SUBUNIT STRUCTURE OF TESTICULAR HYALURONIDASE

James H. GARVIN, Jr. and David M. CHIPMAN*
Department of Biology, University of the Negev, Beer-Sheva, Israel
and
Department of Chemistry, Massachusetts Institute of Technology
Cambridge, Mass., 02139, USA

Received 20 November 1973

1. Introduction

Bovine testicular hyaluronidase demonstrates interesting kinetic behavior and complex patterns of reaction with its oligo- and polysaccharide substrates [1, 2]. The determination of the size of the enzyme and the number of active sites in the molecule is crucial to any attempt to analyze the behavior of this enzyme in terms of a molecular model of its substrate binding site (e.g., as has been done for amylase [3] or lysozyme [4]). Although there has been considerable confusion in the literature about the molecular weight of the enzyme, recent studies by two different groups agree in assigning a molecular weight of about 60 000 daltons to this protein [5, 6]. Khorlin et al. have very recently reported, in the first published study of the quaternary structure of the enzyme, that it is composed of four subunits of molecular weight 14 000 each [6]. We report here conflicting evidence, which we believe indicates that bovine testicular hyaluronidase has only one active site and is probably composed of a single polypeptide chain, with a molecular weight of about 60 000 daltons.

2. Materials and methods

Crude bovine testicular hyaluronidase, Type 1, was obtained from Sigma Chemical Company and purified by the method of Borders and Raftery [5] except that protein peaks were routinely detected by absorption at 280 nm. The method of Lowry was used for determining protein concentration [7]. Hyalyronidase activity was determined by the turbidimetric method of Di Ferrante (using hexadecyltrimethyl-ammonium bromide) [8], and expressed in National Formulary Units (NFU) by comparison with the activity of a reference standard, lot 60572, obtained from the American Pharmaceutical Association. The assay of Margolis and Margolis [9] was used for detecting contaminating ribonuclease activity.

Disc electrophoresis at pH 4.5 on 15% acrylamide gels was carried out as described by Reisfeld [10]. SDS-acrylamide disc gel electrophoresis was based on the procedure of Shapiro et al. [11]. Serum albumin, hemoglobin and lysozyme were used as standards. Gels were scanned after staining using a Gilford spectrophotometer with a cuvette transport accessory. Urea treatments were carried out by the method of Segrest et al. [12]. Incubation in 1% SDS–1% dithiothreitol – 8 M urea (pH 7.1) for 3 hr was also used. Oxidation of the enzyme with metaperiodate was carried out as suggested by Neuberger and Marshall [13]. The protein was reisolated by Sephadex G-25 chromatography, subjected to urea treatment as above, and analyzed by electrophoresis. The procedure of Sia and Horecker was used for the maleic anhydride treatment of the protein [14].

The tetrasaccharide GlcUA-GlcNAc-GlcUA-GlcNAc generally labelled with tritium (2.2×10^6 cpm per

Abbreviations: GlcUA = *D*-glucuronic acid; GlcNAc = *N*-acetyl-*D*-glucosamine; NFU = National Formulary Units (of hyaluronidase activity).

μmole) was provided by S. Highsmith [2]. The plexiglass apparatus used for equilibrium dialysis had four cells, each composed of two half-cells of 0.4 ml vol. separated by a membrane. Equilibration of tetrasaccharide had a half-life of about 80 min using pretreated [15] Union Carbide cellulose dialysis membranes. Experiments were carried out in 0.2 M sodium acetate–0.15 M sodium chloride–0.005 M EDTA, pH 6.0, at 24°C. Hyaluronidase (4×10^{-5} to 3.5×10^{-4} M) was introduced into one side of the cell and tetrasaccharide (5×10^{-4} to 2.3×10^{-3} M) into both sides. After 20 hr aliquots were withdrawn from each compartment with a Chaney adaptor-equipped microsyringe, and the concentration of saccharide determined by scintillation counting in 10 ml of a solution of 7.0 g 2,5-diphenyloxazole, 0.30 g 1,4-bis-[2-(4-methyl-5-phenoxazolyl)] benzene, and 100 g naphthalene in 1 litre dioxane, and protein concentration by the method of Lowry [7]. Measurements were in duplicate at least.

Gray's dansyl chloride method was used for N-terminal group analysis of the protein, with electrophoresis at pH 4.4 [16]. Ritschard's procedure was employed in trypsin digestion and mapping of the resulting oligopeptides [17].

3. Results and discussion

Hyaluronidase was prepared by the method of Borders and Raftery [5] involving cation exchange chromatography and several gel filtration steps. Hyaluronidase with activity in the range 35–41 000 NFU per mg, which migrated as a single band upon disc electrophoresis at pH 4.3 in 15% polyacrylamide gels, was obtained. It showed no contaminating ribonuclease activity [9].

When our purest freshly isolated samples of hyaluronidase (>40 000 NFU per mg) were subjected to standard SDS gel electrophoresis conditions [11] the enzyme revealed a single protein band, which by comparison with standard proteins was found to have a molecular weight of 60 000 ± 2000 daltons. This value is in close agreement with that of 61 000 determined by Borders and Raftery for the intact protein by gel filtration [5], and in reasonable agreement with that of 55 000 determined by Khorlin et al. by sedimentation velocity measurements of their

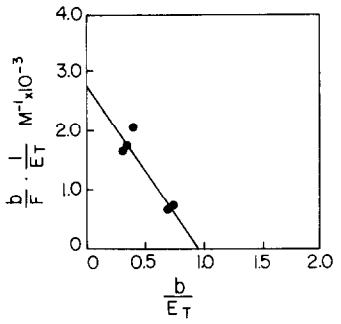

Fig. 1. Scatchard plot of the results of equilibrium dialysis experiments with hyaluronidase and GlcUA-GlcNAc-GlcUA-GlcNAc. b is concentration of bound saccharide, F of free saccharide, and E_T of total protein (assuming mol. wt. 60 000).

'hyaluronidase-1' [6]. When we subjected hyaluronidase to a variety of more drastic conditions expected to promote dissociation into subunits, the denatured protein continued to migrate on SDS-acrylamide gels as a species of 60–65 000 daltons, depending on conditions. Among the conditions used before electrophoresis were incubation in 8 M urea–1% dithiothreitol–1% SDS at 37°C for 3 hr [12], oxidation with periodate followed by urea treatment [13] and maleylation [14]. Samples subjected to urea or maleylation also showed faint bands attributable to a species of 37 000 daltons, but the periodate treated samples did not. The only cases in which bands of molecular weight as low as 12–16 000 daltons were obtained were analyses run on our earliest, somewhat impure samples of the protein prepared from a different lot of crude enzyme.

Studies of the binding of GlcUA-GlcNAc-GlcUA-GlcNAc to hyaluronidase suggest that the enzyme has only one (strong) binding site for this saccharide, the major product of enzymic digestion of hyaluronic acid [1]. A modified Scatchard plot of the equilibrium dialysis data [18] is shown in fig. 1. The data can be fit by a line intersecting the abscissa at 1.0 ± 0.1, implying saturation of the enzyme at one mole of saccharide per mole of protein of 60 000 daltons. The equilibrium constant for this association is found to be $(2.6 \pm 0.2) \times 10^3$ M^{-1}. The data presented do not preclude the presence of additional weaker binding sites (or multiple sites showing negative cooperativity) but demonstrate that the binding of a second saccharide molecule must be at least ten times weaker than that of the first.

active sites in bovine testicular hyaluronidase, and that the enzyme is probably not composed of dissociable subunits.

Preliminary chemical studies do not establish the number of peptide chains in the molecule, but seem to rule out the possibility that it is composed of four equivalent subunits of 14 000 daltons. A single amino terminal residue (leucine?) was detected by dansylation and hydrolysis. The amino acid composition of the enzyme [5] implies that it would contain only 9 lysines and arginines per 14 000 daltons, but a tryptic digest fingerprint [17] too faint for a complete count shows at least 18 spots.

Khorlin and coworkers have reported the separation of crude commercial hyaluronidase (Reanal) into three fractions [19]. Despite our disagreement with their conclusions concerning the quaternary structure of their major fraction E (or 'hyaluronidase-1'), we believe that this fraction is similar to the material we studied, obtained by the procedure of Borders and Raftery [5]. Fraction E requires 0.2 M NaCl for elution from CM-cellulose while the other active fractions obtained appear from their reported chromatographic behavior to be acidic or only weakly basic [19]. On the other hand, the material studied by Khorlin et al. was less extensively purified and had a lower specific activity. (22 000 tru = approximately 32 000 NFU) than that we studied. It is quite possible that the preparations of Khorlin et al. – and our earlier preparations – were contaminated with inactive material. The reported molecular weight of about 14 000 [20] for a protein which had quite low hyaluronidase activity [21] is relevant to this point. The contaminants may be unrelated proteins or hyaluronidase which has undergone partial cleavage in vivo or in the early stages of isolation.

It is not unreasonable that hyaluronidase, despite its size, has no quaternary structure. It is unlikely that an enzyme which degrades a structural polymer would be under metabolic control by allosteric inhibition or activation, the usual role proposed for multiple subunit structures. In addition, since it appears that hyaluronidase interacts simultaneously with a stretch of at least seven or eight monosaccharide units of substrate [22], the active site of the enzyme is expected to be quite large – over 30 Å long.

On the basis of the above data and considerations, we conclude that there is no evidence for multiple

Acknowledgements

Research support from the U.S. National Institutes of Health (PHS Grant No. AM-13590) is gratefully acknowledged. J.H.G. also thanks the N.I.H. for Predoctoral Fellowships from 1969-72.

References

[1] Meyer, K., Hoffman, P. and Linker, A. (1960) in: The Enzymes, 2nd edn (Boyer, P.D., ed.), Vol. 4, p. 447–460, Academic Press, New York.
[2] Highsmith, S. (1972) Ph.D. Thesis, Massachusetts Institute of Technology.
[3] Thoma, J.A., Rao, G.V.K., Brothers, C., Spradlin, J. and Li, L.H. (1971) J. Biol. Chem. 246, 5621–5635.
[4] Chipman, D.M. (1971) Biochemistry 10, 1714–1722.
[5] Borders, Jr., C.L. and Raftery, M.A. (1968) J. Biol. Chem. 243, 3756–3762.
[6] Khorlin, A.Ya., Vikha, I.V. and Milishnikov, A.N. (1973) FEBS Letters 31, 107–110.
[7] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[8] Di Ferrante, N. (1956) J. Biol. Chem. 220, 303–306.
[9] Margolis, R.U. and Margolis, R.K. (1970) Anal. Biochem. 35, 77–81.
[10] Reisfield, R.A., Lewis, U.J. and Williams, D.E. (1962) Nature 195, 281–283.
[11] Shapiro, A.L., Vinuela, E. and Maizel, J.V. (1967) Biochem. Biophys. Res. Commun. 28, 815.
[12] Segrest, J.P., Jackson, R.L., Andrews, E.P. and Marchesi, V.T. (1971) Biochem. Biophys. Res. Commun. 44, 390–395.
[13] Neuberger, A. and Marshall, R.D. (1966) in: Glycoproteins (Gottschalk, A., ed.), p. 235–272, Elsevier, Amsterdam.
[14] Sia, C.L. and Horecker, B.L. (1968) Biochem. Biophys. Res. Commun. 31, 731–737.
[15] Westhead, E.W. and McLain, G. (1964) J. Biol. Chem. 239, 2464–2468.
[16] Gray, W.R. (1967) Methods Enzymol. 11, 139–151.
[17] Ritschard, W.J. (1968) in: Chromatographic and Electrophoretic Techniques, 2nd edn (Smith, I., ed.), Vol. 2, p. 147–165, Wiley-Interscience, New York.
[18] Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660–672.
[19] Vikha, I.V., Fedurkina, N.V. and Khorlin, A.Ya. (1971) Biokhimiya 36, 216–218.
[20] Soru, E. and Ionescu-Stoian, F. (1963) Arch. Roumaines Pathol. Exptl. Microbiol. 22, 783–790 [Chem. Abstr. 60, 9547e].
[21] Soru, E. and Ionescu-Stoian, F. (1963) Biochim. Biophys. Acta 69, 538–543.
[22] Highsmith, S., Garvin, Jr., J.H. and Chipman, D.M., in preparation.