Interfacial behaviour of bovine testis hyaluronidase

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The interfacial properties of bovine testicular hyaluronidase were investigated by demonstrating the association of hyaluronidase activity with membranes prepared from bovine testis. Protein adsorption to the air/water interface was investigated using surface pressure-area isotherms. In whichever way the interfacial films were obtained (protein injection or deposition), the hyaluronidase exhibited a significant affinity for the air/water interface. The isotherm obtained 180 min after protein injection into a pH 5.3 subphase was similar to the isotherm obtained after spreading the same amount of protein onto the same subphase, indicating that bovine testicular hyaluronidase molecules adopted a similar arrangement and/or conformation at the interface. Increasing the subphase pH from 5.3 to 8 resulted in changes of the protein isotherms. These modifications, which could correspond to the small pH-induced conformational changes observed by Fourier-transform IR spectroscopy, were discussed in relation to the pH influ-

ence on the hyaluronidase activity. Adding hyaluronic acid, the enzyme substrate, to the subphase tested the stability of the interfacial properties of hyaluronidase. The presence of hyaluronic acid in the subphase did not modify the protein adsorption and allowed substrate binding to a preformed film of hyaluronidase at pH 5.3, the optimal pH for the enzyme activity. Such effects of hyaluronic acid were not observed when the subphase was constituted of pure water, a medium where the enzyme activity was negligible. These influences of hyaluronic acid were discussed in relation to the modelled structure of bovine testis hyaluronidase where a hydrophobic region was proposed to be opposite of the catalytic site.

Key words: bovine testis hyaluronidase, Fourier-transform IR spectroscopy, hyaluronic acid, Langmuir film.

INTRODUCTION

Hyaluronidases are present in many tissues and organs (such as kidney, liver, skin, testes and uterus), as well as in venom (bees, wasps or cobra) [1]. These enzymes are capable of randomly hydrolysing hyaluronic acid [2], one of the most abundant constituents of the extracellular matrix [3,4]. They also act on chondroitin or chondroitin sulfates [5–8]. Hyaluronidases are implicated in many biological functions, including allergic reactions [9], inflammation [10–13], migration of cancer cells [14] and microvascular permeability [15].

Six human hyaluronidases have been cloned to date [16] and three of them have been expressed in different cell lines but, due to difficulty in isolation and/or purification, the enzymes have not been produced in large enough amounts for enzymatic characterization and structural determination. Bovine testicular hyaluronidase, which is commercially available, has been chosen as a model for several studies. Thus, the hyaluronan hydrolysis catalysed by this enzyme has been studied by investigating the hydrolysis kinetics at pH 5 and 37°C [17]. The authors demonstrated that the hyaluronan hydrolysis by hyaluronidase was inhibited at high substrate concentration and low ionic strength and suggested the formation of non-specific complexes between hyaluronan and hyaluronidase based on electrostatic interactions [17]. Monte Carlo simulation of the mechanism of the bovine testicular hyaluronidase reaction showed that the condensation reaction is much weaker than the transglycosylation reaction, but contributes to product distribution at the final stage of the reaction, preventing complete hydrolysis of the substrates [18].

Even though bovine testicular hyaluronidase and the distantly related bee venom hyaluronidase are well known representatives of the eukaryotic enzymes, structural information is only available for the bee venom hyaluronidase [19]. For this reason we were interested in the characterization of bovine testicular hyaluronidase. In the present study, we looked for interfacial properties of the bovine testicular hyaluronidase because we demonstrated that, in a bovine testis homogenate, a significant amount of the hyaluronidase activity could be recovered associated with membranes. We modelled the bovine testicular hyaluronidase by using the crystal structure of the bee venom hyaluronidase in order to localize the hydrophobic amino acids on the surface of the molecule. Thus using the commercial bovine testicular hyaluronidase, the suspected interfacial properties were studied and the ability of this protein to form films at the air/water interface in different conditions was investigated. The pH-sensitive interfacial properties of the enzyme were compared with the pH-sensitive enzymatic activity of the protein on hyaluronic acid as well as with the pH-induced conformational changes evidenced by FTIR (Fourier-transform IR) spectroscopy. Finally, the influence of hyaluronic acid on the interfacial properties of the bovine testicular hyaluronidase was studied.

MATERIALS AND METHODS

Materials

Hyaluronic acid from human umbilical cord and hyaluronidase from bovine testis (type IV-S) were purchased from

Abbreviations used: FTIR, Fourier-transform IR; GPI, glycosylphosphatidylinositol; Ks, surface elasticity modulus.

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Sigma-Aldrich and deuterated water ($^2\text{H}_2\text{O}$) was purchased from Merck. All organic solvents were analytical grade.

Isolation of bovine testis membranes

Total membranes were prepared as described previously for bovine kidney [20] with minor modifications. Bovine testis tissue was cut into small pieces and homogenized in 10 mM Tris buffer (pH 7.4) containing 150 mM NaCl and 0.25 M sucrose with pepstatin and leupeptin (0.25 mM each). The homogenate was centrifuged at 150 000 *g* for 30 min at 4 °C. The pellets containing total membranes were washed a further three times at 4 °C with the same buffer and centrifuged at 150 000 *g* as described above.

Protein and enzyme assays

Protein concentrations were determined using the Bradford method [21] using BSA as the standard. Hyaluronidase activity was measured in a 100 mM phosphate buffer (pH 5.3) containing 150 mM NaCl as described previously [22]. One enzyme unit hydrolyses half of the hyaluronic acid present in the assay during the 10 min reaction time. Alkaline phosphatase activity was measured at 37 °C in a 25 mM glycine buffer (pH 11) containing *p*-nitrophenyl phosphate as substrate. Release of *p*-nitrophenolate was followed at 420 nm. One enzyme unit hydrolyses one μmol of *p*-nitrophenyl phosphate/min [20].

Monolayer technique

All experiments were performed at a constant temperature of 21.0 ± 0.1 °C. The film balance was built by R&K (Riegler & Kirstein, Wiesbaden, Germany) and equipped with a Wilhelmy-type surface-pressure measuring system as described previously [23]. The subphase buffer used was a 100 mM phosphate buffer with or without 150 mM NaCl at different pH. In all experiments, the subphase was continuously stirred with a magnetic stirrer spinning at 100 rev./min. In order to measure the hyaluronidase adsorption at a constant surface area, the experiments were performed on a small Teflon dish (diameter, 3 cm) with a subphase volume of 7 ml. The enzyme was injected in the subphase at a final concentration of 1.4 $\mu\text{g}/\text{ml}$. Hyaluronidase adsorption at the air/water interface, measured by tensiometry, was followed as an increase in surface pressure. The π -*A* isotherms of the protein were measured either after deposition or after injection of a known quantity of hyaluronidase either on or into the subphase buffer of the Langmuir trough (dimensions: 165 cm² and 120 ml subphase). After 15 min, the surface was compressed at a velocity of 6 cm²/min. The adsorption times indicated on the compression-decompression-recompression π -*A* isotherms correspond to the cumulated adsorption time at zero surface pressure. Indeed, we consider that the protein is weakly adsorbed at the interface during the decompression.

K_s (surface elasticity moduli) were calculated from the pressure-area data obtained from the monolayer compressions using the following equation:

$$K_s = -A d\pi/dA,$$

where *A* is the molecular area at the indicated surface pressure π .

FTIR spectroscopy measurements

IR data were acquired, as described previously [24], with a Nicolet 510M FTIR spectrometer continuously purged with dry filtered air (Balston regenerating desiccant dryer). Spectra were recorded at 25 °C with 128 interferograms and Fourier transformed. The resolution was 4 cm⁻¹. 20 μl of a hyaluronidase solution at

10 mg/ml, freshly prepared in a 100 mM phosphate $^2\text{H}_2\text{O}$ buffer containing 150 mM NaCl at different pH, were deposited into a CaF₂ cell. The final spectrum was fitted by the mean of a curve fitting program computing the Lorentzian band shape components in the amide I region. The wavenumber position of each component band was fixed while the width and the height varied in order to obtain the best fit. The wavenumber position and the number of component bands were determined by using the second derivative spectra. After decomposition, the percentage of each component band was calculated as 100 (*A_x*/*A_I*), where *A_x* is a component band area and *A_I* is the amide band area. IR difference spectra were obtained by using the intensity of the characteristic tyrosine band at 1515 cm⁻¹ as reference for the hyaluronidase concentration.

Modelling of the three-dimensional structure of bovine testicular hyaluronidase

Bovine testicular hyaluronidase (sequence accession code Q7YS45; [25]) was modelled with version 4.0 of the MODELLER program [26] using the hyaluronidase from bee venom, the closest three-dimensional structure available (1FCU Protein Data Bank accession code; [19]). The sequence alignment is shown in Figure 1.

RESULTS

Hyaluronidase from bovine testis is membrane-bound

During assays for the preparation of hyaluronidase from bovine testis, an unexpected result was obtained. Only about 50 % of the hyaluronidase activity of the testicular homogenate was recovered in the soluble protein fraction. To test the efficiency of the membrane preparation, the activity of alkaline phosphatase, a GPI (glycosylphosphatidylinositol)-anchored membrane marker [20], was titrated in parallel with the activity of hyaluronidase. About 80 % of the alkaline phosphatase activity of the homogenate was recovered associated with membranes, showing a good separation of the membranes. Furthermore, three washings of total membranes with buffer did not induce a release of both enzymes. Since it had been demonstrated that the bovine testicular hyaluronidase does not possess a lipidic modification [27], its membrane association could be due to amphiphilic properties.

Hyaluronidase modelling

We then looked for the positioning of hydrophobic amino acids (potent candidates to induce amphiphilic properties of proteins) on the surface of the bovine testicular hyaluronidase molecule after constructing the three-dimensional model of this enzyme based on the known crystal structure of bee venom hyaluronidase [19]. The sequence alignment used is given in Figure 1 showing that there is an N-terminal region (corresponding to 12 amino acids) and a C-terminal region (corresponding to approx. 100 amino acids) both of which were not to be modelled. Figure 1 also shows the presence of several groups of hydrophobic amino acids along the sequence and Figure 2 indicates the positioning of the hydrophobic amino acid residues on the surface of the modelled molecule of testicular hyaluronidase. The top view shows the presence of a hydrophobic patch around the N- and C-terminal regions of the testicular enzyme and the lateral view shows that these hydrophobic regions are located more or less at the opposite of the catalytic site containing two conserved negatively charged amino acids.

HYA_BOVINE	<u>LD</u> FRAPPLISNT <u>S</u> FLWAWNAPVERCVNRRFQLPDDLRLFSVKGSPOKSAT 50
HYA_BEE	---TPDNNKTVREENVYWNVPTFMCHKYGLRFEEVSEKYGIQNWMDKFR 47
HYA_BOVINE	GQFITL <u>F</u> YADRLGYYPHID E KTGKTVFG---GIPQLGNLKSHLEKAKNDI 97
HYA_BEE	GEEIAILYDP--GMFPALLKDPNGNVVARNGGVPQLGNLTKHLQVFRDHL 95
HYA_BOVINE	AYYIPNDSVG-LAVI <u>D</u> WENRPTWARNWPKDVYRDESVELVQKNPQLS 146
HYA_BEE	INQIPDKSFPGVGVID <u>D</u> FSWRPIFRQNWASLQPYKKLSVEVVRREHPFWD 145
HYA_BOVINE	FPEASKIAKVDFETAGKSFMQETLKLGLLRPNHLWGYYLFPDCYNHNHN 196
HYA_BEE	DQRVEQEAQRREFEYQGLFMEETLKAARMRPAANWGYAYPYCYNLTEN 195
HYA_BOVINE	QPTYNGNCPDVEKRRNDLEWLWKESTALFPSVYLNIRLKSTQNAALYVR 246
HYA_BEE	QP--SAQCEATTMQENDKMSWLFESQEDVLLPSVYLRWNLTSGERVGLVG- 242
HYA_BOVINE	NRVQEAIRLSKIASVESPLPVFVYARPVFTDGSSTYLSQGDVNSVGEIV 296
HYA_BEE	GRVKEALRIARQMTTSRKK-VLPYYWYKYQDRDRLSRADLEATLRKIT 291
HYA_BOVINE	SLGASGIIMWGSNLNLSVQSCMNLTGTYLNTTLNPYIINVTLAAKMCSQV 346
HYA_BEE	DLGADGFIWGSDDINTKAKCLQFREYLNNELGPAVKRIALNNANDRI 341
HYA_BOVINE	<u>L</u> CHDGGVCTRKHWNSDYHLNPNMFAIQTGEGGKYTPGTLTLEDLQKE 396
HYA_BEE	<u>T</u> VDVSVQV----- 350
HYA_BOVINE	<u>SDTFYCSCYSNLSCKKRVDIKNVHSVDVCMEDVCIDAFLEKPE</u> 439
HYA_BEE	-----

Figure 1 Sequence alignment of bovine testis hyaluronidase with bee venom hyaluronidase used for modelling

HYA_BOVINE refers to bovine testis hyaluronidase (sequence accession code Q7YS45) and HYA_BEE to bee venom hyaluronidase (Q08169). Regions in grey boxes correspond to undefined structures in the bovine hyaluronidase model (the current study) or the bee hyaluronidase crystal structure (1FCU Protein Data Bank accession code). Conserved residues of the active site (D112 and E114 in HYA_BOVINE, corresponding to D111 and E113 in HYA_BEE) are shown with bold letters. Hydrophobic amino acids of HYA_BOVINE, identified according to <http://www.inrp.fr/Acces/BioGeo/model3d/chimdata/scripts/globine/aa.htm>, are underlined.

Figure 2 Hydrophobic residues in the model of bovine testis hyaluronidase

(A) Top view of the N- and C-terminal regions indicating C_{ter}, N_{ter} and hydrophobic amino acids (black area). (B) View showing the N-terminal region opposite to the aspartate and glutamate conserved residues of the active site, as well as the hydrophobic amino acids (black area). This modelled structure of bovine testis hyaluronidase did not take account of the amino acids in grey boxes for HYA_BOVINE in Figure 1.

Hyaluronidase adsorption at the air/water interface

The hyaluronidase amphiphilic properties suggested by its membrane association and its modelling led us to investigate possible interfacial properties of the protein. This was studied by determining the adsorption of protein at the air/water interface. The same amount of protein (12 µg) was injected into different pH subphases, giving a final enzyme concentration of 1.4 µg/ml. The adsorption was monitored by measuring the surface pressure at constant area as a function of time. When injected into the subphase at pH 5.3 (Figure 3), a lag time of about 20 min was

necessary to detect the protein at the interface. The surface pressure then increased gradually until a maximum was reached after about 70 min, indicating that a stable monolayer was formed at the interface. Varying the pH subphase did not significantly modify the kinetics of the protein adsorption (Figure 3).

If our results are compared with the air/water interface adsorption of the amyloid precursor protein, which has been shown to exhibit interfacial properties [28], it appears that, at similar concentrations, hyaluronidase and amyloid precursor protein show similar adsorption kinetics. This indicates that hyaluronidase effectively exhibits interfacial properties.

Figure 3 Kinetics of surface pressure increase due to hyaluronidase adsorption at the air/water interface at 21 °C

Protein (12 μ g) was injected into a 100 mM phosphate buffer containing 150 mM NaCl at pH 5.3 (○), pH 6.2 (■) and pH 7.2 (◆).

Figure 4 Successive π -A isotherms during hyaluronidase adsorption at the air/water interface at 21 °C

(A) π -A isotherms measured at 15 min (curve a), 35 min (curve b), 65 min (curve c), 125 min (curve d) and 180 min (curve e) after 12 μ g protein injection into pH 5.3 buffer subphase at zero surface pressure or after 12 μ g protein spreading (curve f). (B) Monolayer K_s calculated from the curve e isotherm and represented as a function of surface pressure.

Hyaluronidase isotherms

The adsorption of the protein at the air/water interface was then followed by measuring successive π -A isotherms after injection of 12 μ g of hyaluronidase into the trough subphase buffer to reach a final concentration of 0.1 μ g/ml. The trough surface was compressed after various delays to obtain π -A isotherms presented in Figure 4(A). The molecular area at each surface pressure increased with increasing time, indicating that the protein was adsorbed at the air/water interface [29,30]. The protein adsorption was not measured over 180 min since the isotherm obtained after that time was similar to that obtained when the 12 μ g of protein was deposited onto the subphase buffer, indicating that, after a 180 min delay, roughly all the protein had reached the interface (Figure 4A).

Figure 5 Influence of pH on the hyaluronidase isotherms after 12 μ g protein spreading onto different buffer subphases at 21 °C

Subphases were 100 mM phosphate buffer containing 150 mM NaCl buffer at pH 5.3 (■), pH 6.2 (△), pH 7.2 (●), pH 8 (◇) and pH 5.6 water (▲).

The K_s of the last π -A isotherm was represented against the surface pressure (Figure 4B). The variation of K_s during the compression exhibits three phases. During the first phase, the K_s increased linearly until the surface pressure reached 5 mN/m. For pressures between 8 and 12 mN/m, the K_s increase was lower and stabilized, whereas for pressure higher than 12 mN/m the K_s decreased.

Influence of the buffer composition on the hyaluronidase isotherm

We were then interested in the influence of pH on the interfacial properties of the hyaluronidase. The protein was dissolved in the corresponding subphase buffer and spread at the air/water interface. Measuring π -A isotherms after spreading the protein on different buffered subphases tested the influence of pH on the interfacial properties of hyaluronidase (Figure 5). The hyaluronidase isotherms were shifted to a lower molecular area at pH 5.3 and pH 6.2 as compared with pH 7.2 and pH 8. These shifts decreased during the compression of the hyaluronidase monolayer and at the highest pressures, all of the curves tended towards the same value. Similar experiments were performed with hyaluronidase solubilized in water with a water subphase (Figure 5). In these conditions the shift towards a higher molecular area is observed all along the isotherm.

Influence of hyaluronic acid on the hyaluronidase adsorption

First the absence of interfacial properties of hyaluronic acid was tested. Hyaluronic acid was injected at 8.3 μ g/ml into pH 5.3 buffer or water subphases. Thirty minutes after the injection and at 30 min intervals, the interface was compressed and the pressure was measured during the compression. No variation of the pressure was observed whatever the subphase or the waiting time, indicating the absence of interfacial properties for the hyaluronidase substrate (results not shown).

The influence of hyaluronic acid on the ability of the protein to reach the air/water interface was then tested. Successive π -A isotherms were measured after injecting 0.1 μ g/ml hyaluronidase into pH 5.3 buffer subphase containing 3.3 μ g/ml hyaluronic acid and were compared with those obtained in the absence of enzyme substrate. For each adsorption delay, isotherms obtained with or without hyaluronic acid were practically superposed. Increasing the hyaluronidase concentration up to 0.2 μ g/ml allowed the detection of small differences in the adsorption kinetics (Figure 6).

Similar experiments were performed with hyaluronidase solubilized in water and injected at 0.2 μ g/ml in a water subphase with or without 3.3 μ g/ml hyaluronic acid. Successive π -A isotherms were measured during the protein adsorption and the

Figure 6 Kinetics of hyaluronidase adsorption at 21 °C

The pressures induced by the protein adsorption (0.2 µg/ml) at a 20 cm² area (obtained from the π -A isotherms) were plotted against the adsorption delays. Assays with pH 5.3 buffer (▲) or pH 5.6 water (●) subphases containing no (full lines) or 3.3 µg/ml (dashed lines) hyaluronic acid.

pressures induced by the protein adsorption at an area of 20 cm² were plotted versus the adsorption delays (Figure 6). In the absence of hyaluronic acid, the protein in a water subphase reached the interface more slowly than it did at the same concentration in a pH 5.3 subphase. In the presence of hyaluronic acid, the protein in water induced no significant pressure during interface compression, indicating that the protein remained in the subphase.

Adsorption of hyaluronic acid on the hyaluronidase monolayer

We then tested if the interaction between the enzyme and its soluble substrate would destabilize hyaluronidase films at the air/water interface. The hyaluronidase was spread at the surface of the pH 5.3 buffer subphase that contained different concentrations of hyaluronic acid (1.7 or 3.3 µg/ml) and the isotherms were measured 30 min after the protein deposition. For the two hyaluronic acid concentrations, the isotherms measured in the presence of hyaluronic acid were compared with the isotherm measured in its absence (Figure 7A). In all of the cases, the isotherms measured with hyaluronic acid shifted towards a higher molecular area as compared with the isotherm without hyaluronic acid. These shifts increased with increasing hyaluronic acid concentration. Furthermore, when the isotherms were measured 60 min after the injection of hyaluronic acid no significant changes were observed as compared with the isotherms obtained 30 min after the injection (results not shown). The Ks values of the π -A isotherm before and after hyaluronic acid injection were represented against the surface pressure (Figure 7B). For the lowest surface pressures (until about 7 mN/m), Ks was increased in the presence of hyaluronic acid, confirming the interaction between the hyaluronidase and its substrate.

The influence of the subphase on the interaction between hyaluronic acid and hyaluronidase was then tested. When the pH of the buffer subphase was increased from 5.3 to 8 and the protein was dissolved in the corresponding buffer, the isotherm shifts towards a higher molecular area were still observed in the presence of hyaluronic acid (results not shown). In contrast, such a shift of the isotherm toward a higher molecular area was not observed when the protein was dissolved in water and was spread on a water subphase (Figure 7A).

FTIR spectroscopy studies of the secondary structure of hyaluronidase

The differences observed in the interfacial properties of the enzyme with the wide variations of pH, as well as the interaction

Figure 7 Influence of hyaluronic acid on preformed hyaluronidase films at 21 °C

(A) π -A isotherms of hyaluronidase dissolved in pH 5.3 buffer (closed symbols) or in pH 5.6 water (open symbols) and spread onto pH 5.3 buffer or water subphases containing no hyaluronic acid (squares), 1.7 µg/ml (diamonds) or 3.3 µg/ml (triangles) hyaluronic acid. Isotherms were measured 30 min after the protein deposition. (B) π -A isotherm-derived Ks of hyaluronidase in pH 5.3 buffer subphases containing no (curve a) or 1.7 µg/ml (curve b) hyaluronic acid.

Figure 8 ²H₂O buffer-subtracted FTIR spectrum of hyaluronidase at pH 5.3

Original spectrum (full line), best-fitted individual component bands of the amide I and II regions (full lines) and their resulting summation (dashed line). The protein concentration was 10 mg/ml.

with hyaluronic acid, may influence the conformation of the bovine hyaluronidase. Indeed some amino acid residues may change their local charges and potentially flexible hydrophobic segments buried in the molecule may move to the surface of molecule during its movement to the interface. For this reason, FTIR spectroscopy was used to look for pH-induced changes in the conformation of hyaluronidase.

The ²H₂O buffer-subtracted FTIR spectrum of hyaluronidase at pH 5.3 (p²H 5.5) and its deconvolution are shown in Figure 8. Amide I bands, at 1700–1610 cm⁻¹, are principally C=O stretching vibrations with some N-H bending and C-H stretching vibrations, whereas amide II bands, at 1575–1480 cm⁻¹, are due principally to N-H bending vibrations and to a lesser extent with

Figure 9 Influence of pH on the FTIR spectra of hyaluronidase

(A) $^2\text{H}_2\text{O}$ buffer-FTIR spectra of hyaluronidase at pH 5.3 (a), 6.2 (b), 7.2 (c) and 8.0 (d). (B) IR difference spectra obtained by subtracting the FTIR spectrum at pH 5.3 to FTIR spectra of hyaluronidase at pH 6.2 (a), 7.2 (b) and 8.0 (c).

some $\text{C}\equiv\text{N}$ stretching vibrations. The remaining intense amide II component band at 1545 cm^{-1} indicated an incomplete $\text{NH}/\text{N}^2\text{H}$ exchange. This incomplete deuteration corresponds to N-H residues buried in the hydrophobic regions of the protein, since increasing the incubation time in the $^2\text{H}_2\text{O}$ buffer did not modify the amide II band intensity. The deconvolution of the amide I band gives four component bands at 1634 , 1650 , 1657 and 1675 cm^{-1} . The 1634 cm^{-1} band associated with the 1675 cm^{-1} band corresponds to intramolecular β -sheets, although overlap from β -turns can also contribute to this last band. The 1657 cm^{-1} band in $^2\text{H}_2\text{O}$ is usually produced by α -helices, but it cannot be excluded that it may originate from turns or 3_{10} -helices. The 1650 cm^{-1} band in $^2\text{H}_2\text{O}$ corresponds to unordered structures. Besides, it is well known that some amino acid side chain groups absorb in amide I and II regions [31]. The 1515 cm^{-1} band, corresponding to tyrosine residues, was easily recognized, whilst the bands at 1585 and 1565 cm^{-1} might correspond to aspartate and glutamate carboxy residues. The four amide I component bands at 1675 , 1657 , 1650 and 1634 cm^{-1} , represent 6, 20, 33 and 41 % of the original band respectively.

In order to explain the pH-sensitivity of the hyaluronidase, we looked for hypothetical pH-induced conformational changes of this protein. $^2\text{H}_2\text{O}$ buffer-subtracted FTIR spectra of hyaluronidase with increasing pH showed a slight decrease in the maximum of the amide I band (from 1643 cm^{-1} at pH 5.3, to 1642 cm^{-1} at pH 6.2, 1641 cm^{-1} at pH 7.2 and 1640 cm^{-1} at pH 8) and a decrease in intensity of the amide II band, indicating a $\text{NH}/\text{N}^2\text{H}$ exchange increase (Figure 9A). The pH-induced conformational changes of hyaluronidase could be seen more clearly on the IR difference spectra, obtained by subtracting the FTIR spectrum of hyaluronidase at pH 5.3 from the FTIR spectrum at another pH (Figure 9B). When pH was varied from 6.2 to

8, the negative bands of the IR difference spectra at 1657 cm^{-1} , corresponding to α -helices, decreased in intensity while the positive bands at 1625 cm^{-1} , corresponding to intermolecular β -sheets, increased. This indicates that intermolecular β -sheets appeared at the expense of α -helices. At pH 8, the positive peak of the IR difference spectrum at 1625 cm^{-1} involved 2–3 % of the amino acids of the protein, meaning that about 11 amino acids were implicated in the formation of intermolecular β -sheets. Furthermore the FTIR spectrum of hyaluronidase measured in non-buffered $^2\text{H}_2\text{O}$ was compared with the spectrum measured at pH 5.3 and the resulting IR difference spectrum was similar to that obtained by subtracting the FTIR spectrum at pH 5.3 from the FTIR spectrum at pH 6.2 (results not shown).

DISCUSSION

Sperm and testicular hyaluronidases have the same amino acid sequence but differ by their post-translational modification [27]. Indeed the sperm hyaluronidase possesses a GPI-anchor while the testicular hyaluronidase does not. In the current study, we have shown that the total membranes, obtained by classical centrifugation at $150\,000\text{ g}$ of a bovine testis homogenate, contained hyaluronidase and that three washings of total membranes with buffer did not induce a release of the enzyme. This unexpected membrane association could be explained if bovine testicular hyaluronidase possesses amphiphilic properties. In order to test this hypothesis, the presence of hydrophobic areas at the surface of the bovine testicular hyaluronidase molecule was estimated after modelling the protein with the crystal structure of the bee venom hyaluronidase. A hydrophobic region, more or less opposite the catalytic site, was evidenced at the surface of the bovine testicular hyaluronidase molecule. This region could be responsible for the amphiphilic properties of the protein, but it must be remembered that the modelling did not involve all of the amino acids of the protein. Indeed the non-modelled C-terminal peptide part, which is not significantly enriched in hydrophobic amino acids, could mask the hydrophobic region.

One of the factors contributing to the affinity of the protein for the interface is the presence of hydrophobic areas on the surface of the protein [32]. The monolayer technique, which is a powerful method for assaying the surface activities of proteins [33], was applied to bovine testicular hyaluronidase. Another important advantage of this method is that it requires only small amounts of protein. Following the protein adsorption by measuring directly the pressure increase after injection into a small trough shows that bovine testicular hyaluronidase exhibits a significant surface activity. Measuring successive isotherms after protein injection was the other way chosen to determine the enzyme adsorption at the air/buffer interface in a time-dependent manner. Furthermore, it was shown that hyaluronidase remains at the interface when spread and the surface pressure developed by the protein after injection, as a consequence of its adsorption at the interface, leads to values similar to those obtained by direct spreading onto the surface. This indicates that, as it has been shown for other proteins [33–36], the arrangement adopted by the bovine testicular hyaluronidase at the interface is independent of the method used to form the film. This was confirmed by the similarities in the pressure-dependent variations of Ks observed after injection or spreading. Furthermore, the Ks variations suggest that compression induces a marked rearrangement of the organization and distribution of the protein at the interface. One may consider that the plateau, observed between 8 and 12 mN/m , could be due to equilibrium between the protein desorption and adsorption at the air/water interface. It seems that above 12 mN/m , there was

either a protein desorption or a reorganization of the protein at the air/water interface during the monolayer compression. It is interesting to note that the maximum value of the K_s (at about 30 mN/m) was obtained at a pressure (10 mN/m) similar to those observed for the phospholipid-hydroperoxide glutathione peroxidase [37] and for the creatine kinase [34].

Several factors (pH, salts and hyaluronic acid concentration as well as the glycosaminoglycan microenvironment of hyaluronidase) are known to affect the enzymatic activity of bovine testicular hyaluronidase [5,17,38]. Increasing the subphase pH from 5.3 to 8 (corresponding to a decrease of the optimal conditions for the enzymatic activity of hyaluronidase) induces small but significant differences in the π -A isotherms. This might reflect pH-induced differences either in the desorption of protein segments or in the short-range intermolecular interactions. Indeed decreasing the pH could decrease the repulsive forces between the protein molecules by neutralizing some ionic charges on the protein surface since the pI of the bovine testicular hyaluronidase is about 5 [39]. Similarly, the larger shift observed in the isotherm measured on water (a condition which gives only 7% of the enzymatic activity measured at pH 5.3) could be explained by the absence of salts. Furthermore the variations observed in the isotherms could result from the small structural changes observed by FTIR spectroscopy.

Since hydrophobic interactions on the surface of the hyaluronidase molecule have been previously suggested to play a role during the formation of conjugates between hyaluronidase and glycosaminoglycans [40], we looked for the surface activity of the protein in the presence of its substrate, hyaluronic acid. In a first set of experiments, the influence of hyaluronic acid on the ability of hyaluronidase to reach the interface was followed by measuring successive π -A isotherms after injection of the protein into a pH 5.3 buffered subphase in the absence or presence of the enzyme substrate. In these conditions (corresponding to the optimal conditions for the enzymatic activity), hyaluronic acid did not significantly decrease the amount of hyaluronidase detected at the interface. The presence of the catalytic site opposite the hydrophobic region (as suggested by protein modelling) could explain the persistence of the adsorption of the protein in the presence of hyaluronic acid.

In a second set of experiments, injecting the enzyme substrate under a preformed film of hyaluronidase on a pH 5.3 buffered subphase allowed us to follow the influence of hyaluronic acid on the ability of hyaluronidase to remain at the air/water interface. Whatever the substrate concentration, the isotherms measured in the presence of hyaluronic acid shifted towards a higher molecular area as compared with the isotherm in its absence. These shifts increased with increasing hyaluronic acid concentration. This indicates that the presence of the water-soluble hyaluronic acid in the subphase did not induce a delocalization of the protein from the air/water interface to the bulk subphase (there was no shift of the isotherm towards a lower molecular area). Furthermore, the isotherm shift towards a higher molecular area showed that the protein at the interface was still able to interact with the hyaluronic acid, the complex 'hyaluronidase-hyaluronic acid' having a higher molecular area than the protein alone. Moreover, the absence of significant changes between the isotherms measured at different times after the hyaluronic acid injection might suggest that the enzyme binding to its substrate occurred faster than the hyaluronic acid hydrolysis by hyaluronidase. The temperature (21 °C) used in the experiment which is lower than the physiological temperature could explain this result.

Moreover, the subphase influences the interaction between hyaluronidase and its substrate. When the pH of the buffer

subphase was varied from 5.3 to 8 (the pH range allowing the hydrolysis of hyaluronic acid by the enzyme, even if there is a decrease of enzymatic activity), the presence of hyaluronic acid induces isotherm shifts towards a higher molecular area. In contrast, when the subphase contained only water (a condition which did not induce significant hyaluronic acid hydrolysis), such a shift towards a higher molecular area was not observed. Furthermore, when hyaluronidase was injected into a water subphase, it reached the interface more slowly than the protein injected into the subphase at pH 5.3. The pI of the protein, and the addition of hyaluronic acid induced the remaining protein into the subphase. The hyaluronic acid-induced inhibition of the adsorption of the protein in water could be due either to an increase of the charges at the surface of the molecule or to a partial denaturation of the hyaluronidase, masking the hydrophobic region of the protein.

In conclusion we have shown that bovine testicular hyaluronidase exhibits surface activity depending on the composition of the medium in which it was solubilized. The maximum interfacial properties were observed in conditions corresponding to the maximum of enzymatic activity. At this stage, a deeper knowledge of the influence of the pH as well as a better characterization of the interactions between the protein and hyaluronic acid at the air/water interface, which might influence the three-dimensional structure of the bovine hyaluronidase are necessary. Such studies would require the use of specific interfacial biophysical techniques.

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