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How hyaluronan–protein complexes modulate the hyaluronidase activity: The model

Jean-Claude Vincent *, Hélène Lenormand

Laboratoire "Polymères, Biopolymères, Surfaces", FRE 3101 CNRS – Université de Rouen, 76821 Mont-Saint-Aignan cedex, France

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ABSTRACT

Hyaluronan (HA) is the substrate of hyaluronidase (HAase). In addition, HA is able to form electrostatic complexes with many proteins, including HAase. Experiments have shown the strong inhibition of the HA hydrolysis catalyzed by HAase when performed at low HAase over HA concentration ratio and under low ionic strength conditions. Non-catalytic P proteins are able to compete with HAase to form electrostatic complexes with HA and thus to modulate HAase activity. We have modeled the HA–HAase–P system by considering the competition between the two complex equilibria HA–P and HA–HAase, the Michaelis–Menten type behavior of HAase, and the non-activity of the electrostatically complexed HAase. Simulations performed by introducing experimental data produce a theoretical behavior similar to the experimental one, including all the atypical phenomena observed: substrate-dependence, enzyme-dependence and protein-dependence of HAase. This shows that our assumptions are sufficient to explain the behavior of the system and allow us to estimate unknown parameters and suggest new developments.

1. Introduction

Hyaluronidase (HAase) is an enzyme involved in several fundamental biological phenomena, such as fertilization and cancer [1–4]. Its natural substrate is hyaluronan (HA) which is hydrolyzed into HA oligosaccharides. HA is a linear high molar mass polysaccharide composed of D-glucuronic acid-β(1,3)-N-acetyl-D-glucosamine disaccharide units linked together through β(1,4) glycosidic bonds. It is a major constituent of the extracellular matrix (ECM) of vertebrates and is involved in many biological processes, such as cellular adhesion, mobility and differentiation processes [5–9]. Its properties are a function of its chain length: HA oligosaccharides (4 to 25 disaccharides) have an angiogenic action [9–11] contrary to native HA [12]. In the ECM, the action of HAase produces HA oligosaccharides which favor the production of new blood-vessels facilitating the development of cancer tumors. In addition to many polysaccharides, a lot of proteins are components of the ECM [13,14].

Several authors have shown that non-catalytic proteins, like bovine serum albumin (BSA), are able to increase the activity of HAase in order to enhance the sensitivity of its assay or detection [15]. They have shown that an optimal BSA concentration exists for enhancing the HAase activity: low BSA concentrations activate HAase, whereas high concentrations inhibit HAase. Some other works also report that BSA can activate the HAase activity. Gacesa et al. [16] report that serum proteins are able to enhance the HAase catalytic activity, and show that among the serum proteins, albumin has the greatest effect. Gold [17] observes that BSA is able to activate both human liver HAase and bovine testicular HAase at pH 4. Maingonnat et al. [15] show that other proteins such as hyaluronectin, hemoglobin and immunoglobulins are also able to enhance HAase activity and so, to increase the sensitivity of the HAase detection. All these results have been reported without any definite explanation. We have also shown that, in vitro, proteins like BSA or lysozyme (LYS) modulate the activity of HAase. All these observations have recently received an explanation when we have shown that the positive charges of HAase interact with the negative charges of HA to form non-specific electrostatic HA–HAase complexes in which HAase is no more catalytically active, and that the non-catalytic proteins compete with HAase to form electrostatic complexes with HA, resulting in the modulation of the HAase activity [18–22]. We have also shown that the phenomena of complex formation between HAase and HA is maximum at low ionic strength and pH 4 which is the optimal pH for HAase [23], but also exists at higher ionic strength including 150 mM [21] and higher pH values including pH 7 [23].

The mechanism we proposed can be summarized as follows [22]: At low HAase over HA stoichiometric ratio, the binding of HAase to HA is total and the HAase activity is nil. When the concentration of the non-catalytic protein is increased and if its net charge is positive, the protein is able to form complexes with HA by occupying the free HA complexation sites, non-occupied by HAase. When there are no more free sites and if the protein has a higher affinity for HA than that of HAase, the non-catalytic protein competes with HAase and forms complexes with HA by releasing HAase. HAase being free, recovers its catalytic activity. This phenomenon exists as long as HAase molecules form complexes with HA. The complex formation system is thus a ternary system involving HA, HAase and the non-catalytic protein [22]. When all the HAase molecules are released, they are all active and the HA hydrolysis rate is maximum. However, the non-catalytic protein continues to form complexes with HA. The complex formation system is

* Corresponding author. Tel.: +33 2 35 14 67 42; fax: +33 2 35 14 67 04.
E-mail address: jean-claude.vincent@univ-rouen.fr (J.-C. Vincent).

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now a binary system involving HA and the non-catalytic protein. At each time that one protein molecule forms a complex with HA, it occupies a given number of negative carboxyl groups on the HA molecule and hides a given number of potential cleavable β(1,4) sites for hydrolysis by HAase. These potential cleavable β(1,4) sites being the actual substrate of HAase [24], the concentration of the HAase substrate decreases. The HAase activity thus decreases at high concentrations of the non-catalytic protein [21,22].

To our knowledge, this is the only explanation for the dual role of inhibition/activation of the HAase activity by proteins. The facts were observed by several authors but no explanation was reported in the literature. As the reason lies in the competition between HAase and other proteins for their binding to HA, we propose here a simple and original modeling for this mechanism by using the physicochemical equations at equilibrium.

2. The experimental work

The experimental work concerning the dependence of the HAase activity with respect to the BSA concentration has been reported in previous papers [19,22,23]. The generalized shape of the BSA-dependence is drawn in Fig. 1a and shows four intervals separated by three critical points: i) Point A, at the interface between intervals ① and ②, corresponded to the concentration of the BSA molecules able to form additive complexes with the HA molecules already complexed with the HAase present in the system. This BSA concentration was noted $[BSA]_{HAase\ zero}$ because all the HAase molecules were complexed with HA and no free HAase molecules remained in solution leading to a quasi zero hydrolysis rate. ii) Point B, at the interface between intervals ② and ③, corresponded to the minimum BSA concentration needed so that the HA molecules were complexed with BSA alone. In B, all the HAase molecules were free in solution and able to catalyze the HA hydrolysis. The HA hydrolysis rate was maximum. This BSA concentration was noted $[BSA]_{min}$. In B, all the HA molecules are complexed with BSA with a characteristic BSA over HA ratio noted ψ_{min} [22]. iii) Point C, at the interface between intervals ③ and ④, corresponded to the highest BSA concentration able to produce a hydrolysable complexed HA. This BSA concentration was noted $[BSA]_{max}$. At this point, the HA molecules were too tightly complexed with BSA to be accessible to HAase and the HA hydrolysis rate was nil. All the potentially cleavable HA sites were hidden by the BSA molecules.

Our experimental studies [22] have also shown that we have to consider two types of system: i) a ternary HA/HAase/BSA system when the BSA concentration is lower than that corresponding to the B point, and ii) a binary HA/BSA system with all the HAase molecules free in solution when the BSA concentration is higher than that corresponding to the B point. When the ternary system is considered, we have shown that one HA molecule of 1 MDa can form complexes with approximately either 10 HAase molecules or 10 BSA molecules [22]. It means that statistically one HAase molecule, or one BSA molecule, forms an electrostatic complex with an HA fragment of about 265 disaccharides. When the binary system is considered, all the HAase molecules are free in solution and do not interact with HA for complex formation. In that case, we have shown that one HA molecule of 1 MDa can interact with a maximum of 64 BSA molecules [22]. It means that statistically one BSA molecule forms a non-substrate electrostatic complex with an HA fragment of about 38 disaccharides. The complexed HA fragment can no more be hydrolyzed by HAase because there is no place for HAase to catalytically interact with the corresponding cleavable β(1,4) sites of the HA fragment.

Other experiments, performed in the presence of 0.15 $mol\,L^{-1}$ ionic strength have shown that when the concentration of small ions in the medium is high enough to screen the charges on the two biopolymers, the HA/HAase system behaves as a Michaelis–Menten type enzyme [20]. This Michaelis–Menten type behavior is also observed at low ionic strength in the presence of BSA when the HA molecule complexed with BSA in a constant BSA over HA ratio is considered as the substrate entity.

The formation of electrostatic complexes between HA and proteins and the modulation of the HAase activity by proteins are not specific to BSA and have been observed with other proteins such as hyaluronectin [15], immunoglobulins [15] and lysozyme [23]. The modulation of the HAase activity in the presence of non-catalytic proteins requires two conditions: i) the protein has to be able to form a complex with HA and ii) this complex has to be more stable than the electrostatic complex formed between HA and HAase [22]. In order to reflect the non-specificity of the protein forming electrostatic complexes with HA, we shall use in our model the generic symbol of P for protein. For the ternary system, we assume that an HA fragment of n carboxyl groups, written HA_n, is able to form an electrostatic complex with either one HAase molecule or one P molecule. For the binary system, we assume that an HA fragment of m carboxyl groups, written HA_m, is able to form an electrostatic complex with one P molecule.

3. Theory

We assume that the enzymatic system is governed by the classical Michaelis–Menten equation giving the initial hydrolysis rate, V_i, as a function of the substrate S concentration:

$$V_i = k_2 \times [HAase] \times [S] / (Km + [S]) \tag{1}$$

Where [HAase] is the concentration of the free active enzyme and [S] is the substrate concentration which is equal to the concentration of the potentially cleavable β(1,4) bonds of HA [24]. Modeling of the system takes into account the two intervals successively, before the B point where the system is a ternary system and after the B point where the system is a binary system, HAase being no more involved in the complexes with HA.

3.1. Modeling of the ternary HA/HAase/P system: an expanded complex

Two complex formation equilibria exist in the system:

$$HA_n + HAase \rightleftarrows HA_n - HAase\ electrostatic\ complex \tag{2}$$

this complex is a potential substrate for HAase, but the complexed HAase is not active.

$$HA_n + P \rightleftarrows HA_n - P\ electrostatic\ complex \tag{3}$$

this complex is a potential substrate for HAase. The two equilibria are characterized by their dissociation constants K_{HAase} and K_P:

$$K_{HAase} = [HA_n] \cdot [HAase] / [HA_n - HAase] \tag{4}$$

$$K_P = [HA_n] \cdot [P] / [HA_n - P] \tag{5}$$

In addition to these equations, the mass conservation laws give:

$$[HAase] + [HA_n - HAase] = [HAase]_0 \tag{6}$$

$$[P] + [HA_n - P] = [P]_0 \tag{7}$$

$$[HA_n] + [HA_n - HAase] + [HA_n - P] = [HA_n]_0 \tag{8}$$

By expressing $[HA_n]$, $[HA_n - HAase]$ and $[HA_n - P]$ as a function of [HAase] by using Eqs. (4)–(8), we obtain the following third degree equation in [HAase]:

$$\begin{aligned}[HAase]^3 \times [K_{HAase} - K_P] + [HAase]^2 \times [K_{HAase}^2 - K_P \cdot K_{HAase} \\ + K_P \cdot [HAase]_0 - K_{HAase} \cdot [P]_0 - 2K_{HAase} \cdot [HAase]_0 - K_P \cdot [HA_n]_0 \\ + K_{HAase} \cdot [HA_n]_0] + [HAase] \times [HAase]_0 \times [K_P \cdot K_{HAase} - 2K_{HAase}^2 \\ + K_{HAase} \cdot [HAase]_0 + K_{HAase} \cdot [P]_0 - K_{HAase} \cdot [HA_n]_0] \\ + K_{HAase}^2 \cdot [HAase]_0^2 = 0\end{aligned} \tag{9}$$

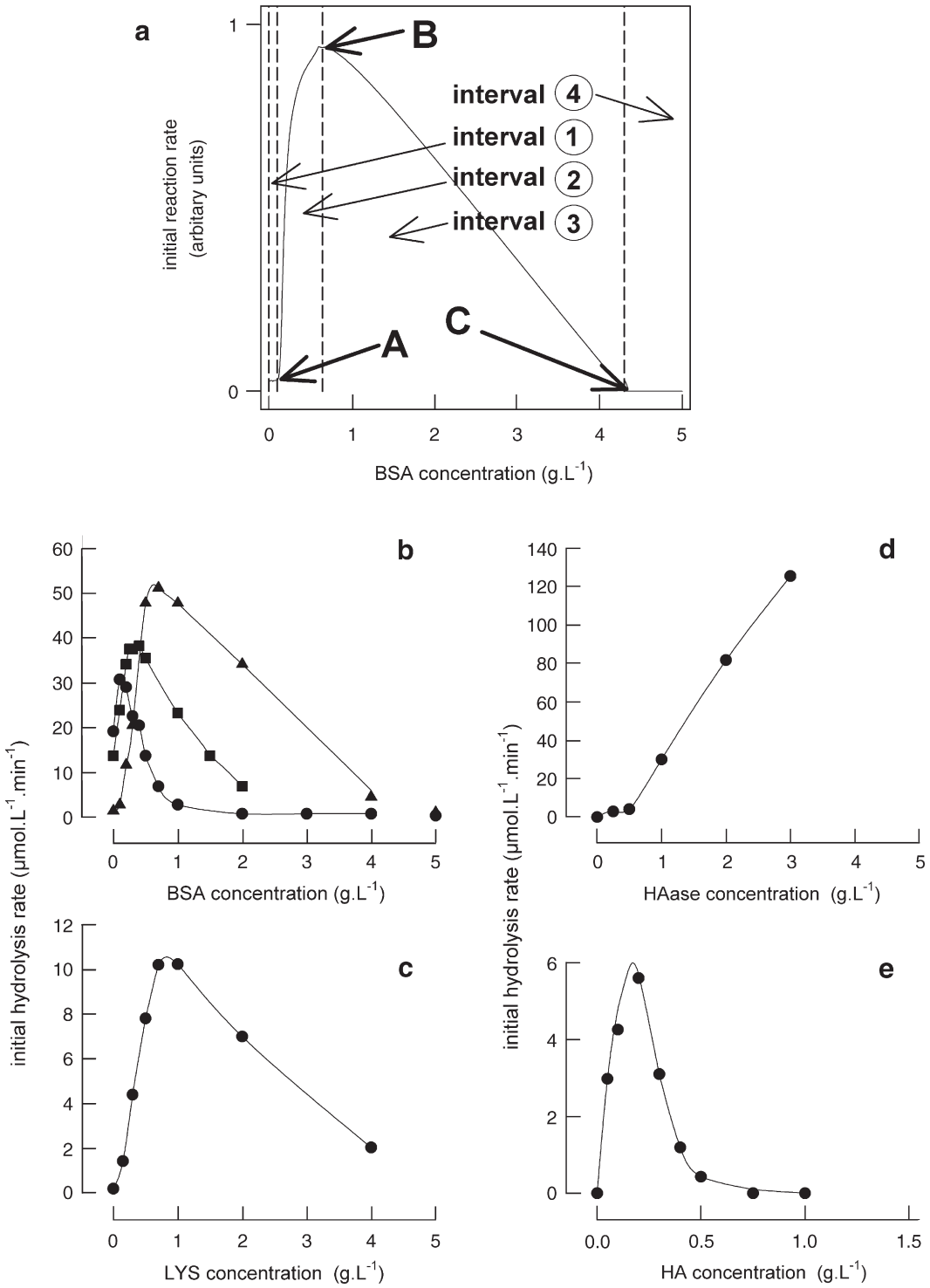

Fig. 1. a) Generalized experimental BSA-dependence of the HAase activity with the four intervals separated by the three noticeable points. Interval ① with no influence of BSA, interval ② with increase in the HAase activity by BSA, interval ③ with decrease in the HAase activity by BSA, and interval ④ with no influence of BSA. b) BSA-dependence of HAase at pH 4 for an HAase concentration of 0.5 $g L^{-1}$ and different HA concentrations: 0.25 $g L^{-1}$ (●), 0.50 $g L^{-1}$ (■), and 1 $g L^{-1}$ (▲) [22]; c) LYS-dependence of HAase at pH 5.25 for an HAase concentration of 0.5 $g L^{-1}$ and an HA concentration of 1 $g L^{-1}$ [23]; d) enzyme-dependence of HAase at pH 4 in the absence of BSA for an HA concentration of 1 $g L^{-1}$ [19]; e) substrate-dependence of HAase in the absence of BSA [20]. (All the experimental data used in this figure have been extracted from our previous work [19,20,22,23]).

where $[HA_n]_0$ is the initial concentration of HA fragments containing n carboxyl groups (i.e. containing n disaccharides of 401 g molar mass), $[P]_0$ the initial protein concentration and $[HAase]_0$ the initial HAase concentration ($M_w = 57{,}000\ g\,mol^{-1}$), expressed by:

$$[HA_n]_0 = [HA]_0(\text{in } g{\cdot}L^{-1}) / 401 / n \tag{10}$$

$$[P]_0 = [P]_0(\text{in } g{\cdot}L^{-1}) / M_w(\text{molar mass})\text{of P} \tag{11}$$

$$[HAase]_0 = [HAase]_0(\text{in } g{\cdot}L^{-1}) / 57,000 \tag{12}$$

The solution of Eq. (9) being a concentration, only the positive solution was considered. Moreover, the solution was always lower than or equal to $[HAase]_0$.

3.2. Modeling of the binary HA/P system: a dense complex

Once all the HAase molecules are released from HA and are free in the solution, the system becomes a binary HA/P system governed by the following equations:

$$HA_m + P \rightleftarrows HA_m - P \text{ electrostatic complex} \quad (13)$$

This dense complex is not a potential substrate for HAase. It is characterized by its dissociation constant:

$$K_{sat} = [HA_m] \cdot [P] / [HA_m - P] \quad (14)$$

In addition to Eq. (14), the mass conservation laws give:

$$[P] + [HA_m - P] = [P]_0 \quad (15)$$

$$[HA_m] + [HA_m - P] = [HA_m]_0 \quad (16)$$

By expressing [P] and [HA_m–P] as a function of [HA_m] by using Eqs. (14)–(16), we obtain the following second degree equation in binding site concentration [HA_m]:

$$[HA_m]^2 + [HA_m] \times [K_{sat} + [P]_0 - [HA_m]_0] - [HA_m]_0 \times K_{sat} = 0 \quad (17)$$

The solution of Eq. (17) being a concentration, only the positive solution was considered. Moreover, the solution was always lower than or equal to $[HA_m]_0$.

As only the free HA_m fragments are hydrolysable by HAase, the β(1,4) bond concentration, which is the actual substrate concentration, is then calculated as being $[S] = m \times [HA_m]$:

$$[S] = \left[-(K_{sat} + [P]_0 - [HA_m]_0) + \sqrt{(K_{sat} + [P]_0 - [HA_m]_0)^2 + 4[HA_m]_0 K_{sat}}\right] \times m/2 \quad (18)$$

where $[HA_m]_0$ is the initial concentration of HA fragments containing m carboxyl groups (i.e. containing m disaccharides of 401 g molar mass), and $[P]_0$ the initial protein concentration, expressed by:

$$[HA_m]_0 = [HA]_0 (\text{in g·L})^{-1} / 401 / m \quad (19)$$

$$[P]_0 = [P]_0 (\text{in g·L})^{-1} / M_w (\text{molar mass}) \text{ of P} \quad (20)$$

Finally, the whole system is governed by Eq. (1) in which [HAase] is given by Eq. (9) and [S] by Eq. (18).

In the case where the protein is BSA, m equals 38 and n equals 265 [22].

4. *In silico* experiments

Although any set of data may be used to run the model, we paid attention to use experimentally compatible data. The set of data used was a set of real experimental data: i) the *m* and *n* stoichiometric constants and the K_m constant were deduced from our experimental work using BSA as P protein [22]: m = 38, n = 265 and $K_m = 0.5\ gL^{-1}$, ii) the total concentrations, $[HA]_0$, $[P]_0$ and $[HAase]_0$, were deduced from the experiments described in our previous papers [19,20,22]: $[HA]_0$ was ranged from 0 to 5 gL^{-1}, 1 gL^{-1} was chosen when only one value was required; $[HAase]_0$ was ranged from 0 to 5 gL^{-1}, 0.5 gL^{-1} was chosen when only one value was required; $[P]_0$ was ranged from 0 to 5 gL^{-1}, and iii) the dissociation constants K_P and K_{HAase} were as suggested by the literature. Although there is no data concerning the HA complexes formed with either BSA or HAase in the literature, a value of $10^{-7}\ molL^{-1}$ was attributed to K_P, as suggested by the work of Van Damme et al. [25] concerning the HA–LYS complexes. A value of $10^{-5}\ molL^{-1}$ for K_{HAase} was deduced from the fact that both BSA and LYS are able to compete with HAase [23] in forming complexes with HA, suggesting a value of K_{HAase} higher than that of K_P.

4.1. The protein-dependence curve

Variations of the free HAase concentrations as a function of the protein concentration are shown in Fig. 2a for different HA concentrations. It shows that: i) at low HA concentrations, the free HAase concentration increased when the protein concentration was increased and stabilized at its maximum equal to $[HAase]_0$. The HA concentration was not sufficiently high to form complexes with the whole HAase. Even without any protein P, there were some HAase molecules free in solution and catalytically active, producing a non-zero hydrolysis activity. When the protein concentration was increased, the protein molecules exchanged with the HAase molecules and the free HAase concentration increased and stabilized at the $[HAase]_0$ level. ii) at middle and high HA concentrations, the free HAase concentration first remained at its minimum value (zero) when the protein concentration was increased up to a critical value, then increased when the protein concentration was increased above the critical value. In that case, the HA concentration was sufficiently high to form complexes with the whole HAase in the absence of

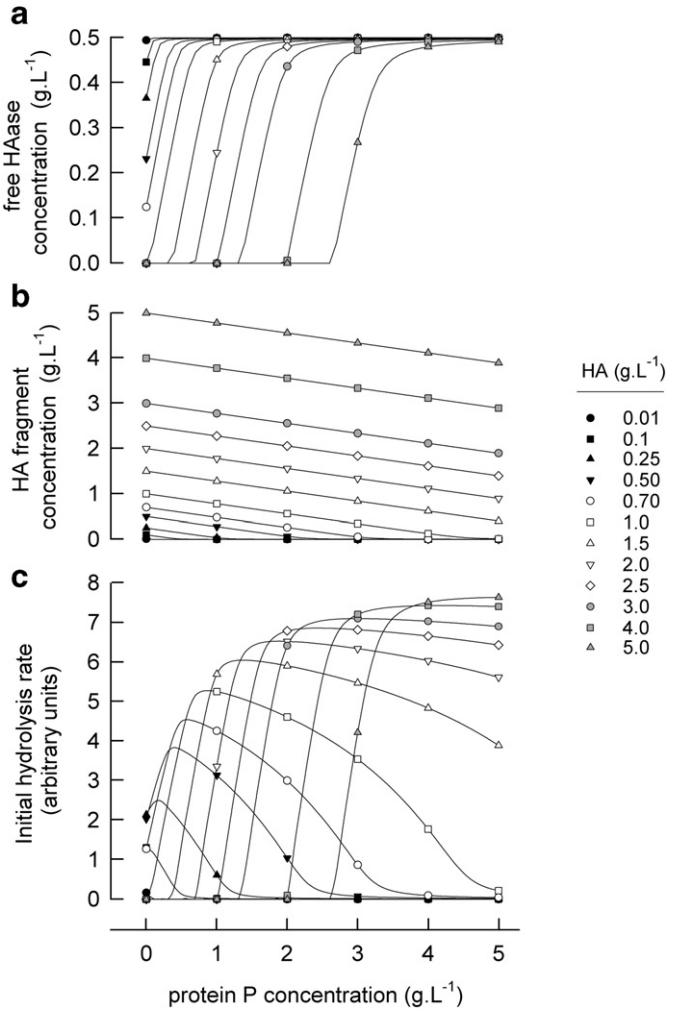

Fig. 2. Superimposition of the *in silico* protein-dependences of the HAase activity: Free HAase concentration (part a), potentially cleavable β(1,4) bond concentration (part b), and HAase activity (part c), as a function of the protein P concentration for different HA concentrations ranging from 0.01 to 5 gL^{-1}. Calculations were performed with the following constants: $K_{HAase} = 10^{-5}\ molL^{-1}$; $K_P = 10^{-7}\ molL^{-1}$; $K_{sat} = 0.1\ molL^{-1}$; $[HAase]_0 = 0.5\ gL^{-1}$; and $K_m = 0.5\ gL^{-1}$.

protein. All the HAase molecules formed complexes with HA and the free HAase concentration was nil. However, there were some places on the HA molecule allowing the protein P to form complexes. A low amount of protein P was thus not able to release HAase from the electrostatic complexes, and the free HAase concentration remained nil when low protein concentrations were present in the system. When higher protein concentrations were present in the system, HAase molecules were released and the free HAase concentration increased and stabilized at the $[HAase]_0$ level.

Variations of the potentially cleavable β(1,4) bond concentration, [S], as a function of the protein concentration are shown in Fig. 2b for different HA concentrations. It shows that the cleavable β(1,4) bond concentration was a quasilinear function of the protein concentration. Statistically, the higher the protein P concentration, the more dense the HA–P complexes and the lower the accessible cleavable β(1,4) bond concentration.

Finally, the combination of the two equations, (Eqs. (9) and (18)), gives the variation of the HAase activity as a function of the protein P concentration for different HA concentrations (Fig. 2c). The curve generally shows four intervals, as for example for an HA concentration of 1 gL^{-1}: i) at low protein concentrations, lower than 0.1 gL^{-1}, the HAase activity was nil because all the HAase molecules are complexed with HA, ii) at protein concentrations ranging from 0.1 to 0.7 gL^{-1}, the HAase activity strongly increased up to a maximum because HAase molecules are released, iii) at protein concentrations ranging from 0.7 to 5 gL^{-1}, the HAase activity decreased down to zero because, although all the HAase molecules are free, the potentially cleavable sites on HA strongly decrease because of the fixation of P, and iv) at protein concentrations higher than 5 gL^{-1}, the HAase activity remained nil because the P molecules entirely cover the HA molecule. 0.7 gL^{-1} is thus the optimal P concentration when the HA concentration is 1 gL^{-1}. Thus 0.7 is the optimal BSA over HA mass ratio. The theoretical curve was similar to the experimental one (Fig. 1a), signifying that the HA–P complexes actually modulate the HAase activity. Taken together, these theoretical results were closely similar to those experimentally observed (Fig. 1b) [22].

4.2. Stoichiometry of the HA–P complexes at the B point

All the protein-dependence curves corresponding to different HA concentrations showed a maximum which is equivalent to the B point of the generalized experimental curve (Fig. 1a). In these B points, all the HAase molecules were free in solution (Fig. 2) and HA was complexed with the protein P only. When the protein P concentrations corresponding to these B points were plotted against the HA concentration, a straight line was obtained (Fig. 3). It means that, in B, the HA–P complexes were always in the same stoichiometric ratio, which is optimal for the HAase activity. In the *in silico* experiments described here, the optimal stoichiometric ratio was equal to 0.7. These theoretical results were similar to those obtained experimentally with BSA at pH 4 and 37 °C [22], where the optimal stoichiometric ratio was equal to 0.65.

4.3. Michaelis–Menten behavior of the model

When the hydrolysis rates corresponding to the B points were plotted against the HA concentration, a hyperbolic curve was obtained (Fig. 4) signifying that the model obeyed the Michaelis–Menten type law. It means that, according to our modeling, HAase actually behaved as a Michaelis–Menten type enzyme when HA was complexed with the protein P in the optimal stoichiometric ratio. When other values of the P over HA ratio were considered, ranging from 0.7 to 2 (0.7; 1; 1.5; and 2), HAase also behaved as a Michaelis–Menten type enzyme (results not shown). More generally, HAase behaves as a Michaelis–Menten type enzyme when the HA–protein complex in constant stoichiometric ratio (a given complexation state) is considered as being the substrate. These theoretical results were similar to those obtained experimentally with BSA at pH 4 and 37 °C [22].

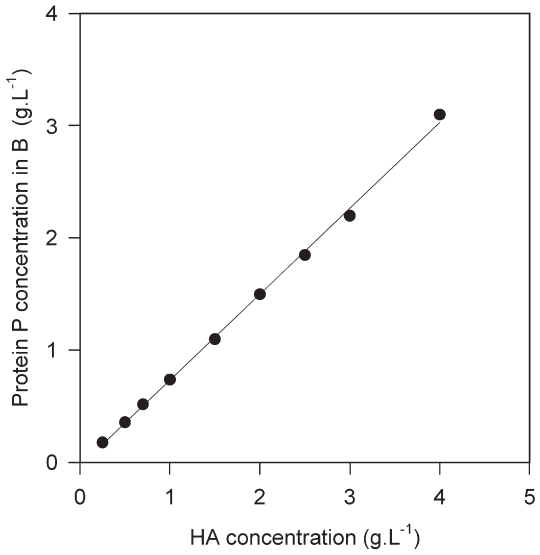

Fig. 3. Stoichiometry of the HA–P complexes in B. Protein P concentration corresponding to the B points plotted against the HA concentration. The slope represents the protein P over HA stoichiometric ratio in B. Constants and data were: $K_{HAase} = 10^{-4}$ mol L^{-1}; $K_P = 10^{-7}$ mol L^{-1}; $K_{sat} = 0.1$ mol.L^{-1}; $[HAase]_0 = 0.5$ g L^{-1}; and $K_m = 0.5$ g L^{-1}.

4.4. Behavior of the model in the absence of protein P

4.4.1. Substrate-dependence of the HAase activity

Fig. 5 represents the variations of the HAase activity as a function of the HA concentration in the absence of protein P for an HAase concentration of 0.5 gL^{-1}. The HAase activity first increased when the HA concentration was increased, then reached a maximum and finally decreased down to zero at high substrate concentration, instead of reaching a plateau as for a Michaelis–Menten type enzyme. It shows that the theoretical atypical substrate-dependence of HAase is similar

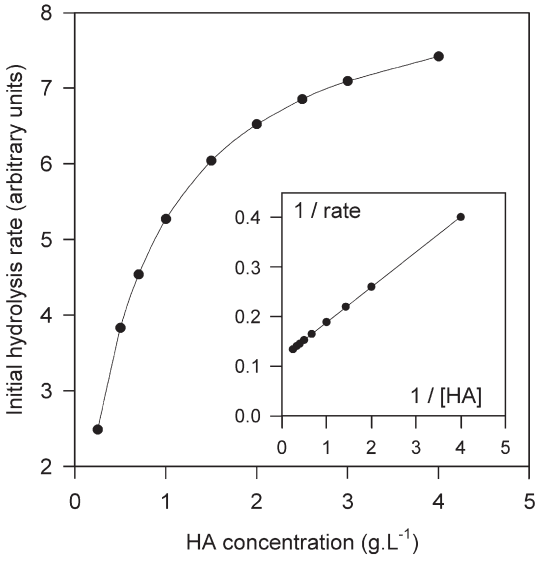

Fig. 4. HAase activity as a function of the substrate concentration in B points: Michaelis–Menten type behavior of the *in silico* substrate-dependence of the HAase activity. The insert shows the Lineweaver and Burk linearization of the same data. Constants and data were: $K_{HAase} = 10^{-5}$ mol L^{-1}; $K_P = 10^{-7}$ mol L^{-1}; $K_{sat} = 0.1$ mol L^{-1}; $[HAase]_0 = 0.5$ g L^{-1}; and $K_m = 0.5$ g L^{-1}.

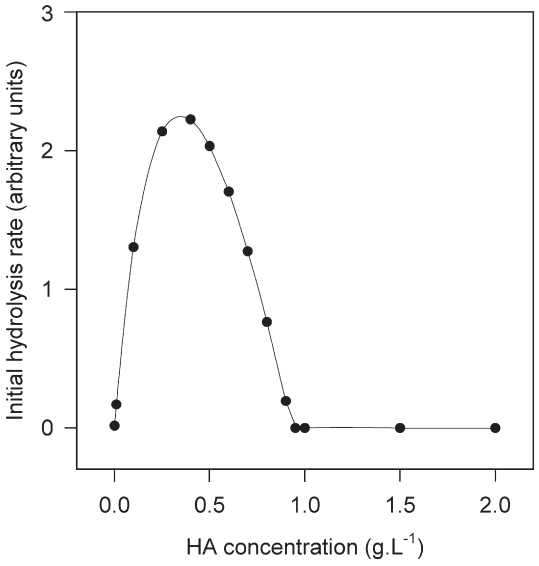

Fig. 5. *In silico* substrate-dependence of the HAase activity in the absence of protein P. HAase activity as a function of the HA concentration for an HAase concentration of 0.5 gL^{-1}. Constants and data were: $K_{HAase} = 10^{-5}$ $molL^{-1}$; $K_P = 10^{-7}$ $molL^{-1}$; $K_{sat} = 0.1$ $molL^{-1}$; and $K_m = 0.5$ gL^{-1}.

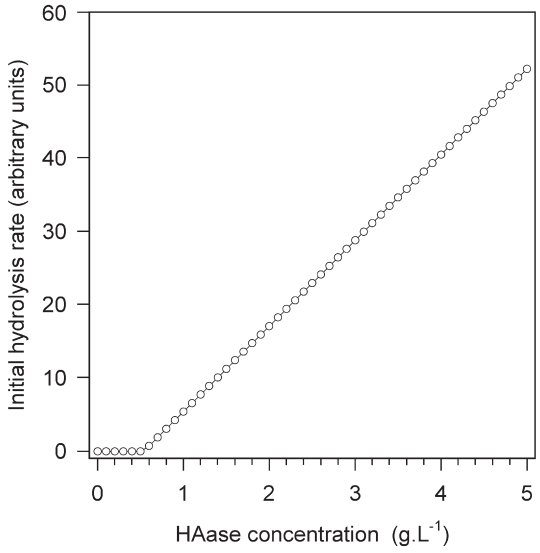

Fig. 6. *In silico* enzyme-dependence of the HAase activity in the absence of protein P. HAase activity as a function of the HAase concentration for an HA concentration of 1 gL^{-1}. Constants and data were: $K_{HAase} = 10^{-5}$ $molL^{-1}$; $K_P = 10^{-7}$ $molL^{-1}$; $K_{sat} = 0.1$ $molL^{-1}$; and $K_m = 0.5$ gL^{-1}.

in shape to the experimental curve already reported in our previous paper (Fig. 1e) [20]. This theoretical substrate-dependence of HAase shows that the electrostatic HA–HAase complexes are definitely responsible for the inhibition of the HAase activity by high substrate concentrations. The curve also shows that 0.5 gL^{-1} of HAase was entirely complexed by 1 gL^{-1} of HA. This corresponds to 287 HA carboxyl groups per HAase molecule, close to the assumptions used in the modeling.

4.4.2. Enzyme-dependence of the HAase activity

Fig. 6 represents the variations of the HAase activity as a function of the HAase concentration in the absence of protein P for an HA concentration of 1 gL^{-1}. The HAase activity first remained nil when the HAase concentration was increased from 0 to 0.6 gL^{-1}. Then, it increased when the HAase concentration was increased from 0.6 to 5 gL^{-1}. The curve shows that 0.6 gL^{-1} of HAase were complexed by 1 gL^{-1} of HA, and this quantity of enzyme was not catalytically active, as stated in our assumptions. This represents the binding capacity of 1 gL^{-1} of HA. For higher HAase concentrations, the hydrolysis rate increased with the HAase concentration. Fig. 6 shows that the theoretical atypical enzyme-dependence of HAase is similar in shape to the experimental curve already reported in our previous paper (Fig. 1d) [19]. This theoretical enzyme-dependence of HAase shows that the electrostatic HA–HAase complexes are definitely responsible for the inhibition of the HAase activity by high substrate over HAase concentration ratios.

4.5. Extensions of the model

To build a model for a biological or physicochemical process is useful to test our understanding of the process and to identify the elementary mechanisms and the main laws involved. In the present system, this step can be considered satisfactory as we have shown how the HA–protein complexes actually modulate the HAase activity. To build a model can also be used to test the behavior of the process when one or several of its elements are changed, for example how it works when another protein forming a complex with HA with a different dissociation constant is considered. It is thus interesting to predict the behavior of the system for different values of the parameters. The system being controlled by the competition between HAase and the other protein to form electrostatic complexes with HA, the main parameters are the dissociation constants of the HA–protein complexes.

4.5.1. Variations of K_P and K_{HAase}

The constants K_p and K_{HAase} mainly concerned interval ② as they controlled the exchange between the protein P and HAase in forming complexes with HA. When K_P was high, the protein P was not a good competitor of HAase for the formation of complexes with HA and HAase was released with difficulty, leading to a relatively low HAase activity in B. Conversely, a low value of K_P led to a complete complexation of the protein P, a complete release of HAase and thus, a high HAase activity in B (Fig. 7a). For K_{HAase}, the behavior was symmetrical: a high value of K_{HAase} led to an easy and complete release of HAase and a high HAase activity in B. Conversely, a low value of K_{HAase} led to a partial release of HAase, a high percentage of HAase complexed with HA, and a low HAase activity in B (Fig. 7b). In fact, K_P and K_{HAase} essentially concerned the HAase activity in the vicinity of point B in intervals ② and ③. A high K_P over K_{HAase} ratio led to a low HAase activity in B and a smooth transition between intervals ② and ③; a low K_P over K_{HAase} ratio led to a high HAase activity in B and an angular transition between intervals ② and ③.

4.5.2. Variations of K_{sat}

The constant K_{sat} mainly concerned intervals ③ and ④ as it controlled the inhibition of the enzymatic system by over-binding of HA. Low values of K_{sat} signify that the dense HA–protein complexes were very stable. In that case, the protein concentration required for complete inhibition of the system was low. High values of K_{sat} signify that the dense HA–protein complexes were formed with difficulty; even at high protein concentrations, the HAase activity did not reach the zero level and the HAase activity could not be totally suppressed by addition of protein P (Fig. 7c).

4.5.3. Variations of all the dissociation constants: Effect of ionic strength

The increase in ionic strength is characterized by an elevation of the concentration of small ions which screened the fixed charges at the surface of the biomacromolecules, HA, HAase and P. The electrostatic

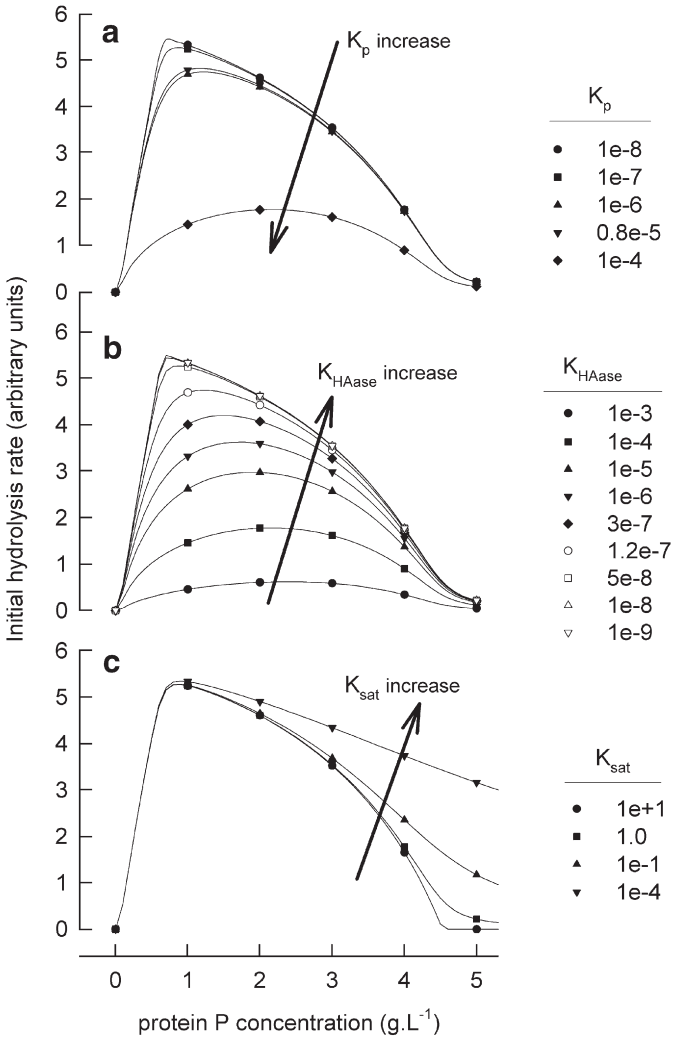

Fig. 7. Superimposition of the *in silico* Protein P-dependences of the HAase activity: HAase activity as a function of the protein P concentration for different values of the dissociation constants: K_P (part a), K_{HAase} (part b), and K_{sat} (part c). Calculations were performed with $[HAase]_0 = 0.5\ g\,L^{-1}$; $[HA]_0 = 1\ g\,L^{-1}$; and $K_m = 0.5\ g\,L^{-1}$. The dissociation constants were equal to: $K_P = 10^{-7}\ mol\,L^{-1}$; $K_{HAase} = 10^{-5}\ mol\,L^{-1}$; and $K_{sat} = 0.1\ mol\,L^{-1}$, except when the constant was the adjustable parameter.

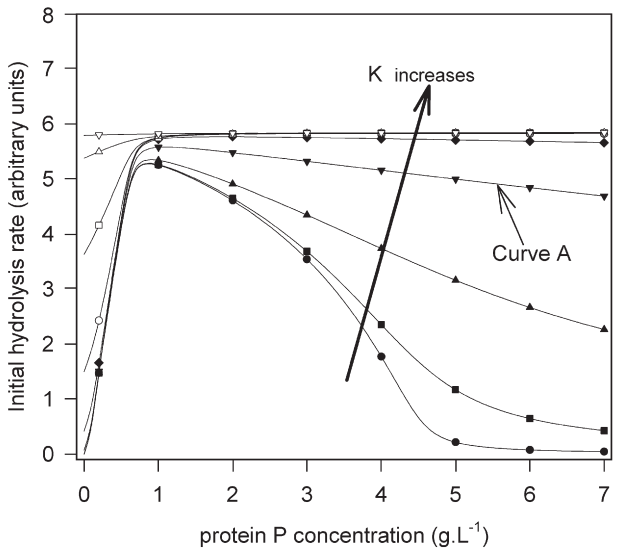

Fig. 8. Superimposition of the *in silico* Protein P-dependences of the HAase activity: HAase activity as a function of the protein P concentration for different values of the dissociation constants. Calculations were performed with the following constants: $K_{HAase} = 10^{-5}\ mol\,L^{-1}$; $K_P = 10^{-7}\ mol\,L^{-1}$; $K_{sat} = 0.1\ mol\,L^{-1}$; $[HAase]_0 = 0.5\ g\,L^{-1}$; $[HA]_0 = 1\ g\,L^{-1}$; and $K_m = 0.5\ g\,L^{-1}$. For the different curves, all the dissociation constants were multiplicated by the same factor: 1 (●), 10 (■), 10^2 (▲), 10^3 (▼), 10^4 (◆), 10^5 (○), 10^6 (□), 10^7 (△), 10^8 (▽).

interactions between two biomacromolecules thus decreased and the dissociation constants of the complex equilibrium increased. Here, the increase in the ionic strength can thus be modeled by an increase in all the dissociation constants K_{HAase}, K_P and K_{sat}. Fig. 8 shows that such an increase in these dissociation constants progressively erased the effect of the protein P on the HAase activity. The inhibiting effect at high P concentrations was first erased, then the enhancing effect at low P concentrations. At very high dissociation constants, there was no more effect of P proteins. Nevertheless, according to Lenormand et al. [20], an ionic strength close to 150 $mmol\,L^{-1}$, which is the physiological ionic strength, corresponds to a K_{HAase} value of the order of magnitude of $10^{-3}\ mol\,L^{-1}$, represented by curve A in Fig. 8. In that case, activation of the HAase activity due to low protein P concentrations was still observable but not its inhibition by high protein P concentrations.

5. Materials and methods

The paper is essentially concerned by the theoretical model and the corresponding equations. The experimental curves are only used to illustrate the different behaviors of the system. All the experimental data have been extracted from previous papers and the complete experiments, including the materials and the methods used, have been described in detail in these papers. Here, only the materials and methods for computations are this study's concern.

Computations have been performed with a PC equipped with Microsoft Excel 97 software. Two sets of values (ranging from 0 to $5\ g\,L^{-1}$) were considered for $[HA]_0$ and $[P]_0$ with a fixed $[HAase]_0$ value. For each couple, $[HA]_0$ and $[P]_0$, the Excel module, written with Visual Basic (Microsoft), first calculates the HAase concentration by using the trigonometric resolution (Cardan) of Eq. (9) and considering only the positive solution. $[HA_n]$, $[HA_n - HAase]$, [P] and $[HA_n - P]$ were then successively calculated by using a combination of Eqs. (4)–(8). $[HA_m]$ was then calculated by using Eq. (17) and considering the positive solution. [S] was calculated by using Eq. (18). Finally, the hydrolysis rate was calculated by using Eq. (1) with an arbitrary value of k_2.

6. Discussion and conclusion

The fact that the theoretical protein-dependence curves of HAase are closely similar to the experimental curves [22] shows that our modeling is consistent with the physicochemical HA/HAase/BSA system that we have studied experimentally. The assumptions consisting in the formation of electrostatic complexes between HA and proteins, including HAase, and the non-activity of the complexed HAase are sufficient to explain the complete behavior of the system. From a general point of view, this concerns the modulation of the catalytic activity of a positively charged enzyme by another positively charged protein (or a polycation) through a competition to form electrostatic complexes with a polyanionic substrate. The HA/HAase system is one of the systems concerned, the systems concerning RNA or DNA with the numerous associated enzymes could be another among them.

Without any added non-catalytic protein, this system is characterized by an inhibition of the HAase activity at high HA over HAase concentration ratios, not due to a classical inhibition of the enzyme by an excess of substrate mechanism, but by a sequestration of the enzyme at high HA

concentration by electrostatic binding with HA. For the substrate-dependence, the HAase activity first increases when the HA concentration is increased, then reaches a maximum and finally decreases down to zero at high substrate concentrations. For the enzyme-dependence, the HAase activity shows two phases: it remains nil at low HAase concentrations because of its non-catalytic binding to HA and increases with its concentration at high values. When a non-catalytic protein P is added, the HAase activity is modulated by the competition between the complexes formed between HA and HAase on one hand, and between HA and the protein P on the other hand. This modulation occurs when: i) the interactions involved in the HA–P complexes are stronger than those involved in the HA–HAase complexes and ii) the complexed HAase is no longer catalytically active. This modulation is thus non-specific with respect to the protein, but depends on the charge and on the pI value of the protein, including HAase, and on the value of the dissociation constant of the HA–P complexes.

The non-specificity of the HAase modulation with respect to the protein has been experimentally confirmed by the fact that LYS, which has a high pI value, behaves as BSA even at a pH higher than 4 (Fig. 1c) [23]. LYS and BSA exactly behave as the generic P protein [23]. On a general point of view, our model is based on the existence of HA–protein complexes. This could be confirmed by experimental determination of the temperature-dependence of the HAase catalyzed reaction in the presence or absence of the P protein. This could also be shown by chemical analysis of the precipitates obtained. The experimental demonstration of the existence of such HA–protein complexes has been performed by separation, extraction and chemical analysis of the precipitates. The conclusion was that precipitates always contain both HA and the protein [21]. This fact also shows that HA, at least at the concentrations used in the experiments, does not induce or facilitate protein aggregation/precipitation. All of this allows us to fix the limits of validity of our model which considers a competition between the two electrostatic HA–HAase and HA–P complexes, assuming that the two proteins are positively charged. It thus excludes interactions between the two proteins, P and HAase, which could occur at higher pH values where one of the proteins could be negatively charged, and other phenomena such as protein aggregation or changes in protein structure (not experimentally observed).

The generalized protein-dependence of the HA hydrolysis catalyzed by HAase shows four intervals delimited by three noticeable points. i) Point A, at the interface between intervals ① and ②, corresponds to the concentration of the protein P molecules able to form additive complexes with the HA molecules already complexed with the whole HAase present in the system. All the HAase molecules are complexed with HA and no free HAase molecules remain in solution leading to a quasizero hydrolysis rate. ii) Point B, at the interface between intervals ② and ③, corresponds to the minimum protein P concentration needed so that the HA molecules are complexed with P alone. All the HAase molecules are free in solution and are able to catalyze the HA hydrolysis. In B, the HA hydrolysis rate is maximum. iii) Point C, at the interface between intervals ③ and ④, corresponds to the highest protein P concentration able to produce a hydrolysable complexed HA. In C, the HA molecules are too tightly complexed with the protein P to be accessible to HAase and the HA hydrolysis rate is nil. All the potentially cleavable HA sites are hidden by the P molecules.

Intervals ① and ② are characterized by a high and quasiconstant cleavable HA site concentration and a variable free HAase concentration. The HAase activity is thus controlled by the free HAase concentration. In intervals ① and ②, the system is a ternary HA/HAase/Protein P system since there is a co-complexation of HAase and P on the HA molecules. Interval ③ is characterized by a high and constant, free HAase concentration and a variable cleavable HA site concentration. The HAase activity is thus controlled by the non-complexed cleavable HA site concentration. In interval ③, the system is a binary HA/protein P system associated with a constant HAase concentration since HAase is no more complexed with HA. In our simulations, the P over HA stoichiometry is 10 proteins per HA molecule in B and 64 proteins per HA molecule in C. The low P over HA stoichiometry in intervals ① and ②, associated with the high P over HA stoichiometry in interval ③ may give rise to an interesting question. It may suggest that a change in the HA conformation may occur in the vicinity of the B point, such as an unfolding of the HA molecule due to the screening of a sufficient number of negative charges by the protein P. We may imagine that the low stoichiometry concerns the BSA molecules in electrostatic interaction with the HA molecule in the random coil conformation, and that the high stoichiometry concerns the BSA molecules in electrostatic interaction with the HA molecule in the unfolded conformation. This may justify the use of two different dissociation constants for the complexes formed between HA and P. This may also suggest electrostatic interactions between P proteins themselves, which bear both positive and negative charges.

Since the theoretical protein-dependence of the HA hydrolysis catalyzed by HAase is very similar to the experimental protein-dependence, our model can also be used to estimate some unknown parameters such as the dissociation constant of the HA–HAase electrostatic complex. According to our *in silico* experimentations, the shape of the protein-dependence of the HA hydrolysis depends on the dissociation constants and on their ratio. Fig. 7 (parts a and b) shows that the shape of the experimental BSA-dependence of the HA hydrolysis catalyzed by HAase [22] in the vicinity of the B point is very similar to the shape of the theoretical protein-dependence when the K_{HAase} over K_P dissociation constant ratio is close to 100. As the value of the dissociation constant for the complex formed between HA and LYS (another protein able to modulate the HAase activity) has been estimated to 10^{-7} M or 10^{-8} M [25], we suggest that the value of the dissociation constant for the HA–HAase complex is close to 10^{-6} M. From a general point of view, the shape of the protein-dependence can furnish information about the values of the dissociation constants of the different HA–protein complexes under various ionic strength conditions.

P can thus be considered as both an activator for the HA/HAase system when considered at low concentration by avoiding the binding of HAase on the HA molecule and an inhibitor when considered at high concentration by covering the HA molecule. In fact, the enhancement of the HAase activity in the presence of P, is not an activation of the enzyme by itself, but rather the suppression of its inhibition due to the abolishment of the binding of the enzyme molecules to HA. Because of the electrostatic nature of the HA–P interactions, this occurs mainly at low levels of ionic strength, but recent experiments have shown that the phenomenon of complex formation still exists at 0.15 M ionic strength, which is usually considered as the physiological ionic strength, and at low HAase over HA ratios [20,21]. The apparent activation/inhibition of HAase by proteins, such as BSA, have been reported several times in the literature without any definitive explanation. Our results have very important implications on the measure of the HAase activity in biological fluids, and especially in serum or plasma, since it has been shown that the HAase activity measured in serum could be a signal of liver damages [26]. When measured in urine, the HAase activity is a bladder cancer indicator [3] and when measured in plasma, it may be a tumor indicator [27]. This explains why several research groups are nowadays involved in measuring the exact value of the HAase activity in biological fluids.

Moreover, our good understanding of the possible mechanism for the modulation of the HAase activity by proteins is of great interest in cancer biology since the enhancement/suppression of the HAase activity in the ECM of tumor cells may be involved in the tumor development. The atypical behavior of the initial reaction rate of the HA hydrolysis as a function of the HA concentration under low ionic strength conditions is characterized by a highly non-linear and non-monotonous relationship. Such behavior could be of great importance

in extracellular matrix regulation, under either normal or pathological conditions. On one hand, although tumoral HAase [28] is different from testicular HAase, there are many similarities between the two enzymes, particularly concerning the kinetic behavior [29]. Atypical behavior such as that ascribed for HAase could be of great interest in cancer research since many authors have already mentioned that there are high HA and HAase concentrations in tumor tissues compared to normal tissues [30–34]. Our results are also very important for cancer research for which the problem of inhibition of HAase is nowadays crucial [35]. The actual tendency is to consider that HAase inhibitors are ubiquitous in mammalian tissues and biological fluids such as plasma, serum, urine and saliva. In serum, studies suggest that the circulating inhibitor has the characteristics of the inter-α-inhibitor family [36] and that a covalent linkage with polysaccharides, such as chondroïtin sulfate, is required for HAase inhibition. However, the inhibitors of the HAase activity are still uncharacterized and many studies were undertaken in the last decade to understand the inhibition phenomenon and define the inhibitor. Our studies suggest that high molar weight HA could be a good candidate for this type of ubiquitous and non-specific inhibition which can be reversed by pulses of ionic strength or protein fluxes, and involved in the regulation of HAase in the ECM.

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