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In vitro mutagenesis of PH-20 hyaluronidase from human sperm

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The cDNA encoding PH-20 hyaluronidase from human sperm has been mutated at five positions by *in vitro* mutagenesis. We have changed three acidic amino acids and two arginine residues that are conserved in the sequence of mammalian PH-20 polypeptides as well as in the hyaluronidases from bee and hornet venom. Of the former, the mutants [Gln113]PH-20 and [Gln249]PH-20 had no detectable enzymatic activity; the mutant [Asn111]PH-20 had about 3% activity. The mutant [Thr252]PH-20 was also inactive, while [Gly176]PH-20 had only about 1% activity. This indicates that the PH-20 hyaluronidases, like numerous enzymes that hydrolyze glycosidic bonds, have acidic amino acids in their active site. Moreover, for the binding of the substrate, the polyanion hyaluronan, arginine residues appear to be essential.

Keywords: PH-20 hyaluronidase; *in vitro* mutagenesis; spermatozoa.

Hyaluronan (hyaluronic acid) is a ubiquitous component of the extracellular matrix of vertebrates. This glycosaminoglycan, which is composed of alternating units of *N*-acetylglucosamine and glucuronic acid, can form highly viscous solutions and thereby influence the properties of this matrix. Hyaluronan has been implicated in many biological processes including fertilization, embryonic development, cell migration, and differentiation, wound healing, inflammation, and growth and metastasis of tumor cells (Laurent and Fraser, 1992). Three types of hyaluronidases are known which are widely distributed in nature (see Meyer, 1971; Kreil, 1995; Frost et al., 1996 for reviews). One group are endo-*N*-acetylhexosaminidases that hydrolyze hyaluronan with a molecular mass of over 1×10^6 Da mostly to tetrasaccharides. These enzymes have been detected in diverse sources such as mammalian testes and insect venoms. A few years ago, the amino acid sequence of bee venom hyaluronidase was elucidated via cDNA cloning (Gmachl and Kreil, 1993). The sequence of this enzyme was found to be homologous to PH-20, a polypeptide localized on the head of guinea pig sperm (Lathrop et al., 1990). It could subsequently be demonstrated that PH-20 from different mammalian species has hyaluronidase activity (Gmachl et al., 1993; Lin et al., 1994a; Cherr et al., 1996).

A comparison of the amino acid sequences of bee and hornet venom hyaluronidases (Gmachl and Kreil, 1993; Lu et al., 1995) and those of the human, monkey, mouse, and guinea pig PH-20 proteins (Lathrop et al., 1990; Gmachl et al., 1993; Lin et al., 1994a, b) demonstrated that in a common region encompassing about 340 amino acids, 57 amino acids are conserved in all of these proteins (Fig. 1). These include four cysteine residues forming two disulfide bridges. In addition, we assume that the residues essential for substrate binding and catalysis are also conserved between the hymenopteran and mammalian enzymes.

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At present, nothing is known about the active site of hyaluronidases. Based on a few assumptions, we have now addressed the question which amino acids in these enzymes are essential for the hydrolysis of hyaluronan. Binding of the acidic glycosaminoglycan to the enzyme probably involves ionic interaction with basic amino acids. Indeed, a comparison of the mammalian PH-20 proteins and the enzymes from insect venom demonstrates that three arginine residues are conserved in all these polypeptides. As for the active site of hyaluronidases, we assumed that it may be similar to that of other polysaccharide hydrolases. It has been shown that in many enzymes of this type, such as lysozymes, chitinases, xylanases and others a pair of acidic residues, mostly glutamic acids, serves as the nucleophile and the acid/base catalyst (see Withers and Aebersold, 1995 for a recent review). Three glutamic and three aspartic acids are present at homologous positions in the insect and the mammalian hyaluronidases. Following these conjectures, we have changed several of these common amino acids in the human PH-20 hyaluronidase by *in vitro* mutagenesis.

EXPERIMENTAL PROCEDURES

***In vitro* mutagenesis.** The clone pCM19/24 (Gmachl et al., 1993) containing the human PH-20 cDNA in the Bluescript vector (Stratagene) was amplified twice by PCR with suitable oligonucleotides as primers (Higuchi et al., 1988). For the first rounds of PCR, these were GCCAGTATACAATTTGGGAATGGC (5′-outside primer), which hybridizes to the 5′-end of the *Acc*I fragment of the PH-20 cDNA and which contains a restriction site for this enzyme (underlined), and a 3′-mismatch primer with the desired mutation. In a parallel PCR, the same mutation was present in a 5′-mismatch primer; the oligonucleotide CGCCAAATGTATACACAAGTTC, which hybridizes to the 3′-end of the *Acc*I fragment, was used as the second primer (3′-outside primer). PCR was performed with 2 μg DNA, 25 pmol of each primer, 0.2 mM dNTPs, 0.5 U Tth DNA polymerase (Promega), 0.05 U Deep Vent DNA polymerase, and 0.1 vol. Deep Vent DNA polymerase buffer (Biolabs) in a final volume of 50 μl. After denaturating the DNA for 2 min at 92°C, 10

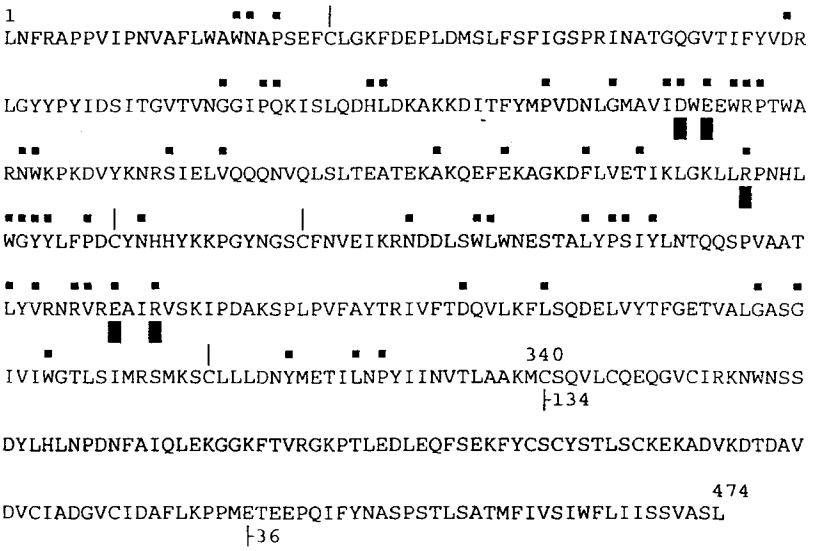

Fig. 1. Amino acid sequence of human PH-20 hyaluronidase. Amino acids conserved between mammalian and insect venom hyaluronidases are marked (■), the four conserved cysteine residues are marked (|). The five mutated amino acids are indicated by (▮). The deletions are marked (⊢).

cycles of PCR were performed (30 s at 92 °C, 30 s at 48 °C, 15 s at 72 °C) followed by 3 min of polymerization at 72 °C. Amplified fragments were purified by agarose gel electrophoresis and eluted with the Qiaex II agarose gel extraction kit (Qiagen).

The fragments obtained in the two PCRs were combined and amplified under the same conditions except that only the outside primers were used. The products were again purified by agarose gel electrophoresis and digested with the restriction endonuclease *Acc*I. This yielded a central fragment, nucleotides 784–1341, of the mutated PH-20 cDNAs, which was then inserted into the *Acc*I site of the plasmid pAta-HPH (see below).

Preparation of PH-20 mutants with C-terminal deletions. The mutant des-(439–474)-PH-20 was generated by digestion of the human PH-20 cDNA with the endonuclease *Sty*I. The truncated cDNA was blunt ended with T4 DNA polymerase and ligated into the *Sma*I site of pgpt-Ata-18. The mutant des-(341–474)-PH-20 was created by the introduction of a stop codon at the position of the Cys341 (TGT→TGA) using the same method as for the other mutants.

Ligation of PCR fragments into the vaccinia virus expression vector pgpt-Ata-18. The human PH-20 cDNA had earlier been inserted into the pgpt-Ata-18 vector (Stunnenberg et al., 1988) to yield p-Ata-HPH (Gmachl et al., 1993). To remove the *Acc*I restriction site in the polylinker of the vector, p-Ata-HPH was digested with *Bam*HI and *Pst*I. After incubation with T4 DNA polymerase (10 min at 16 °C, 5 min at 37 °C, 20 min at 65 °C), the blunt-ended vector was ligated with T4 DNA ligase. The modified p-Ata-HPH plasmid and the PCR products were digested with *Acc*I and ligated for 2 h at room temperature. Inserts of positive clones were checked by sequencing the region corresponding to the *Acc*I fragments.

Preparation of recombinant vaccinia virus. Recombinant virus expressing the different PH-20 mutants were prepared as described previously (Gmachl et al., 1993; Stunnenberg et al., 1988). HeLa cells were infected with recombinant vaccinia virus at a multiplicity of infection of 0.1 or 1. After different times, cells were lysed by freezing and thawing, and tested for expression of wild-type and mutant PH-20 polypeptides.

Western blots. Cells were lysed by freezing and thawing and crude membrane fractions were isolated by centrifugation at 900 *g* for 10 min. Membranes were solubilized in NaCl/P_i (137 mM NaCl, 2.7 mM KCl, 7.5 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.5) containing 1% Triton X-100 and used for western blot analysis. Prior to digestion with glycosidase F (Boehringer), samples were denatured in NaCl/P_i/0.1% sodium dodecylsulfate/25 mM EDTA for 5 min at 95 °C. Digestion was performed with 0.4 U enzyme at 37 °C for 90 min. The recombinant human PH-20 polypeptides were detected with a polyclonal antiserum against PH-20 from cynomolgus macaques (kindly provided by Dr Paul Primakoff) and a second antibody labelled with peroxidase (Bio-Rad). The antiserum was diluted 1000-fold.

Hyaluronidase assays. HeLa cells infected with recombinant vaccinia virus were centrifuged at 900 *g* for 10 min, washed with NaCl/P_i, and lysed by freezing and thawing. Crude membranes were pelleted, washed with NaCl/P_i and PH-20 polypeptides were solubilized by addition of 1% Triton X-100 (1 ml Triton for about 2×10^7 cells harvested from one large Petri dish). Supernatants were stored at −20 °C. Aliquots of 20 µl were used for different hyaluronidase assays (Dorfman, 1955; Guntenhöner et al., 1992; Lee and Cowman, 1994).

Hyaluronan from human umbilical cord was dissolved at a concentration of 0.25 mg/ml in 0.02 M sodiumphosphate, pH 6.9, containing 75 mM NaCl and 0.01% bovine serum albumin. Aliquots of the Triton extracts were added to 20 µl hyaluronan and incubated for 2 h at 37 °C. Samples were then boiled and fractionated by agarose gel electrophoresis (0.5% agarose). Hyaluronan and its fragments were detected by staining the gel with the cationic dye Stains-All (3,3′-dimethyl-9-methyl-4,5,4′,5′-dibenzothiacarbocyanine) as described by Lee and Cowman (1994). Membranes prepared from rabbit kidney RK13 cells expressing the DG42 polypeptide were incubated in the presence of UDP-GlcNAc and [^{14}C]UDP-glucuronic acid as described (Meyer and Kreil, 1996). At the end of the reaction, the solution was centrifuged and from the supernatant, low-molecular-mass components were removed by filtration through a 30 kDa filter (Centricon 30, Amicon).

Hyaluronidase activity was determined as described above except that about 30 ng [^{14}C]hyaluronan (3000 cpm) were included in the reaction. At different times, aliquots were removed, boiled for 5 min and fractionated by paper chromatography (Whatman 3MM paper) using 7:13 (by vol.) 1 M ammonium acetate (pH 5.5)/ethanol as solvent. Radioactivity remaining at the origin was detected with a liquid scintillation counter.

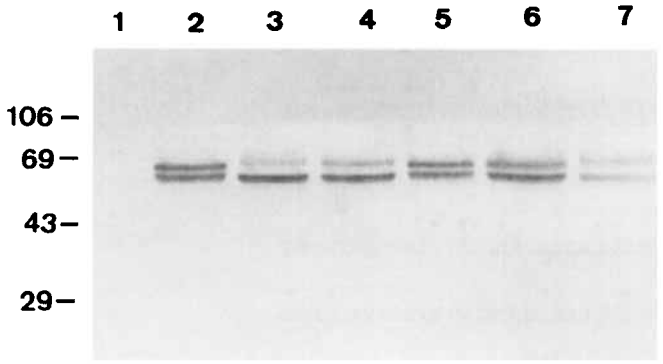

Fig. 2. Western blot analysis of membranes from cells expressing PH-20 mutants. HeLa cells were infected with recombinant vaccinia virus at a multiplicity of infection of 0.1. After 2 days, cells were lysed, crude membranes were prepared and used for western blots with the polyclonal anti cPH-20 antiserum as described in the Experimental Procedures section. Lane 1, wild-type vaccinia virus; lane 2, vaccinia virus expressing the human PH-20 polypeptide; lane 3, PH-20 mutant [Gly176]PH-20; lane 4, mutant [Thr252]PH-20; lane 5, mutant [Gln113]PH-20; lane 6, mutant [Asn111]PH-20; lane 7, mutant [Gln249]PH-20. Molecular mass markers (kDa) are indicated.

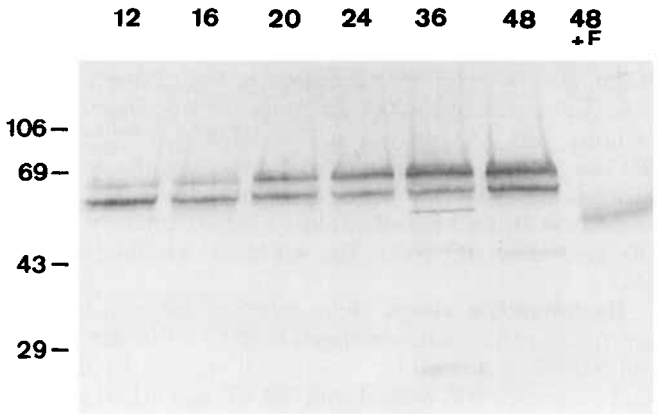

Fig. 3. Western blot analysis of human PH-20 protein expressed in HeLa cells. Cells were infected with recombinant vaccinia virus at a multiplicity of infection of 1. At the times indicated (hours post infection), cells were lysed and used for western blot analysis. One sample collected at 48 h post infection was treated with glycosidase F. Numbers on the left indicate molecular mass markers (kDa).

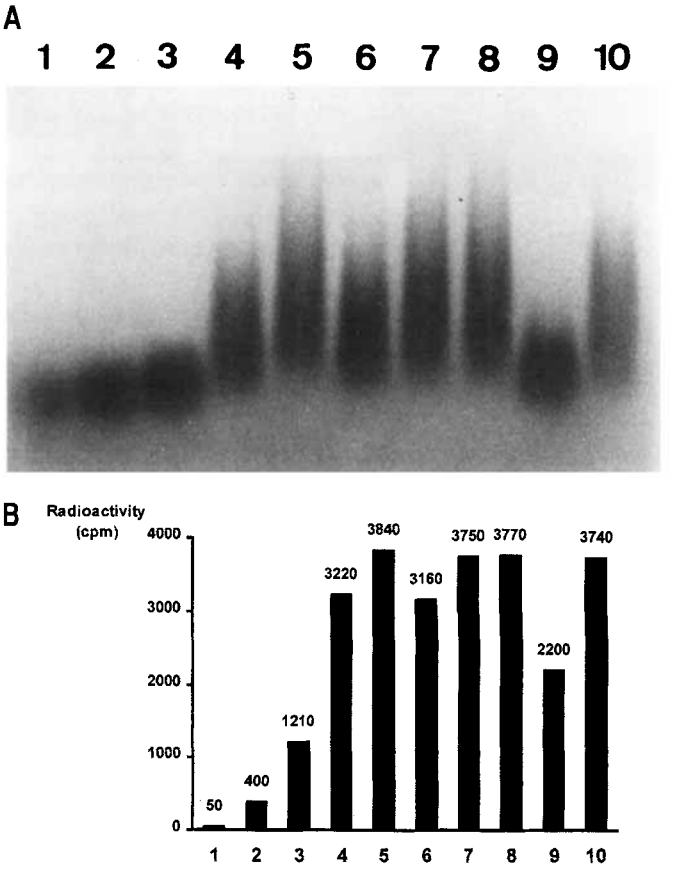

Fig. 4. Assays for hyaluronidase activity. (A) Hyaluronan was incubated for 2 h with 20 µl Triton X-100 extracts, which corresponds to about 4×10^5 cells expressing mutant PH-20 proteins. The remaining hyaluronan was fractionated by agarose gel electrophoresis and gels were stained with Stains-All (see Experimental Procedures section). The assay with [^{14}C]hyaluronan is shown in B. Triton X-100 extracts prepared from cells infected with recombinant vaccinia virus were incubated with radioactive hyaluronan for 2 h. After paper chromatography, radioactivity remaining at the origin, expressed as cpm, was measured (number above the bars). For comparison, decreasing amounts of wild-type PH-20 hyaluronidase were assayed under the same conditions. Lanes 1–4, aliquots of 1/5, 1/10, 1/20 and 1/100 the amount of extract from cells expressing wild-type PH-20 hyaluronidase; lane 5, blank without enzyme; lane 6, mutant [Gly176]PH-20; lane 7, mutant [Thr252]PH-20; lane 8, [Gln113]PH-20; lane 9 [Asn111]PH-20; lane 10, [Gln249]PH-20. In three separate experiments, 97.2–98.3% of the counts were recovered at the origin for mutants in lane 7, 8, and 10; 79.3–84.0% in mutant [Gly176]PH-20 (lane 6, five experiments, average 82.1); 51.2–59.2% for mutant [Asn111]PH-20 (lane 9, five experiments, average 54.2).

RESULTS

The amino acid sequence of the human PH-20 protein starting with the expected amino end (von Heijne, 1986) is shown in Fig. 1. Amino acids conserved between insect venom hyaluronidases and the mammalian PH-20 proteins are marked. Of these invariant residues, we have selected two glutamic, one aspartic acid, and two arginines. These were changed to neutral amino acids by *in vitro* mutagenesis of the *Acc*I fragment of the cloned cDNA encoding the human PH-20 protein (see Experimental Procedures section). The mutations were checked by sequencing. Subsequently, the *Acc*I fragment was excised from the pAta-HPH vector encoding the human PH-20 cDNA and replaced by *Acc*I fragments containg the desired mutations. Recombinant vaccinia virus was prepared and used to infect HeLa cells. Expression of the wild-type and mutated PH-20 proteins was checked by western blotting. As shown in Fig. 2, comparable amounts of PH-20 protein could be detected in all cases. Only for the mutant [Gln249]PH-20 was expression somewhat lower. Two bands with an apparent molecular mass around 65 kDa were observed, which probably represent polypeptides with different glycosylation patterns. Indeed, the relative amount of the larger, presumably more highly glycosylated form, increases with time after infection by the recombinant vaccinia virus (Fig. 3). Treatment with endoglycosidase F yields a single polypeptide. This pattern is similar to that observed with PH-20 from macaque sperm (Cherr et al., 1996).

Cells infected with the different recombinant vaccinia virus at a multiplicity of infection of 0.1 were collected after 48 h, lysed, and crude membrane fractions were tested for hyaluronidase activity. With the five mutants, enzymatic activities were too low to obtain reliable values with the assay developed by Dorfmann (1955). For a first estimate, we therefore used a gel assay with decreasing amounts of wild-type PH-20 hyaluronidase as a standard (Fig. 4A). This indicated that three of the

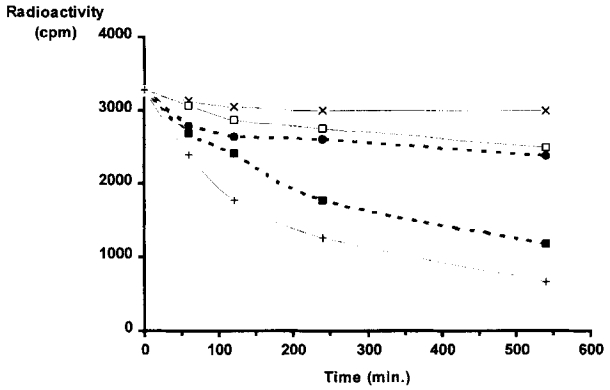

Fig. 5. Hyaluronidase activity of PH-20 mutants [Asn111]PH-20 (–■–) and [Gly176]PH-20 (–●–). Radioactive hyaluronan was incubated with extracts from HeLa cells infected with recombinant vaccinia virus for various periods of time. Radioactivity remaining at the origin after chromatographic separation is plotted against time. As a control, a blank with no enzyme (–×–) and samples with 1/100 (–□–) and 1/20 (–+–) the amount of extract from cells expressing the wild-type PH-20 enzyme were included.

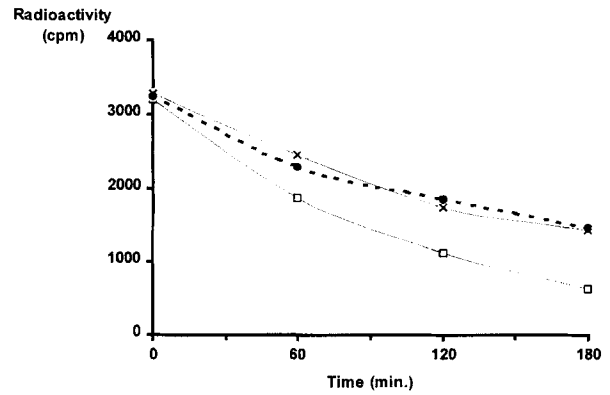

Fig. 6. Hyaluronidase activity of a truncated form of the human PH-20 protein. An extract from HeLa cells expressing the mutant des-(439–474)-PH-20 of human PH-20 hyaluronidase (–●–) was incubated with radioactive hyaluronan. Controls were aliquots of 1/4 (–×–) and 1/2 (–□–) the amount of extract from cells expressing wild-type PH-20. Conditions are as described in the legend of Fig. 5.

mutants, [Gln113]PH-20, [Gln249]PH-20, and [Thr252]PH-20 were devoid of enzymatic activity, while the two other mutants, [Asn111]PH-20 and [Gly176]PH-20, had residual activities in the range of one to a few percent of wild-type PH-20 hyaluronidase.

This was tested in more detail with a radioactive assay as used for measuring the biosynthesis of hyaluronan (Meyer and Kreil, 1996). Upon paper chromatography in a suitable solvent system, high-molecular mass polysaccharides stay at the origin, while UDP-sugars and oligosaccharides migrate away. Radioactive hyaluronan was incubated with equal-volume aliquots from lysates prepared from cells infected with recombinant vaccinia virus encoding the different mutants. As controls, a series of dilutions from lysates obtained from cells expressing the wild-type PH-20 hyaluronidase were included. As shown in Fig. 4B, the mutants [Gly176]PH-20 and [Asn111]PH-20 had about 1% and 3% of the wild-type enzyme activity, respectively. This was corroborated by a time-course experiment (Fig. 5). For the other three mutants, enzymatic activity could not be detected with this assay using a radioactive substrate.

We also wanted to test whether PH-20 polypeptides with deletions at the C-terminus had any enzymatic activity. One such mutant that lacked the last 36 amino acids had about 25% of the hyaluronidase activity exhibited by the intact molecule (Fig. 6). The PH-20 protein is anchored to the head of spermatozoa via a glycosyl-phosphatidylinositol anchor. The des-(439–474)-PH-20 mutant should lack this anchor and would thus be expected to be a secreted polypeptide. Interestingly, it was found that upon expression of this mutant in HeLa cells, the protein was not secreted but retained inside the cells (data not shown).

A larger deletion at the C-terminus that lacked 134 amino acids was also devoid of enzymatic activity. As checked by Western blotting, the mutant protein was not secreted from infected cells (data not shown).

DISCUSSION

Since the elucidation of the three-dimensional structure of lysozyme 30 years ago (Philips, 1967), many of the enzymes that degrade different polysaccharides have been studied in detail (see recent reviews by Henrissat and Bairoch, 1993; Withers and Aebersold, 1995). Apparently without exception, the carboxyl groups of two acidic amino acids function as acid/base catalysts in the hydrolytic reaction. Moreover, in some instances a third residue of this type is also important as it apparently modulates the ionization of one of the other carboxyl groups (Klein et al., 1992; Qian et al., 1994). In this study, we present evidence that suggests that this is also true for the human PH-20 hyaluronidase. *In vitro* mutagenesis of the Glu113 or Glu249 to glutamine yielded PH-20 polypeptides without detectable enzymatic activity in two different assay systems. A third mutant, where Asp111 was changed to asparagine, had about 3% of the activity of the wild-type enzyme. These three acidic amino acids lie within clusters of amino acids that are conserved between mammalian and hymenopteran hyaluronidases. There are three more conserved residues of this type, Asp59, Asp275, and Glu158, but these are located outside the constant sequence segments and were thus considered to be less likely candidates for being part of the active site.

In view of the fact that the substrate of hyaluronidases is a polyanion, it seemed likely that its binding to the enzyme involves ionic interaction with positively charged amino acids. Of the conserved residues of this group, three arginines and one histidine, we have mutated two arginines. Of these, the [Thr252]PH-20 mutation yielded an enzyme without detectable activity. This residue is close to Glu249, which appears to be part of the active site. The second mutant, [Gly176]PH-20, has residual enzymatic activity about 1% of the wild-type enzyme, indicating that this amino acid is also important for substrate binding. It has been shown that the testicular hyaluronidase interacts with a hexasaccharide as the smallest substrate (Takagaki et al., 1994). It thus seems likely that at least one more positively charged amino acid interacts with a carboxyl group of a glucuronic acid of the substrate. Finally, we have generated two truncated versions of the human PH-20 hyaluronidase. In one version, we only removed 36 amino acids from the carboxyl end. This should yield a secreted polypeptide without the glycosyl-phosphatidylinositol anchor of the native form. This rather small deletion yields a protein with about one quarter of the enzymatic activity of wild-type PH-20. However, the mutated polypeptide is not secreted from HeLa cells but remains inside the cells. A similar deletion in the PH-20 protein from cynomolgus monkey was constructed by Lin et al. (1994a). This truncated protein was secreted by insect cells and could be purified with an antibody column from media. Apparently, this mutant retains full

activity. A second, larger deletion in the human protein was generated to obtain a derivative of the testis enzyme closely resembling the bee venom enzyme. The des-(341−474)-PH-20 mutant terminates in the same region as the latter; in addition, only the four cysteine residues common to all these hyaluronidases are present in this fragment. However, contrary to our expectations we could not detect enzymatic activity in cells expressing the des-(341−474)-PH-20 mutant.

In this study, we present data on amino acids that are apparently essential for binding or hydrolysis in a mammalian hyaluronidase. It has previously been shown that guinea pigs immunized with their own PH-20 protein are sterile but regain fertility when the antibody titer decreases (Primakoff et al., 1988). Should such a method ever be applied in human contraception, it would clearly be advantageous to use a polypeptide for immunization that is devoid of enzymatic activity. Within the limits of the hyaluronidase assays used in this study, three such mutants are now available.

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