IMMUNOCHEMICAL STUDIES ON HEN'S EGG-WHITE LYSOZYME
Effect of formylation of the tryptophan residues

Arthur D.STROSBERG and Louis KANAREK
Laboratorium voor Chemie der Proteinen, Vrije Universiteit te Brussel, Brussel

Received 10 October 1969

1. Introduction

Specific chemical modification of amino acid residues of egg-white lysozyme has provided valuable information concerning the nature and location of some antigenic determinants of this enzyme. It has been demonstrated that some of the ϵ-amino groups [1], carboxyl groups [2], the three tyrosine residues [3,4] and the methionine residues [5,6] are part of antigenic sites of lysozyme. In contrast, the α-amino group, the carboxy-terminal leucine residue and the histidine residue do not seem to be involved in the reaction of the enzyme with the anti-lysozyme antibodies [7,8]. The present work describes the involvment of the tryptophan residues in the antigenic structure of lysozyme.

2. Materials and methods

Lysozyme (three times recrystallized) was obtained from Sigma Chemical Co. Lyophilized *Micrococcus lysodeikicus* cells were freeze-dried vials from Worthington Biochemical Corp.

2.1. *Preparation of formylated lysozyme*

The indole side-chains of lysozyme were modified by the method of Previero et al. [9], using 98–100% formic acid saturated with gaseous HCl. These authors have shown that no other amino acid residue reacted under these conditions. The product obtained had identical chemical, physical and antigenic properties as a sample sent to us by Dr. Previero.

2.2. *Analytical methods*

The extent of modification of the tryptophan residues was established by measuring the extinction of the lysozyme derivative 25 μM in 8 M urea, pH 4, at 298 mμ. ϵ is 4,880. Transition temperature and b_0 were obtained from 0.25 mM protein solutions, in 0.1 M KCl, pH 5 on a Perkin Elmer 141 M polarimeter. Protein concentrations were determined photometrically assuming that $E^{280\ m\mu}_{1\ mg/ml}$ is 2.60 for lysozyme and 1.40 for IgG antibodies. Disc electrophoresis was performed in 7.5% polyacrylamide gel by a modification of the method of Ornstein [10] and Davis [11]; the pH was 4.5 and the gels were 8 M urea. The samples were subjected to 2.5 mA per tube for 1.5 hr, and stained with amido black.

2.3. *Enzymic activity*

Enzymic activity of lysozyme was based on the rate of lysis of *M. lysodeikticus* as described in a previous publication [12].

2.4. *Immunochemical methods*

Rabbit (R) and goat (G) antisera were prepared against lysozyme by repeated weekly injections of 2 ml of a water in oil emulsion containing 20 mg enzyme. All the sera gave single line by agar double diffusion.

Double diffusion in agar was performed by the method of Ouchterlony [13]. Precipitin experiments were performed according to the procedure of Heidelberger and Kendall [14], with 0.5 ml of antiserum and increasing amounts of antigen in 0.5 ml of 0.15 M NaCl. In all sera used, anti-lysozyme antibodies moved as a single peak in the analytical ultracentrifuge Spinco Model E, and their sedimentation constant was 6.5 S.

Antibodies were separated from the other serum proteins by an immunoadsorbent prepared by the procedure of Avrameas and Teirnynck [15].

North-Holland Publishing Company – Amsterdam

3. Results and discussion

5 of the 6 tryptophan residues of lysozyme [16] were formylated under our conditions. The spectral properties of 1-formyl-lysozyme were the same as those described by Previero and coworkers [9]. This derivative moved as a single band in polyacrylamide gel electrophoresis, and its mobility at pH 4.5 was 50% that of native lysozyme. The derivative was enzymically inactive. This is expected, since 3 of the 6 tryptophan residues of lysozyme are involved in substrate binding [17].

The transition temperature in 0.1 M KCl at pH 5 was 45°C for the modified protein compared with 75°C for the native enzyme. b_0 was -140 as compared with -150 for native lysozyme. Thus the overall conformation seems to be little affected by formylation of the indole side chains, although the stability of the tertiary structure is drastically lowered by the modification.

The role of the tryptophan residues in the antigenic properties of the enzyme was studied both by agar double immunodiffusion and quantitative precipitin reaction with anti-lysozyme antibodies. Double diffusion in agar showed complete fusion of the precipitin lines obtained with 1-formyl-lysozyme and lysozyme. Quantitative precipitin curves of modified and native lysozyme with anti-lysozyme goat G1 antiserum are shown in fig. 1. The ability of 1-formyl-lysozyme to precipitate with various rabbit and goat antisera was decreased by about 15% (table 1).

Antibodies which did not precipitate with formylated lysozyme were separated from the other serum proteins by an immunoadsorbent containing native lysozyme. These antibodies were unable to precipitate unmodified lysozyme, although they completely inhibited the catalytic activity of the enzyme in a one to one molar ratio. The inhibition of the lytic activity could be explained either by specific binding to essential residues of lysozyme, as are for example tryptophan 62,63 or 108, or by steric hindrance.

The six tryptophan residues are more or less buried in the interior of the molecule and it is difficult at this stage to decide which of the residues could be part of an antigenic determinant. However, oxidation of tryptophan 62 by *N*-bromosuccinimide did not modify the antigenic properties of the enzyme [6]. This would imply that tryptophan 62 is not a residue whose formylation decreases precipitation of the antibody.

Table 1
Relative amounts of precipitation formed by 1-formyl-lysozyme with various rabbit (R) and goat (G) anti-lysozyme antisera[a].

Antiserum	1-Formyl-lysozyme
R 118	89
R 184	85
R 188	89
G 1	85

[a]Values are expressed in per cent precipitation of homologous reaction and represent the average of three independent determinations which varied ± 3% or less.

All six tryptophan residues of lysozyme were modified by reaction with 2-nitrophenylsulfenyl chloride [18]. Significant conformational changes as well as a marked decrease in the ability of the lysozyme derivative to react with anti-lysozyme antibodies were observed. Apparently far more antigenic determinants of the enzyme were modified by this method, probably as a consequence of conformational reorganization of the molecule.

From our results we conclude that at least one of the six tryptophan residues is involved in the binding of the anti-lysozyme antibodies.

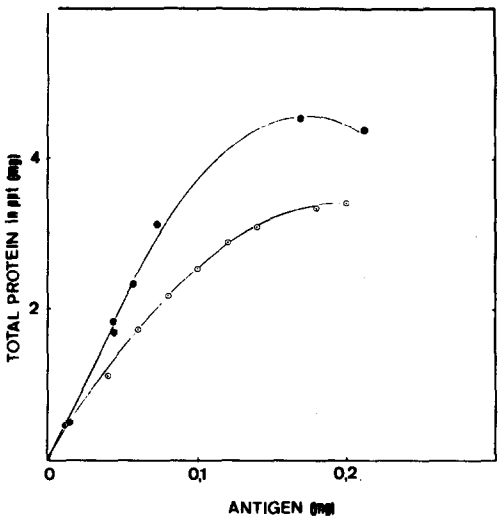

Fig. 1. Precipitation studies on lysozyme • and 1-formyl-lysozyme ○. Reactions were with goat G 1 antiserum to native lysozyme.

Acknowledgements

This research was aided by a grant form EURATOM: ABIB 016-61-10. A.D.S. is a predoctoral fellow of the "Institut pour l'encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture. We thank Dr. Previero from Montpellier for providing a sample of formylated lysozyme which could be compared with our preparation.

References

[1] A.D.Strosberg, L.Kanarek, Arch. Intern. Physiol. Biochim. 76 (1968) 949.
[2] H.Fujio, S.Kishiguchi, Y.Saiki, T.Amano, Biken's J. 2 (1959) 56.
[3] A.D.Strosberg, L.Kanarek, Abstracts Fifth Meeting F.E.B.S. Prague 1968 p. 92.
[4] M.Z.Atassi, A.F.S.A.Habeeb, Biochemistry 8 (1969) 1385.
[5] A.D.Strosberg, L.Kanarek, Arch. Intern. Physiol. 76 (1968) 950.
[6] B.Bonavida, A.Miller, E.Sercarz, Biochemistry 8 (1969) 968.
[7] A.D.Strosberg, L.Kanarek, Arch. Intern. Physiol. Biochim. 76 (1968) 202.
[8] A.D.Strosberg, L.Kanarek, Arch. Intern. Physiol. Biochim. 75 (1967) 898.
[9] A.Previero, M.A.Coletti-Previero, J.C.Cavadore, Biochim. Biophys. Acta 147 (1967) 453.
[10] L.Ornstein, Ann. N.Y. Acad. Sci. 121 (1964) 321.
[11] B.J.Davis, Ann. N.Y. Acad. Sci. (1964) 404.
[12] R.A.Bradshaw, L.Kanarek, R.L.Hill, J. Biol. Chem. 242 (1967) 3789.
[13] O.Ouchterlony, Acta Pathol. Microbiol. Scand. 26 (1949) 507.
[14] M.Heidelberger, F.E.Kendall, J.Exptl. Med. 62 (1935) 6971.
[15] S.Avrameas, T.Ternynck, J. Biol. Chem. 242 (1967) 1651.
[16] P.Jollès, J.Jauregui-Adell, I.Bernier, P.J.Jollès, Biochim. Biophys. Acta 78 (1963) 668.
[17] C.C.F.Blake, L.N.Johnson, G.A.Mair, A.C.T.North, D.C. Philips, V.R.Sarma, Proc. Roy. Soc. (London) Ser. B 167 (1967) 378.
[18] A.F.S.A.Habeeb, M.Z.Atassi, Immunochemistry 6 (1969) 555.