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MODIFICATION OF FUNCTIONAL ARGININE RESIDUES IN PURIFIED BOVINE TESTICULAR HYALURONIDASE WITH BUTANE-2,3-DIONE

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Purified bovine testicular hyaluronidase (hyaluronate 4-glycanohydrolase, EC 3.2.1.35) was inactivated by butane-2,3-dione in either borate or Hepes buffer, pH 8.3. The presence of borate enhanced the inactivation process which followed pseudo-first-order kinetics with a calculated second-order rate constant of 13.54 $M^{-1} \cdot min^{-1}$. Using kinetic data it was estimated that the modification of 1 mol arginine per mol enzyme was sufficient for inactivation to occur, whereas amino acid analysis indicated that 4 mol arginine had been modified. The inactivation process was partially prevented by using either competitive inhibitors or substrates of the enzyme, thus indicating that the essential arginine residue is close to the active site of hyaluronidase. A full kinetic analysis of the enzyme with either hyaluronic acid or chondroitin 6-sulphate as substrate showed that the activity of hyaluronidase was uncompetitively activated by either protons or NaCl. The product obtained by reduction of the carboxyl groups of hyaluronic acid to the corresponding alcohol groups was a competitive inhibitor. The possibility that the microenvironment of hyaluronic acid was responsible for the observed kinetic effects of pH and ionic strength was dispelled. It is concluded that these data are compatible with a mechanism that involves an ionic interaction between a carboxyl group on the substrate and an arginine residue on the enzyme.

Bovine testicular hyaluronidase (hyaluronate 4-glycanohydrolase, EC 3.2.1.35) is an endoglycosaminidase that can hydrolyse the β-(1 → 4) glycosidic bonds in a number of different substrates such as hyaluronic acid, chondroitin 4-sulphate and chondroitin 6-sulphate. Although a number of investigations have been made of the properties of this enzyme [1] very little definitive information is available about the mechanism of action or the nature of the catalytic site. In part, this reflects the difficulties in obtaining pure preparations of the enzyme, and the lack of a suitable routine assay.

However, studies with purified enzyme preparations have indicated that the enzyme possesses a large active site which exhibits increasing reactivity towards larger oligosaccharides up to at least an octasaccharide [2], and evidence has been obtained to show that there are four or five subsites for hyalobiuronate residues (the repeating disaccharide unit) within the single catalytic site [3]. No data are yet available to indicate which amino acids in the enzyme may be involved in binding or catalysis, other than the observation that the enzyme is insensitive to diisopropylfluorophosphate [4].

Chemical modification studies have shown that arginine residues play an essential role in many enzymes that utilise anionic substrates or ligands. These arginine residues act as positively charged binding sites for a wide range of enzyme substrates, including polyanions [5], phosphorylated glycolytic intermediates [6] and compounds containing carboxyl groups [7]. This information has been obtained by modifying various enzymes with diketones, particularly butane-2,3-dione, which interact specifically with arginine residues [8].

As bovine testicular hyaluronidase is able to hy-

Abbreviations: dansyl chloride, 5-dimethylaminonaphthalene-1-sulphonyl chloride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

drolyse several polyanions it is conceivable that positively charged residues on the enzyme may interact with the negative charges on the substrates. This paper describes the results of experiments in which the arginine residues of highly purified enzyme are selectively modified with butane-2,3-dione. Preliminary results have been published elsewhere [9]. The effects of pH and ionic strength on these enzyme-substrate interactions are also evaluated.

Materials and Methods

Materials

Hyaluronic acid, dermatan sulphate (chondroitin sulphate B) and chondroitin 6-sulphate (chondroitin sulphate C) were purchased from Miles Laboratories Ltd., Slough, Berks., U.K. [G-^{3}H]Dansyl chloride (specific activity 18 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks. and PCS scintillant from Baird and Tatlock, Swansea, U.K. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride was purchased from Calbiochem. Ltd., Bishops Stortford, Herts. and sodium D-glucuronate and D-glucuronamide from Sigma, Poole, U.K. Aristar hydrochloric acid and all other materials were from BDH Chemicals Ltd., Poole, U.K.

Enzyme preparation

Bovine testicular hyaluronidase with a specific activity of approx. 300 units/mg was purchased from Miles Laboratories, Slough, Berks., U.K. and purified by the methods of Gorham [10] and Pope et al. [11] to a specific activity of at least 41 000 units/mg. The activity of the preparation was defined by comparison with a sample of the International Standard for hyaluronidase [12] which was kindly donated by the World Health Organization. A sample of the highly purified enzyme (50 μg) gave a single protein band on polyacrylamide gel electrophoresis at pH 8.5. With respect to other enzymes that might be involved in proteoglycan degradation this final preparation had no detectable β-*N*-acetylglucosaminidase, β-glucuronidase [13] or neutral proteinase [14] activity using glucopyranoside, phenolphthalein β-D-glucuronoside and azocasein as substrates. With prolonged incubations trace amounts of arylsulphatase activity could be detected [15] but were considered to be of no significance.

Enzyme inactivation

The stock solution of enzyme (5 mg/ml 0.1 M NaCl) was diluted to give a final concentration of 10 μg (410 units) in either 50 mM sodium borate buffer, pH 8.3, or 50 mM Hepes buffer, pH 8.3 (total volume of incubation mixture, 0.7 ml). The enzyme was inactivated at 20°C using a final concentration of 16.3 mM-butane-2,3-dione. In certain experiments inhibitors of bovine testicular hyaluronidase were present or the concentration of butane-2,3-dione was altered.

At intervals 0.1 ml samples were withdrawn and residual enzyme activity was measured in a standard assay system (total volume 0.5 ml) containing 0.5 mg of hyaluronic acid in a final concentration of 0.1 M sodium citrate buffer, pH 4.0, and 0.15 M NaCl. After incubating for 10 min at 37°C, the reaction was stopped by the simultaneous addition of 100 μl 0.7 M potassium tetraborate, pH 9.2, and 10 μl of 6 M Na_2CO_3. The liberated reducing *N*-acetyl-D-hexosamine was assayed by the method of Reissig et al. [16].

Reduction of hyaluronic acid

The D-glucuronic acid residues in hyaluronic acid were converted to D-glucose by $NaBH_4$ reduction of a carbodiimide intermediate [17]. The product was extensively dialysed against distilled water and freeze-dried. The extent of reduction was measured using the modified carbazole method [18] and it was established that 96% of the D-glucuronic acid residues had disappeared.

Amino acid analysis

The amino acid composition of various enzyme hydrolysates was measured by reaction with [G-^{3}H]-dansyl chloride and chromatography on polyamide sheets (7.5 × 7.5 cm). Enzyme (250 μg) was inactivated with 16.3 mM butane-2,3-dione in 50 mM sodium borate buffer, pH 8.3, as described previously. At intervals, 0.1 ml samples were pipetted into 1 ml 6 M HCl and hydrolysed in sealed ampoules for 18 h at 105°C. Aliquots (10 μl) were removed from the ampoules, concentrated to dryness in a desiccator over NaOH and P_2O_5, dansylated with [G-^{3}H]dansyl chloride (spec. act. 18 mCi/mmol) and chromatographed on polyamide sheets [19]. In order to prevent the possibility of tritium exchange [20]

the samples were not incubated in 6 M HCl after dansylation. After development of the chromatograms the dried sheets were examined under an ultraviolet lamp and the fluorescent spots corresponding to dansyl-isoleucine and dansyl-arginine were cut out and placed in vials. Ethanol (0.1 ml) was added to each vial and after 30 min 4 ml PCS scintillant were added and the piece of polyamide sheet was removed with the aid of a needle. Samples were counted in an Intertechnique PG 4000 scintillation counter. The amount of arginine in the protein samples was calculated from the ratio of counts of isoleucine to arginine assuming that the isoleucine counts represented 25 mol Ile per mole protein as determined by automated amino acid analysis [21]. The dansyl chloride-TLC method was used in preference to automated amino acid analysis because only small quantities of the purified enzyme were available for use in these experiments. However, for comparison of results two samples of hydrolysate were analysed using standard techniques on a Locarte amino acid autoanalyser.

Kinetic data

Enzyme activity was measured over a range of pH values at several different concentrations of NaCl and in the absence of buffers. The pH of each incubation mixture was measured before and during the reaction and no change in pH values was apparent. Initial rates of enzyme activity were measured and kinetic information obtained by using direct linear plots [22].

Potentiometric measurements

An attempt was made to measure the proton concentration gradient across a concentrated solution of hyaluronic acid in order to obtain information about the ionic composition of the microenvironment surrounding this polymer. To facilitate measurement a simple apparatus was devised (Fig. 1) combining two pH (combination) microelectrodes in an electrically continuous solution, but separated from each other by a semi-permeable membrane. One of the electrodes was inserted into a pre-treated dialysis sack [23] that contained 1 ml of a solution of potassium hyaluronate and the top of the dialysis sack was sealed with an elastic band. The sealed electrode, together with a second electrode, was placed in water (400 ml). To enhance equilibration across the membrane the contents of the outer compartment were mixed using a magnetic stirrer. The system was then modified by the addition of small quantities of 0.1 M HCl or 0.1 M NaOH to the outer compartment and, after allowing time for equilibration, the differential pH recorded by the two electrodes was related to the pH value of the outer compartment. This system enabled a semiquantitative estimation to be made of the microenvironmental pH surrounding potassium hyaluronate. The effect of NaCl concentration on the system could be observed by addition of either the solid or a concentrated solution to the outer compartment. In all experiments both electrodes were calibrated with two solutions of standard pH and controls were run in which hyaluronate solution was replaced with water.

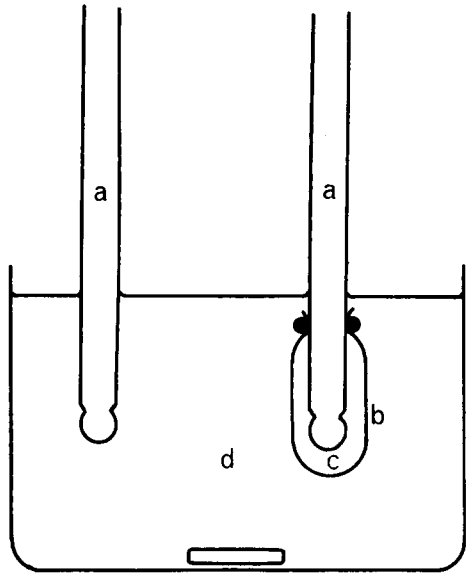

Fig. 1. Potentiometric measurements. Measurement of the microenvironment pH in a solution of hyaluronic acid was obtained by entrapping the polymer within the dialysis sack and recording the pH difference between the two Pye Ingold micro combination electrodes. (a) pH electrodes, (b) dialysis membrane, (c) hyaluronic acid solution, (d) bulk solution.

Results

Inactivation by butane-2,3-dione

Highly purified bovine testicular hyaluronidase was preincubated with 16.3 mM butane-2,3-dione

and aliquots were removed at intervals and assayed for residual enzyme activity. The results in Fig. 2 show that the inactivation process follows pseudo-first-order kinetics and is dependent on the nature of the buffer used. The presence of borate buffer increases the rate of inactivation. If chondroitin 6-sulphate is used as a substrate for the enzyme instead of hyaluronic acid similar $t_{1/2}$ values are obtained (borate buffer, $t_{1/2}$ = 2.7 min; Hepes buffer, $t_{1/2}$ = 11.5 min).

Values of $t_{1/2}$ for enzyme inactivation were obtained using a series of concentrations of butane-2,3-dione (1.63 mM to 16.3 mM) in the presence of borate buffer, pH 8.3. These data were further analysed by plotting values of log $t_{1/2}^{-1}$ against log butanedione concentration (Fig. 3a). The slope of the line, 0.95, is an estimate of the number of molecules of inhibitor reacting per active unit of the enzyme, suggesting that one butane-2,3-dione molecule per active unit of the enzyme is responsible for the observed inactivation process. When the $t_{1/2}$ values obtained with different concentrations of butanedione are converted to pseudo-first-order rate constants, k_i, and plotted against butane-2,3-dione concentration a straight line is obtained (Fig. 3b). The slope of the line gives an estimate of the second-order rate constant for the inactivation process and the extrapolated intercept on the ordinate axis is an estimate of the first-order rate constant for the reverse reac-

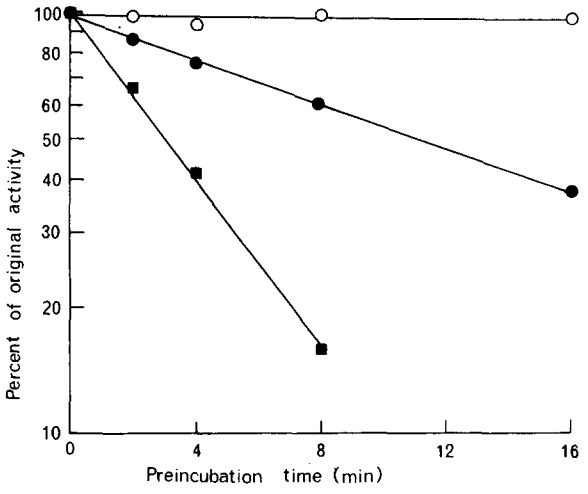

Fig. 2. Semilogarithmic plot of hyaluronidase inactivation by butane-2,3-dione. The enzyme (10 μg, 410 units) was preincubated with 16.3 mM butane-2,3-dione in the presence of either 50 mM borate (■) or 50 mM Hepes buffer (●), pH 8.3. Control incubation mixtures were subjected to the same conditions but in the absence of butane-2,3-dione (○).

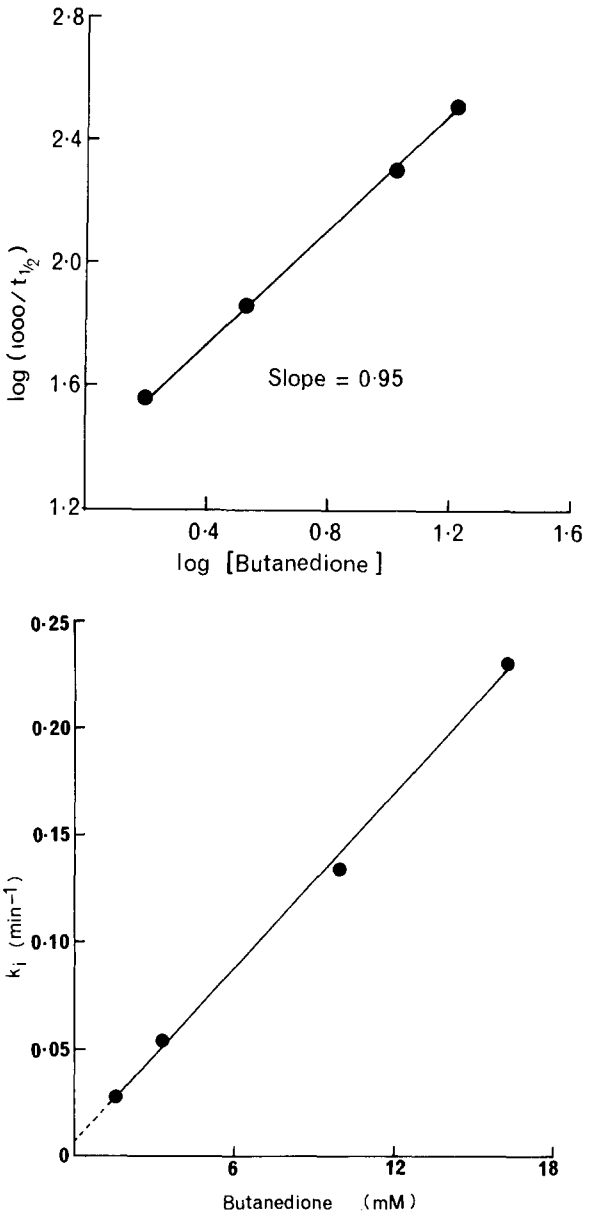

Fig. 3. Kinetic analysis of the inactivation process. Values of $t_{1/2}$ were obtained using a variety of butane-2,3-dione concentrations (1.63–16.3 mM) in the presence of 50 mM borate buffer, pH 8.3. (a) Apparent order of reaction between hyaluronidase and butane-2,3-dione. (b) Determination of the rate constant for the inactivation process.

tion. These results are compatible with the reaction mechanism (1) where $k_1 = 13.54\ M^{-1} \cdot min^{-1}$.

$$\text{enzyme} + \text{butanedione} \xrightarrow{k_1} \text{enzyme-butanedione complex} \quad (1)$$

Amino acid analysis

The results in Fig. 4 show that the changes in activity correlate with the loss of more than one arginine residue. Total loss of activity correlated with an extrapolated value of 4 mol arginine residues modified out of a total of 28 mol residues per mol protein. No change was observed in the ultraviolet spectrum (250–300 nm) of the enzyme, indicating that there were no side reactions of butane-2,3-dione with the aromatic amino acids.

Protection against inactivation by substrates and inhibitors

Residual enzyme activity was determined at a series of time points after the addition of 16.3 mM-butane-2,3-dione, in the presence of several substrates and inhibitors. The effect of various monosaccharides (20 mM) on the $t_{1/2}$ values in the presence of borate or Hepes buffer are presented in Table I. Both sodium D-glucuronate and D-glucuronamide are known inhibitors of enzyme activity, whereas D-glucose is not an inhibitor. Macromolecular substrates, hyaluronic acid and chondroitin 6-sulphate, and the competitive inhibitor dermatan sulphate were also able to protect the enzyme against inactivation.

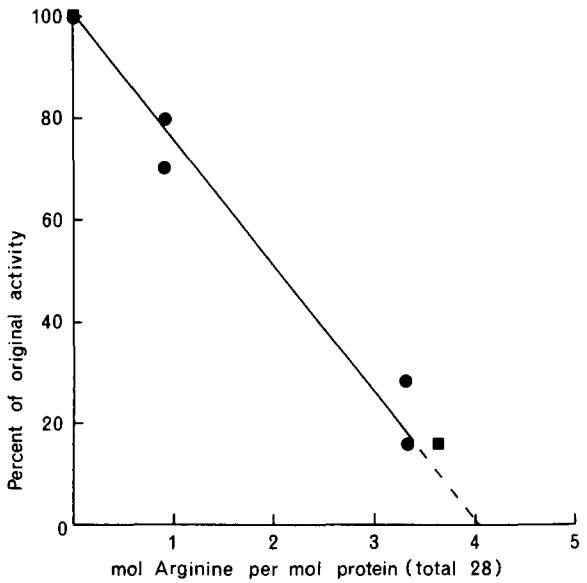

Fig. 4. Determination of the number of arginine residues modified. Enzyme (250 μg, 10 250 units) was incubated with 16.3 mM butane-2,3-dione in 50 mM borate buffer, pH 8.3. Aliquots (0.1 ml) were withdrawn at intervals and processed for amino acid analysis by dansylation (•) or autoanalysis (■). Separate aliquots were taken to determine enzyme activity.

Properties of reduced hyaluronic acid

Incubation of bovine testicular hyaluronidase with reduced hyaluronic acid (1.54 mg/ml) produced no detectable reducing end-groups by the method of Reissig et al. [16]. Competition studies in which both hyaluronic acid and the reduced analogue were present clearly indicated that the reduced analogue of hyaluronic acid was a competitive inhibitor with a K_i of 0.66 mg/ml in the presence of 0.1 M sodium citrate buffer (pH 4.0)/0.15 M NaCl.

Kinetic parameters of testicular hyaluronidase

Values of K_m and V were derived from initial rate measurements and were plotted as a function of pH and NaCl concentration. Similar results were obtained when either hyaluronic acid (Fig. 5a, b) or chon-

TABLE I

PROTECTION OF HYALURONIDASE FROM INACTIVATION BY BUTANE-2,3-DIONE

Various monosaccharides (20 mM) and polysaccharides (0.1 mg/ml) were tested for their ability to protect hyaluronidase from inactivation by 16.3 mM butane-2,3-dione in the presence of either 50 mM Hepes or 50 mM borate buffer, pH 8.3.

Addition to preincubation mixture	$t_{1/2}$ values (min)	
	Borate buffer	Hepes buffer
None	3.0	11.5
D-Glucuronic acid	9.0	27.0
D-Glucuronamide	5.0	11.5
D-Glucose	6.0	11.5
Hyaluronic acid	4.0	35.5
Chondroitin 6-sulphate	4.5	47.0
Dermatan sulphate	4.2	39.5

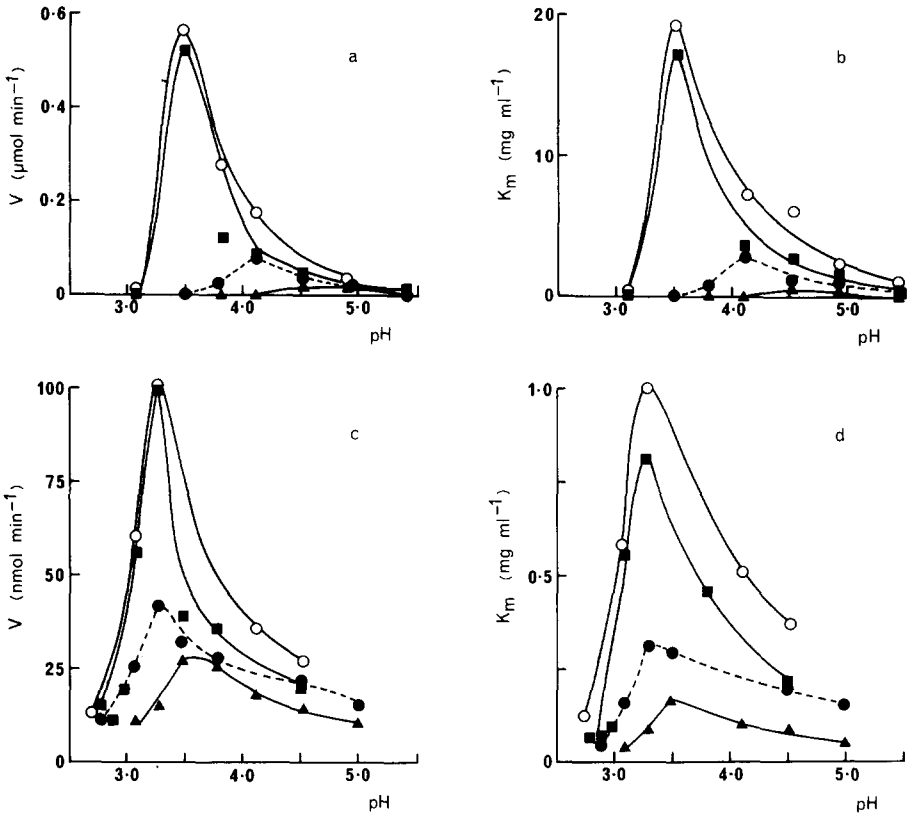

Fig. 5. Dependence of kinetic constants on pH. Enzyme activity was measured at each pH in the presence of 0.1 M (▲), 0.2 M (●), 0.3 M (■) or 0.4 M (○) NaCl but in the absence of buffers. Kinetic constants were determined from initial rates measured at a minimum of five different concentrations of substrate. (a) V vs. pH for hyaluronic acid; (b) K_m vs. pH for hyaluronic acid; (c) V vs. pH for chondroitin 6-sulphate; (d) K_m vs. pH for chondroitin 6-sulphate.

droitin 6-sulphate (Fig. 5c, d) was used as substrate. Both K_m and V increased with increasing ionic strength and a shift in the pH profile also occurred, resulting in constant values of V/K_m as a function of either pH or ionic strength.

The effect of ionic strength on the substrate

Using the apparatus described in Fig. 1, the pH of the microenvironment surrounding hyaluronic acid was measured at a variety of bulk solution pH values. The results (Fig. 6) show that the gradient of proton activity, as measured by pH, increased at the surface of hyaluronic acid. As either the pH of the bulk solution or the concentration of hyaluronic acid was increased, so the gradient became more pronounced. The addition of 0.1 M NaCl to the bulk solution was sufficient to destroy this differential pH effect.

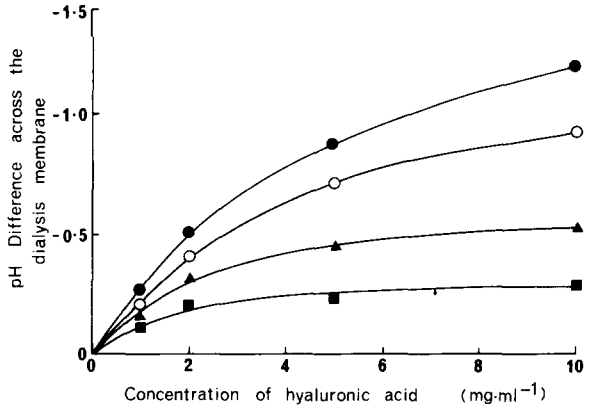

Fig. 6. Effect of hyaluronic acid concentration on microenvironment pH. The dialysis membrane contained 1 ml of a concentrated solution of hyaluronic acid. The pH difference between the bulk solution and the hyaluronic acid solution was measured at a series of bulk pH values: pH 4.0 (■), 5.0 (▲), 6.0 (○) and 7.0 (●).

Discussion

Inactivation of bovine testicular hyaluronidase occurred at pH 8.3 in the presence of butane-2,3-dione and was enhanced by borate buffer, as may be predicted from the known mechanism of this reaction [7]. Kinetic analysis of the data indicates that the loss of activity is caused by the modification of a single arginine residue (Fig. 3a). However, this value should be regarded as a minimum and does not take into account the possibility that other residues may have reacted at a considerably higher rate [24]. Amino acid analysis of the enzyme during the inactivation process (Fig. 4) suggests that approximately four arginine residues are modified during the inactivation process, although only one of these appears to be essential for activity as indicated by the straight line obtained in Fig. 4. The value for the second-order rate constant of the inactivation process was obtained by measuring the loss of enzyme activity at a variety of concentrations of butane-2,3-dione (Fig. 3b), and it is comparable with rate constants that have been obtained by other workers for a variety of enzymes [25,26] undergoing the same modification.

Bovine testicular hyaluronidase was significantly protected against inactivation by a variety of substrates or inhibitors. Sodium D-glucuronate was effective in increasing the $t_{1/2}$ values in both borate and Hepes buffers (Table 1). The uncharged competitive inhibitor, D-glucuronamide, was unable to protect against inactivation in the presence of Hepes buffer, although a measurable protective affect was noticeable in the presence of borate buffer. Assuming that the mechanism of inactivation proposed by Riordan [7] is correct then the apparent protective effect in the presence of borate most probably reflects a partial complexing of the buffer with the monosaccharide. This would reduce the effective borate concentration available for stabilisation of the arginine-butane-2,3-dione intermediate and thus increase the value of $t_{1/2}$. Further evidence of this is shown by the results obtained with D-glucose, which is not an inhibitor of enzyme activity but is still able to cause an increase in the value of $t_{1/2}$ obtained in borate buffer. These results show that only the anionic monosaccharide inhibitor is capable of genuinely protecting against inactivation by butane-2,3-dione.

The macromolecular substrates (hyaluronic acid, chondroitin 6-sulphate) and inhibitor (dermatan sulphate) are all capable of significantly protecting bovine testicular hyaluronidase from inactivation by butane-2,3-dione (Table I). Any interaction between these compounds and borate is not likely to be significant as two of the hydroxyl groups on each monosaccharide unit are involved in glycosidic links and are therefore unavailable for complexing with the buffer.

The results obtained from the inactivation experiments strongly suggest that one arginine residue is involved in the binding of substrates to hyaluronidase. Unpublished data (Gacesa, Earnshaw, Dodgson and Olavesen) indicate that lysine residues are not involved in the catalytic activity or the binding of anionic substrates to the enzyme and that the ionic-type interactions are confined to arginine residues. In this context it is of interest to note that the derivative of hyaluronic acid in which the D-glucuronic acid residues have been reduced to D-glucose is a competitive inhibitor of the enzyme and not a substrate.

The concept of a partially ionic interaction between bovine testicular hyaluronidase and its substrates, hyaluronic acid and chondroitin 6-sulphate may well explain the conflicting data that have been published on the pH and salt effects of this enzyme [27,28]. A full kinetic analysis of the enzyme activity shows that V/K_m remains constant over a range of pH values (approx. 3–5) and NaCl concentration (0.1–0.4 M), although the value is dependent on the substrate used. This is indicative of an uncompetitive activation mechanism as shown in Eqn. 2 in which either H^+ or Na^+ may act as an activator of enzyme activity. In this mechanism enzyme and substrate combine to form a

$$\begin{array}{l} \overset{k_1}{\underset{k_{-1}}{E + S \rightleftharpoons ES}} \\ \qquad\quad \Downarrow \; k_{cat} \\ \qquad\quad EAS \longrightarrow \text{products} \end{array} \qquad (2)$$

complex, ES, that is not capable of forming products without the prior addition of activator. This mechanism would be entirely compatible with an ionic interaction between substrate and enzyme.

Studies on the substrates in the absence of enzyme show that there is a possibility that a microenvironmental pH effect (Fig. 6) may account for the effect of NaCl on hyaluronidase activity. However, it seems

unlikely that the microenvironment is important in the activity of the enzyme, as the effect is completely destroyed by 0.1 M NaCl. Furthermore, if the microenvironment effect was significant then it would be expected that both titration curves that constitute the pH profile (Fig. 5) would alter with ionic strength in the same manner to give a new pH optimum without the activation effect. The values of V/K_m would not be constant under these circumstances.

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References

1 Meyer, K. (1971) in The Enzymes (Boyer, P.D., ed.), Vol. 5, pp. 307–320, Academic Press, New York
2 Gorham, S.D., Olavesen, A.H. and Dodgson, K.S. (1975) Connect. Tissue Res. 3, 17–25
3 Highsmith, S., Garvin, J.H. and Chipman, D.M. (1975) J. Biol. Chem. 250, 7473–7480
4 Rhodes, C., Dodgson, K.S., Olavesen, A.H. and Högberg, B. (1971) Biochem. J. 122, 575–582
5 Borders, C.L., Jr., Riordan, J.F. and Auld, D.S. (1975) Biochem. Biophys. Res. Commun. 66, 490–496
6 Riordan, J.F., McElvany, K.D. and Borders, C.L., Jr. (1977) Science 195, 884–886
7 Riordan, J.F. (1973) Biochemistry 12, 3915–3923
8 Yankeelov, J.A., Jr. (1972) Methods Enzymol. 25, 566–579
9 Gacesa, P., Dodgson, K.S. and Olavesen, A.H. (1979) Biochem. Soc. Trans. 7, 1058–1060
10 Gorham, S.D. (1974) Ph.D. Thesis, University of Wales
11 Pope, D.J., Rhodes, C. and Gorham, S.D. (1976) British Patent 1425918
12 Humphrey, J.H. (1957) Bull. World Health Org. 16, 291–294
13 Levvy, G.A. and Conchie, J. (1966) Methods Enzymol. 8, 571–584
14 Beynon, R.J. and Kay, J. (1978) Biochem. J. 173, 291–298
15 Dodgson, K.S. and Spencer, B. (1954) in Methods of Biochemical Analysis (Glick, D., ed.), Vol. 4, pp. 211–255, Wiley Interscience, New York
16 Reissig, J.L., Strominger, J.L. and Leloir, L.F. (1955) J. Biol. Chem. 217, 959–966
17 Taylor, R.L. and Conrad, H.E. (1972) Biochemistry 11, 1383–1388
18 Bitter, T. and Muir, H. (1962) Anal. Biochem. 4, 330–334
19 Brown, J.P. and Perham, R.N. (1973) Eur. J. Biochem. 39, 69–73
20 Venn, R.F., Basford, J.M. and Curtis, C.G. (1978) Anal. Biochem. 87, 278–282
21 Borders, C.L., Jr. and Raferty, M.P. (1968) J. Biol. Chem. 243, 3756–3762
22 Eisenthal, R.S. and Cornish Bowden, A. (1974) Biochem. J. 139, 715–720
23 McPhie, P. (1971) Methods Enzymol. 22, 23–32
24 Levy, H.M., Leber, P.D. and Ryan, E.M. (1963) J. Biol. Chem. 238, 3654–3659
25 Cardemil, E. and Eyzaguirre, J. (1979) Arch. Biochem. Biophys. 192, 533–538
26 Borders, C.L., Jr. and Riordan, J.F. (1975) Biochemistry 14, 4699–4704
27 Alburn, H.E. and Whitley, R.W. (1951) J. Biol. Chem. 192, 379–393
28 Bollet, A.J., Bonner, W.M., Jr. and Nance, J.L. (1963) J. Biol. Chem. 238, 3522–3527