 days. Varying amounts of native and modified enterotoxin were precipitated by the addition of a constant amount of antibody. Precipitates were washed, dissolved in 0.1 N NaOH and measured by the method of Lowry et al. (12) using bovine serum albumin as a standard.

that the modification of five or six histidine residues caused a significant loss in the toxic activity of enterotoxin A.

Discussion

The histidine residues were investigated because of their presence in the active sites of some enzymes (13,14) and the availability of chemical modification procedures relatively specific for histidine (15). The procedure of Harris and Hill (8) for modification of proteins with bromoacetic acid at pH 7.0 was chosen because of its specificity for L-histidine residues. This procedure has provided us with a useful means of studying the role of the

Table 1. Effect of chemical modification of histidine residues on the emetic activity of staphylococcal enterotoxin A.

No. histidine residues modified	Bromoacetic acid treatment (pH 7.0) days	Enterotoxin A µg/monkey	Activity*
None		20	2/6
None**		30	3/6
5	8	75	0/6
6	14	50	0/6
6	14	150	0/6

*Number of monkeys showing positive reaction vs. number of monkeys challenged.
**Enterotoxin was allowed to stand 14 days at room temperature in phosphate buffer at pH 7.0.

histidine residues in the enterotoxins. Only one other residue, a lysine, was affected after prolonged treatments. The 14-day treatment required to inactivate all six histidine residues of enterotoxin A did not significantly affect the stability of the enterotoxin A molecule. Control solutions of enterotoxin A held at room temperature under reaction conditions for 14 days showed no loss in ability to react with antibody to native enterotoxin A (Figure 2) or in ability to cause emesis in monkeys (Table 1).

The change in the immunological and biological activity of the derivatives do not appear to be due to the gross conformational changes because the fluorescence spectra of the modified toxins were identical to that of the native enterotoxin A (Figure 1). This is the first instance in which a staphylococcal enterotoxin has been inactivated in the absence of gross conformational changes. Although alkylation of five to seven methionine residues of enterotoxin B (12), guanidation of 31 of 32 lysine residues of enterotxoin B (16) and acetylation or succinylation of amino groups of enterotoxin B (17) caused reduction in the emetic activity of the enterotoxin molecule, all of these modifications were accompanied by gross conformational changes, as measured by their fluorescent spectra.

The loss in toxicity of the enterotoxin A molecule in the absence of a gross conformational change could be due to the modification of an amino acid residue located within the toxic site of the enterotoxin molecule or close

enough to the toxic site to cause a localized change in conformation. Alternately, the modified amino acid residue could be located within or near a site that binds to the affected cells in the body. The amino acid residue responsible for the loss of the toxicity is probably one of the histidines, not the single lysine which is also modified. Earlier studies have shown that lysine probably is not involved in the toxic activity of enterotoxin B (16,17). Because the reaction of bromoacetic acid with each of the histidine residues of enterotoxin A is relatively slow, derivatives of enterotoxin A having from one to four modified histidines can be obtained and assayed for toxicity; thus, the histidine residue involved in the toxicity of the molecule should be identifiable.

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