PREPARATION, CHARACTERIZATION AND ANTIGENIC SPECIFICITY OF A TYROSINE–LYSINE CROSS-LINKED HEN EGG WHITE LYSOZYME DERIVATIVE

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1. Introduction

Characterization of antigenic determinants through chemical modification of amino acid side chains of hen egg white lysozyme have been reported by this laboratory [1, 2]. It was found for instance that 20% of the anti-lysozyme antibodies did not react with the lysozyme derivative nitrated at tyrosine 20 and/or tyrosine 23. Secondly, the lysozyme derivative having 5 out of 6 lysine residues acetylated, reacted only with about 50% of all anti-lysozyme antibodies. Both results were obtained through precipitation curve analysis, and confirmed by immunoadsorbent experiments. Since none of the modifications resulted in any conformational change of the molecule, as controlled through optical rotatory dispersion and circular dichroism measurements [1–3] it was concluded that both tyrosine 20 and 23 are part of the same antigenic determinant, and secondly that at least one of the lysine residues is implicated in the antigenic specificity.

In order to situate more accurately the tyrosine 20–23 determinant, we prepared a derivative modified at two positions, namely tyrosine 23 and lysine 97. It was possible to cross-link both residues through a dinitrobenzene group. As both residues seem therefore to be in close juxtaposition on the surface of the molecule, and since tyrosine as well as lysine are implicated in the antigenic specificity, we propose they can be part of the same antigenic determinant.

In addition it is known, from X-ray analysis, that tyrosine 20 and lysine 96 are also in juxtaposition [4]. Hence we suggest that all four residues may be part of the same antigenic determinant of hen egg white lysozyme.

2. Materials and methods

Three times recrystallized egg white lysozyme was purchased from Sigma Chemical Co. (St. Louis, Missouri), and was used without further purification. 1,5-Difluoro-2,4-dinitrobenzene was purchased from Fluka.

Quantitative precipitation tests, using goat anti-lysozyme antiserum, amino acid analysis and gel electrophoresis were performed as described previously [1].

The method used for cross-linking one tyrosine with one lysine side chain was a modified procedure of the one used by Cuatrecasas et al. [5]. Mononitro lysozyme nitrated at position 23, prepared and purified as described [2], was found to be slightly contaminated with the derivative nitrated at tyrosine 20. Mononitro lysozyme (1.75×10^{-4} M) in 0.05 M Tris buffer, pH 8.0, was reduced with $Na_2S_2O_4$ (3×10^{-4} M) at 23°C during 10 min. Amino acid analysis and spectral analysis show complete disappearance of nitro tyrosine. Cross-linkage with difluorodinitrobenzene is performed at a concentration of 2×10^{-4} M in 0.05 M phosphate buffer, pH 7.0. Lysozyme–(tyrosine-NH_2)$_1$ concentration was never higher than 2×10^{-5} M in order to prevent intermolecular cross-linkage. The reaction was allowed to proceed in the dark at room temperature for 16 hr, and was followed through change in absorbance at 350 nm.

Position of the bridge is obtained through trypsin digestion of the derivative having its four disulfide bridges reduced and blocked with iodoacetic acid. Separation of the peptides was performed through paper chromatography at pH 3.5.

Table 1
Amino acid composition of native lysozyme and lysozyme-DNB derivative.

	Native lysozyme		
	Theoretical no.	Found+	Lysozyme-DNB
ASP	21	21.3	21.1
THR	7	6.4	6.5
SER	10	8.5	8.7
GLU	5	5.2	5.1
PRO	2	1.9	2.1
GLY	12	12	12
ALA	12	12.0	12.4
1/2 CYS	8	3.5	3.9
VAL	6	5.9	5.7
MET	2	1.7	1.7
ILE	6	5.6	5.1
LEU	8	8.3	7.2
TYR	3	3.1	1.8
PHE	3	3.1	3.0
LYS	6	5.9	5.0
HIS	1	1.0	1.2
ARG	11	10.7	11.0

+ Data were calculated assuming 12 residues of glycine per molecule.

Table 2
Amino acid composition of the major coloured tryptic peptide from lysozyme-DNB.

	Theoretical no. for (22–33)+(74–97)	Theoretical no. for (22–33)+(97–112)	Found+
ASP	5	4	4.3
THR	1	0	0.6
SER	5	2	1.8
GLU	0	0	1.0
PRO	1	0	0
GLY	2	4	4
ALA	5	4	3.7
CM–CYS	4	1	1.4
VAL	2	3	3.7
MET	0	1	0.6
ILE	2	1	1.2
LEU	4	1	1.7
TYR	1†	1†	0.4
PHE	0	0	0
LYS	3†	2†	1.1
HIS	0	0	0
ARG	0	1	1.4

+ Assuming each peptide contains 4 glycine residues.
† No correction is made for possible involvement in cross-link.

3. Results and discussion

The reaction of 1,5-difluoro-2,4-dinitrobenzene with the monoamino lysozyme derivative was followed at 350 nm, and was found to be completed after overnight reaction in the dark. The absorbance spectrum showing a maximum at 350 nm, was reported to be typical for a tyrosine–lysine link through dinitrobenzene [6]. The derivative called lysozyme-DNB, was pure on acrylamide gel electrophoresis.

Amino acid composition (table 1) showed, in addition to the lack of one tyrosine-, the loss of one lysine residue, as expected. Identification of the cross-linking site was performed through trypsin digest of the reduced and carboxymethylated lysozyme-DNB derivative.

Upon paper chromatography of this digest, one large and one minor yellow spot were separated, eluted with 0.1 N acetic acid and hydrolyzed. Table 2 shows the amino acid composition of the major spot. Depending on whether tyrosine 23 is cross-linked to lysine 96 or 97, the tryptic peptides will be (22–33)+(74–97) or (22–33)+(97–112) respectively. Although the peptide seemed to be slightly contaminated, we found it corresponded to sequence (22–33)+(97–112), therefore proving that tyrosine 23 is cross-linked to lysine 97.

The second smaller yellow spot was also assayed for amino acid but found to be contaminated by other tryptic peptides. In view of the fact that our starting product, mononitro lysozyme modified at position 23, was slightly contaminated with the derivative modified at position 20, we assume this peptide to be (22–33)+(74–97), formed by trypsinolysis of the tyrosine 20–lysine 96 lysozyme-DNB derivative. Cross-linking experiments of this sort therefore seem to be a perfect tool to prepare homogeneous protein derivatives modified at two different residues in juxtaposition on the surface of the molecule.

In order to test the influence of this modification on the antigenic specificity, precipitation analysis of this derivative with goat anit-lysozyme antibodies was performed. Fig. 1 shows the precipitation curves of lysozyme, mononitro lysozyme, acetyl lysozyme and lysozyme-DNB, using a goat anti-lysozyme antiserum. As is clear from the figure and from the results sum-

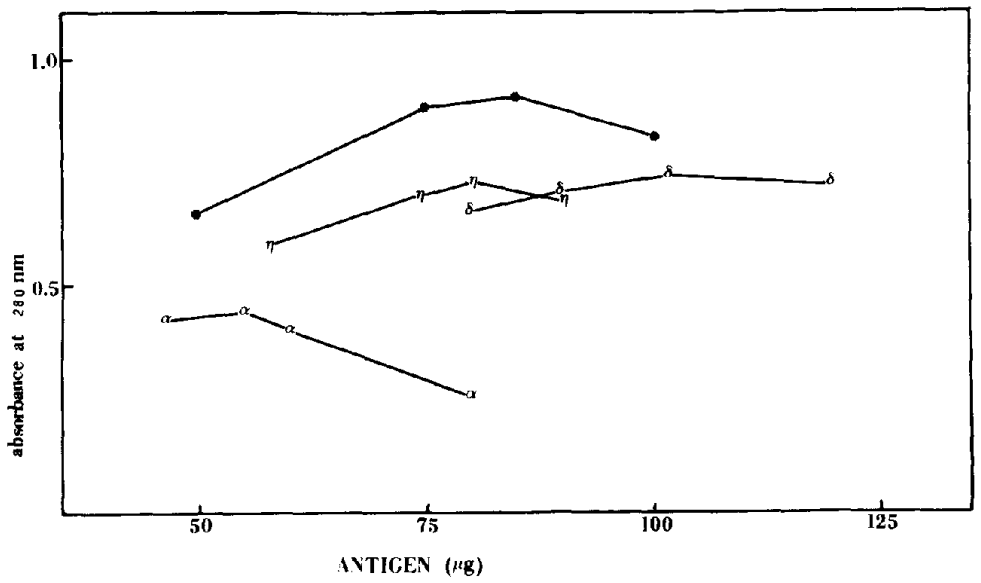

Fig. 1. Precipitation studies on lysozyme and lysozyme derivatives: (⋆—⋆—⋆) native lysozyme; (η—η—η) mononitro lysozyme; (δ—δ—δ) lysozyme-DNB; (α—α—α) acetyl lysozyme. Goat antiserum was obtained by immunization with native lysozyme.

marized in table 3, lysozyme-DNB precipitates the same amount of antibody as mononitro lysozyme, however slightly more antigen was needed to attain the equivalence point. This may suggest that, due to the cross-linking bridge, the affinity of lysozyme-DNB toward specific antibody is slightly lower than for the native enzyme and for mononitro lysozyme.

These results suggest that lysine 97, and eventually lysine 96, can be recognized by specific anti-tyrosine 20–23 antibodies. Indeed, knowing that lysine at position 1 is not implicated [1], and that probably one lysine is not on the surface of the molecule (only 5 out of 6 could be acetylated), this results in three candidate groups of lysines, reacting with 50% of the anti-lysozyme antibodies, and of which only one group is known to be situated in an antigenic reactive region, i.e. lysines 96–97. Stressing the above suggestion are subsequent experiments using fragment (1–27)+(122–129), which reacts with only half of the anti-tyrosine 20–23 antibodies [7]. This suggests that 50% of the antibodies recognizing tyrosine 20–23, i.e. 10% of all anti-lysozyme antibodies, recognize additional residues in this region of the lysozyme molecule.

In conclusion we suggest therefore that nitration seems to result in the inability for 20% of the anti-lysozyme antibodies to react, half of that amount recognizing specific residues in fragment (1–27)+(122–129), the other half recognizing residues 96–97 principally.

Table 3

Relative amounts of specific antibodies formed against the amino acid residues specifically modified in the lysozyme-derivatives.

Lysozyme antigen	Modified amino acids	Specific antibodies[+]
Native lysozyme	–	100
Acetyl lysozyme	5 Lysines out of 6	50
Mononitro lysozyme	Tyrosine 23	20
Lysozyme-DNB	Tyrosine 23 and lysine 97	18

[+] Values are expressed as percentage specific antibody relative to 100% anti-lysozyme (native) antibodies.

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