Designed human serum hyaluronidase 1 variant, HYAL1ΔL, exhibits activity up to pH 5.9
Stephan Reitinger 1,2, Johannes Müllegger 3, Brigitte Greiderer 1, Jens Erik Nielsen 4, Günter Lepperdinger 1

From The Extracellular Matrix Research Group, Institute for Biomedical Aging Research, Austrian Academy of Sciences, Rennweg 10, 6020 Innsbruck, Austria 1, present address: Department of Chemistry, University of British Columbia, 2036 Main Mall, Vancouver, B. C., V6T 1Z1, Canada 2, Zymeworks Inc., 540-1385 West 8th Avenue, Vancouver, B. C., V6H 3V9, Canada 3, School of Biomolecular and Biomedical Science, Centre for Synthesis and Chemical Biology, UCD Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland 4

Address correspondence to: Günter Lepperdinger, Institute for Biomedical Aging Research of the Austrian Academy of Sciences, Rennweg 10, A-6020 Innsbruck, Tel: 0043 512 5839 1940; Fax 0043 512 5839 198; email: Guenter.Lepperdinger@oeaw.ac.at

Hyaluronidases from diverse species and sources have different pH optima. Distinct mechanisms with regard to dynamic structural changes, which control hyaluronidase activity at varying pH are unknown. Human serum hyaluronidase 1 (HYAL1) is active solely below pH 5.1. Here we report the design of a HYAL1 variant, that degrades hyaluronan up to pH 5.9. Besides highly conserved residues in close proximity of the active site of most hyaluronidases, we identified a bulky loop formation located at the end of the substrate binding crevice of HYAL1 to be crucial for substrate hydrolysis. The stretch between cysteine residues 207 and 221, which normally contains 13 amino acids could be replaced by a tetrapeptide sequence of alternating glycine serine residues, thereby yielding an active enzyme with an extended binding cleft. This variant exhibited hyaluronan degradation at elevated pH. This is indicative for appropriate substrate binding and proper positioning being decisively effected by sites far off from the active center.

Hyaluronan (HA), a linear polysaccharide found in the extracellular matrix of most tissues and body fluids of vertebrates is enzymatically degraded by hyaluronidases (1). Mammalian-type hyaluronidases are grouped into EC 3.2.1.35 (2,3) or the glycoside hydrolase family 56 (4). Members of this enzyme family hydrolyze the 1,4-β-glycosidic linkage between N-acetyl-D-glucosamine and D-glucuronate within HA polymers (5). In mammalians, hyaluronidases have been found in testis, liver lysosomes, and serum. They are involved in controlling HA levels and are thus implicated in various diseases related to defects of HA metabolism (6).

The crystal structures of hyaluronidase from bee (7), wasp (8), and only recently that of human serum hyaluronidase 1 (HYAL1) (9) have been deciphered. In addition to the N-terminal catalytic domain of the insect enzymes, which resembles a distorted (β/α)8 barrel, HYAL1 contains yet another domain. HA hydrolysis is achieved by a pair of acidic amino acids via a retaining double-displacement mechanism and a substrate-assisted catalysis, in which the carbonyl oxygen of the N-acetyl group of the cleaved HA subunit acts as the catalytic nucleophile (7).

Mammalian-type hyaluronidases display different pH optima. HYAL1 (10) and hyaluronidase 2 (HYAL2) (11) exhibit highest activities at acidic conditions, whereas the hyaluronidase found in Xenopus laevis kidney is only active at neutral pH (12). Bee venom hyaluronidase (13) as well as sperm hyaluronidase, PH-20 (SPAM1) (14) are capable of degrading HA over a broad pH range. Up to three PH-20 isoforms with greatly different pH-optima could be found in protein preparations from bovine testis (15). Extensive analysis of hyaluronidase structures did not bring forward any insights as to what residues or regions of the enzymes specify a specific pH optimum.

Profiles of pH dependent activities can be assigned by computing the enzyme's electrostatic interactions, which are primarily determined by the ionization states of its amino acid side chains.
The enzyme's pKa values of titratable groups reflect pH-dependent properties such as stability, enzymatic interaction, and substrate interactions (16). Here we present computational and experimental data on the replacement of a loop region located at the end of the substrate binding groove yielding a variant hyaluronidase with an altered pH profile.

Experimental Procedures

Molecular Modeling - Tertiary structure models were generated (17) and Deep View/Swiss-Pdb Viewer version 3.7 (18) using the 3D-coordinates of bee hyaluronidase (PDB accession 1fcv) and HYAL1. (PDB accession 2pe4). Images were generated with the aid of Open-Source PyMOL 0.99rc6 (19).

Mutagenesis of HYAL1 - Coding regions of wildtype and mutated HYAL1 (accession number NM_007312) were inserted into the plasmid pT7TS (P. A. Krieg, University of Arizona) using restriction sites EcoRV and BcuI, respectively. Four-primer PCR technology was applied to substitute the native amino acid sequence between cysteine residues at position 207 and 221. Flanking primers together with the primer pair 5’–
TGGGCAGGAGCCACTACCGCAGTCAGGG
AAGCC–3’ and 5’–
GACTGCGGTAGTGGCTCCTGCCCATCAGG
CATC–3’ were used to replace the original loop by the sequence of the four amino acid residues, glycine serine glycine serine resulting in a cDNA encoding HYAL1Δ.

Hyaluronidase expression in Xenopus laevis oocytes - Frog surgery, oocytes preparation, and cRNA injection was accomplished as described previously (20). The coding regions of HYAL1 and HYAL1Δ in pT7TS vector linearized with BamHI were transcribed in vitro. 50 ng of capped cRNA were injected into freshly prepared oocytes. The cells were kept in culture medium O-R2 containing antibiotics at 16°C for two days. Western blot analysis - Homogenized Xenopus oocytes were mixed with SDS-loading buffer (315 mM Tris-HCl pH 6.8, 50% (v/v) glycerol, 10% (w/v) SDS, 25% (v/v) 2-mercaptoethanol) and separated on a 10% polyacrylamide gel prior to semi-dry blotting onto nitrocellulose membrane (PROTRAN, Schleicher and Schuell). Hyaluronidase was detected using specific antibody raised in rabbits as described previously (13).

pKα calculations and electrostatic potential calculations- Titration curves for all titratable groups were calculated using the WHAT IF pKα calculation package, as described previously (21) except using a single uniform dielectric constant of 8. Calculations for HYAL1 and HYAL1Δ are available (see: http://enzyme.ucd.ie/pKD, accession numbers 2pe4_apo.pka.pdb and hyal_deltaL_apo.pka.pdb). Titration curves and pKα values were analyzed with pKaTool (16).

Electrostatic potential maps at pH 3.8 and pH 5.5 were calculated by linearly scaling the OPLS charges (22) for each titratable group by the fractional degree of protonation of that group at the desired pH value, which were directly derived from the titration curves calculated in the pKα calculations. The hydrogen bond network in each structure was optimized as described previously (23) and the electrostatic potential was solved using DelPhi 2 (24) using a 65-cubed grid, a protein dielectric constant of 8, solvent dielectric of 80, ionic strength of 0.144 M and an ion exclusion layer of 2.0 Å. Electrostatic potential maps were visualized using PyMOL (19).

Hyaluronidase activity measurement and evaluation - After cRNA injection and culture healthy oocytes were pooled and homogenized prior to enzymatic analysis. As described previously (12,25), the amount of lysate and the time of hydrolysis were titrated to specify the protein amount and assay duration, at which cleavage rates were in the linear range appropriate for subsequent comparative analyses of the respective pH profiles. Hydrolysis rates were quantified by determining the relative median length along with the amount of the remaining hyaluronan applying histogrammatic characterization of the digitized blots using ImageJ (NIH). A paired, two-tailed Student’s T test was applied for statistical evaluation.

RESULTS
HYAL1 was initially expressed in E.coli. The expressed recombinant protein was exclusively found as an insoluble protein aggregate, and no enzymatic activity could be obtained following solubilization and refolding. After separation by SDS-PAGE, recombinant HYAL1 was used to raise polyclonal antibodies in rabbits, which were applicable for Western analysis.

Bee hyaluronidase has been co-crystallized in the presence of HA tetrasaccharides (PDB accession code: 1fcv) (7). Due to the polymeric structure of HA, substrate binding is mediated alongside one half of the globular protein, opposite to the side, where N- and C-termini are located. In HYAL1 across from the active center and located at the end of the substrate binding groove, a loop, which is positioned between two cysteine residues at position 207 and 221, leads to a tapering geometry thereof. Hence, we altered the native sequence between the two cysteines both in silico (Fig. 1) as well as in reality (table 1) by replacing it with either one of the respective sequence of the bee venom (HYAL1∆beeh) or that of the testicular (human PH20) hyaluronidase (HYAL1∆PH20), or by mutating the potential N-glycosylation site at position 218 from a threonine to an alanine HYAL1∆glyc. Employing a protein structure modeling approach, this loop region may be replaced without distorting the enzyme’s structural conformation by the shortest possible stretch of four amino acids. In the model the sequence glycine-serine-glycine-serine (GSGS) was introduced and the resulting protein model was termed HYAL1∆L (Fig. 1). The latter could be also expressed as an active enzyme.

In previously analyses, we showed that highly active hyaluronidase can be reliably expressed by injection of cRNA into X.laevis oocytes (12,20,26). Here, we continued to employ this method to express a variety of HYAL1 variants. The activity of the variants was first examined at pH 4.5 and 5.5, the former being optimal, the latter inhibitory for wildtype HYAL1. Apart from HYAL1∆beeh, all variant forms were active at pH 4.5. Only HYAL1∆L hydrolyzed hyaluronan at pH 5.5, albeit at a decreased rate (table 1). The mutated region appears to be neither part of the active center, nor is it conceivable that direct interactions with residues of the active center do occur. To corroborate this assumption we calculated pKa values for all residues and analyzed the titration curves of the two carboxyl groups of the active site amino acids, aspartate (D129) and glutamate (E131). The active site pKa values appeared relatively insensitive to the deletion. In HYAL1, pKa of D129 is 6.1 and that of E131 is 9.4. Similarly, the respective values in HYAL1∆L are 6.3 and 9.5. The calculated pKa values are in good agreement with the observed pH-activity profiles of both enzymes, although the magnitude of the pH-shift for HYAL1∆L is not accurately captured by these calculations. Since the deletion is a substantial modification, it is possible that structural relaxation, which is neglected in the HYAL1∆L model, causes minor conformational rearrangements at the active site that further alters the pKa values of D129 and E131.

The mutated loop is demarcating the substrate binding crevice at one of its ends. Appropriate binding of HA, in particular the site-specific positioning of the N-acetyl group at the active center is pivotal for efficient hydrolysis of the polymer. Hence not only steric hindrance, but also a modified intermolecular binding capacity may contribute to the observed change in the pH-activity profile of the enzyme. We therefore calculated the surface electrostatic potential for HYAL1 and HYAL1∆L at pH 3.8 and pH 5.5 using calculated pKa values (electrostatic potential, data not shown) (21,27). HYAL1∆L exhibited a more positive surface electrostatic potential at pH 5.5 than wildtype HYAL1. Thus, besides the protonation states of the active site residues, HA association and dissociation may be rate limiting for HYAL1 activity. Not only the absence of the loop in HYAL1∆L makes it conceivable that the binding crevice is rendered more accessible to the HA polymer (Fig.1) but also changes in the electrostatic potential maps
suggest that the pKa values in HYAL1<sup>ΔL</sup>, which govern substrate binding and product release, are altered.

**DISCUSSION**

Insect hyaluronidases fold into a single domain. This supports the notion that the homologous part of other hyaluronidases is also sufficient for HA hydrolysis. Specific properties of mammalian-type hyaluronidases such as their pH optima may however be effectively influenced by the C-terminal extensions. Vertebrate enzymes come in various isoforms, most likely resulting from posttranslational proteolytic cleavage(s). For instance, mature hyaluronidases can be liberated from their GPI-anchored state by proteolysis close to the C-terminus. In some cases this first processing is followed by a second proteolytic cleavage. In case of HYAL1, both processing steps occur: a 57 kDa form is the only isoform found in plasma; besides the 57, also a 45 kDa form is found in urine (28). A soluble form of PH-20 is also well documented (29,30). The processed 53 kDa form has a pH optimum of 4.0, whereas the unprocessed form is most active at neutral pH (15). The conformational differences at the active center and variations regarding substrate positioning in order to achieve such differences have not been investigated.

Performing (site-directed) mutagenesis and recombinant expression in *Xenopus* oocytes, we studied the serum enzyme, HYAL1, which as shown by others previously is only active at pH below 5.1 (10,31,32). Glutamate at position 131 is the proton donor in the proposed catalytic mechanism for hyaluronidases (7), but E131 is unlikely to be able to donate a proton to the substrate without D129 being protonated. The lowest pKa value in the pair D129 and E131 will therefore govern the pH activity profile of the enzyme. Experimental data on the catalytically competent protonation state in xylanase variant Bcx N35D (family 11 glycosidases) supports the conclusion that only the doubly protonated state is catalytically active (33,34). Many residues in the proximity of the active site are conserved within hyaluronidases. In most cases, mutation at these positions rendered HYAL1 inactive, presumably due to steric hindrance and/or incorrect folding (unpublished results). Since HA is a charged polymer, the pH dependency for substrate association as well as product dissociation must not be neglected. In order to change the gross geometry of the binding cleft, we mutated the bulky epitope at the tapering end of the binding cleft, which folds up between cysteine residues 207 and 221 and contains 13 amino acids. Mutation of the potential glycosylation site (T218A) within the loop region did not greatly alter the enzyme's activity with respect to pH. Similar results were obtained when the stretch betwixt the two cysteines was replaced with the interjacent sequence of PH20/SPAM1, an hyaluronidase that also exhibits activity at neutral pH. In addition of being exactly 13 amino acids long, it also bears four positive charges (H210, H211; K214, K215) compared to one negative charge (D211) present in the HYAL1 loop. It is therefore conceivable that due to stronger ionic interactions with the hyaluronan polymer at positions a long way off from the active center, substrate binding at elevated pH may be actually enforced. Although active at pH 4.5, HYAL1<sup>ΔPH20</sup> was found inactive at pH above 5. Another family member of the mammalian-type hyaluronidases, which exhibits a broad pH profile still being active at neutral pH, is the enzyme secreted into bee venom (13). Its loop sequence is by two amino acid moieties shorter and actually contains no charged residues. Unexpectedly, this variant although exhibiting some activity at pH 3.8 turned out to be inactive at pH 4.5, demonstrating that the mutated region appears to be either essential for direct substrate binding, or is involved in proper positioning of the polymer with respect to the catalytic center.

We next applied computer-assisted protein modeling to design a G-S-G-S interposed variant which displays a greatly enlarged binding crevice. This variant was found active, and over and above, it processed HA also at elevated pH. We therefore conclude that raising pH does not induce major conformational or chemical changes at the active center of HYAL1<sup>ΔL</sup>, and HA can still be sufficiently bound and properly positioned there to be efficiently hydrolyzed. By performing pKa calculations, we were able to qualitatively predict the upshift in the pH-activity profile, but only inadequately predict the
magnitude of the pH-change. It is likely that the deletion in HYAL1∆L causes minor structural relaxation that affects the packing around D129 and E131 thus causing the pKa values of these residues to shift even more than computed. Since structural rearrangements are not accurately comprehended by homology modeling methods, we cannot take these into account in the pKa calculations. However, it is well documented that even small changes in protein structures can lead to large changes in pKa value calculations (16,35). It is furthermore possible that interactions between the protein and the glycan side chains are effected by secondary sites, which induce long range effects on substrate accommodation at the active site.

Acknowledgments
S.R. is FWF Schrödinger fellow. Peter Lackner (University of Salzburg) is acknowledged for assistance in computational protein modeling.

REFERENCES
1. Lepperdinger, G., Fehringer, C., and Reitinger, S. (2004) Biodegradation of hyaluronan. In: Hari, G. (ed). Chemistry and Biology of Hyaluronan, Elsevier
2. Bairoch, A. (2000) Nucleic Acids Res 28(1), 304-305
3. Kreil, G. (1995) Protein Sci 4(9), 1666-1669
4. Henrisat, B., and Bairoch, A. (1996) Biochem J 316, 695-696
5. Lepperdinger, G., and Kreil, G. (2004) Functional, structural and biological properties of hyaluronidase. In: Hari, G. (ed). Chemistry and Biology of Hyaluronan, Elsevier
6. Menzel, E. J., and Farr, C. (1998) Cancer Lett 131(1), 3-11
7. Markovic-Housley, Z., Miglierini, G., Soldatova, L., Rizkallah, P. J., Muller, U., and Schirmer, T. (2000) Structure 8(10), 1025-1035
8. Skov, L. K., Seppala, U., Coen, J. J., Crickmore, N., King, T. P., Monsalve, R., Kastrup, J. S., Spangfort, M. D., and Gajhede, M. (2006) Acta Crystallogr D Biol Crystallogr 62(Pt 6), 595-604
9. Chao, K. L., Muthukumar, L., and Herzberg, O. (2007) Biochemistry 46(23), 6911-6920
10. Frost, G. I., Csoka, A. B., Wong, T., and Stern, R. (1997) Biochem Biophys Res Commun 236(1), 10-15
11. Lepperdinger, G., Müllegger, J., and Kreil, G. (2001) Matrix Biol 20(8), 509-514
12. Reitinger, S., Müllegger, J., and Lepperdinger, G. (2001) FEBS Lett 505(2), 213-216
13. Reitinger, S., Boroviak, T., Laschober, G. T., Fehringer, C., Müllegger, J., Lindner, H., and Lepperdinger, G. (2008) Protein Expr Purif 57(2), 226-233
14. Gmachl, M., Sagan, S., Ketter, S., and Kreil, G. (1993) FEBS Lett 336(3), 545-548
15. Öttl, M., Höchstetter, J., Asen, I., Bernhardt, G., and Buschauer, A. (2003) Eur J Pharm Sci 18(3-4), 267-277
16. Nielsen, J. E. (2007) J Mol Graph Model 25(5), 691-699
17. Arnold, K., Bordoli, L., Kopp, J., and Schwede, T. (2006) Bioinformatics (Oxford, England) 22(2), 195-201
18. Guex, N., and Peitsch, M. C. (1997) Electrophoresis 18(15), 2714-2723
19. DeLano, W. (2002) The PyMOL Molecular Graphics System. In., www.pymol.org
20. Reitinger, S., Laschober, G. T., Fehringer, C., Greiderer, B., and Lepperdinger, G. (2007) Biochem J 401(1), 79-85
21. Nielsen, J. E., and Vriend, G. (2001) Proteins 43(4), 403-412
22. Jorgensen, W. L., and Tirado-Rives, J. (1988) J. Am. Chem. Soc. 110, 1657-1666
23. Nielsen, J. E., Andersen, K. V., Honig, B., Hooft, R. W., Klebe, G., Vriend, G., and Wade, R. C. (1999) **Protein engineering** **12**(8), 657-662
24. Nicholls, A., and Honig, B. (1991) **Journal of Computational Chemistry** **12**, 435-445
25. Müllegger, J., Reitinger, S., and Lepperdinger, G. (2001) **Anal Biochem** **293**(2), 291-293
26. Müllegger, J., and Lepperdinger, G. (2002) **Mechanisms of development** **111**(1-2), 25-35
27. Vriend, G. (1990) **J Mol Graph** **8**(1), 52-56, 29
28. Csoka, A. B., Frost, G. I., Wong, T., and Stern, R. (1997) **FEBS Lett** **417**(3), 307-310
29. Cherr, G. N., Meyers, S. A., Yudin, A. I., VandeVoort, C. A., Myles, D. G., Primakoff, P., and Overstreet, J. W. (1996) **Dev Biol** **175**(1), 142-153
30. Meyer, M. F., Kreil, G., and Aschauer, H. (1997) **FEBS Lett** **413**(2), 385-388
31. Podyma, K. A., Yamagata, S., Sakata, K., and Yamagata, T. (1997) **Biochem Biophys Res Commun** **241**(2), 446-452
32. Natowicz, M. R., and Wang, Y. (1996) **Clinica chimica acta; international journal of clinical chemistry** **245**(1), 1-6
33. Joshi, M. D., Sidhu, G., Nielsen, J. E., Brayer, G. D., Withers, S. G., and McIntosh, L. P. (2001) **Biochemistry** **40**(34), 10115-10139
34. Søndergaard, C. R., McIntosh, L. P., Pollastri, G., and Nielsen, J. E. (2008) **Journal of molecular biology** **376**(1), 269-287
35. Nielsen, J. E., and McCammon, J. A. (2003) **Protein Sci** **12**(2), 313-326
Table 1: Sequence and activity information on HYAL1 variants

|          | loop sequence          | relative activity | pH | 4.5 | 5.5 |
|----------|------------------------|-------------------|----|-----|-----|
| HYAL1<sup>wt</sup> | CYNYDFLSPNYTGQC      | ++++              | 4.5|     |     |
| HYAL1<sup>Δglyc</sup> | CYNYDFLSPNYAGQC      | +++   |     |     |
| HYAL1<sup>ΔPH20</sup> | CYNHHYKKPGYNGSC     | ++    |     |     |
| HYAL1<sup>Δbeeh</sup> | CYNLTPNQPS--AQC    | n/d   |     |     |
| HYAL1<sup>ΔL</sup>   | C---GSGS---C        | +++   |     |  +  |
FIGURE LEGENDS

Figure 1: Structural models: On the basis of the three-dimensional structures of HYAL1 and bee hyaluronidase, models for wildtype hyaluronidase (HYAL1\textsuperscript{wt}) and the loop variants (HYAL1\textsuperscript{\Deltaglyc}: mutated potential glycosylation site (arrow head) within the loop; HYAL1\textsuperscript{\Deltabeeh}: native sequence replaced with loop sequence of that of bee venom hyaluronidase; HYAL1\textsuperscript{\DeltaPH20}: replaced with sequence of human testicular hyaluronidase, PH20; and HYAL1\textsuperscript{\DeltaL}; for detailed sequence information see table 1) with a bound HA tetrasaccharide (stick mode) were generated. The active centre and the loop region are highlighted in color.

Figure 2: Western blot analysis of recombinantly expressed HYAL1 enzymes. Homogenized cell lysates of \textit{X.laevis} oocytes expressing wildtype hyaluronidase 1 (HYAL1\textsuperscript{wt}), the loop-mutant (HYAL1\textsuperscript{\DeltaL}), or uninjected controls were separated on 12% polyacrylamide gel, electroblotted onto nitrocellulose membrane, and immunochemically detected applying our polyclonal antiserum and anti-rabbit-HRP conjugates. Molecular weight is indicated at the left in kDa.

Figure 3: Hyaluronidase activity: Homogenized cell lysates containing wildtype HYAL1 (wt), variant HYAL1\textsuperscript{\DeltaL} (\DeltaL) or mock (m) where incubated with hapten-labeled hyaluronan in citric-phosphate buffer at indicated pH (bottom line). Reactions were electrophoretically separated and blotted onto a nylon membrane and immunochemically detected. (A) representative example of activity assessment at specified pH; (B) pH profiles after histogrammatic quantification of hyaluronidase activity as shown in A, standard deviation are indicated; (C) cumulative evaluation of four independent activity measurements at increasing pH.
Figure 1

| Frontal View | Side View |
|--------------|-----------|
| HYAL1^WT    |           |
| HYAL1^Δglyc |           |
| HYAL1^Δbeeh |           |
| HYAL1^ΔPH20 | HYAL1^ΔL  |
| HYAL1^ΔL    |           |
Designed human serum hyaluronidase 1 variant, HYAL1ΔL exhibits activity up to pH 5.9
Stephan Reitinger, Johnnes Müllegger, Brigitte Greiderer, Jens Erik Nielsen and Günter Lepperdinger

J. Biol. Chem. published online May 28, 2009

Access the most updated version of this article at doi: 10.1074/jbc.C109.004358

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts