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STRUCTURE AND ACTIVITY OF RAGWEED ANTIGEN E—III.

THE ROLE OF THE THIOL GROUP IN THE CONFORMATION AND ALLERGENIC ACTIVITY OF ANTIGEN E*

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Abstract—The free thiol groups of antigen E (AgE) were titrated with *p*-hydroxymercuribenzoate (PHMB) under various conditions to determine the number and accessibility of these groups in solution. The native protein possessed one free thiol group. Reaction with PHMB under dissociating conditions exposed no further thiol reactivity indicating that the thiol group was not buried or partially masked in native AgE. A derivative of AgE, modified at the single thiol group under non-dissociating conditions by PHMB showed little or no conformational changes relative to native AgE as revealed by ORD and CD measurements. AgE and its derivative both had equal rotatory powers showing a negative minimum at 233 nm. In CD measurements, both proteins gave negative ellipticity bands at 205 and 222 nm and the $[\theta']$ values at these minima were comparable. The allergenicity was unaltered in the derivative relative to AgE, as measured by RAST with ragweed sensitive sera. Thus it was concluded that the thiol group of AgE is not involved in a conformationally critical interaction within the molecule and is not involved in the binding of AgE with its immunoglobulin E antibodies.

INTRODUCTION

Ragweed pollen is a major cause of seasonal allergic rhinitis (hay fever) and antigen E (AgE)§ is the main component in ragweed pollen extracts. Most of the sera (97%) from individuals with ragweed hay fever have been shown (Adolphson *et al.*, 1978) to contain IgE antibodies reactive with AgE. Thus antigen E is an allergen of great clinical importance. Since its isolation (King *et al.*, 1962) there has been little investigation into the structure and activity of AgE. Not much is known about AgE other than its amino acid composition and the fact that it is composed of two subunits. The α and β sub-units have been separated by urea, guanidine or

sodium dodecyl sulfate but these procedures yielded in each case inactive subunits (Griffiths, 1973; King *et al.*, 1974; Takatsu *et al.*, 1975).

We have initiated studies aimed at determining the allergenic structure of AgE. Recently we introduced (Paull *et al.*, 1978) a new approach for the isolation of the α and β chains of AgE in their active form (Paull *et al.*, 1978, and in press). The preparation of active sub-units from AgE is a necessary first step for undertaking investigations into the molecular basis of the allergenicity of the subunits. This paper reports on the reactivity and accessibility of the free thiol group(s) of AgE. Native AgE was found to contain a single fully accessible thiol group and the effects of modification of this side chain on the conformation and allergenicity of the protein were studied in detail.

MATERIALS AND METHODS

Materials

Purified AgE and Rye group 1 were kindly provided by the Research Resources Branch of the National Institute of Allergy and Infectious Diseases, Bethesda, MD. AgE was dialyzed against several changes of 0.05 M NH_4HCO_3 (pH 8.0) buffer to remove glycerol and was lyophilized prior to use. Cyanogen bromide and

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§Abbreviations used: AgE, ragweed antigen E; PHMB, *p*-hydroxymercuribenzoate; PMB-AgE, antigen E in which the thiol group is modified by reaction with PHMB in 0.05 M phosphate buffer, pH 7.0; ORD, optical rotatory dispersion; CD, circular dichroism; RAST, radioallergosorbent test.

p-chloromercuribenzoic acid, sodium salt were obtained from Aldrich Chemical Co. Iodoacetic acid was purchased from Sigma Chemical Co.

Sera

Sera used in this study have been described in detail previously (Paull *et al.*, 1978, and in press). Briefly patients were chosen on the basis of the following criteria: past history of seasonal rhinitis, positive immediate intradermal skin test and nasal challenge with crude short ragweed extract, no prior immunotherapy and a preseasonal level of serum IgE antibodies greater than seven times control binding as measured by the RAST. A ragweed serum pool was obtained from five untreated patients with ragweed hayfever. IgE antibodies to ragweed allergens in the pool exceeded 2000 ng/ml (Adolphson *et al.*, 1978).

Modification of the thiol groups

The availability of the thiol groups in AgE was determined under two different conditions and employing two different thiol group reagents. Modification of the thiol groups at neutrality under non-dissociating conditions gave the number of reactive SH groups that are accessible to reagents under these conditions in native AgE. The total number of SH groups in AgE was determined by performing the modification reactions under extreme dissociating conditions.

(a) *Reactions with p-hydroxymercuribenzoate.* Reaction under neutral non-dissociating conditions was carried out by monitoring the increase in absorbance at 255 nm of the protein solution upon the addition of suitable aliquots of PHMB (Boyer, 1954). The procedure previously described (Atassi, 1964) was employed here. In the present work, an aliquot (2 ml) of AgE (1.2–1.6 mg) in 0.05 M sodium phosphate buffer at pH 7.0 was mixed in a 3 ml cuvette with an aliquot (10 μ l) of 1 mM *p*-hydroxymercuribenzoate and, after standing at room temperature for 20 min, the absorbance at 255 nm determined. The addition of 10 μ l aliquots of PHMB solution was continued in this manner until the absorbance at 255 nm levelled off. For determining total SH groups, AgE was dissolved in 7 M urea, pH 7.0 and the solution allowed to stand at room temperature for 4 hr prior to titration with PHMB.

(b) *Reactions with iodoacetic acid.* These were carried out in 0.05 M phosphate buffer at pH 7.0 or in 7 M urea at pH 7.0. A solution (0.5 ml) of antigen E (1.0–1.2 mg) was mixed with 20-fold

molar excess of iodoacetic acid. The reaction mixture was stirred magnetically at room temperature for 4 hr in the dark. After extensive dialysis against distilled water, freeze-drying and hydrolysis of the samples, their amino acid compositions were determined. The number of reactive thiol groups under each of the reaction conditions was determined from the content of S-carboxymethylcysteine.

ORD and CD measurements

Conformational changes were monitored by ORD and CD studies on solutions of AgE or derivative (0.05–0.2 mg/ml) in 0.05 M sodium phosphate buffer, pH 7.0. Measurements were performed at 25°C in a JASCO J-20A spectropolarimeter. The ORD and CD results were corrected for the refractive index dispersion of water and are therefore given here in $[m']$ and $[\theta']$, respectively. Experimental procedure and quantitative treatment of data were carried out as described in detail elsewhere (Singhal & Atassi, 1970). The molecular weights of AgE and PMB-AgE were calculated from their compositions and were: AgE 37,495; PMB-AgE 37,815. The mean residue weight values used here are: AgE 106.22; PMB-AgE 107.12.

Reaction of AgE and derivative with IgE antibodies

Reactions of AgE and PMB-AgE with IgE antibodies in the sera were measured by RAST (Wide *et al.*, 1967). Solid phase antigens were prepared by linking AgE or its PMB-derivative (0.6–0.7 μ g in 50 μ l) to individual cyanogen bromide-activated filter paper discs (dia. = 6.3 mm) (Yunginger *et al.*, 1973). The coupling reaction of proteins to the discs was allowed to continue for 24 hr at 22°C, following which any free reactive groups remaining on the discs were blocked by allowing each disc to stand in 1.0 M ethanolamine at pH 8.5 (500 μ l) for an additional 24 hr. Detection of the reaction of allergens with IgE antibody was performed by direct RAST as described by Yunginger *et al.* (1973).

Analytical methods

Absorbance measurements were carried out in a Zeiss PMQII spectrophotometer. Concentrations of protein solutions were based on their nitrogen contents which were determined by a micro-Kjeldahl procedure as described elsewhere (Atassi & Singhal, 1972). The nitrogen contents of AgE and PMB-AgE, calculated from the amino acid composition (taking all glutamates

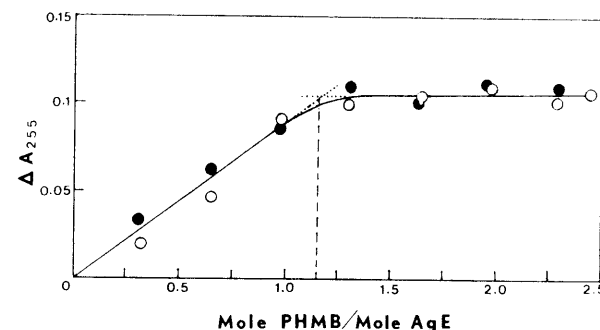


Fig. 1. Titration of the thiol groups with PHMB. (○) Native AgE in 0.05 M sodium phosphate buffer, pH 7.0. (●) AgE dissolved in 7 M urea at pH 7.0 at least 4 hr before the thiol groups were titrated. Note that AgE, under either of these two conditions, has a single reactive thiol group. For details see the text.

and aspartics as the acids and not the amides since these are not yet known), were 16.29% and 16.15%, respectively. Amino acid analyses of protein hydrolysates were done with a Beckmann 119C amino acid analyzer. Procedures for acid hydrolysis have been described (Atassi & Saplin, 1968).

RESULTS

Modification and availability of the thiol groups

Six replicate titrations of AgE with PHMB were performed each under neutral non-dissociating conditions and in 7 M urea. An example of one such titration is shown in Fig. 1. In phosphate buffer at pH 7.0, the titrations showed that 1.01 ± 0.17 SH groups/mole AgE were reactive with PCMB. The number of reactive SH groups in 7 M urea was entirely identical to the value in non-dissociating media (e.g. Fig. 1). On carboxymethylation of AgE by

iodoacetic acid in phosphate buffer at pH 7.0 or in 7 M urea, the two reaction products had identical contents of S-carboxymethylcysteine (1 residue per mole AgE).

Determination of conformational changes

Conformational measurements were carried out on AgE and its PMB derivative to determine if any conformational changes had occurred as a result of thiol group modification in phosphate buffer at pH 7. ORD measurements in the range 280–220 nm showed that AgE and PMB-AgE had equal negative rotation minima at 233 nm ($[m']_{233} = -2300 \pm 150$). Figure 2 shows an example of CD spectra of AgE and PMB-AgE in the range 200–250 nm. In CD studies AgE and PMB-AgE gave negative ellipticity bands at 222 and 205 nm. The $[\theta']$ values given by the two proteins at either of these minima were equal. The results clearly indicated that the derivative suffered little, if any, conformational changes.

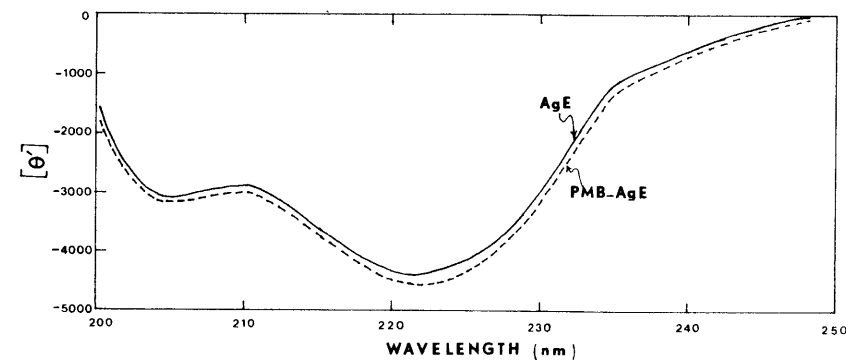


Fig. 2. CD spectra of AgE and PMB-AgE. Measurements were carried out on the protein solutions in 0.05 M sodium phosphate buffer at pH 7.0.

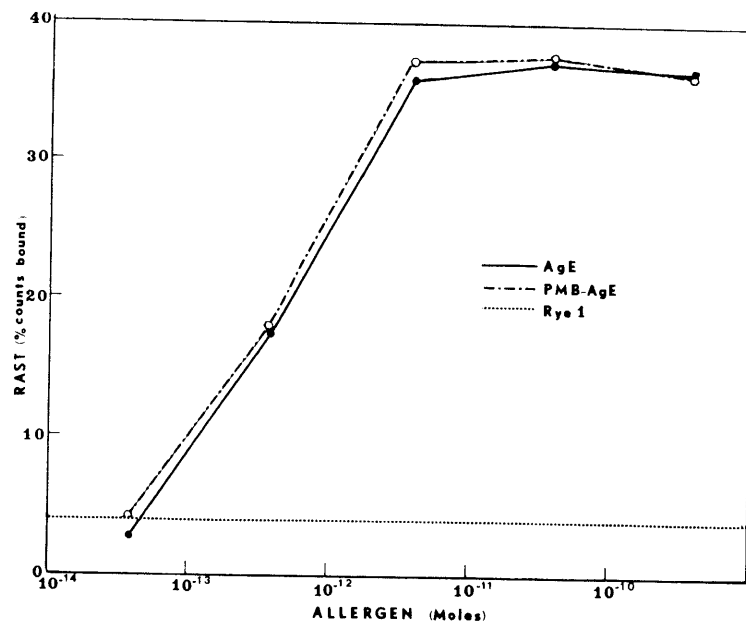


Fig. 3. RAST activity with IgE antibodies in antisera from ragweed sensitive individuals. (○) AgE; (●) PMB-AgE; (.....) Rye 1, an unrelated allergen shown as a control.

The absence of conformational changes in PMB-AgE made this derivative highly suited for immunochemical studies.

Immunochemistry of the derivative

AgE and PMB-AgE had identical allergenic activities as measured by their ability to bind IgE antibodies in the pool of sera from ragweed sensitive individuals. Figure 3 shows the result of IgE antibody binding by direct RAST to various amounts of AgE and PMB-AgE. Specificity of the binding is indicated from the fact that Rye 1, an unrelated allergen, bound no IgE antibodies. RAST inhibition showed that AgE or PMB-AgE inhibited the binding of IgE antibodies to either antigens with equal efficiency whereas Rye 1 had no inhibitory effect on this binding.

DISCUSSION

The strategy previously set forth (Atassi, 1972, 1975) for the delineation of protein antigenic structures relies on several independent approaches. Of these, specific chemical modification of selected amino acid locations has proved to be one of the most effective approaches in determining side chains involved

in binding of antigen with antibody (e.g. Atassi, 1967a, 1968, 1972, 1975, 1978). Useful conclusions can be derived from this approach only when the extent of modification is kept down to a minimum (preferably one or two residues), the products of any side reactions removed and conformational changes do not occur as a result of the modification. In addition, the nature of the modification must be taken into account (Atassi & Habeeb, 1969). The alteration of the modified side chain must be sterically or chemically capable of impairing the participation of the side chains in its normal biological function. The advantages and limitations of chemical modification in the immunochemistry of proteins have recently been discussed in detail together with an extensive review of chemical modification and cleavage reactions (Atassi, 1977). Modification studies thus far carried out on AgE have been crude and drastic in nature such as extensive acetylation, succinylation and alkylation (King *et al.*, 1974). These modifications, as fully expected, inactivated AgE but they provided no information on the role of the modified side chains in the allergenicity of the molecule. Due to the fact that a large number of residues were modified and the conformations of the derivatives were not studied it is not possible

to make functional assignments to any of the modified residues.

The present work has shown that the number of reactive thiol groups of AgE remains unchanged upon unfolding of the molecule in 7 M urea. Recently, others have reported (King *et al.*, 1974) that AgE has 0.4 mole of SH group which is in some way blocked and the roles of the so-called exposed fraction of SH in the allergenicity and conformation of AgE were not defined. Our present findings with two different reagents have clearly shown that AgE has a single free thiol group which is fully accessible and reactive. From amino acid analysis, the α chain has an odd number of half-cystine residues (5), whereas the β chain has an even number (2). Since the two subunits are not linked by interchain disulfide bonds, the single thiol group must reside in the α subunit of AgE. It is well established that organic mercurials exhibit superior selectivity for thiol groups than haloacetate compounds. Haloacetates can modify, in addition to reaction with thiol groups, amino groups and histidine, methionine and tyrosine residues (for review, see Atassi, 1977). Some of these modifications, such as formation of sulfonium salts of methionine, cannot be detected by amino acid analysis of protein hydrolysates. Accordingly, to avoid these complications and to ensure that only effects of thiol group modification on conformation and allergenicity are being investigated, the present studies were carried out on a derivative of AgE in which the single thiol group was modified with PHMB.

Modification of the single thiol group in AgE under neutral non-dissociating conditions caused no detectable conformational changes in the protein. The immune response to native protein antigens is directed against their native three-dimensional structure (Atassi, 1967b; Atassi & Thomas, 1969) and conformational changes are known to influence antigenic reactivity (Atassi, 1967b). In view of the absence of conformational changes in PMB-AgE, changes in the allergenic activity can be readily attributed to the modification and cannot be the by-product of a conformational change. Furthermore, the modification of the thiol group by a *p*-mercuribenzoate substituent ($-\text{Hg}-\text{C}_6\text{H}_4-\text{COOH}$) is obviously a sufficient chemical and steric change to impair the participation of a thiol group in a biological function in which it may be involved. The finding that native AgE and PMB-AgE have identical

activities clearly indicates that the thiol group of AgE does not participate in the binding of the allergen with its IgE antibodies.

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