Unbinding of Hyaluronan Accelerates the Enzymatic Activity of Bee Hyaluronidase

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Hyaluronan (HA), a polymeric glycosaminoglycan ubiquitously present in higher animals, is hydrolyzed by hyaluronidases (HAases). Here, we used bee HAase as a model enzyme to study the HA-HAase interaction. Located in close proximity to the active center, a bulky surface loop, which appears to obstruct one end of the substrate binding groove, was found to be functionally involved in HA turnover. To better understand kinetic changes in substrate interaction, binding of high molecular weight HA to catalytically inactive HAase was monitored by means of quartz crystal microbalance technology. Replacement of the delimiting loop by a tetrapeptide interconnection increased the affinity for HA up to 100-fold, with a $K_D$ below 1 nM being the highest affinity among HA-binding proteins surveyed so far. The experimental data of HA-HAase interaction were further validated showing best fit to the theoretically proposed sequential two-site model. Besides the one, which had been shown previously in course of x-ray structure determination, a previously unrecognized binding site works in conjunction with an unbinding loop that facilitates liberation of hydrolyzed HA.

The HAase isolated from the venom gland of honey bee (bee venom HAase, or BVH) was the first HAase member of the endo-$\beta$-N-acetyl-hexosaminidase family (EC 3.2.1.35), to which also all human HAases belong, that could be molecularly cloned and recombinantly expressed (11). BVH is an endoglycolytic enzyme which catalyzes the hydrolysis of $\beta$-1,4 glycosidic bond of HA, as well as although with consistently less efficient, chondroitin sulfates (12). In case of HA hydrolysis, the reducing end is generated at the terminal N-acetyl-d-glucosamine.

Many structural features of BVH are also present in human HAases such as HYAL1 (13) or HYAL2 (14). The 3-dimensional structure of BVH could be elucidated by means of x-ray crystallography (15). Moreover, the exact positioning of a HA tetramer tethered directly adjacent to the active center could be resolved. This data greatly argued for an acid/base catalytic mechanism, in which glutamic acid acts as the proton donor and the N-acetyl carbonyl group of the substrate as the nucleophilic base (16). The presence of an extended furrow spanning one hemisphere of the globular structure of the bee enzyme further suggested that long parts of the HA chain interact also with sites other than that next to the active center.

In this study, we used BVH as a model enzyme, primarily because we could recently establish methods for high-yield recombinant expression in Pichia pastoris (12). We also wanted to investigate the function of a bulky structure delimiting one end of the putative substrate binding groove in acid-active human serum hyaluronidase 1, and as recently shown by us to be involved in regulating this enzyme’s activity with respect to varying pH (17). Whether structural determinants in the close vicinity to this particular structure are actually involved in regulating substrate binding, or solely influence the active center by long distance conformational changes remained elusive.

To address this question experimentally, we performed extensive kinetic analysis with wild-type recombinant BVH, as well as with a variant lacking the bulky formation. The results obtained suggest a likely role in substrate binding for the region next to the bulky formation. Working along this line we were able to reveal distinct kinetic parameters of substrate binding employing quartz crystal microbalance (QCM) technology (18, 19). Taken together, the presence of a second binding site being located opposite the face of the bulky formation could be demonstrated (Fig. 1A). This site probably controls product release.

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1–3 and Figs. 1–6.

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3 The abbreviations used are: HA, hyaluronan; CS-A, chondroitin sulfate-A; CS-B, chondroitin sulfate-B; CS-C, chondroitin sulfate-C; BVH, bee venom hyaluronidase; QCM, quartz crystal microbalance.
Unbinding Site in Bee Hyaluronidase

EXPERIMENTAL PROCEDURES

Materials—Hyaluronic acid potassium salt from human umbilical cord with an estimated average molecular mass of 750 kDa (H1504, lot no. 097K1495), chondroitin sulfate A (27042, lot STBC0183V) with an estimated average molecular mass of 21 kDa (20, 21), chondroitin sulfate B (C3788, lot no. 080M1668V) of ~42 kDa (21), chondroitin sulfate C (C4384, lot no. 1426300V) of ~60 kDa (22), N-acetyl-glucosamine (A8625), potassium tetraborate tetrahydrate (P5754), and p-di-methyl-benzaldehyde (D2004) were purchased from Sigma.

Molecular Modeling—The three-dimensional structure of BVH has recently been deciphered in the presence of a HA tetrasaccharide (Protein Data Bank code 1FCV) to validate the degree of native-ness of the BVH isoforms compared with the wild-type structure (23).

Mutagenesis of Hyaluronidase—Numbering of the amino acids of BVH starts with Thr in its mature form (15). An inactive variant of BVH named NQ-BVHWT was obtained by mutagenesis yielding a cDNA encoding for the inactive iso- tion vector, pPIC9, in frame with the —factor signal sequence.

This cDNA was further modified by replacement with the endonuclease yielding a cDNA encoding for the inactive iso- tion vector, pPIC9, in frame with the —factor signal sequence.

The initial reaction rates were calculated as the values

Equation 1.

\[
[\text{Ch}]_t = A_1(1 - e^{-kt}) + A_2(1 - e^{-kt})
\]

The Michaelis-Menten kinetic parameters could be calculated by non-linear regression of the data using KaleidaGraph (version 4.1.1, Synergy Software).

Quartz Crystal Microbalance Measurements—Carboxyl sensor chips were obtained from Attana AB (Stockholm, Sweden). The preparation of the sensor chips and the immobilization procedure by amine coupling were performed according to the manufacturer’s instructions. Briefly, a carboxyl chip was pre-wetted with MilliQ water prior to immobilization, inserted in the Attana A100 QCM biosensor instrument (Attana AB, Stockholm, Sweden), and left to stabilize. The immobilization procedure was carried out at a flow rate of 10 μl/min in HBS-T buffer (10 mM HEPES, 150 mM NaCl, 0.005% Tween 20, pH 7.4) at 25 °C. Thereafter, 0.4 mM N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride and 0.1 mM Sulfo-NHS were mixed in 1:1 ratio, and the solution was injected immediately with 300 s of contact time to activate the chip surface. After rinsing the injection loop with MilliQ water the ligand solution containing 20 or 10 μg/ml of purified NQ-BVHWT and NQ-BVHΔL, respectively, in 10 mM sodium acetate buffer, pH 5.0, was loaded to the chip for 300 s. The injection loop was
were performed with a continuous flow (25 μl/min) of running buffer (20 mM citrate buffer, 137 mM NaCl, 2.7 mM KCl, 0.005% Tween 20, pH 4.0) allowing for a contact time of 100 s. Substrate samples were prepared by serial dilutions in running buffer, and the interaction was recorded with the aid of Attener® software (Attana AB) for HA concentrations ranging from 0.05 to 1.0 mg/ml and for chondroitin sulfate concentrations ranging from 0.05 to 0.5 mg/ml injected in a random order at 37 °C. After each measurement the chip surface was regenerated by a 30-s injection of 2 M NaCl to remove any remaining analyte. Two to three independent experiments were performed.

The frequency response curves of the interaction between the various substrates at different concentrations and the immobilized inactive BVH variants, as well as the unmodified surface were analyzed by the Attache Evaluation software (version 3.3.3.1, Attana AB). The curves obtained at distinct substrate concentrations were averaged and corrected by subtracting the nonspecific binding responses obtained from measurements using an unmodified surface. The kinetic data analysis was performed using ClampXP2 (version 3.50) provided by the developers (27) by fitting theoretical models to the experimental data. First, a global analysis attempt was made, which proved insufficient. Next, a local analysis of the association and the dissociation phases was performed. The equilibrium dissociation constants of BVH variants for high molecular weight HA and chondroitin sulfates were calculated from the directly estimated association and dissociation rate constants ($K_D = k_{dis}/k_{as}$).

RESULTS

Enzyme Activity and Kinetic Analysis—The substrate binding groove of HAase is constrained at one end by a bulky noose, which comprises a short stretch of amino acids that loop out at a cystine bridge linking position 189 and 201. By means of computer-aided homology modeling (23), this loop could be generated by substituting the two essential acidic residues of the wild-type and the loop mutant of bee hyaluronidase. FIGURE 1. Three-dimensional models and enzymatic activity of the wild-type and the loop mutant of bee hyaluronidase. The steric models of BVHWT (left) and BVH3L (right) with a bound HA-tetrasaccharide (blue in stick mode) were generated using PyMOL. The wild-type and the mutant loops are highlighted in yellow and green, respectively, and the active centers, Asp-111 and Glu-113 are shown in magenta and red, respectively (A). Expression of the wild-type enzyme and the variant were analyzed on a 12% SDS-PAGE and visualized by Coomassie staining. The arrow indicates the expected molecular masses (inset in B). Determination of pH dependence of BVHWT (filled circle and solid line) and BVH3L (filled square and dashed line) using 0.1 mg/ml of HA concentration and 20 min of incubation time at 37 °C was performed by Reissig assay ($n = 3$). The values represent the mean ± S.E. and in 80% of all data sets S.E. were <10%. The enzyme activity is expressed in units (U), 1 unit equaling 0.1 nmol of reducing N-acetyl-d-glucosamine end groups generated by 1 mg of enzyme (B).

variants showed a progressive decrease of reaction rates at substrate concentrations up to 2 mg/ml. The best fit for all series of experimental data points was a biexponential function, displaying two distinct phases of a putative two-step process: during the early phase, the number of reducing ends increases rapidly, high molecular weight HA is efficiently hydrolyzed and a large amount of oligomeric substrate is generated; thereafter reaction rates gradually decrease. Even in the presence of high HA concentrations, BVHWT could always reach this late phase. In contrast, BVH3L showed reduced hydrolysis rates during the early stage, and kinetics only approached the late phase during the time course of the experiment at low HA concentrations (Fig. 2).

Next, the initial reaction rates were calculated and plotted against HA concentration. The kinetic curves clearly showed that hydrolysis rates gradually decreased at HA concentration higher than 0.5 mg/ml for BVHWT or 0.2 mg/ml for BVH3L. Consequently, the initial reaction rates showed asymmetric, bell-shaped concentration dependences with optima between 0.2–0.5 mg/ml HA for the wild-type enzyme (Fig. 3A). Despite displaying similar characteristics, the initial rate of BVH3L was found to be 10 times lower (Fig. 3B).

The biexponential progression of HA hydrolysis could be resolved into two kinetic phases: a dominant fast-rate step ($v_01$) and a slow process with a distinctly lower rate ($v_02$). Both the
wild-type and the mutant enzyme have comparable $v_{01}$; the
10-fold difference in $v_{02}$ is largely due to a rate difference of
the dominant reaction $v_{01}$ (Fig. 3). During the initial phase
of the reaction, only high molecular weight HA is present, which
is degraded by the wild-type enzyme with high
$V_{max}$ for BVHWT was $1 \times 10^6$ ± 3 $\times 10^4$ units/min.
Unexpectedly, BVHΔL had a lower $K_m$ (0.24 ± 0.01 versus
0.37 ± 0.08 mg/ml), indicative for higher substrate affinity.

Substrate Binding—Under the experimental conditions used
in the previous kinetic analyses, substrate degradation rates
of BVH and the loop variant were too high for binding studies.
Therefore, interactions at varying HA concentrations (0.05–1
mg/ml) were studied with the inactive HAase variants
$NQ$-BVHWT and $NQ$-BVHΔL. Both HAase variants firmly
bound high molecular weight HA. The point mutations intro-
duced in the active center, although located within the sub-
strate binding groove did not induce major conformational
changes, which incommode HA binding. Moreover, the poly-
mer could be detached from both variants. Prior to a successive
measurement, the chip was efficiently regenerated by washes
with high ionic strength buffer (Fig. 4).

Working along these lines, experimental data for HA associ-
ation to and dissociation from the inactive HAase variants were
collected. The results obtained thereby were further validated
using previously established theoretical models. A fit to the
simple Langmuir binding model could not be accomplished,
both the simple binding, as well as the mass transport-limited
models were unable to adequately describe the interaction.
This suggested that interactions between HA and BVH are
more complex. Therefore, various two-state models were chal-
gened (28–30), and finally, a two-site model was computed,
which provided useful fits. When supposing substrate binding
in random order, the estimation of two out of four association
rate constants ($k_{a}$) yielded no meaningful results, suggesting
that distinct binding events are either negligible or random
combinations of binding and unbinding are likely circumstan-
tial. Challenging the alternative proposition that binding may
take place in a directed fashion the following sequential two-
site model was fitted (Equation 2).

$$S1 + HA \rightleftharpoons S1 \cdot HA + S2 \rightleftharpoons S1 \cdot HA \cdot S2$$  \hspace{1cm} (Eq. 2)

$S1$ and $S2$ are putative substrate binding sites, and thus, this
model assumed that the enzyme actually accommodates two
distinct binding sites, which associate with the polymer in a
sequential order. Also, this particular model approximated the
that the putative second binding site is locally close to the bulky first binding site were found comparable, thus indicating released more slowly from the complex as the binding site was distinctly lower. In either variant, the inactive loop variant, NQ-BVH when comparing the derived data with that of the wild-type enzyme, the inactive loop variant, NQ-BVH

TABLE 1

Kinetic parameters of HA binding determined by QCM approach

| Interaction phase | Kinetic parameter | HA concentration (mg/ml) |
|-------------------|------------------|-------------------------|
|                   |                  | 0.05 | 0.1 | 0.2 | 0.5 | 1.0 |

| NQ-BVHWT | Equilibrium | $K_{D1}$ ($\times 10^{-4}$) | M | 3.22 ± 0.12 | 3.05 ± 0.32 | 2.48 ± 0.16 | 9.84 ± 0.61 | 87.7 ± 2.76 |
|----------|-------------|-----------------|---|---------------|---------------|---------------|---------------|---------------|
|          | $K_{D2}$ ($\times 10^{-4}$) | 5.48 ± 0.13 | 12.0 ± 3.70 | 52.0 ± 8.71 | 64.0 ± 17.6 | 0.69 ± 0.35 |
|          | $K_{DISUM}$ ($\times 10^{-9}$) | 1.77 ± 0.43 | 3.66 ± 1.51 | 12.9 ± 3.00 | 62.9 ± 21.1 | 6.10 ± 3.26 |
|          | Association | $k_{a}$ ($\times 10^3$) M$^{-1}$s$^{-1}$ | 66.7 ± 1.52 | 99.2 ± 4.72 | 106 ± 2.69 | 18.5 ± 0.39 | 3.34 ± 0.04 |
|          | $k_{d1}$ ($\times 10^{-2}$) | 54.9 ± 4.95 | 13.5 ± 1.89 | 2.64 ± 0.24 | 0.78 ± 0.14 | 8.62 ± 0.31 |
|          | $k_{d2}$ ($\times 10^{-2}$) | 36.6 ± 4.14 | 13.4 ± 2.51 | 2.80 ± 0.33 | 0.14 ± 0.03 | 0.29 ± 0.01 |
|          | Dissociation | $k_{diss}$ ($\times 10^{-2}$) s$^{-1}$ | 21.5 ± 0.34 | 30.3 ± 1.72 | 26.3 ± 1.04 | 18.2 ± 0.74 | 29.3 ± 0.57 |
|          | $k_{diss}$ ($\times 10^{-2}$) | 30.1 ± 3.50 | 16.3 ± 2.78 | 13.7 ± 1.05 | 5.06 ± 0.48 | 0.00$^{a}$ ± 0.28 |
|          | $k_{diss}$ ($\times 10^{-4}$) | 64.7 ± 8.55 | 50.0 ± 11.3 | 36.0 ± 4.19 | 9.21 ± 1.25 | 1.76 ± 0.85 |

| NQ-BVHΔL | Equilibrium | $K_{D1}$ ($\times 10^{-4}$) | M | 2.40 ± 0.27 | 4.69 ± 0.49 | 2.59 ± 0.29 | 6.34 ± 0.55 | 12.5 ± 6.63 |
|----------|-------------|-----------------|---|---------------|---------------|---------------|---------------|---------------|
|          | $K_{D2}$ ($\times 10^{-4}$) | 0.48 ± 0.09 | 0.91 ± 0.54 | 3.80 ± 1.03 | 1.11 ± 0.59 | 0.04 ± 0.03 |
|          | $K_{DISUM}$ ($\times 10^{-9}$) | 0.11 ± 0.03 | 0.42 ± 0.29 | 0.98 ± 0.37 | 0.70 ± 0.44 | 0.05 ± 0.07 |
|          | Association | $k_{a}$ ($\times 10^3$) M$^{-1}$s$^{-1}$ | 108 ± 9.06 | 78.3 ± 2.37 | 80.9 ± 2.55 | 37.3 ± 0.77 | 23.2 ± 10.3 |
|          | $k_{d1}$ ($\times 10^{-2}$) | 62.0 ± 2.02 | 42.4 ± 15.4 | 9.21 ± 0.58 | 5.41 ± 0.27 | 150 ± 30.9 |
|          | $k_{d2}$ ($\times 10^{-2}$) | 67.0 ± 7.80 | 33.2 ± 13.1 | 7.45 ± 0.70 | 2.01 ± 0.14 | 34.8 ± 22.6 |
|          | Dissociation | $k_{diss}$ ($\times 10^{-2}$) s$^{-1}$ | 25.9 ± 0.69 | 36.7 ± 2.72 | 20.9 ± 1.65 | 23.6 ± 1.58 | 29.0 ± 2.51 |
|          | $k_{diss}$ ($\times 10^{-2}$) | 2.99 ± 0.48 | 3.84 ± 0.89 | 3.50 ± 0.73 | 0.00$^{a}$ ± 0.29 | 0.00$^{a}$ ± 0.37 |
|          | $k_{diss}$ ($\times 10^{-4}$) | 7.74 ± 1.45 | 14.1 ± 4.31 | 7.32 ± 2.10 | 1.42 ± 0.78 | 1.74 ± 1.22 |

$^{a}$ ClampXP2 cannot calculate $k_{diss}$ values $< 10^{-3}$, therefore, instead of zero, an estimated value of 0.6$10^{-4}$ was substituted.

experimental data regarding the interaction of HA with the two inactive variants best, as the kinetics showed congruent matches for all experimental data sets (Fig. 4). Because the global fitting algorithm could not be applied, the association ($k_a$) and dissociation ($k_{diss}$) constants, as well as the equilibrium dissociation constants ($K_D$) were determined for each HA concentration by local fitting (Table 1).

When plotting $k_a$ and $k_{diss}$ rate constants of the two binding sites against substrate concentration, it became apparent that when comparing the derived data with that of the wild-type enzyme, the inactive loop variant, NQ-BVHΔL formed the HA-enzyme complex at a considerably higher rate as reflected by a higher $k_a$ at the second binding site. Moreover, HA was released more slowly from the complex as $k_{diss}$ at the second binding site was distinctly lower. In either variant, $k_a$ and $k_{diss}$ of the first binding site were found comparable, thus indicating that the putative second binding site is locally close to the bulky loop (Fig. 5, A and B). Also, line up of the $K_D$ regarding the first binding sites ($K_{D1}$) showed highly similar results for both variants, with a sole exception of a slight difference at the highest experimental HA concentration. In the case of NQ-BVHΔL, the second binding site exhibited again a significantly lower $K_{D2}$ (Fig. 5C), by and large confirming the aforementioned assumption that the bulky formation solely impinges on the putative second binding site, which has high HA affinity when not affected by the loop.

Furthermore, comparison of the overall affinity constants ($K_{DISUM}$) as calculated from the $K_D$ values of both binding site ($K_{D1} \times K_{D2}$), revealed the highest value for the wild-type protein with 62.9 nM at 0.5 mg/ml HA, whereas the loop variant exhibited 10 to 100 times lower $K_D$ values with a maximum value of 0.98 nM at 0.2 mg/ml HA concentration (Table 1). Thus, replacement of the bulky formation greatly enhanced the overall affinity of the enzyme for high molecular weight HA yielding tight binding and/or impeded product release.

Chondroitin sulfate (CS) A, B, and C are also substrates to BVH. Compared with HA, the hydrolysis rate of CS-C is ~40%, for CS-C 20%, and CS-B is only very slowly degraded (12). Binding of all three CS types to NQ-BVHWT, as well as to NQ-BVHΔL, was evaluated accordingly (supplemental Figs. 1–6). Analysis of $K_{DISUM}$ for

FIGURE 4. QCM sensorgrams for substrate binding. Inactive forms of BVH containing the normal (A) and the mutated loop sequences (B) were used for QCM measurements. Varying HA concentrations (0.05 to 1.0 mg/ml) were loaded onto immobilized BVH in a random order, and interactions were monitored at pH 4.0 and at 37 °C (solid lines) ($n \approx 2–3$). The curves from each HA concentrations were averaged and corrected by subtracting the nonspecific binding responses obtained from the unfunctionalized surface. The sequential two-site model was fitted (dotted lines) to the averaged sensorgrams by local analysis using ClampXP2 software.
the CS types with regard to HA showed that substrate binding is an important actuating value determining turnover rates (supplemental Table 1–3), as CS-B showed the highest $K_{\text{DSUM}}$ value of 801 nM at 0.5 mg/ml for the wild-type and 257 nM at 0.2 mg/ml for the loop variant (supplemental Table 2), and CS-A and CS-C showed $K_{\text{DSUM}}$ values of 172 nM at 0.5 mg/ml and 198 nM at 0.05 mg/ml for NQ-BVHWT, and 42.5 nM at 0.2 mg/ml and 19 nM at 0.1 mg/ml for NQ-BVH3L, respectively. Further kinetic analyses showed best fit of the respective data again to the sequential two-site model. In view of these results, we propose here a model for the cleavage cycle of HAase featuring two distinct binding sites, which sequentially bind and release the products accordingly (Fig. 6).

**DISCUSSION**

The published crystal structures of HAase from bee (15) and wasp venom (31) or that of human serum HAase, human serum

**FIGURE 5. Comparison of the association and dissociation rate constants of HA binding.** The association ($k_a$) (A) and the dissociation rate constants ($k_{\text{diss}}$) (B) of the first (filled circles and dotted line) and the second binding sites (filled squares and solid line) of NQ-BVHWT and the first (filled squares and dotted line) and the second binding sites (filled squares and solid line) of NQ-BVH3L were estimated directly during the fitting procedure. The $K_D$ values of the first (filled circles and dotted line) and the second binding sites (filled circles and solid line) of NQ-BVHWT and the first (filled squares and dotted line) and the second binding sites (filled squares and solid line) of NQ-BVH3L were calculated from the association and the dissociation constants ($K_D = k_{\text{diss}}/k_a$) (C). Values represent the mean ± S.E., and in 78% of all data sets, S.E. were <25%.

**FIGURE 6. Cleavage cycle of bee hyaluronidase.** The schematic representation of the model for the cleavage cycle was built based on HA binding and hydrolysis data. HA sugar residues are drawn according to commonly accepted graphical carbohydrate symbolism (49); N-acetyl-D-glucosamine is depicted as a filled square with solid lines, and D-glucuronic acid is depicted as a diamond with gray upper segment and black outline. Reducing ends of the substrate are indicated by $R$, non-reducing ends are indicated by $N$; in hydrolysis, products $R'$ and $N'$ are used accordingly.
hyaluronidase 1 (32) led to the identification of a bulky knob, which rises at the upper end of the substrate binding groove. Both length and amino acid composition of this unshaped bump are highly variable, with the sole exception of a single asparagine residue which is conserved in most species. In many hyaluronidas from hymenoptera, that asparagine is part of a potential N-glycosylation recognition sequence, which in all cases characterized so far is followed by a proline. This combination, however, renders this particular site highly unlikely for posttranslational glycosylation (33). This assumption was further strengthened by comparing wild-type BVH with BVHSL in which the respective stretch had been replaced with a tetrapeptide sequence comprising alternating Gly and Ser residues. The latter thus lacks the potential glycosylation site. After expression in Pichia pastoris, a microbe known to efficiently glycosylate, the molecular weight of the two secreted proteins was found comparable. Interestingly, enzymatic and kinetic analyses of BVHSL in which the blocking knob has been depleted showed that it has reduced activities.

Previous analyses with bovine testicular hyaluronidase had shown that two phases of HA hydrolysis can be distinguished: an early part, where high molecular weight HA was found to be degraded at a relatively fast rate; thereafter, the resulting oligomeric fragments were hydrolyzed with slow kinetics (26, 34, 35). This was found to be true for wild-type BVH activity. However, the results obtained for BVHSL, which supposedly should offer more open space for a high molecular weight substrate, were unexpected. The rapid early phase of HA hydrolysis was not found for the variant enzyme. Although counterintuitive, this bulky loop actually enhances turnover of polymeric HA.

The initial reaction rates for both wild-type and BVHSL clearly showed an inhibition at high HA concentration. As reported previously, short HA fragments are poor substrates for HAases (34, 35). Provided that the atypical bell-shaped progression of the substrate concentration dependence could be due to increasing amounts of short HA oligomers in concentrated polydisperse substrate solutions. Increasing concentrations of short HA may thus shift to late phase kinetics and greatly decelerate the overall turnover rate. It is, however, also conceivable that similar to steric exclusion of bovine serum albumin in highly concentrated HA solutions (36, 37), the observed suppression of enzyme activity could also be a consequence of crowding out of HAase by closely interacting HA chains.

To address this question, the kinetic data sets were analyzed further. Working along these lines yielded only an approximation of Michaelis-Menten parameters. However, acceptable estimates for $V_{\text{max}}$ and $K_m$ could still be achieved. An important result was that $K_m$ for BVHSL was found to be much lower, thus indicating enhanced affinity for HA.

More detailed binding studies were performed by means of QCM technology, which is capable of monitoring the interaction in real-time as a change in frequency of a surface-coated quartz crystal resonator. In solution, this method is highly effective at determining the affinity of molecules, in particular for large binding partners, such as high molecular weight HA. So far, no experimental results based on QCM were available for HA-HAase interaction. Surface plasmon resonance has previously been introduced to monitor the interactions of bovine testicular hyaluronidase with sulfated glucosaminoglycans. Notably, however, HA as the major binding partner for HAase has for unknown reasons not been studied (38).

When using enzymatically active HAase in QCM measurements, binding could be examined, but due to hydrolysis taking place at the same time with concomitant generation of highly polydisperse HA, no distinct models for subsequent data analysis could be built. Stable interaction of HA, and other substrates such as CS with the enzyme could only be studied for HAase lacking hydrolysis activity. Mutations of specific catalytic residues in the active center were previously reported to inactivate human testicular hyaluronidase (24), as well as human serum hyaluronidase 1 (39). Point mutations introduced into BVH also yielded an inactive variant, which still bound high molecular weight substrate efficiently. From data obtained with this variant, a sequential two-site model could be deduced that features a dynamic mechanism involving a second potential binding site, which only becomes available after a first site has been successfully occupied by an uncleaved polymeric substrate. According to this model, we propose the existence of two kinetically distinct binding sites, which are both located in the substrate binding groove on either side of the active center. Following the nomenclature for sugar binding sites in glycosyl hydrolases (40), the first binding site (S1) is located adjacent to the non-reducing end away from the point of cleavage and therefore labeled as the $-n$ subsites, whereas the second binding site (S2), which we mapped next to the bulky formation interacts with HA at $+n$ subsites, with cleavage taking place in between $-1$ and $+1$.

The model of the cleavage cycle as depicted in Fig. 6 states that initial attachment at S1 is mandatory for HA to subsequently interact with S2. After completion of the hydrolytic reaction the HA fragment bound to S2 will be released first, and only thereafter the remaining fragment detaches from S1. Replacement of the loop yielded a variant with lower enzymatic activity but with up to 100 times higher affinity in HA-binding. The equilibrium dissociation constant, $K_d$ for high molecular weight HA was found to be 0.05 to 0.98 nM, whereas the $K_d$ for the wild-type enzyme was only 1.77 to 62.9 nM. Previously obtained $K_d$ for HA-binding proteins or hyalectins were 5–150 μM for CD44 (41–43), 11–36 μM for LYVE-1 (43), 0.2–55 μM for TSG-6 (44, 45), 226 nM for aggrecan, and exactly 82 nM for Link protein (46). The HA receptor for endocytosis has the highest affinity for HA ranging 5 to 23 nM (47, 48). Compared with these specific binding proteins, the BVH loop variant shows the highest avidity for HA.

Indeed, the bulky formation interferes with HA binding but probably promotes the unbinding of short oligomeric chains tethered to the $+n$ site. The penultimate step of the catalytic cycle had actually been depicted before by x-ray structure determination of BVH co-crystallized with an HA hexasaccharide. In the crystals, the HA hexasaccharide had been degraded, and the HAase only retained a tetrasaccharide fragment bound to the subsites $-4$ to $-1$. At the $-n$ subsites amino acids, which directly interact with HA such as Tyr-55, Asp-111, Glu-113, Tyr-184, Tyr-227, Trp-301, Ser-303, and Ser-304 could be identified (15). In turn, however, the situation regarding the $+n$ subsites was less clear. To gain more insight, the three-dimen-
sional coordinates of HA were derived from the crystal structure of the BVH-HA complex. These were ASP-129, GLU-131, TYR-202, TYR-247, SER-245, and ARG-265 (39). The latter two correspond to SER-225 and ARG-244 of the bee enzyme. However, no direct interaction with the HA tetrasaccharide had been reported in the previous study (15). Also in close vicinity to the bulky knob, SER-225 and ARG-244 are likely residues of the +n subsites and thus shape the second binding site.

In conclusion, we report here that the bulky formation, which at first glance appears to block the substrate binding groove, actually contributes to a second HA binding site in HAase. The results presented in this study demonstrate that this loop is involved in modulation of the enzymatic activity, which besides substrate binding also requires efficient clearing of the products after catalytic processing. In turn, when a product is transiently retained at the second site through strong binding, further progression through the catalytic cycle is retarded. Through the bulky loop in BVH unbinding and release of the cleaved HA fragment from the enzyme is enhanced, altogether resulting in a higher rate of hydrolysis.

The various bulky formations in different hyaluronidase species may have evolved to efficiently hydrolyze high molecular weight HA in different biological processes. The bee venom enzyme is considered to be a spreading factor. At the site of a bee sting, rapid hydrolysis of HA chains in the dermis facilitates the diffusion of other venom constituents, mainly the lytic peptide mellitin and phospholipase, thereby augmenting local damage and inflammation.

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REFERENCES

1. Hascall, V., and Esko, J. D. (2009) in Essentials of Glycobiology (Varki, A., Cummings, R. D., Esko, J. D., Freeze, H. H., Stanley, S., Bertozzi, C. R., Hart, G. W., and Etzler, M. E., eds.), 2010/03/20 Ed., pp. 219–228, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
2. Wang, A., de la Motte, C., Lauer, M., and Hascall, V. (2011) FEBS J. 278, 1412–1418
3. Jiang, D., Liang, J., and Noble, P. W. (2011) Physiol. Rev. 91, 221–264
4. Delmage, J. M., Powars, D. R., Jaynes, P. K., and Allerton, S. E. (1986) Annu. Clin. Lab. Sci. 16, 303–310
5. West, D. C., Hampson, I. N., Arnold, F., and Kumar, S. (1985) Science 228, 1324–1326
6. Noble, P. W. (2002) Matrix Biol. 21, 25–29
7. Xu, H., Ito, T., Tawada, A., Maeda, H., Yamanokuchi, H., Ishara, K., Yoshida, K., Uchiyama, Y., and Asari, A. (2002) J. Biol. Chem. 277, 17308–17314
8. Tammi, R. H., Passi, A. G., Rilla, K., Karousou, E., Vigetti, D., Makkonen, K., and Tammi, M. I. (2011) FEBS J. 278, 1419–1428
9. Stern, R. (2003) Glycobiology 13, 105R–115R
10. Stern, R., Asari, A. A., and Sugahara, K. N. (2006) Eur. J. Cell Biol. 85, 699–715
11. Gmachl, M., and Kreil, G. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 3569–3573
12. Reitinger, S., Boroviak, T., Laschober, G., T., Fehrer, C., Müllegger, J., Lindner, H., and Lepperding, G. (2008) Protein Expr. Purif. 57, 226–233
13. Frost, G. I., Csóka, A. B., Wong, T., and Stern, R. (1997) Biochem. Biophys. Res. Commun. 236, 10–15
14. Lepperding, G., Strobl, B., and Kreil, G. (1998) J. Biol. Chem. 273, 22466–22470
15. Markovic-Housley, Z., Miglierini, G., Soldatova, L., Rizkallah, P. N., Müller, U., and Schirmer, T. (2000) Structure 8, 1025–1035
16. Markovic-Housley, Z., and Schirmer, T. (eds) (2002) Structural Evidence for Substrate Assisted Catalytic Mechanism of Bee Venom Hyaluronidase, a Major Allergen of Bee Venom, pp. 19–27, The Royal Society of Chemistry, MPG Books Ltd., Bodmin, Cornwall, UK
17. Reitinger, S., Müllegger, J., Greiderer, B., Nielsen, J. E., and Lepperding, G. (2009) J. Biol. Chem. 284, 19173–19177
18. Sauerbrey, G. (1959) Zeitschrift für Physik 155, 206–222
19. Bruckenstein, S., and Shai, M. (1985) Electrophor. Acta 30, 1295–1300
20. Jumel, K., Harding, S. E., Sobol, E., Omel’chenko, A., Svirdov, A., and Jones, N. (2002) Carbohydr. Polym. 48, 241–245
21. Hess, R., Douglas, T., Myers, K. A., Rentsch, B., Rentsch, C., Worch, H., Shrive, N. G., Hart, D. A., and Scharnweber, D. (2010) J. Biomech. Eng. 132, 021001
22. Rees, S. G., Shellis, R. P., and Embery, G. (2002) Biochem. Biophys. Res. Commun. 292, 727–733
23. Benkert, P., Biasini, M., and Schwede, T. (2011) Bioinformatics 27, 343–350
24. Arming, S., Strobl, B., Wechselberger, C., and Kreil, G. (1997) Eur. J. Biochem. 247, 810–814
25. Reissig, J. L., Storminger, J. L., and Leloir, L. F. (1955) J. Biol. Chem. 217, 959–966
26. Vincent, J. C., Asteriou, T., and Deschrevel, B. (2003) J. Biol. Phys. Chem. 3, 35–45
27. Myszka, D. G., and Morton, T. A. (1998) Trends Biochem. Sci. 23, 149–150
28. Morton, T. A., Myszka, D. G., and Chaiken, I. M. (1995) Anal. Biochem. 227, 176–185
29. Karlsson, R., and Fält, A. (1997) J. Immunol. Methods 200, 121–133
30. De Crescenzo, G., Grotto, S., Lortie, R., Debanne, M. T., and O’Connor-McCourt, M. (2000) Biochemistry 39, 9466–9476
31. Skor, L. K., Seppälä, U., Coen, J. J., Crickmore, N., King, T. P., Monsalve, R., Kastrup, I. S., Spangfort, M. D., and Gajbe, H. (2006) Acta Crystallogr. D Biol. Crystallogr. 62, 595–604
32. Cho, K. L., Muthukumar, L., and Herzberg, O. (2007) Biochemistry 46, 6911–6920
33. Gavel, Y., and von Heijne, G. (1990) Protein Eng. 3, 433–442
34. Deschrevel, B., Tranchepain, F., and Vincent, J. C. (2008) Matrix Biol. 27, 475–486
35. Cramer, J. A., Bailey, L. C., Bailey, C. A., and Müller, R. T. (1994) Biochim. Biophys. Acta 1200, 315–321
36. Ogston, A. G., and Preston, B. N. (1966) J. Biol. Chem. 241, 17–19
37. Shaw, M., and Schy, A. (1977) Biochim. 17, 47–55
38. Shen, B., Shimmon, S., Smith, M. M., and Ghosh, P. (2003) J. Pharm. Biomed. Anal. 31, 83–93
39. Zhang, L., Bharadwaj, A. G., Casper, A., Barkley, J., Barycki, J. J., and Simpson, M. A. (2009) J. Biol. Chem. 284, 9433–9442
40. Davies, G. J., Wilson, K. S., and Henriksson, B. (1997) J. Biol. Chem. 321, 557–559
41. Skelton, T. P., Zeng, C., Nocks, A., and Stamenkovic, I. (1998) J. Cell Biol. 140, 431–446
42. Banerji, S., Noble, M., Teriet, P., Wright, A. J., Blundell, C. D., Campbell, I. D., Day, A. J., and Jackson, D. G. (eds) (2005) Structure of the CD44 Hyaluronic-binding Domain and Insight into Its Regulation by N-Glycosylation, Vol. II, pp. 625–630, Matrix Biology Institute, Edgewater, NJ
43. Banerji, S., Hid, B. R., James, J. R., Noble, M. E., and Jackson, D. G. (2010) J. Biol. Chem. 285, 10724–10735
44. Kohda, D., Morton, C. J., Parker, A. A., Hatanaka, H., Inagaki, F. M., Campbell, I. D., and Day, A. J. (1996) Cell 86, 767–775
45. Kahmann, J. D., O’Brien, R., Werner, J. M., Heinegård, D., Ladbury, J. E.,
Campbell, I. D., and Day, A. J. (2000) *Structure* **8**, 763–774
46. Watanabe, H., Cheung, S. C., Itano, N., Kimata, K., and Yamada, Y. (1997)
*J. Biol. Chem.* **272**, 28057–28065
47. Harris, E. N., Kyosseva, S. V., Weigel, J. A., and Weigel, P. H. (2007)
*J. Biol. Chem.* **282**, 2785–2797
48. Harris, E. N., Parry, S., Sutton-Smith, M., Pandey, M. S., Panico, M., Mor-
ris, H. R., Haslam, S. M., Dell, A., and Weigel, P. H. (2010) *Glycobiology* **20**, 991–1001
49. Varki, A., Cummings, R. D., Esco, J. D., Freeze, H. H., Stanley, P., Marth,
J. D., Bertozzi, C. R., Hart, G. W., and Etzler, M. E. (2009) *Proteomics* **9**, 5398–5399