UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

MERCK SHARP & DOHME LLC,
Petitioner

v.

HALOZYME, INC.,
Patent Owner

Case PGR2025-00050
U.S. Patent No. 12,077,791

DECLARATION OF GREGORY A. PETSKO, PH.D. IN SUPPORT OF PATENT OWNER'S RESPONSE

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Halozyme EX2070
Merck v. Halozyme
PGR2025-00050

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I, Gregory A. Petsko, Ph.D., hereby declare as follows.

I. INTRODUCTION

1. I have been retained by Halozyme, Inc. ("Patent Owner") to provide expert opinions regarding protein structure, the disclosure of U.S. Patent No. 12,077,791("the '791 patent"),[1] and to evaluate and respond to opinions of Dr. Michael Hecht and Dr. Sheldon Park as set forth in their declarations regarding the '791 patent.

2. My opinions in this declaration are based on the documents I cite along with my education, experience and knowledge that I have acquired working in the fields of protein structure and enzymology over the past 55 years.

3. I am being compensated for my services as an expert at my standard consulting rate of $1750 per hour. My compensation is in no way contingent on the substance of my opinions or the outcome of this case.

II. BACKGROUND AND QUALIFICATIONS

4. I am currently Professor of Neurology at the Ann Romney Center for Neurologic Diseases at Harvard Medical School and Brigham and Women's Hospital. I also am Adjunct Professor of Neuroscience at Weill Cornell Medical College and Adjunct Professor of Biomedical Engineering at Cornell University,

[1] I understand that the 791 patent is related to U.S. Patent Application No. 13/694,731, which was filed on December 28, 2012. Dr. Hecht refers to the disclosures of both applications as "the common disclosure."

and I am the Gyula and Katica Tauber Professor, Emeritus, in Biochemistry and Chemistry at Brandeis University.

5. Halozyme has requested that I provide this opinion as an expert in the field of structural biochemistry. My qualifications in these areas, as well as other areas, are established below and by my *curriculum vitae*. As an expert, I have offered opinions throughout this declaration from the perspective of a person of ordinary skill in the art ("POSA"), defined in Section IV below.

6. My major research interests revolve around protein structure and function, in recent years especially in regards to neurodegenerative diseases. Throughout my career I have studied various types of protein interactions from the macromolecular to the atomic level.

7. I graduated *summa cum laude* with a B.A. in Chemistry from Princeton University in 1970.

8. Then as a Rhodes Scholar, I earned a D. Phil. (the Oxford Ph.D.) in Molecular Biophysics from Oxford University in 1973.

9. I then pursued post-doctoral training at the Institut de Biologie Physico-Chemique at the Universite de Paris in Paris, France before beginning my faculty career at Wayne State University School of Medicine in 1974.

10. I have held a number of other academic appointments over my career, including serving as Chair of the Department of Biochemistry at Brandeis

University from 2008-2011; as Director of the Rosenstiel Basic Medical Sciences Research Center at Brandeis University from 1994-2007; as a Professor or Associate Professor of Chemistry at the Massachusetts Institute of Technology from 1979-1990; as the Arthur J. Mahon Professor of Neurology and Neuroscience at Weill Cornell Medical College from 2012-2018, and the Director of the Helen and Robert Appel Alzheimer's Disease Research Institute at Weill Cornell Medical College from 2014 to 2018.

11. As an undergraduate in 1970, I published my first research paper in the prestigious American journal, SCIENCE (Petsko, G.A. "Proton Magnetic Resonance Spectrum of 'Anomalous' Water," 167 SCIENCE 171-72 (1970)), and have gone on to author or co-author more than 350 peer-reviewed articles, most detailing the relationship between protein structure and function.

12. Much of my work involves the use of X-ray crystallography to determine the atomic structure of proteins.

13. I have been the recipient of the Pfizer Award in Enzyme Chemistry from the American Chemical Society. I am generally considered one of the founders of the field of structural enzymology.[2] I have published hundreds of papers on enzyme structure and mechanism, and have contributed a number of

[2] *See., e.g.,* EX2163 (https://nationalmedals.org/laureate/gregory-petsko/?amp=1) (recognizing my "role in founding structural enzymology").

principles to our general understanding of the structural basis of the catalytic power of enzymes. I have also published studies on enzyme stability and engineering.

14. In 2003, I published a textbook (alongside Prof. Dagmar Ringe) covering basic principles of protein structure, folding, and function, including enzymology, entitled PRIMERS IN BIOLOGY: PROTEIN STRUCTURE AND FUNCTION. It was reprinted in 2009.

15. Leading scientific professional organizations have recognized my work as a structural biochemist. I am an elected member of both the National Academy of Sciences (1995) and the Institute of Medicine (the National Academies' health arm, now called the National Academy of Medicine) (2001), as well as the American Philosophical Society, the oldest honorary society in the United States (2010).

16. I have also been elected as a member of the American Academy of Arts and Sciences (2002) in recognition for my work which "[p]ioneered the development of low-temperature X-ray crystallography" and "considerably broadened our view of the dynamic nature of proteins."[3]

17. The American Crystallographic Association awarded me the Siddhu Award for outstanding contributions to the development of X-ray diffraction in

[3] EX2168, https://www.amacad.org/person/gregory-petsko.

1980, and in 2015 bestowed on me the Martin J. Buerger Award for "contributions of exceptional distinction" in the area of crystallography.

18. I also served as President of the American Society for Biochemistry and Molecular Biology from 2008-2010 and as President of the International Union of Biochemistry and Molecular Biology from 2012-2015.

19. In October of 2023, I was awarded the National Medal of Science by President Joe Biden for my work in advancing understanding of neurodegenerative diseases such as Alzheimer's, Parkinson's, and ALS (amyotrophic lateral sclerosis, also known as Lou Gehrig's disease), and for "his role in founding structural enzymology". The National Medal of Science, established in 1963 by President John F. Kennedy, is the highest honor the United States can bestow on a scientist or engineer.

20. I have served on the editorial boards of peer-reviewed journals such as the *Proceedings of The National Academy of Science*, *Science Advances*, *Trends in Biochemical Sciences*, and *Current Opinion in Structural Biology*. I was the co-founding editor of the journal *Protein Engineering* and was a co-founding editor of the PLoS family of journals.

21. I have also founded or co-founded therapy development companies and served on the scientific advisory boards of life sciences organizations such as

Ironwood Pharmaceuticals, Amicus Therapeutics, Pfizer, MeiraGTx, Retromer Therapeutics, Graviton Biosciences, and the Howard Hughes Medical Institute.

22. I have engaged in public service from 2001 to 2015 as a member of scientific committees of the National Research Council and the U.S. Department of Energy.

23. Further details regarding my background, publications and experience are provided in my *curriculum vitae*, which is appended to this Declaration as EX2071.

III. MATERIALS CONSIDERED

24. The information that I have relied upon in forming my opinions for this declaration, in addition to my professional and academic experience, is set forth throughout the report and in the Table of Exhibits.

IV. POSA DEFINITION

25. I have been asked to apply Merck's definition of a person of ordinary skill in the art ("POSA") for purposes of my analysis in this Declaration:

> A person of ordinary skill in the art would have had an undergraduate degree, a Ph.D., and post-doctoral experience in scientific fields relevant to study of protein structure and function (*e.g.*, chemistry, biochemistry, biology, biophysics). From training and experience, the person would have been familiar with factors influencing protein structure, folding and activity, production of modified proteins using recombinant DNA techniques, and use of biological assays to characterize protein function, as well with techniques used to analyze protein

structure (*i.e.*, sequence searching and alignments, protein modeling software, etc.).

26. Separate from Merck's POSA definition, I note that a POSA could have worked as part of a multidisciplinary team, and that team could include at least one person with hyaluronidase experience.

27. I understand that the '791 patent claims priority to a patent application filed on December 28, 2012 and to provisional patent applications filed on November 1, 2012 and December 30, 2011. I had at least the qualifications of a POSA as of each of those dates.

28. Throughout my declaration, my opinions are given from the perspective of a POSA as of December 28, 2012, unless I have specified a different date.

V. LEGAL STANDARD

29. For purposes of this declaration, I have been informed by counsel for Patent Owner about certain aspects of the law that are relevant to my analysis and opinions.

30. In formulating my opinions set forth in this Declaration, I applied the following legal principles:

A. Burden of Proof

31. I have been informed that the party challenging the claim's patentability in a post-grant review proceeding ("PGR") must establish

unpatentability by a preponderance of the evidence. I understand that "preponderance of the evidence" means more likely than not.

B. Written Description

32. I understand that the written description requirement under the patent laws requires a patent specification to include a written description sufficient to show a POSA that the inventor had possession[4] of the claimed subject matter as of the filing date of the patent, or as of the filing date of an earlier related application if the patent is to receive the benefit of that earlier date. Whether this requirement is satisfied is determined through an objective inquiry into the disclosure in the specification, evaluated from the perspective of a POSA at the relevant time.

33. I further understand that this written description requirement is assessed in view of the state of the knowledge of a POSA in the art. In addition, I understand that a patent specification is written for a POSA and that such a hypothetical person is presumed to have all of the knowledge of the state of the art as of the relevant date. Well-known concepts, structures, and techniques are part of that knowledge and need not be explicitly stated in the specification.

34. I understand that, for a claim that covers more than one embodiment (*i.e.*, a genus claim), which is the case here, written description requires the

[4] I understand "possession" does not mean physical possession and does not require making or testing the invention.

disclosure of either a representative number of species (*i.e.*, embodiments) falling within the scope of the genus or structural features common to the members of the genus so that one of skill in the art can visualize or recognize the members of the genus.

35. I also understand that adequate written description requires a precise definition, such as by structure, formula, chemical name, physical properties, or other properties, of species falling within the genus sufficient to distinguish the genus from other materials.

36. I understand that a patent specification can provide adequate written description support for functional claim language (*i.e.*, claim language that describes how the invention functions rather than what the invention is) when the specification, read in light of the art, has established a correlation between structure and function.

37. I understand that the specification need not disclose every embodiment within the scope of the claims to satisfy the written description requirement.

38. Finally, I understand that merely drawing a fence around the outer limits of a purported genus is not an adequate substitute for describing a variety of materials constituting the genus and showing that one has invented a genus and not just a species.

C. Enablement

39. I understand that to satisfy the enablement requirement, the specification must provide sufficient information to allow a POSA to make and use the full scope of the claimed invention without undue experimentation as of that application's filing date.

40. I understand that whether a claim is enabled without undue experimentation is evaluated based on several non-exhaustive factors, commonly referred to as the *Wands* factors: (1) the breadth of the claims; (2) the nature of the invention; (3) the state of the prior art; (4) the level of ordinary skill in the art; (5) the predictability of the art; (6) the amount of guidance provided in the specification; (7) the presence or absence of working examples; and (8) the quantity of experimentation needed.

41. I understand that even if some experimentation is required, a claim may still be enabled so long as the experimentation is routine and not undue. The inquiry is not purely quantitative; a substantial amount of experimentation may be acceptable if that type of experimentation is routine in the art.

42. I understand that enablement does not require that the claims expressly exclude inoperative embodiments. A claim that covers inoperative embodiment is enabled so long as a POSA can identify the inoperative embodiments using routine experimentation.

43. I also understand that a patent need not explicitly teach a POSA how to make every possible composition, permutation, or form covered by the claims, but the patent must enable a POSA to make and use the full scope of the claimed subject matter.

VI. SUMMARY OF OPINIONS

44. The ’791 patent provides a comprehensive and systematic analysis of each position on PH20, achieved through large-scale mutagenesis and enzymatic activity testing of thousands of variants, with up to 18 mutations per position. These data provide a functional map of PH20 that identifies for the POSA the extent to which each position in the sequence tolerates substitutions and which do not, which types of residues at each position are likely to be successful in terms of maintaining activity, and which positions in particular could be targeted to maintain or improve activity. The patent also teaches which positions to change to inactivate PH20.

45. The ’791 patent also teaches many substitutions that retain or improve PH20 activity. For example, the patent provides data showing that the I309E, I309G, I309H, I309L, I309M, I309N, I309Q, I309R, I309S and I309T substitutions are all active mutants, and improve activity. EX1001, Table 9.

46. Claim 1, which I understand is the broadest claim, recites “[a] modified PH20 polypeptide comprising an amino acid sequence, wherein:” (a) “the

modified PH20 polypeptide comprises an amino acid modification at a position corresponding to position 309 … [that is] selected from among E, G, H, L, M, N, Q, R, S, and T," (b) "at least 95% of the residues of the amino acid sequence of the modified PH20 polypeptide are identical to the residues in the amino acid sequence of SEQ ID NO: 35" and (c) "the modified PH20 polypeptide exhibits increased hyaluronidase activity compared to the hyaluronidase activity of the polypeptide of SEQ ID NO: 3". EX1001, Claim 1.

47. SEQ ID NOs: 3 and 32-66 are C-terminal truncation variants. The sequences are identical up to position 430. They then differ by one amino acid from position 430 to 465 in the mature PH20 sequence.[5] For example, SEQ ID NO: 32 ends at position 430, SEQ ID NO: 33 ends at position 431, and so on.

48. The POSA would have understood from the disclosure in the '791 patent and in the art (*e.g.*, the '457 publication that is incorporated by reference into the '791 patent) that SEQ ID Nos. 36-55 & 3 have hyaluronidase activity.

49. The claims thus cover a narrow set of proteins—modified PH20 polypeptides that share at least 95% identity with SEQ ID NO: 35 and one of ten permitted mutations at position 309. The claimed set of proteins share 95% of their primary structure, including the substitution at position 309. The 95%

[5] I use the term "mature" to refer to the PH20 sequence without the 35 amino acid signal peptide. I use the term "precursor" to refer to the PH20 sequence with the 35 amino acid signal peptide.

sequence identity and substitution at position 309 are common structural features shared by the set of claimed modified PH20 polypeptides.

50. POSAs could have readily visualized and recognized the set of modified PH20s with 95% primary sequence identity and the recited modification at position 309. POSAs would have understood that the set of claimed proteins share at least 95% of their primary structure, with less than 5% variation, and the recited modification at position 309. POSAs would have been able to recognize the types of modifications permitted to achieve at least 95% sequence identity and would have been able to visualize the sequences that have these features. And POSAs could have readily determined whether any sequence meets the 95% identity requirement and the 309 substitution with a simple sequence alignment.

51. The POSA would have understood that properly folded PH20s adopt the same tertiary structure-an alpha-beta barrel fold. The claimed modified PH20s (that fold) thus share this fundamental common structural feature (the PH20 alpha-beta barrel fold).

52. The alpha-beta barrel fold structure is a robust tertiary structure that often is resilient to modification without inactivating the protein. However, the patent data show that this is not the case for PH20. The patent data show which positions on PH20 do not tolerate substitutions or tolerate very few substitutions, and the types of substitutions that are tolerated. This extensive, virtually

unparalleled information (for an enzyme of this size) present in the specification permits a POSA to predictably make and use PH20 variants with 95% identity to the recited sequences and also containing the recited 309 mutation.

53. Enzymatically active modified PH20s within the scope of the claims share a common mechanism of action in that they cleave hyaluronan ("HA") through a substrate-assisted hydrolysis reaction. All share the same active site.

54. A POSA would have understood that the '791 patent teaches a correlation between the structure of modified PH20 and the hyaluronidase function of modified PH20. Through comprehensive mutagenesis data (Tables 9-10), the patent shows how mutations of specific amino acid positions affect hyaluronidase enzymatic activity. The patent data show, for each of the 447 positions in SEQ ID NO: 3, the tolerance of each position to substitution. The data show which positions are critical to activity, which are generally intolerant in that they permit only very limited substitution, and which are generally tolerant in that they permit a broad range of substitutions. The data also identify tolerant positions that retain activity when different types of residues are substituted (*e.g.*, negatively charged, positively charged, large, small, hydrophobic, hydrophilic).

55. The POSA would expect that single or multiple changes in positions that tolerate mutations will generally maintain activity. The POSA would expect that single or multiple changes in positions that are generally tolerant are likely to

maintain activity, depending in part on where the changes are and which are made, and the ’791 patent provides the information the POSA requires to make these judgments. The ’791 patent provides the information a POSA requires to identify both single and multiple mutations that are likely to produce the desired activity.

56. The POSA would expect that single or multiple changes in positions that do not tolerate substitution or that generally do not tolerate substitution are likely to inactivate the protein, with the caveat that it may be possible to introduce additional mutations that restore activity. Again, the patent provides the information the POSA requires to make these determinations.

57. In addition to providing data on 5847 single substitution variants, the patent also provides proof of principle for several multiply modified PH20s, including N47A/N131A, N47A/N219A, N131A/N219A and N47A/N131A/N219A, which were shown to be active. The POSA would expect that adding the recited mutations at position 309 in those variants would maintain or improve activity (because the POSA would expect the change at position 309 to operate independently of the changes at positions 47, 131 and 219). Similarly, the patent teaches primate PH20 sequences that the POSA would expect would have similar or improved activity with the recited mutations at position 309 and that satisfy the 95% sequence identity requirement (*i.e.,* chimpanzee PH20, gibbon PH20 and orangutan PH20).

58. A POSA would have understood that the I309E, I309G, I309H, I309L, I309M, I309N, I309Q, I309R, I309S and I309T variants disclosed in the specification are representative of the narrow scope of modified PH20 polypeptides that satisfy the claims. The claimed modified PH20 polypeptides share at least 95% of their primary amino acid sequence. From a protein structure perspective, that is a tiny amount of variability. All folded PH20s that meet the claims will have the same alpha-beta barrel fold. All will share the recited mutation at position 309. All enzymatically active modified PH20s within the scope of the claims will cleave HA through the same mechanism of action using the same chemistry carried out by the same active site residues oriented in about the same way. The claims cover a homogeneous set of proteins, and the disclosed examples are representative of this homogeneous set, which share common structure-function relationships.

VII. TECHNICAL BACKGROUND

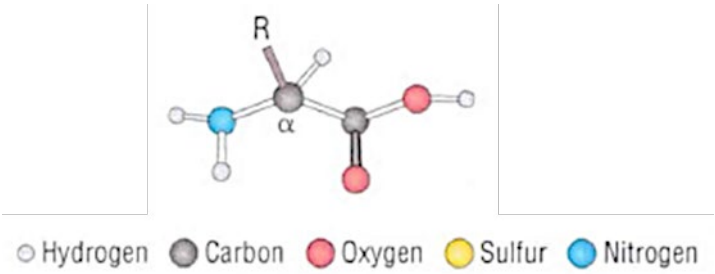
59. Below, I describe foundational principles of biology that would have informed POSAs interpreting the patent disclosure and data and seeking to make modified PH20s as of 2012.

A. Protein Structure

60. "It is a fundamental axiom of biology that the three-dimensional structure of a protein determines its function." EX2164 (Petsko), 50.

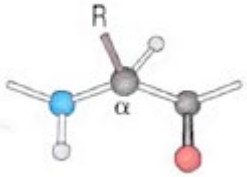
1. Amino Acids

61. "Proteins are polymers of 20 different amino acids joined by peptide bonds." EX2164 (Petsko), 3. The chemical structure of an amino acid is shown below:



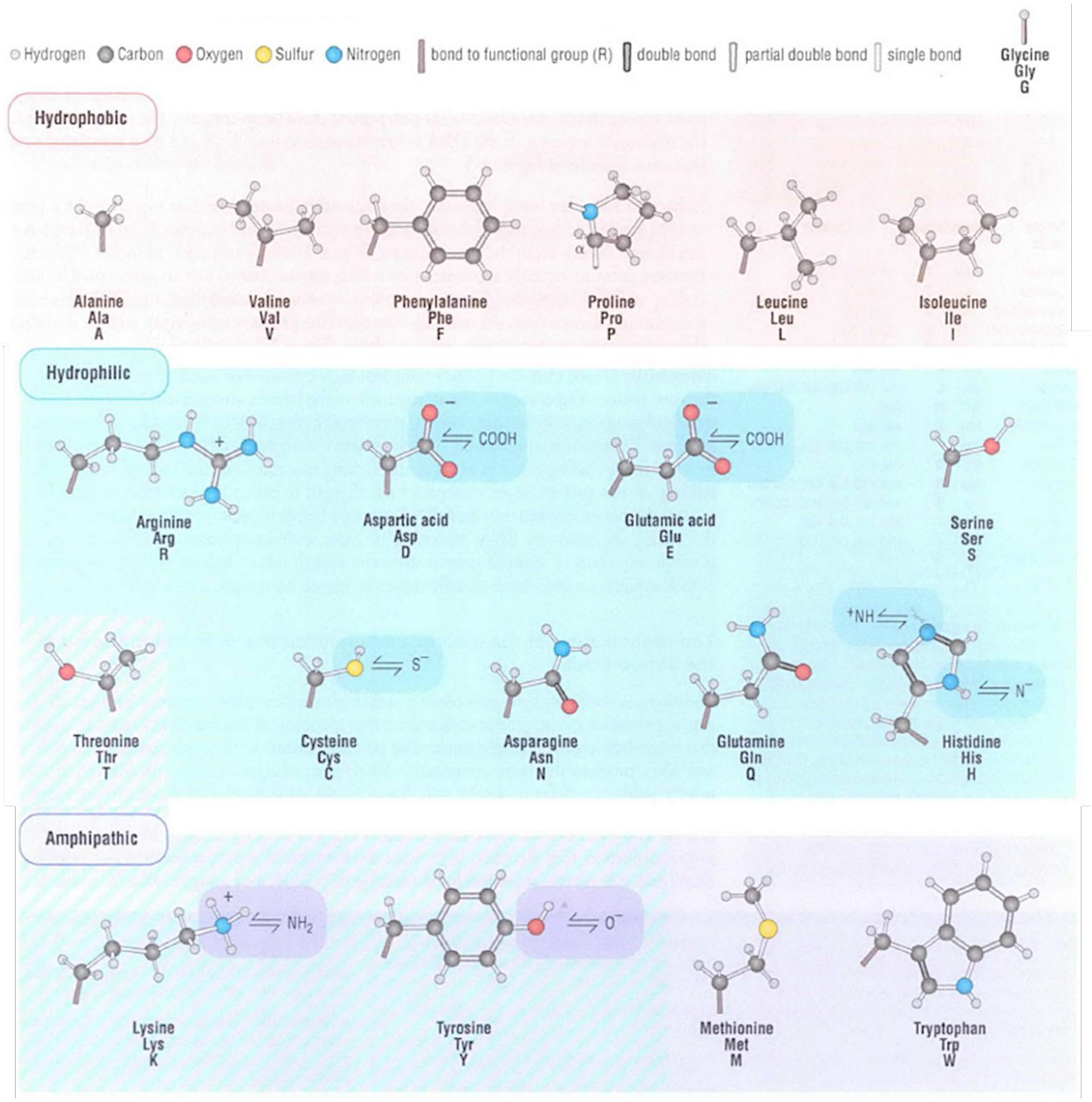
EX2164 (Petsko), 5. "The backbone[6] is the same for all amino acids and consists of the amino group (NH2), the alpha carbon and the carboxylic acid group (COOH). Different amino acids are distinguished by their different side chains, R." *Id.*

62. The figure below shows an "amino-acid residue as it is incorporated into a polypeptide chain. The R group is the side chain." EX2164 (Petsko), 5.



[6] The "backbone" is the "regularly repeating part of a polymer. In proteins it consists of the amide -N-H, alpha carbon -C-H and the carbonyl -C=O groups of each amino acid." EX2164 (Petsko), 3. The backbone for a proline residue is slightly different. *Id*., 4.

63. "The chemical characters of the amino-acid side chains have important consequences for the way they participate in the folding and functions of proteins." EX2164 (Petsko), 4. The amino acid side chains are shown in Figure 1-3, reproduced below. Figure 1-3 shows the name of each amino acid as well as the one letter and three letter abbreviation for each amino acid.



64. **"Hydrophobic** amino-acid **residues** engage in *van der Waals* interactions only. Their tendency to avoid contact with water and pack against each other is the basis for the *hydrophobic effect.*" EX2164 (Petsko), 4. "Alanine and leucine are strong helix-favoring residues, while proline is rarely found in helices because its backbone nitrogen is not available for the hydrogen bonding required for helix formation. The aromatic side chain of phenylalanine can sometimes participate in weakly polar interactions." *Id.*

65. "**Hydrophilic** amino-acid residues are able to make *hydrogen bonds* to one another, to the peptide backbone, to polar organic molecules, and to water. This tendency dominates the interactions in which they participate." EX2164 (Petsko), 4.

66. Some of the hydrophilic residues "can change their charge state depending on their pH or the microenvironment. Aspartic acid and glutamic acid have pKa values near 5 in aqueous solution, so they are usually unprotonated and negatively charged at pH 7. But in the hydrophobic interior of a protein molecule their pKa may shift to 7 or even higher (the same effect occurs if a negative charge is placed nearby), allowing them to function as proton donors at physiological pH." EX2164 (Petsko), 4. This effect is seen in the PH20 active site, in which a glutamate functions as a proton donor to hydrolyze HA at neutral pH.

67. **"Amphipathic** residues have both polar and nonpolar character, making them ideal for forming interfaces. It may seem surprising to consider the charged side chain of lysine as amphipathic, but its long hydrophobic region is often involved in van der Waals interactions with hydrophobic side chains. Tyrosine does not usually ionize at physiological pH (its pKa is about 9) but in some enzyme active sites it can participate in acid-base reactions because the environment can lower this pKa. The -O-H group is able both to donate and to accept hydrogen bonds, and the aromatic ring can form weakly polar interactions." EX2164 (Petsko), 4.

2. Primary Structure

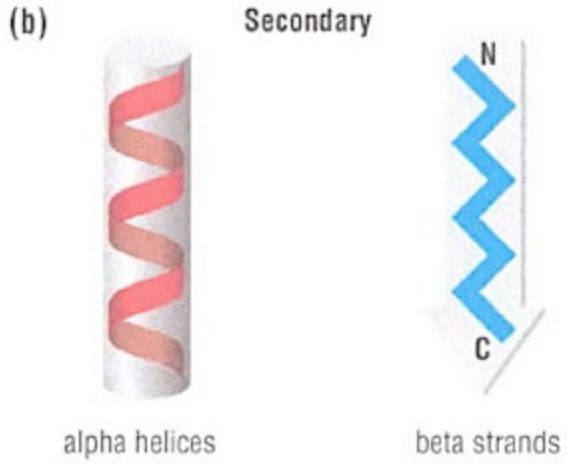
68. "The sequence of the different amino acids in a protein, which is directly determined by the sequence of nucleotides in the gene encoding it, is its *primary structure* (Figure l-2a)." EX2164 (Petsko), 3. Figure 1-2a is reproduced below.



69. The primary structure "in turn determines how the protein folds into higher level structures." EX2164 (Petsko), 3.

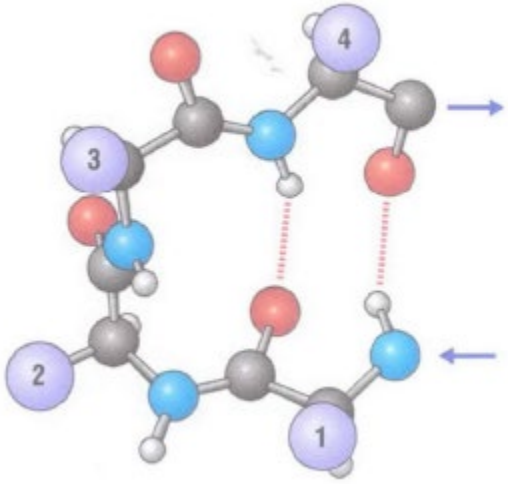
3. Secondary Structure

70. "The *secondary structure* of the polypeptide chain can take the form either of alpha helices or of beta strands, formed through regular hydrogen-bonding interactions between -N-H and C=O groups in the invariant parts of the amino acids in the polypeptide **backbone** or main chain (Figure 1-2b)." EX2164 (Petsko), 3. Figure 1-2b is reproduced below.



71. "The simplest secondary structure element usually involves four residues but sometimes requires only three. It consists of a hydrogen bond between the carbonyl oxygen of one residue (n) and the amide N-H of residue n+3, reversing the direction of the chain (Figure 1-12). This pattern of hydrogen bonding cannot ordinarily continue because the turn is too tight. This tiny element of secondary structure is called a **beta turn** or **reverse turn** or, sometimes, a

hairpin turn based on its shape." EX2164 (Petsko), 13. Figure 1-12 is reproduced below:

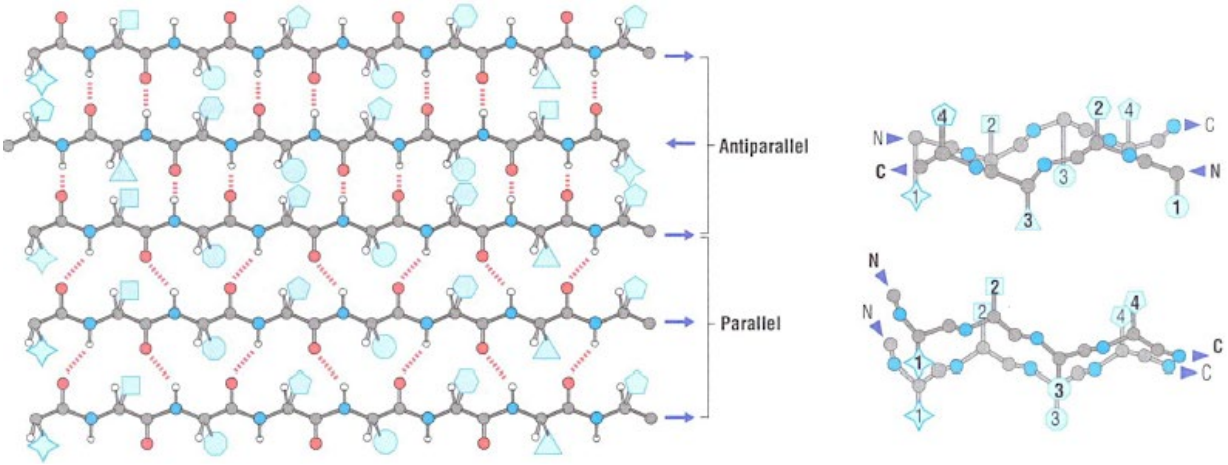


72. "[B]eta turns are found on the surfaces of folded proteins, where they are in contact with the aqueous environment, and by reversing the direction of the chain they can limit the size of the molecule and maintain a compact state." EX2164 (Petsko), 13.

73. "Alpha helices are the commonest secondary structural elements in a folded polypeptide chain, possibly because they are generated by local hydrogen bonding between C=O and N-H groups close together in the sequence." EX2164 (Petsko), 14. "The result is a cylindrical structure where the wall of the cylinder is formed by the hydrogen-bonded backbone, and the outside is studded with side chains. The protruding side chains determine the interactions of the alpha helix both with other parts of a folded protein chain and with other protein molecules." *Id.*

74. "In contrast to the alpha helix, the beta pleated sheet, whose name derives from the corrugated appearance of the extended polypeptide chain (Figure 1-17), involves hydrogen bonds between backbone groups from residues distant from each other in the linear sequence. In beta sheets, two or more strands that may be widely separated in the protein sequence are arranged side by side, with hydrogen bonds between the strands (Figure 1-17)." EX2164 (Petsko), 16.

75. Figure 1-17 is reproduced below:



EX2164 (Petsko), 16.

76. "Certain amino acids are more usually found in alpha helices, others in beta sheets." EX2164 (Petsko), 18. "For example, long side chains such as those of leucine, methionine, glutamine and glutamic acid are often found in helices, presumably because these extended side chains can project out away from the crowded central region of the helical cylinder. In contrast, residues whose side chains are branched at the beta carbon, such as valine, isoleucine and

phenylalanine, are more often found in beta sheets, because every other side chain in a sheet is pointing in the opposite direction, leaving room for beta-branched side chains to pack." *Id.* However, there are no invariable rules regarding the types of residues that can be included in each type of secondary structure.

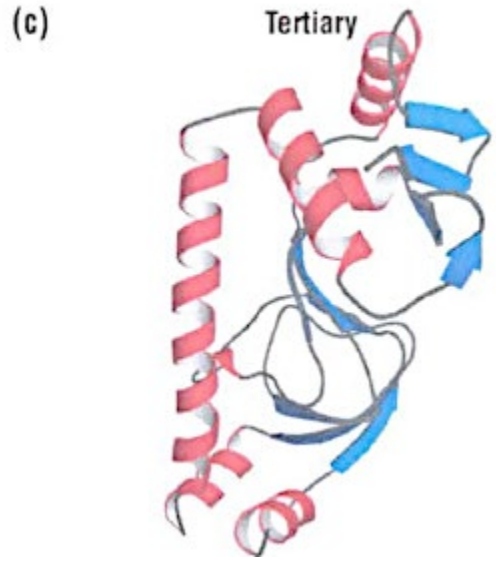
77. The table below shows "conformational preferences of the amino acids. The normalized frequencies for each conformation were calculated from the fraction of residues of each amino acid that occurred in that conformation, divided by this fraction for all residues. Random occurrence of a particular amino acid in a conformation would give a value of unity. A value greater than unity indicates a preference for a particular type of secondary structure." EX2164 (Petsko), 18.

Conformational Preferences of the Amino Acids

Amino acid	Preference α-helix	β-strand	Reverse turn
Glu	**1.59**	0.52	1.01
Ala	**1.41**	0.72	0.82
Leu	**1.34**	1.22	0.57
Met	**1.30**	1.14	0.52
Gln	**1.27**	0.98	0.84
Lys	**1.23**	0.69	1.07
Arg	**1.21**	0.84	0.90
His	**1.05**	0.80	0.81
Val	0.90	**1.87**	0.41
Ile	1.09	**1.67**	0.47
Tyr	0.74	**1.45**	0.76
Cys	0.66	**1.40**	0.54
Trp	1.02	**1.35**	0.65
Phe	1.16	**1.33**	0.59
Thr	0.76	**1.17**	0.90
Gly	0.43	0.58	**1.77**
Asn	0.76	0.48	**1.34**
Pro	0.34	0.31	**1.32**
Ser	0.57	0.96	**1.22**
Asp	0.99	0.39	**1.24**

4. Tertiary Structure

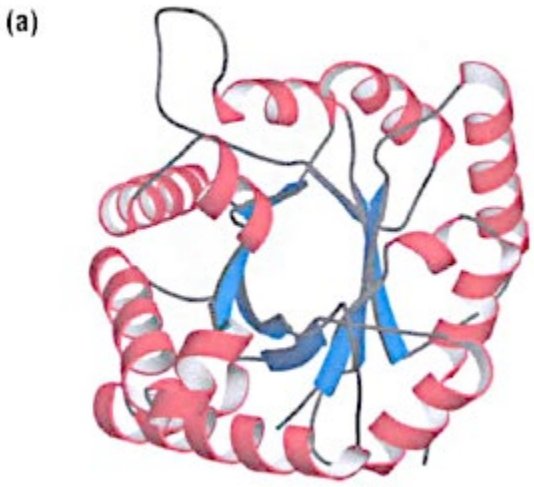
78. "In a folded protein, the secondary structure elements fold into a compact and nearly solid object stabilized by weak interactions involving both polar and nonpolar groups. The resulting compact folded form is called the **tertiary structure** of the protein." EX2164 (Petsko), 22. The tertiary structure of a protein is illustrated in Figure 1-2c of Petsko, reproduced below:



EX2164 (Petsko), 3.

79. "One way to characterize tertiary structure is by the topological arrangement of the various secondary structure elements as they pack together." EX2164 (Petsko), 22. As I explain below, PH20 adopts an alpha-beta barrel fold.

80. An exemplary alpha-beta barrel motif is illustrated in Figure 1-61a of Petsko, which I have reproduced below:



EX2164 (Petsko), 38.

81. "The individual secondary structure elements in a protein pack together in part to bury the hydrophobic side chains, forming a compact molecule with very little empty space in the interior (Figure 1-27)." EX2164 (Petsko), 23. Figure 1-27 is reproduced below:



Figure 1-27 Cut-away view of the interior of a folded protein The atoms in the interior of a protein are packed almost as closely as in a solid. Note that there are a few cavities and small channels in some parts of the structure. These packing defects provide room for neighboring atoms to move, allowing the structure to have some flexibility.

82. "Noncovalent forces are the principal interactions that stabilize protein tertiary structure but they are not the only ones. Many proteins also are stabilized by additional, covalent interactions that provide a form of cross-linking between segments of secondary structure in the native state. The most common of these covalent bonds is the disulfide bridge that can form between two cysteine

side chains that are brought close together by the tertiary structure (Figure 1-37)." EX2164 (Petsko), 28. Figure 1-37 is reproduced below:



Figure 1-37 The structure of the small protein bovine pancreatic trypsin inhibitor, BPTI The three disulfide bonds are yellow, beta strands are blue, and alpha helices are red. If these disulfide bonds are reduced this small protein unfolds, presumably because there is not enough secondary structure to stabilize the fold without them. (PDB 1bpi)

B. Enzymes

83. "The catalytic efficiency of enzymes is remarkable: reactions can be accelerated by as much as 17 orders of magnitude over simple buffer catalysis. Many structural features contribute to the catalytic power of enzymes: holding reacting groups together in an orientation favorable for reaction (proximity); binding the transition state of the reaction more tightly than ground state complexes (transition state stabilization); acid-base catalysis, and so on." EX2164 (Petsko), 2.

84. "Specific binding occurs at sites on the protein that provide the complementarity for the ligand. [The sites are called] ligand-binding sites if their sole function is molecular recognition … or **active sites** if they promote chemical

catalysis. Such sites are formed as a consequence of the three-dimensional structure of the protein." EX2164 (Petsko), 52 (emphasis added). In other words, an enzyme's three-dimensional structure directly affects its catalysis. These active sites create "specialized microenvironments that result from protein tertiary structure" which "contribute to catalysis." EX2164 (Petsko), 52.

85. "Specificity of binding comes from the close fit of the substrate within the active-site pocket, which is due primarily to van der Waals interactions between the substrate and non-polar groups on the enzyme, combined with complementary arrangements of polar and charged groups around the bound molecule." EX2164 (Petsko), 64.

86. "Reactive groups in enzyme active sites are optimally positioned to interact with the substrate." EX2164 (Petsko), 64. The chemistry of the enzyme-substrate interaction is controlled because "[m]ost enzymes operate, in part, through *general acid-base catalysis*, in which protons are transferred between donating or accepting atoms on the substrate and key basic and acidic side chains in the enzyme active site." *Id.*, 52. "Groups that are already part of the active-site structure in the folded protein before the substrate binds are already oriented properly for catalysis, or become so as the enzyme binds its substrate.… Every substrate molecule that forms the enzyme-substrate complex will therefore be

exposed to an environment in which the catalytic groups are positioned correctly, relative to the substrate, for the desired reaction to take place." *Id*., 65.

C. Sequence Alignments And The Concept of Homology

87. Comparing amino acid sequences to "to find the degree of similarity between them is a key technique." EX2164 (Petsko), 132. "A marked similarity between two gene or protein sequences may reflect the fact that they are derived by evolution from the same ancestral sequence. Sequences related in this way are called **homologous** and the evolutionary similarity between them is known as **homology**." *Id.*

88. "A key step in comparing two sequences is to match them up to each other in an **alignment** that shows [ ] any similarity that is present. Alignments work on the general principle that two homologous sequences derived from the same ancestral sequence will have at least some identical residues at the corresponding positions in the sequence; if corresponding positions in the sequence are aligned, the degree of matching should be statistically significant compared with that of two randomly chosen unrelated sequences." EX2164 (Petsko), 132.

89. "As a quantitative measure of similarity, a pairwise alignment is given a score, which reflects the degree of matching. In the simplest case, where only identical matched residues are counted, the fraction of identical amino acids or

nucleotides gives a similarity measure known as **percent identity."** EX2164 (Petsko), 132.

90. "In practice, insertions and deletions as well as substitutions will have occurred in two homologous sequences during their evolution. This usually results in two gene or protein sequences of different lengths in which regions of closely similar sequence are separated by dissimilar regions of unequal length. In such cases, portions of the sequence are slid over each other when making the alignment, in order to maximize the number of identical and similar amino acids. Such sliding creates gaps in one or other of the sequences (Figure 4-2). Experience tells us that closely related sequences do not, in general, have many insertions or deletions relative to each other. Because any two sequences could be broken up randomly into as many gaps as needed to maximize matching, in which case the matching would have no biological significance, gaps are subject to a penalty when scoring sequence relatedness." EX2164 (Petsko), 132. Figure 4-2 is reproduced below:

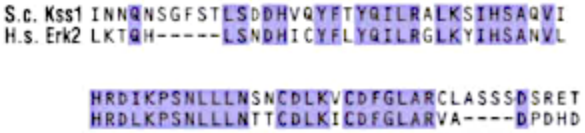


Figure 4-2 Pairwise alignment Part of an alignment of the amino-acid sequences of the kinase domains from two ERK-like kinases of the MAP kinase superfamily, Erk2 from humans and Kss1 from yeast. The region shown covers the kinase catalytic loop and part of the activation loop (see Figure 3-24). Identical residues highlighted in purple show the extensive similarity between these two homologous kinases (their evolutionary relationship can be seen in Figure 4-5). To maximize similarity, a small number of gaps have had to be inserted in the human sequence.

91. "The alignment process can be expanded to give a **multiple sequence alignment**, which compares many sequences (Figure 4-4). Such multiple sequence alignments are arrived at by successively considering all possible pairwise alignments.… From this alignment one can see that certain residues are identical in all the sequences. Any residue, or short stretch of sequence, that is identical in all sequences in a given set (such as that of a protein family) is said to be **conserved**. Multiple alignments tend to give a better assessment of similarity than pairwise alignments and can identify distantly related members of a gene family that would not be picked up by pairwise alignments alone." EX2164 (Petsko), 133. Figure 4-4 is reproduced below:

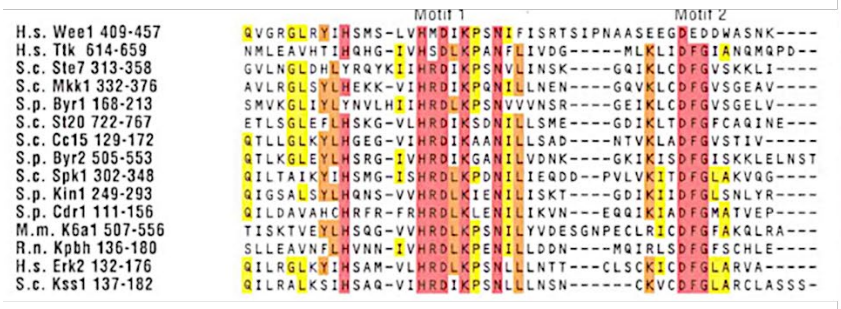


Figure 4-4 Multiple alignment A small part of a large multiple alignment of more than 6,000 protein kinase domains in the Pfam database (http://pfam.wustl.edu), displaying part of the region shown in Figure 4-2. Residues identical in all or almost all sequences in the complete alignment are highlighted in red, the next most highly conserved in orange and those next most conserved in yellow. The alignment reveals residues and sequence motifs that are common to all protein kinase catalytic domains and can be used to identify additional members of the family. One is motif 1, which identifies the catalytic loop and contains a conserved aspartic acid (D) important to catalytic function. H.s.: human; S.c.: *Saccharomyces cerevisiae*; S.p.: *Schizosaccharomyces pombe*; M.m.: *Mus musculus*, mouse; R.n.: *Rattus norvegicus*, rat.

92. "During evolution, certain positions in a sequence change more rapidly than others. Functionally and structurally important residues tend to be conserved." EX2164 (Petsko), 135. "Because functionally important residues must necessarily be conserved over evolution, when multiple sequences from different organisms can be aligned, the probability of recognizing related proteins or a similar biochemical function even at very low overall sequence identity increases dramatically." *Id*.

93. Dr. Park used Clustal Omega for his multiple sequence alignment analysis, and he testified at deposition that a POSA seeking to perform a multiple sequence alignment in 2011 would have used Clustal Omega. EX2077 (Park I Tr.), 96:5-12. I agree that in 2011 a POSA seeking to perform a multiple sequence alignment would have used Clustal Omega.

94. Dr. Park testified that a POSA seeking to perform a pairwise sequence alignment in 2011 would have used BLAST. EX2077 (Park I Tr.), 96:13-15. I

agree that in 2011 a POSA seeking to perform a pairwise alignment would have used BLAST.[7]

D. Homologous Proteins With High Sequence Identity (Typically More Than 40%) Adopt The Same Fold

95. "Structure can be derived from sequence by reference to known protein folds and protein structures. Because structure changes more slowly than sequence, if there is a high degree of sequence identity between two proteins, their overall folds will always be similar. But at sequence identity of less than around 40% (see Figure 4-19), structures can be markedly different from each other. In practice, however, structural similarity often extends to lower levels of sequence identity, depending on how the identical residues are distributed." EX2164 (Petsko), 142. Figure 4-19 and its legend are reproduced below:

[7] Clustal Omega and BLAST were also available in 2012.

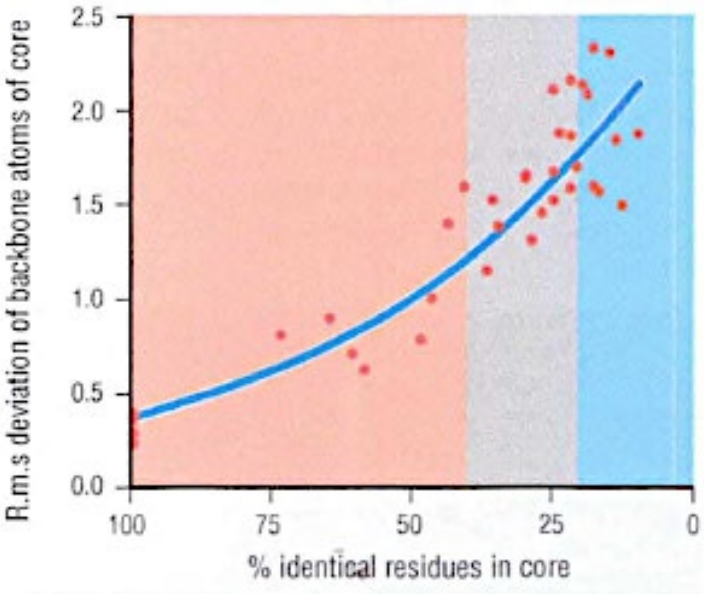


Figure 4-19 Relationship between sequence and structural divergence of proteins The percent identity of the protein cores of 32 pairs of proteins from eight different structural families was plotted against their structural divergence as measured by the root-mean-square difference in spatial positions of backbone atoms. A striking relationship is found, which holds for all the families studied. As the sequences diverge, the structures diverge, but not at the same rate. Small differences in sequence have little effect on structure, but structural divergence increases exponentially as sequence divergence becomes greater. Sequences with greater than 40% identity are generally considered to be homologous and the probability that they will have the same overall structure is also very high. For proteins with sequence identities below about 20%, evolution has usually altered much of the structure, and homology cannot be determined with any certainty. In between is a "grey area", where the overall identity between two sequences is less than about 40% but greater than about 20%, and when it may be impossible from sequence comparisons alone to determine that two proteins are related. Data from Lesk, A.M., *Introduction to Protein Architecture* (Oxford University Press, Oxford, 2001).

EX2164 (Petsko), 140.

96. Figure 4-19 and its legend provide critical foundational principles of structural biology that underpin my analysis that are worth emphasizing. Specifically, as stated in the legend, with respect to naturally occurring proteins, "***[s]mall differences in sequence have little effect on structure***, but structural divergence increases exponentially as sequence divergence becomes greater." EX2164 (Petsko), 140 (emphasis added). To put what is meant by "small differences in sequence" and "little effects on structure" in context, the root mean square difference in spatial positions of backbone atoms was less than 1 Ångstrom for sequences that were between 50% and 75% identical. Because the change in structure is so small at these levels of identity, sequences "with greater than 40% identity are generally considered to be homologous and the probability that they will have the same overall structure is also very high." EX2164 (Petsko), 140.

97. Dr. Park described these foundational principles in his deposition:

> If you ask a practitioner in the field: I have two proteins that are homologous and have high sequence similarity, would I expect them to fold to the same structure, of course I would say yes.

EX2077 (Park I Tr.), 60:9-13.

98. Dr. Park also explained as follows:

> Protein structures, if one compares the folding structure to an entire space of – conformational space that can be adopted by the polypeptide, it's a very small fraction.

> Now, if you have homologous proteins, meaning they came from the same ancestor, and they have maintained similar sequence similarity, they would be obligated to explore that very small sliver of the conformational space. They would not be able to go and randomly find another island of stability in that conformational space that can be occupied by that particular sequence. So that restricts what the protein can do. ***So this is the foundation behind the assumption that homologous highly similar sequences would adopt the same conformational three-dimensional structure.***

EX2077 (Park I Tr.), 60:16-61:10 (emphasis added).

99. Consistent with these principles, Dr. Park testified that he expected the homologous hyaluronidases "to all adopt the same fold, to all fold in the same way" and to "fold to the same structure." EX2077 (Park I Tr.), 75:5-12, 77:10-11.

100. Similarly, Dr. Hecht testified as follows:

> When you have a family of homologous proteins that share common ancestry and share considerable sequence identity, share evolutionary history, and in most cases share enzymatic activity, then one infers from that that they're going to share structure.

EX2076 (Hecht Tr.), 80:9-15.

101. As I will explain in detail below, based on these principles, a POSA would have expected that folded, secreted proteins that are 90-95% identical in sequence adopt the same fold. In other words, with respect to PH20, a POSA would have expected that a folded modified PH20 that is 95% identical in sequence to wild type ("WT") would adopt the same alpha-beta barrel fold as WT

PH20. The POSA would not expect the 95% identical protein to adopt a completely different protein fold. Put another way, if the 95% identical modified PH20 folds, it will fold into the same alpha-beta barrel motif as the WT.

1. Homology Modelling

102. Based on the expectation that homologous proteins with high sequence identity will adopt the same fold, it is possible to generate a model of a protein structure using an experimentally determined structure of a homologous protein as the template. This is referred to as homology modeling.

103. "The technique of **homology modeling** aims to produce a reasonable approximation to the structure of an unknown protein by comparison with the structure of a known sequence homolog (a protein related to it by divergent evolution from a common ancestor)." EX2164 (Petsko), 142. "In practice, a sequence with greater than about 40% amino-acid identity with its homolog, and with no large insertions or deletions having to be made in order to align them (Figure 4-22), can usually produce a predicted structure equivalent to that of a medium-resolution experimentally solved structure." *Id.* " Higher-resolution models can be obtained, in principle at least, when there are a number of aligned sequences." *Id.* Figure 4-22 is reproduced below:

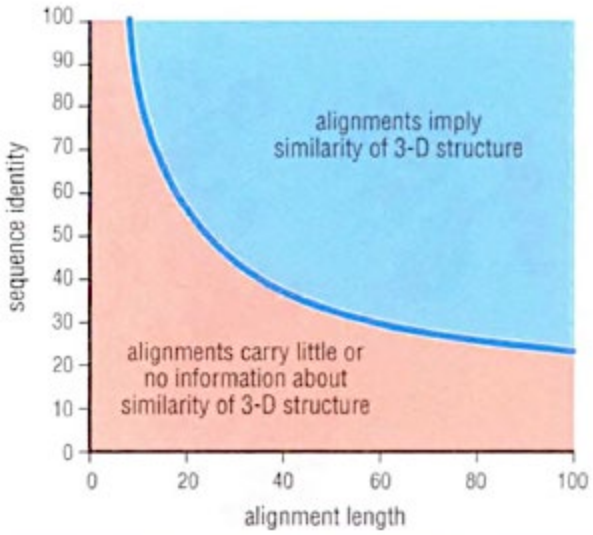


Figure 4-22 The threshold for structural homology Sequence space, plotted as a function of length of the segment being aligned and the percent identity between the two sequences, can be divided into two regions. The upper region (above the curve) shows where sequence similarity is likely to yield enough structural similarity for homology modeling to work. The lower region is highly problematic. At present 25% of known protein sequences fall in the safe area, implying 25% of all sequences can be modeled reliably.

104. I note that the 40% sequence identity figure and comments regarding the homology model above were written in the context of the state of the art in 2003. In 2011-2012, POSAs would have expected to generate a reasonably reliable model for homologous sequences having 40% or better sequence identity. Consistent with this, Dr. Park testified as follows:

> Q Is there a minimum amount of sequence identity that you need to satisfy in order to use SWISS-MODEL to generate a homology model?
>
> A This is another one of those questions where one doesn't have a solid number, except a number that's

> commonly used and understood. A 30 percent identity between two proteins that are homologous would be assumed to fold the same structure.
>
> Q Okay.
>
> A So if it's 31 percent, I don't know. But if it's 45 percent, that seems like a pretty safe place to be.
>
> Q …And so the 30 percent identity between two proteins that are homologous, they would be assumed to fold the same structure? Is that 30 percent number, is that well-known in the field?
>
> A If you ask a POSA to put a number on that, I think they would name 30 percent.
>
> Q Okay. And was that the case in 2011?
>
> A The understanding of 30 percent being sort of the cut-off has been around for a long, long time, including 2011, yes.

EX2077, (Park I Tr.), 93:9-94:12. Although it is possible in some cases to obtain a homology model with less than 30% sequence identity, I generally agree with Dr. Park's assessment above.

105. Dr. Park explained that "[t]he absence of an experimentally determined structure for [human PH20] protein does not preclude the use of structure-based modeling methods. That is because the structure of a protein can be modeled in a process called 'homology modeling.' Homology modeling was routinely used by 2011 and provided an 'accurate computational method to generate reliable structural models.'" EX1004 (Park Decl.), ¶ 36. I agree with Dr. Park.

106. Dr. Park explained why POSAs generate homology models in the testimony below:

> Q So what's the purpose of generating a homology model for a protein structure?
>
> A It allows you to look at the molecule, as opposed to imagining it in your head.
>
> Q And it's useful to be able to look at the molecule, right?
>
> A Absolutely.
>
> Q And you can use homology modeling to predict the impact of substitutions in a protein?
>
> A A homology model can help you to evaluate the impact of a substitution. Yes.

EX2077 (Park I Tr.), 53:15-54:5. I agree with Dr. Park that homology models provide a useful tool for POSAs to analyze protein structure and evaluate potential substitutions.

107. Swiss-Model is a widely used platform for generating three-dimensional homology models of proteins and was available in 2011. Dr. Park testified at his deposition that Swiss-Model was the only homology modeling tool a POSA would have been able to use because the others required specialized knowledge. EX2077 (Park I Tr.), 49:5-50:8.

108. I agree that Swiss-Model was widely used for homology modelling in 2011 (or 2012), but I disagree that that is the only program a POSA could have

used to generate a homology model in that timeframe. POSAs also could have used Modeller, which was another well-established and capable homology modeling software. Dr. Park indicated that Modeller required some specialized knowledge. EX2077 (Park I Tr.), 43:17-44:4. I disagree that Modeller required specialized knowledge above and beyond a POSA's capabilities. A POSA could readily have used Modeller to generate homology models, and in my experience Modeller was commonly used in industry and academia to generate homology models in the 2011-2012 timeframe.

109. Consistent with my opinion, Dr. Hecht testified at deposition that his students used Modeller. EX2076, (Hecht Tr.), 38:4-13.

2. PyMOL

110. PyMOL is a widely used program that enables a POSA to visualize and analyze a protein structure or model, and manipulate protein structural models in three dimensions. This software provides graphical representations of protein structures that allow a POSA to examine spatial relationships between amino acids and understand the structural context of individual residues. With PyMOL, a POSA could examine where each residue appears in the structure and understand the structural context of the residue, including whether it is part of the active site, whether it is in the hydrophobic core of the protein, whether it is involved in a secondary structure like an α helix and/or β sheet, or whether it is part of an

unstructured loop on the protein surface. A POSA can also use PyMOL's measurement tools to measure distances between atoms to evaluate potential interactions between residues.

111. POSAs routinely used PyMOL to visualize and analyze structures and models in the 2011/2012 timeframe.

VIII. KNOWLEDGE OF HYALURONIDASE STRUCTURE/FUNCTION AS OF DECEMBER 2012

112. Before I discuss the disclosure of the '791 patent, I first will summarize the knowledge in the art as of December 28, 2012 regarding the structure of hyaluronidases, their mechanism of action and the amino acids known to be important for activity. Understanding the state of the art provides perspective regarding the importance of the '791 patent disclosure to the POSA seeking to make and use modified PH20 polypeptides.

A. Hyaluronidases

113. PH20 is a member of a class of enzymes called hyaluronidases, and it degrades hyaluronan. Hyaluronan, or HA, is a linear, negatively charged glycosaminoglycan made of repeating disaccharide units. EX1006 (Chao), 6911.

114. Humans express five proteins that are categorized as "hyaluronidases": PH20, Hyal-1, Hyal-2, Hyal-3 and Hyal-4. EX1006 (Chao), 6911. Even though they all are called "hyaluronidases," not all of them actually exhibit hyaluronidase activity. For example, "Hyal-4 appears to be a

chondroitinase, with high specificity for Ch and ChS." EX1008 (Stern), 820. And the role of Hyal-3 is unclear. EX1006 (Chao), 6911.

B. Bee Venom Hyaluronidase and Hyal-1 Crystal Structures and PH20 Model

115. By 2012, the crystal structures for bee venom hyaluronidase and human Hyal-1 had been solved. The bee venom hyaluronidase structure was disclosed in 2000 in a paper by Marković-Housley (Marković-Housley et al., "Crystal Structure of Hyaluronidase, a Major Allergen of Bee Venom," Structure, 8:1025-1035 (2000)). EX1033. I downloaded the pdb file (1FCV) (EX2179) for the bee venom hyaluronidase structure from the Protein Data Bank website and used it in my analysis, as I explain below.[8]

116. The Hyal-1 structure was disclosed in a 2007 paper by Chao (Chao et al., "Structure of Human Hyaluronidase-1, a Hyaluronan Hydrolyzing Enzyme Involved in Tumor Growth and Angiogenesis," Biochemistry, 46:6911-6920 (2007)). EX1006. I downloaded the pdb file (2PE4) (EX2180) for the Hyal-1 structure from the Protein Data Bank website and used it in my analysis, as I explain below.

[8] Three structures were deposited in the Protein Data Bank as part of the bee venom hyaluronidase work—a structure of the monoclinic crystal form and two structures of the trigonal crystal form, one of which includes a hyaluronic acid tetramer. I rely on the structure with the tetramer (1FCV) for my analysis.

117. As of 2012, the structure for PH20 had not been solved. As Dr. Park explains in his Declaration, however, the "absence of an experimentally determined structure for a protein does not preclude the use of structure-based modeling methods." EX1004, ¶ 36. That is because the PH20 structure can be modeled. *Id.* I agree with Dr. Park that in 2011 (and 2012) it was routine for those skilled in the art to generate structural models using homology modelling, particularly in cases where the sequence identity and similarity is high between the template protein whose structure is known and the protein whose structure is to be modeled.

118. I also agree with Dr. Park and Dr. Hecht that the POSA seeking to evaluate potential substitutions in PH20 would have used the Hyal-1 crystal structure as a template and generated a homology model of PH20. For example, Dr. Park testified as follows:

> A In the year 2011, to consider a mutation, it would have been very common and expected for a POSA to generate a model and consider the substitution in that structural context.

EX2077 (Park I Tr.), 176:2-5.

119. Similarly, Dr. Hecht testified as follows:

> Q So a POSA in 2011 seeking to evaluate potential substitutions in PH20 would have generated a homology model using HYAL1 as the template; is that right?
>
> A Correct.

> Q So in 2011 a POSA seeking to evaluate potential substitutions in PH20 likely to be tolerated would have generated a homology model; is that right?
>
> A Yes. Among other things, yes.
>
> Q And the POSA would have used that homology model to evaluate the particular substitutions?
>
> A Would have used the homology model and also used other input.
>
> Q And what other input?
>
> A As we said before, the sequence alignment of many sequences, not just those in the figure, and just expertise and experience in the field of protein structure and engineering and mutagenesis.

EX2076 (Hecht Tr.), 93:4-22.

120. Dr. Park used SWISS-MODEL to generate a homology model of PH20 using the Hyal-1 structure as template. I agree that a POSA in 2012 (or 2011) would have been able to readily obtain a PH20 homology model using SWISS-MODEL. Alternatively, the POSA could have used Modeller, which was commonly used in industry at the time, to generate a homology model of PH20.

121. I independently recreated the PH20 homology model using Swiss-Model. To do this, I went to the Swiss-Model server (https://swissmodel.expasy.org/interactive) and uploaded the amino acid sequence

of PH20 as the target sequence in FASTA[9] format. For the template, I used two methods, both of which were available in 2012. In the first method, Swiss-Model searches the database of experimentally determined protein structures for a close sequence match to the target sequence. In the second, the user can specify a specific template structure as a pdb format atomic coordinate file. As the closest related structure, I selected Hyal-1 (PDB entry 2PE4, 40% identical in sequence to PH20) as a desired template).[10]

122. After I selected the template (Hyal-1), the model-building process is essentially automatic.

---

[9] "A sequence in FASTA format begins with a single-line description, followed by lines of sequence data. The description line (defline) is distinguished from the sequence data by a greater-than (">") symbol at the beginning." EX2169, https://blast.ncbi.nlm.nih.gov/doc/blast-topics/. An example sequence in FASTA format is shown below:

>P01013 GENE X PROTEIN (OVALBUMIN-RELATED)

QIKDLLVSSSTDLDTTLVLVNAIYFKGMWKTAFNAEDTREMPFHVTKQES
KPVMMCMNNSFNVATLPAEKMKILELPFASGDLSMLVLLPDEVSDLERIE
KTINFEKLTEWTNPNTMEKRRVKVYLPQMKIEEKYNLTSVLMALGMTDLF
IPSANLTGISSAESLKISQAVHGAFMELSEDGIEMAGSTGVIEDIKHSPESEQ
FRADHPFLFLIKHNPTNTIVYFGRYWSP

[10] In 2012, Hyal-1 was the only human hyaluronidase for which a crystal structure had been solved, so in 2012 the Hyal-1 structure would have been the best template for the model. I note that Dr. Park also used Hyal-1 as a template for the PH20 homology model; Dr. Park states in his declaration that "HYAL1 is the only suitable template structure for PH20 and was used as the template of the PH20 model I prepared." EX1004 (Park Decl.), ¶ 146. I agree that in the 2011-2012 timeframe, Hyal-1 would have been the best template for a PH20 model.

123. Since 2012, some of the internal algorithms used by the program have doubtless changed somewhat, but I do not expect that to have much effect on the result in a case such as this, where the template sequence and the target sequence are closely related. Dr. Park similarly opined that "overall, the PH20 model from SWISS-MODEL that I used in my analysis should be very similar to what a skilled artisan would have obtained in 2011. Changes in the modeled structures between today and in 2011, if any, would be very subtle and would not impact my analysis, which was based on my visual analysis of the structures at the positions where substitutions were being assessed." EX1004 (Park Decl.), ¶ 150. I agree with Dr. Park's assessment.

124. Swiss-Model reports a few measures of the quality of the resulting homology model of PH20, and I note that these values are essentially identical to those obtained by Dr. Park and reported in his testimony (*e.g.*, QMEANDisCo Global = 0.74 +/-0.05).[11]

[11] Minor variation in the quality measures are due to the fact that there are portions of the PH20 and Hyal-1 sequences where there essentially is no sequence similarity, and the POSA would expect some minor variation in the resulting model of those regions, which occur chiefly in loops and at the extreme ends of the protein. Consistent with my analysis, Dr. Park refers to "3 insertions and 3 deletions between HYAL1 and PH20" that "introduce variation in the main chain conformation, but these constitute only minor perturbations because every insertion and deletion between HYAL1 and PH20 only involve one amino acid." EX1004 (Park Decl.), ¶ 147. I agree that such "perturbations" are "minor," and they would not meaningfully impact a POSA's analysis.

125. I have used the Swiss-Model derived PH20 model along with the bee venom hyaluronidase structure (1FCV) and the Hyal-1 structure (2PE4) in my analysis below.

C. Common Features of PH20, Hyal-1 and Bee Venom Hyaluronidase

126. As I explain in detail below, PH20, Hyal-1 and bee venom hyaluronidase share several common features, including a common mechanism of action, common active site, and common critical residues involved in structure and function. Hyal-1 and PH20 also share a common alpha-beta barrel fold, with bee venom hyaluronidase adopting a similar fold.

127. These three proteins are considered homologous with high sequence identity (30-40%). EX1006 (Chao), 6912; EX1008 (Stern), 826; *see also* EX1004 (Park), ¶ 40. The similarities shared by these three different proteins provide key insight into how POSAs in the 2012 time frame would think about concepts like common structural features, structure/function correlation and representative examples disclosed across the scope of a claimed set of protein variants.

128. To reiterate, PH20, Hyal-1 and bee venom hyaluronidase share significant and critical common features with a 30-40% primary sequence identity. They are not variants of one another; they are different proteins (*e.g.,* one is a bee protein found in bee venom and two are human proteins). And yet they share common features. By contrast, the claims cover certain PH20 variants—modified

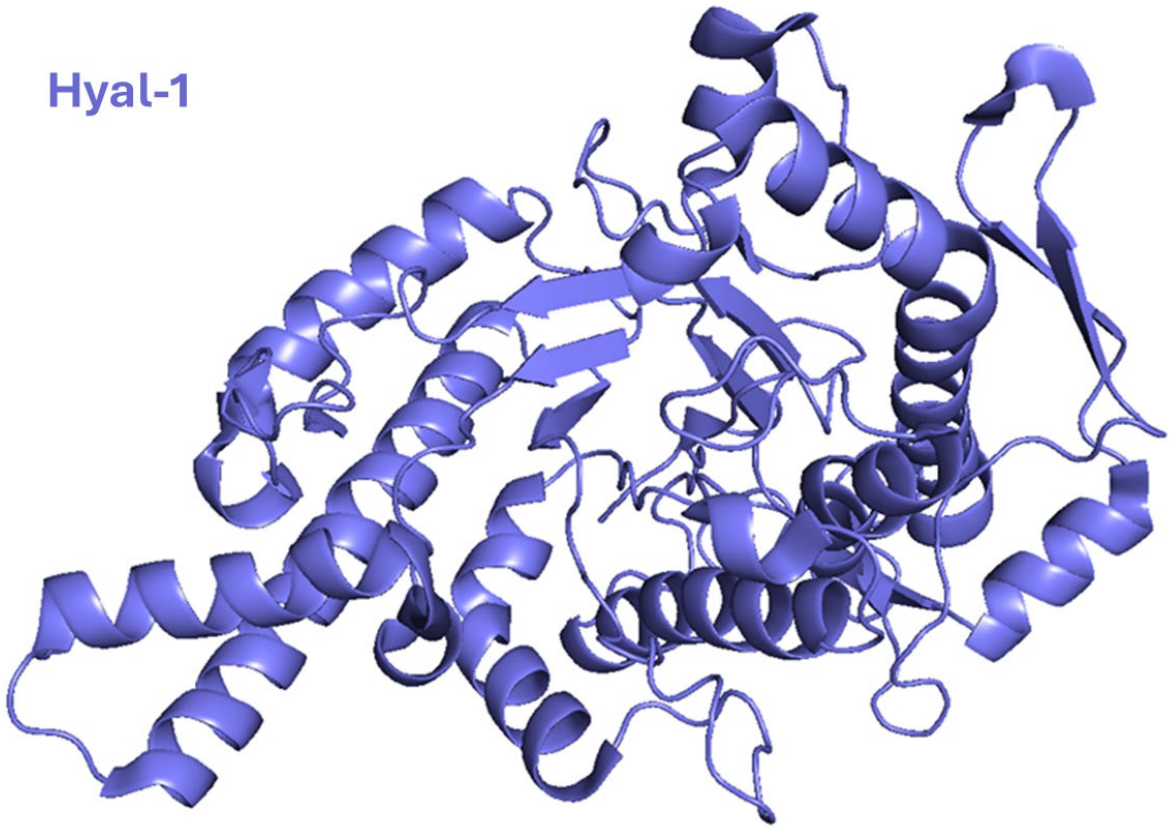
PH20s with 95% sequence identity and the specified change at position 309. As I will explain below, 95% identical PH20 variants also share common structural features; indeed by definition they share 95% of their primary structure. And enzymatically active[12] 95% identical PH20 variants share a common tertiary structure (the PH20 alpha-beta barrel fold) as well as other common structural features I will describe in detail below.

1. PH20, Hyal-1 and Bee Venom Hyaluronidase Adopt An Alpha-Beta Barrel Fold

129. For my analysis, I superimposed the bee venom crystal structure (1FCV) on the Hyal-1 structure (2PE4) in PyMOL. In addition, I superimposed the PH20 model on the Hyal-1 structure.

130. The Hyal-1 structure is shown in "cartoon" format in the figure below. As is shown in this figure, Hyal-1 has a tertiary structure that the POSA would characterize as an alpha-beta barrel fold:

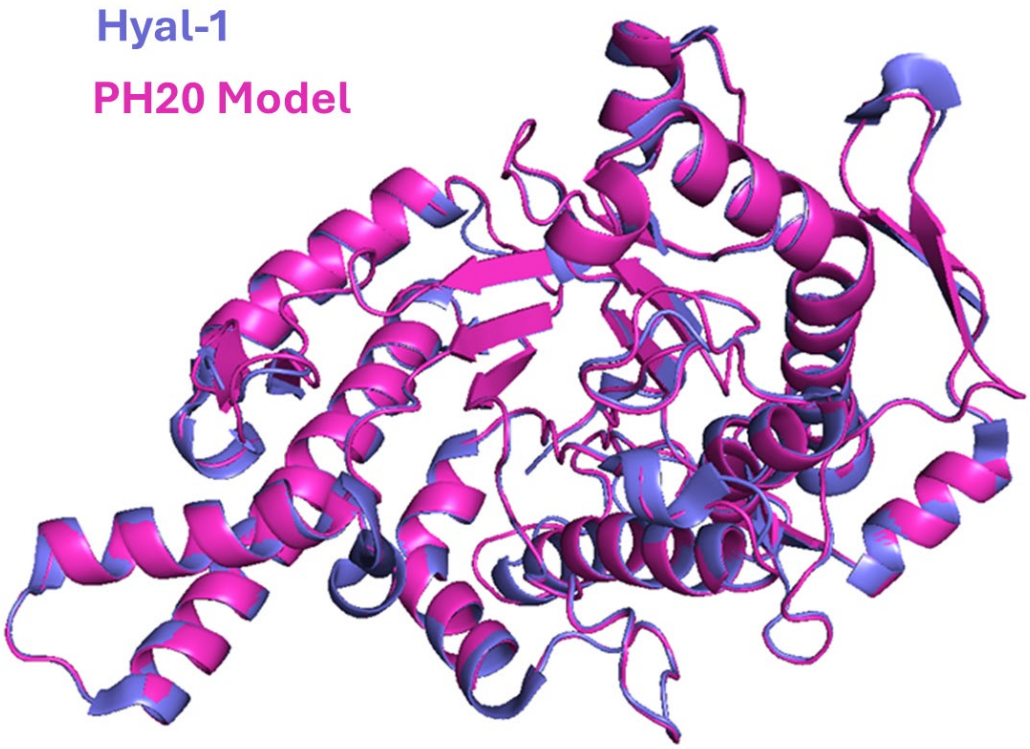
[12] When I say "enzymatically active" in this context, I am not referring to a specific level of enzymatic activity. Rather, I am using enzymatic activity as a proxy for a properly folded protein. Secretion is another proxy that could be used for a properly folded protein; proteins that do not fold will generally not be secreted.



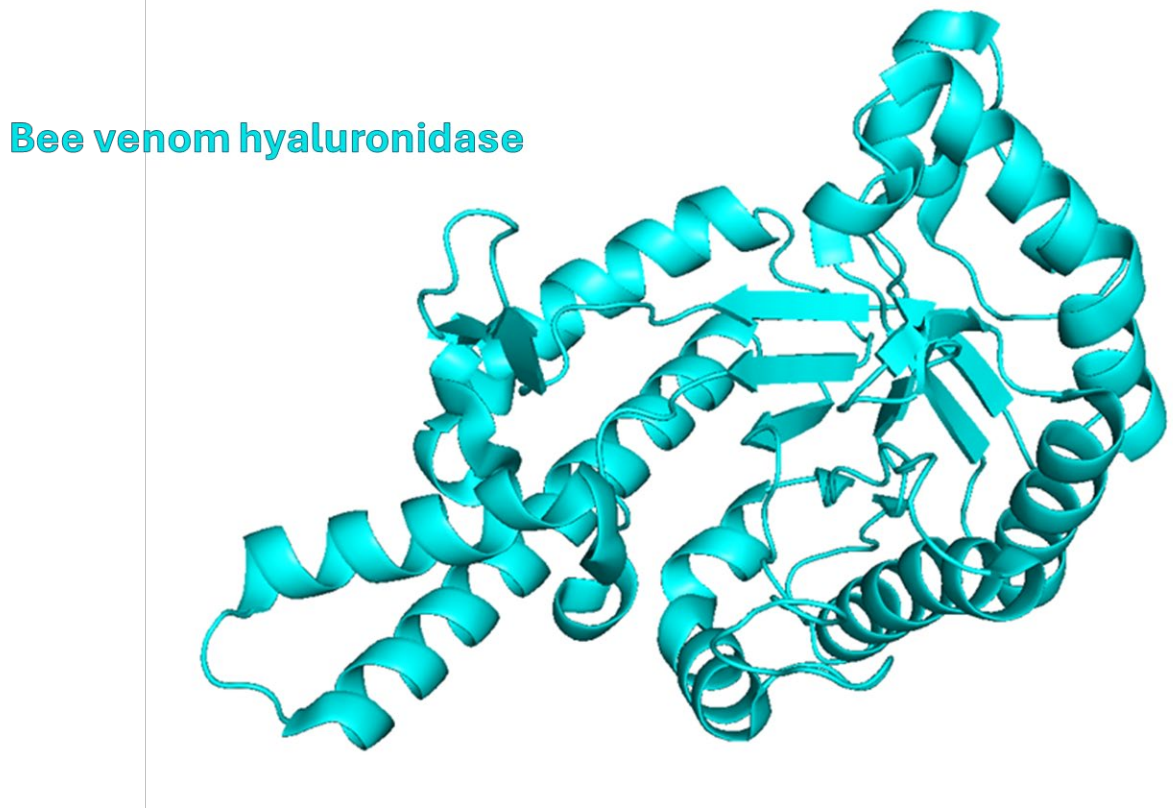
131. Based on the PH20 model, PH20 also has an alpha-beta barrel fold, as shown in the figure below.



132. When the PH20 model is superimposed on the Hyal-1 structure in PyMOL, as in the figure below, it is evident that both proteins adopt essentially the same alpha-beta barrel fold.



133. As shown in the figure below, bee venom hyaluronidase also has a tertiary structure that the POSA would characterize as an alpha-beta barrel fold:



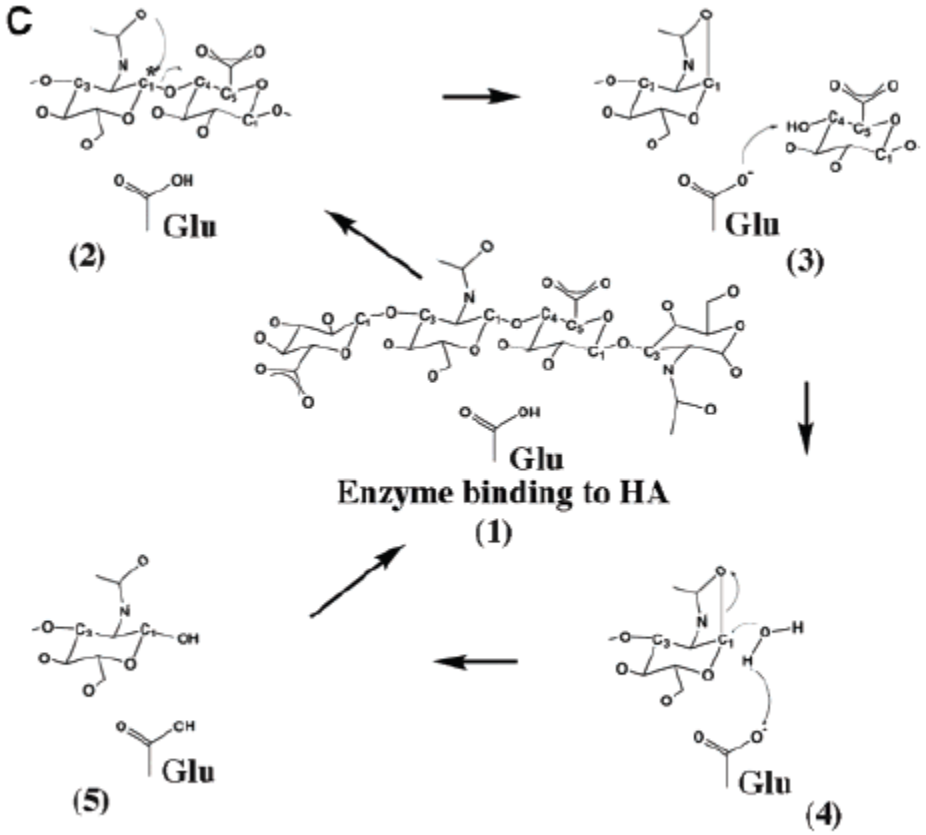
134. Thus, all three proteins have a tertiary structure that is an alpha-beta barrel fold.

2. PH20, Hyal-1 and Bee Venom Hyaluronidase Share A Common Mechanism Of Action

135. PH20, Hyal-1 and bee venom hyaluronidase share a common mechanism of action in that they all cleave HA through the same chemical reaction. Specifically, all three are glycoside hydrolase enzymes that cleave the 1–4 linkage between the N-acetylglucosamine and glucuronic acid components of the hyaluronan through substrate-assisted catalysis. EX1010 (Zhang), 9433; EX1033 (Marković-Housley), 1025; EX1008 (Stern), 832-33.

136. The hydrolysis reaction "takes place via general acid catalysis that requires two critical residues: a proton donor and a nucleophile/base." EX2091 (Davies 1995), 853. A conserved glutamic acid residue acts as the proton donor, and the nucleophile/base is the carbonyl oxygen of the C-2 *N*-acetyl group on the substrate (which is why the reaction is categorized as "substrate-assisted"). EX1008 (Stern), 832. For example, in bee venom hyaluronidase, "Glu113 act[s] as the proton donor and the *N*-acetyl carbonyl group of the substrate act[s] as the nucleophilic base." EX1033 (Marković-Housley), 1030.

137. Stern illustrates the mechanism of action as follows (EX1008, Figure 6(c)):



138. The "process of HA degradation occurs through a random bite type of catalysis" and through an "endolytic, nonprocessive mechanism, with the products of each degradation reaction becoming substrates for further cleavage." EX1008 (Stern), 833. "Endolytic" means that the hyaluronidases cleave HA in the middle of the carbohydrate chain, not the end of the chain. The reaction is not processive, which means the enzyme does not walk along HA and repeatedly cleave it but instead releases the HA and binds it again and cleaves in another random site (hence the term "random bite type of catalysis").

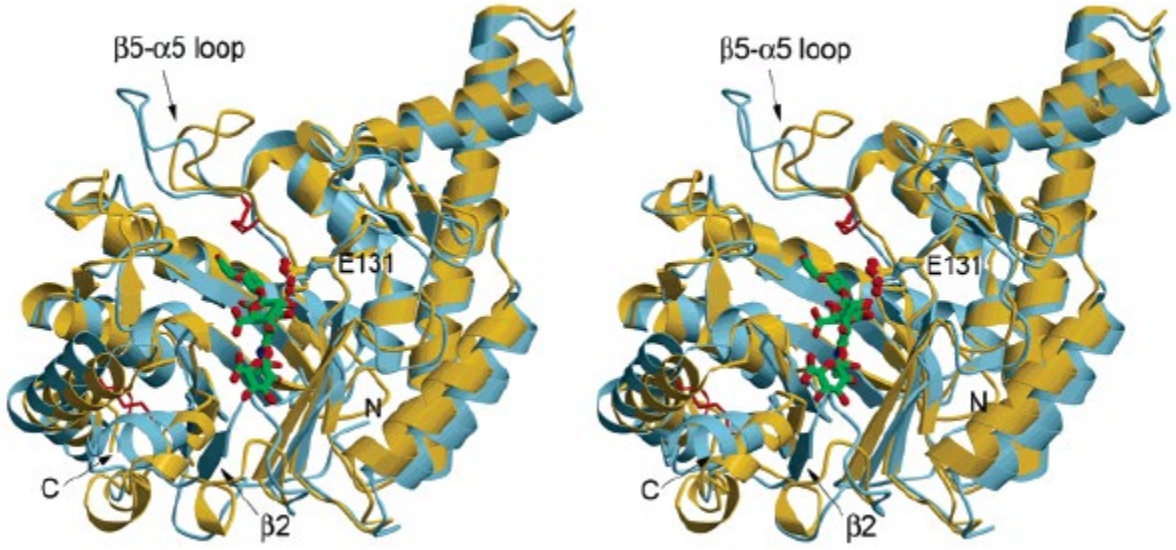
3. PH20, Hyal-1 and Bee Venom Hyaluronidase Share A Common Active Site Cleft/Groove[13]

139. PH20, Hyal-1 and bee venom hyaluronidase all achieve the function of cleaving HA using a common structure—the active site in all three proteins is a long groove and the same critical residues (oriented in about the same positions) catalyze the reaction in all three proteins.

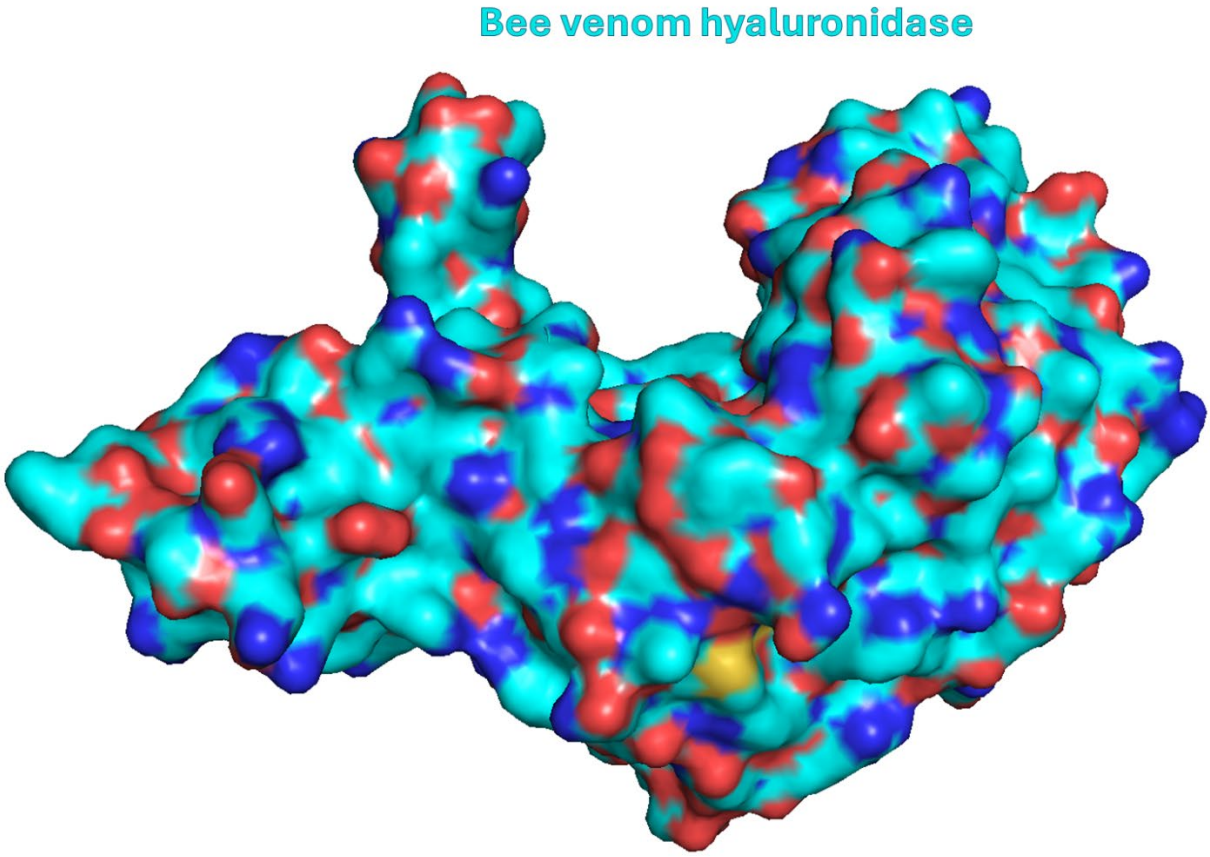
140. I note that this common structural feature was recognized in the literature: Stern teaches that "HA-degrading enzymes" share a "common structural feature," which is an "elongated cleft for accommodating substrate" EX1008 (Stern), 833. Consistent with this, Marković-Housley reports that the "dominant feature of the [bee venom hyaluronidase] protein surface is a large groove" that forms the active site and "would be large enough to accommodate a hexasaccharide" of HA. EX1033 (Marković-Housley), 1028.

141. Similarly, Chao superimposed the bee venom hyaluronidase and Hyal-1 structures and concluded that "that the active site clefts are similar in size and shape," as shown in Figure 2C, which I've reproduced below. EX1006 (Chao), 6914.

[13] The literature uses the terms "cleft" and "groove" interchangeably. So do I.

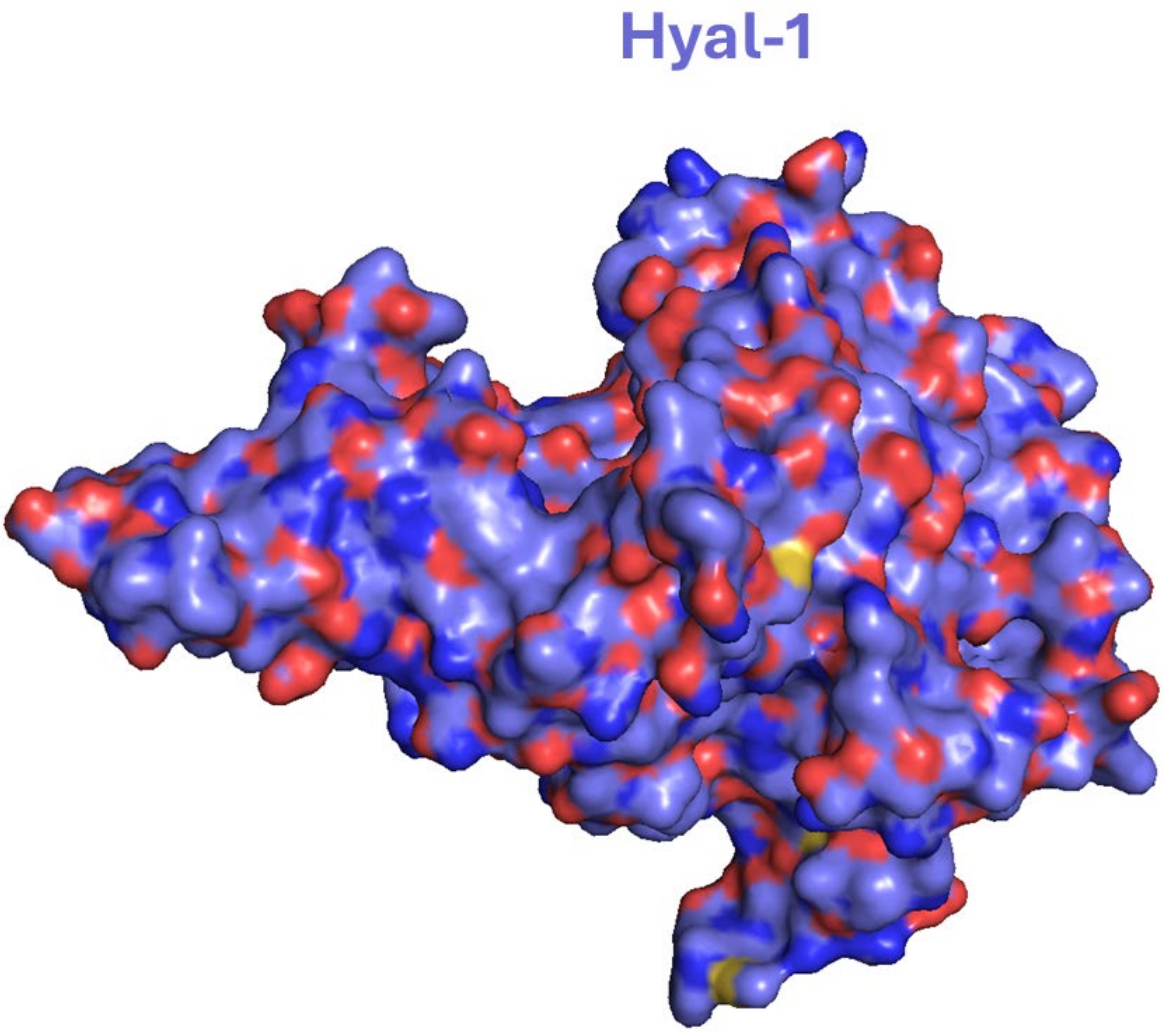


142. In the figure below, I show the active site groove of the bee venom hyaluronidase structure:



I note that in this figure, the carbon atoms are colored cyan, the oxygen atoms are colored red, the nitrogen atoms are colored blue and the sulfur atoms are colored yellow. The groove is the U-shaped feature at the top of the structure.

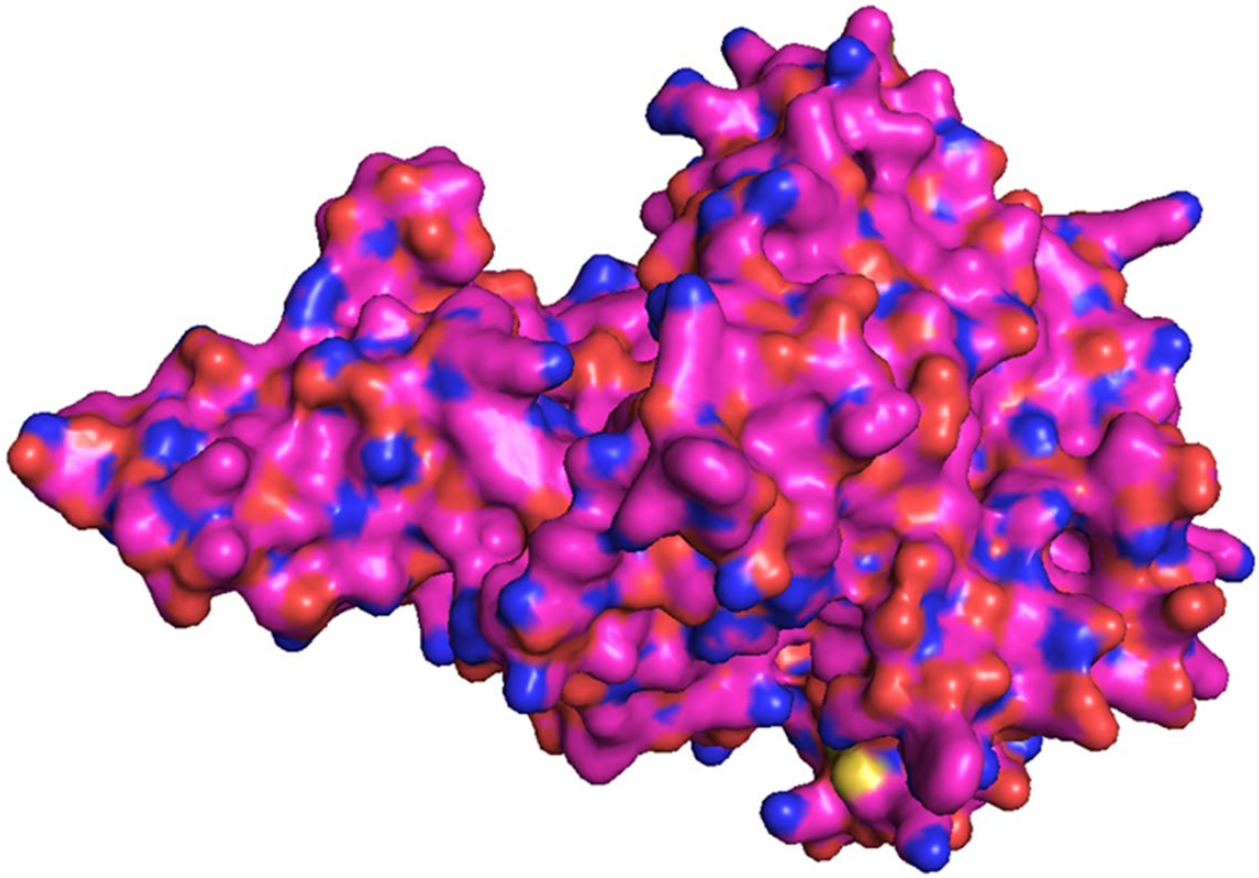
143. Like bee venom hyaluronidase, "the active site of hHyal-1 spans an elongated cleft." EX1006 (Chao), 6914. The elongated cleft is clearly visible in the image below:



I note that in this figure, the carbon atoms are colored slate, the oxygen atoms are colored red, the nitrogen atoms are colored blue and the sulfur atoms are colored yellow.

144. And the PH20 model shows that the active site on PH20 forms an elongated cleft/groove:

PH20 Model



I note that in this figure, the carbon atoms are colored magenta, the oxygen atoms are colored red, the nitrogen atoms are colored blue and the sulfur atoms are colored yellow.

4. PH20, Hyal-1 and Bee Venom Hyaluronidase Share Residues Known To Be Important For Structure/Function

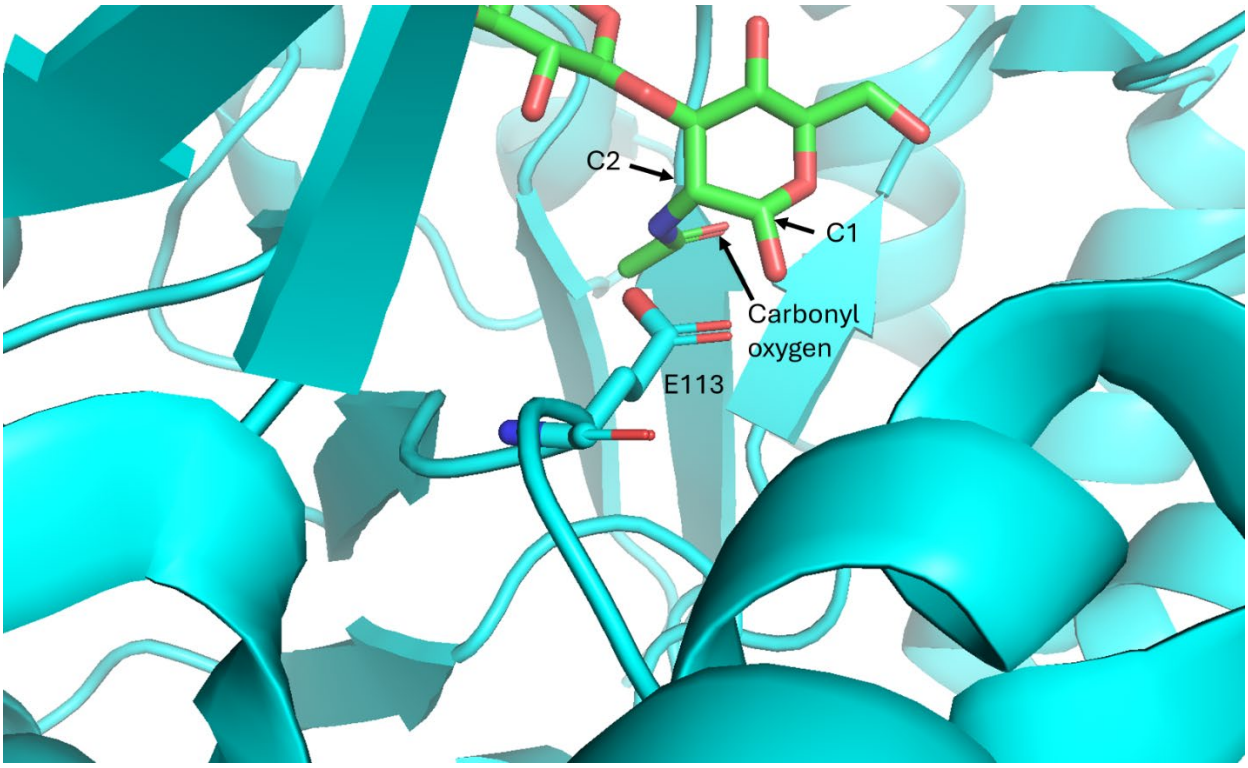
145. As noted above, the critical active site residues in Hyal-1, bee venom hyaluronidase and PH20 are conserved in terms of their identity, their positioning

and their interactions with HA. Thus, all three enzymes share a common function (hydrolyzing HA) that is achieved through shared structural features.

146. These three proteins are 30-40% identical in sequence[14] but share a common function and common structural features. Thus, they exhibit a common structure/function relationship.

147. In the figure below, bee venom hyaluronidase is shown in cyan and the HA carbohydrate is shown in green. I've labeled Glu113 and the carbonyl oxygen of the C-2 *N*-acetyl carbonyl that act as the proton donor and nucleophile, respectively, during the hydrolysis reaction. The carbohydrate shown in this structure is the end product of the cleavage reaction, so according to this mechanism of action, the carbonyl oxygen I label below would have already acted as the nucleophile in the hydrolysis that produced this end product.

[14] EX1006 (Chao), 6912; EX1008 (Stern), 826; *see also* EX1004 (Park), ¶ 40.



(a) Four Critical Active Site Residues

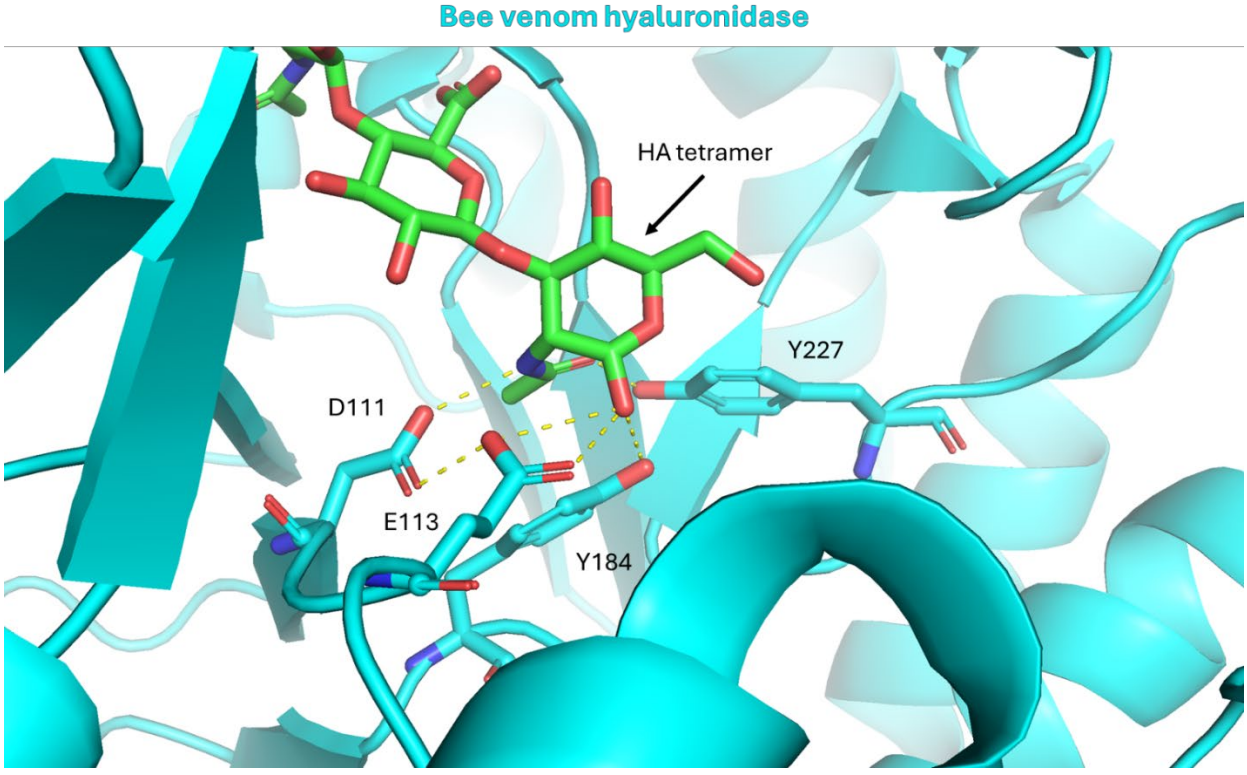
148. I start by showing four critical residues in the active site of the three proteins, and then I gradually add additional residues to the analysis. I do this in order to zoom in closely on the most critical residues that are directly involved in catalysis and then gradually broaden the field of view to include conserved residues that are in the active site, and then residues found to be important for positioning the critical active site residues or to help establish the chemical nature of the catalytic groups.

149. The residues I describe as critical are identified in the literature, and specifically in the Marković-Housley, Chao, Zhang and Arming references. Zhang

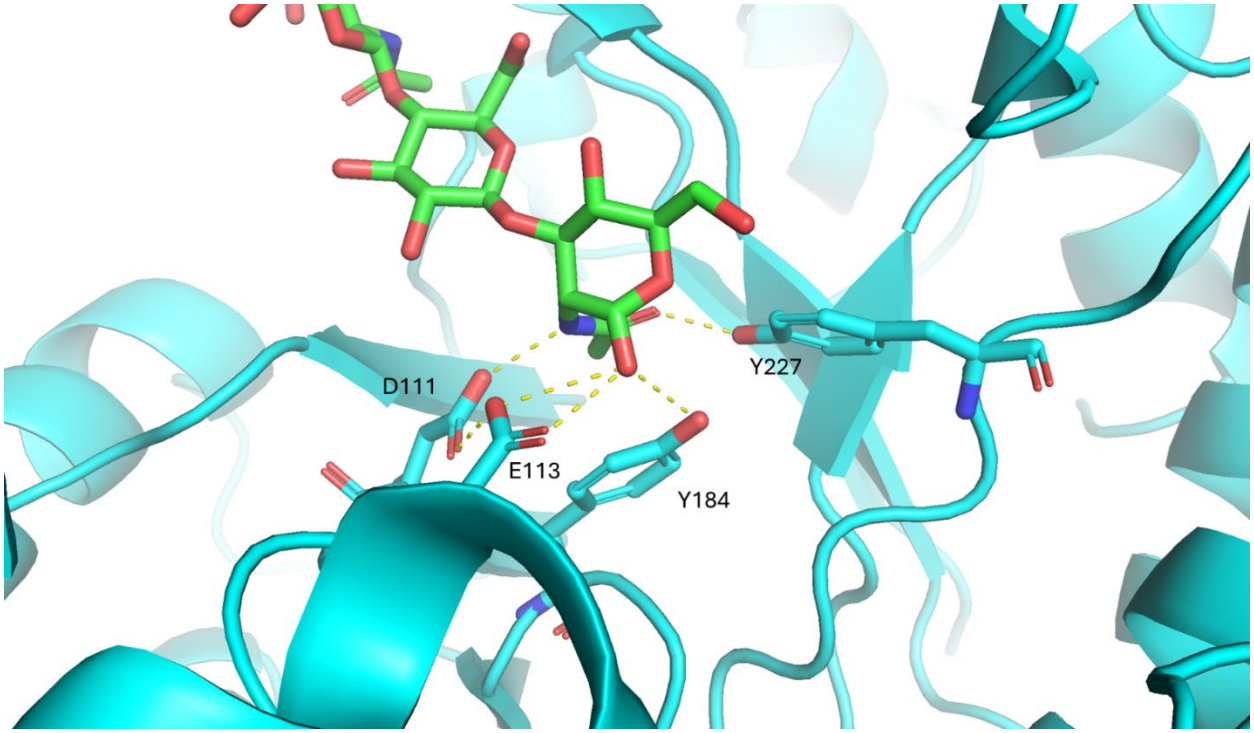
and Arming disclose mutagenesis data for specific residues; Zhang discloses data for Hyal-1 and Arming for PH20.

150. The fact that these critical residues are conserved in identity and orientation across all three proteins is evidence that these three proteins achieve a common function (hydrolysis of HA) through a common chemical mechanism (substrate-assisted general acid catalysis) performed using common structural features. This is a general principle in enzymology.

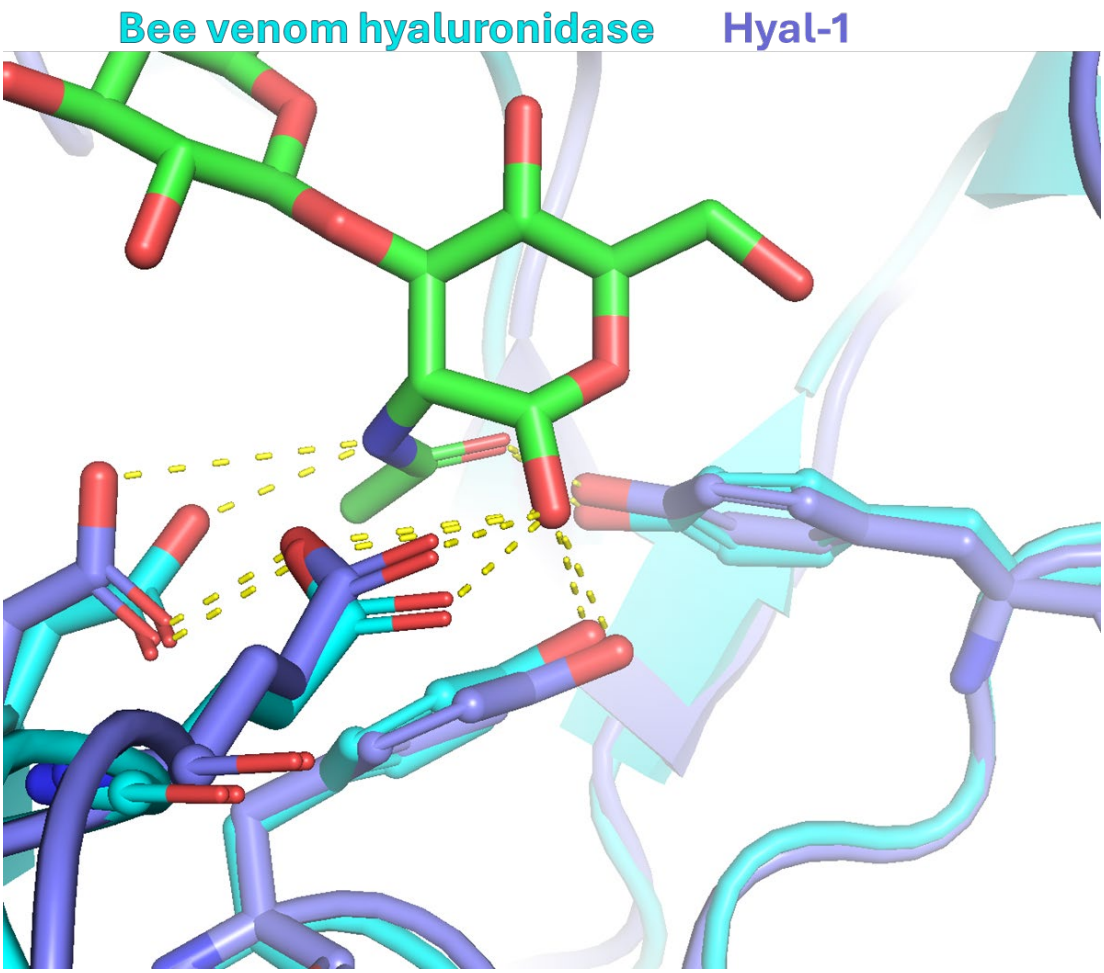
151. In the figures below of the bee venom hyaluronidase structure, Asp111, Glu113, Tyr184 and Tyr227 are shown in stick form (colored cyan), as is the HA product (colored green) and interactions are shown as yellow dashed lines:



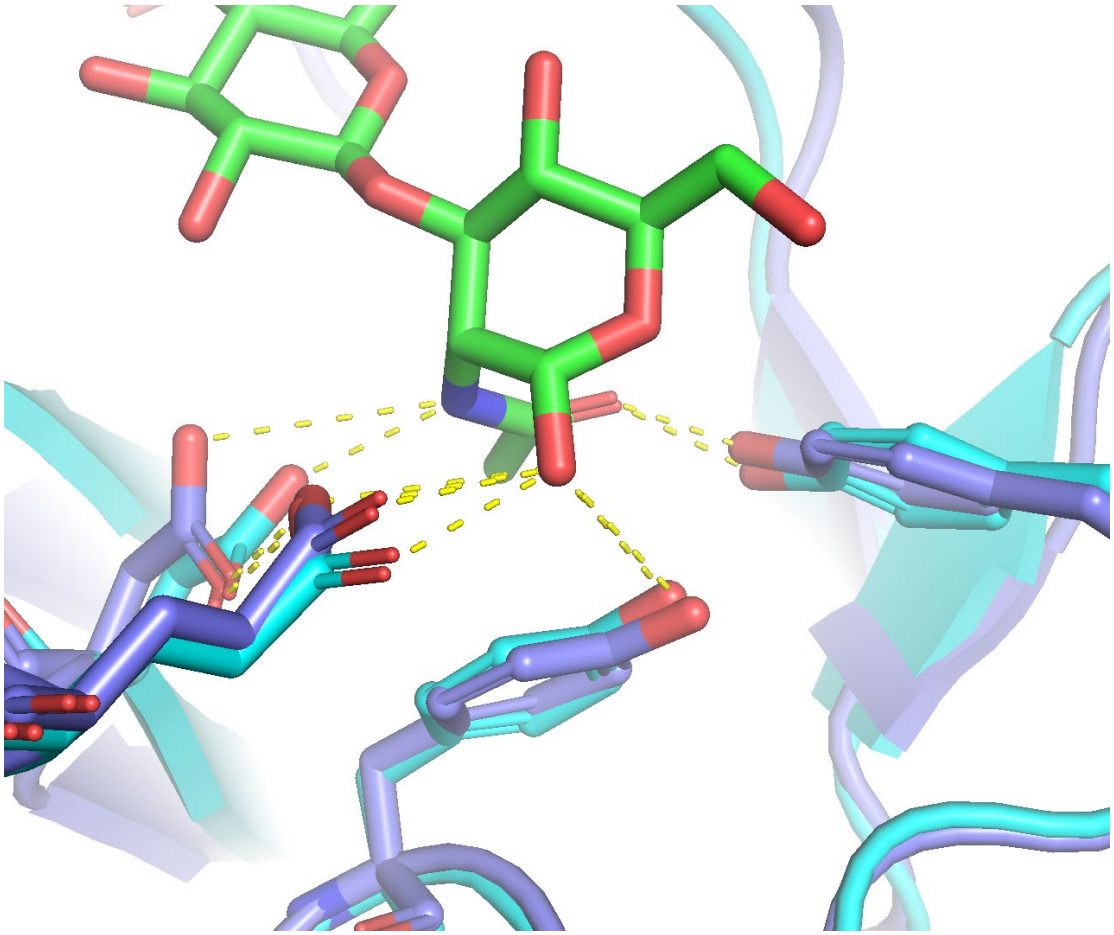
152. In the figure below, I have rotated the structure clockwise around the Y axis slightly so that the interaction between Tyr227 and the HA tetramer is visible.



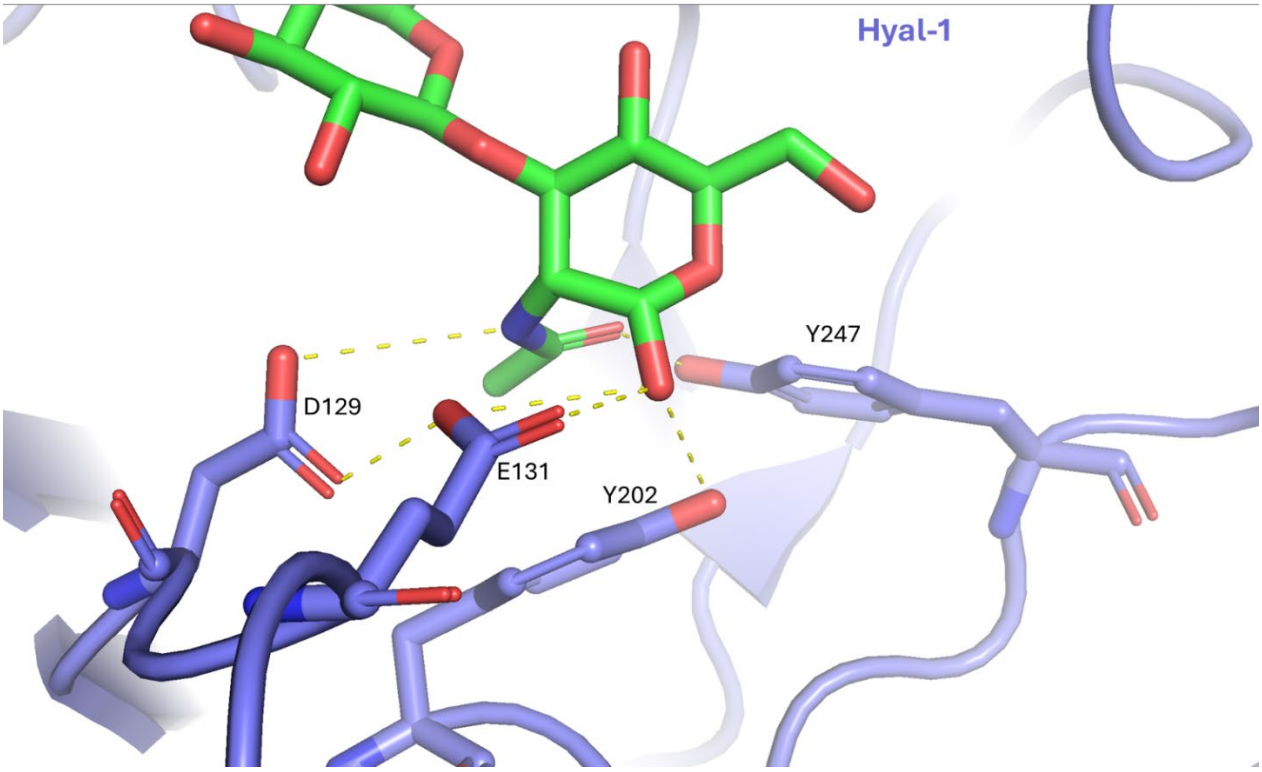
153. To illustrate that these four residues appear in a similar orientation in the Hyal-1 structure and form similar interactions, the figures below show the bee venom hyaluronidase structure superimposed on the Hyal-1 structure. In the figures below, residues Asp111, Glu113, Tyr184 and Tyr227 in the bee venom hyaluronidase structure (colored cyan) and residues Asp129, Glu131, Tyr202 and Tyr247 (colored slate) are shown in stick format, and interactions with the carbohydrate are shown as yellow dashed lines.



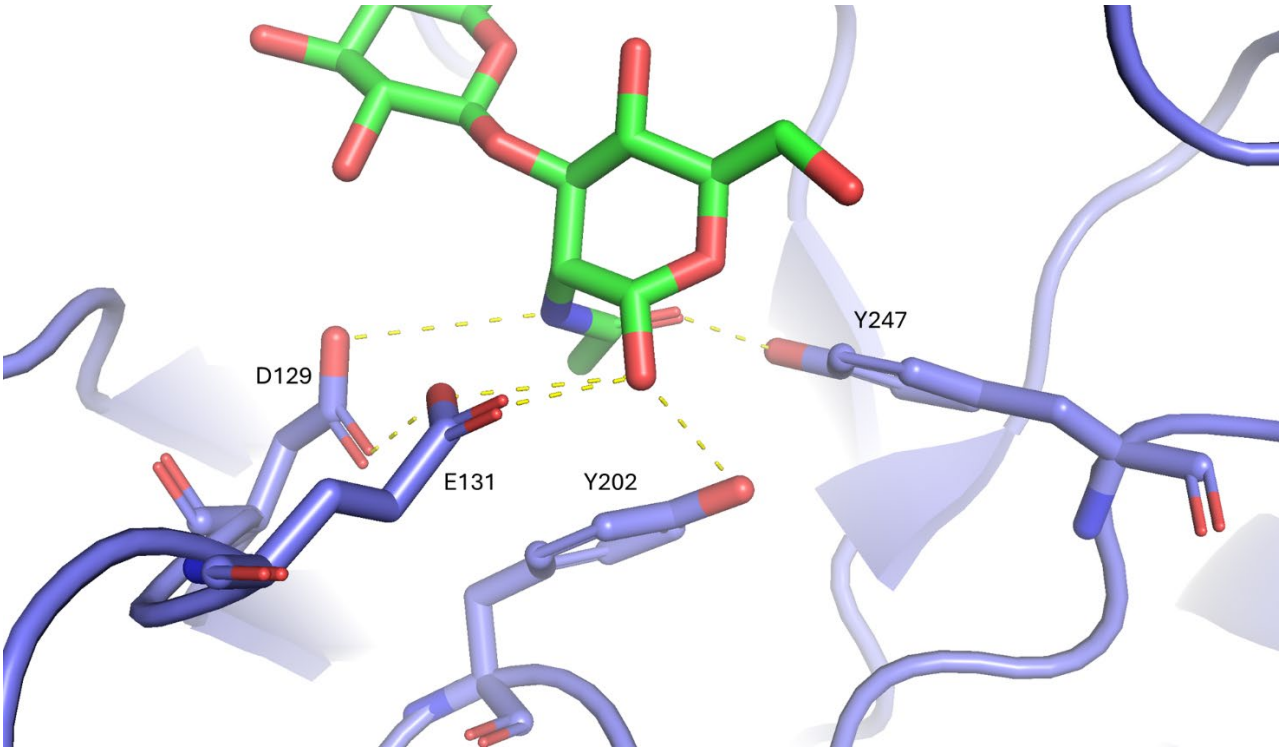
154. In the figure below, I have rotated the structures clockwise around the Y-axis slightly so that the interaction between the tyrosine (position 227 in bee venom hyaluronidase and position 247 in Hyal-1) and the HA tetramer is visible.



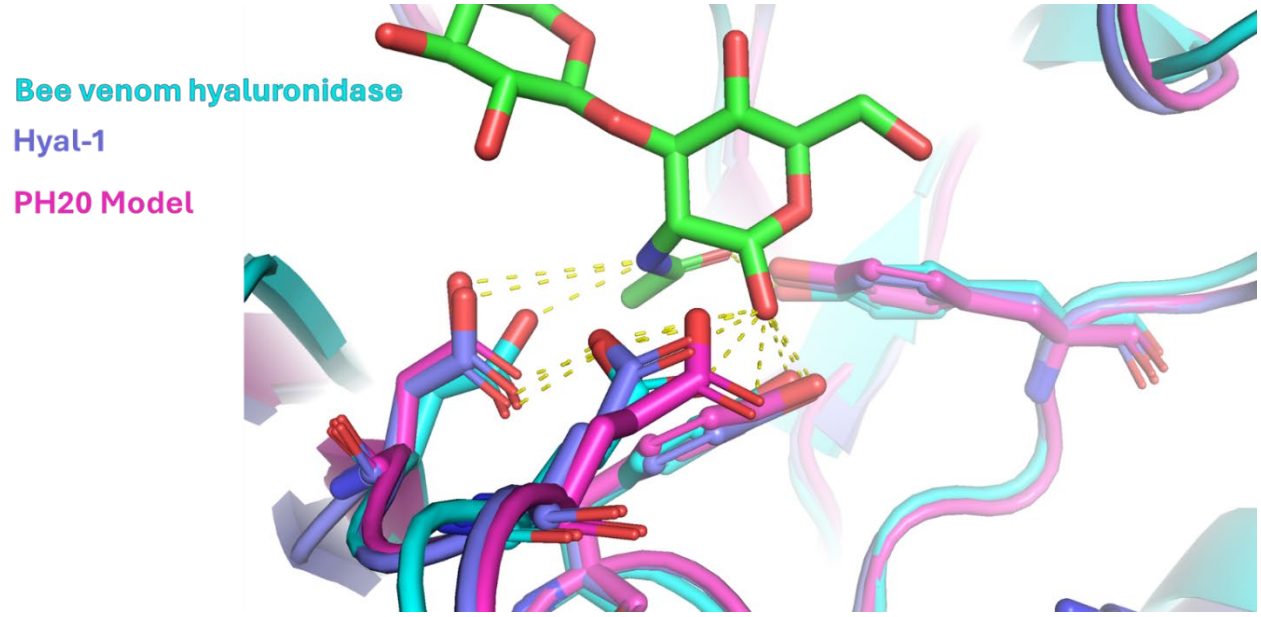
155. In the images below, I have hidden the bee venom hyaluronidase structure so that only the Hyal-1 structure is visible, and I have labeled the residues in the Hyal-1 structure:



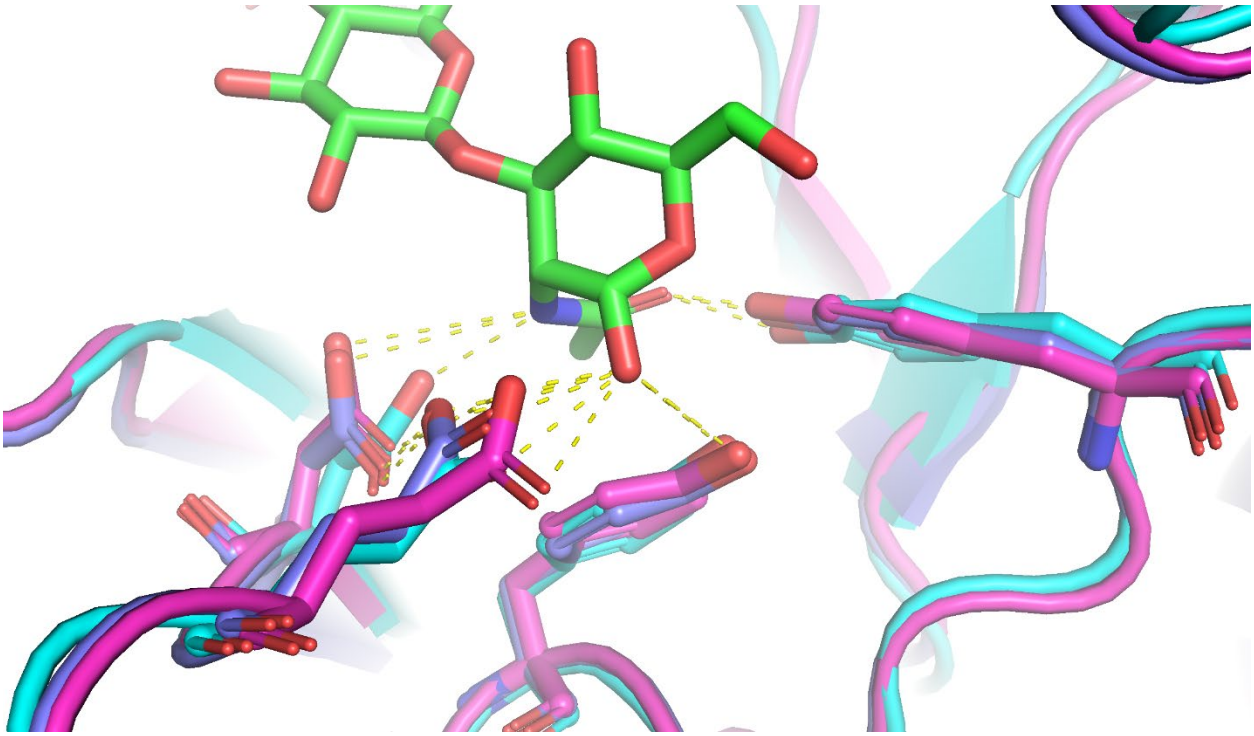
156. In the figure below, I have rotated the structures clockwise around the Y-axis slightly so that the interaction between the Tyr247 and the HA tetramer is visible.



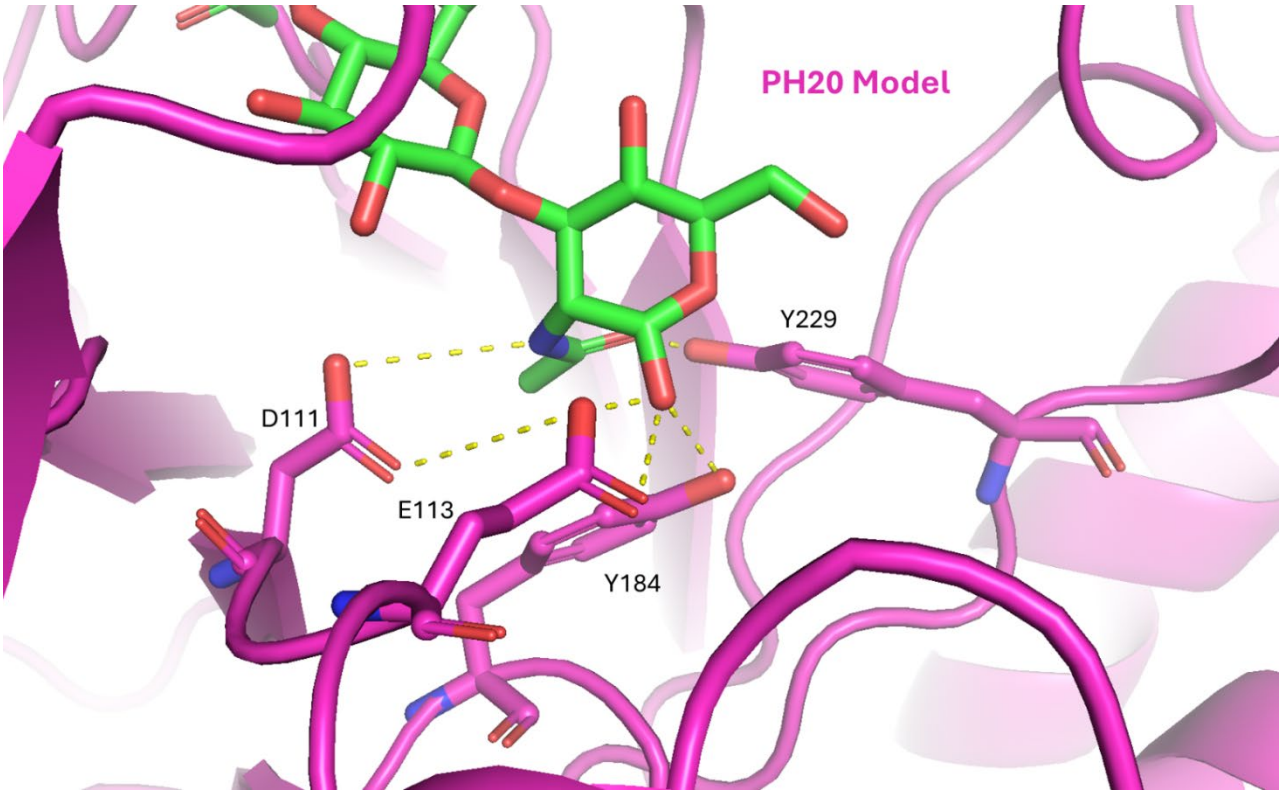
157. Finally, to illustrate that these four residues adopt about the same positions and form similar interactions in the PH20 model as they do in Hyal-1 and bee venom hyaluronidase, the figures below show both the PH20 model and the bee venom hyaluronidase structure superimposed on the Hyal-1 structure:



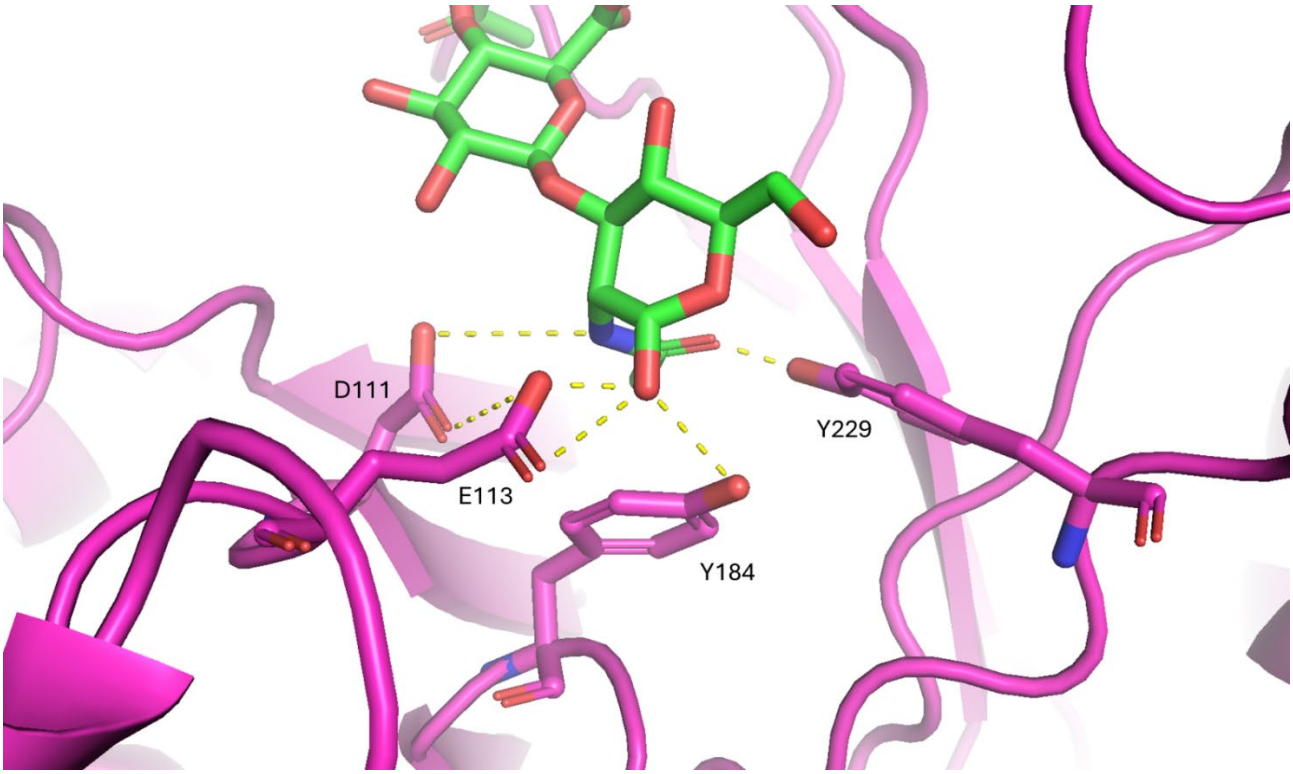
158. In the figure below, I have rotated the structures clockwise around the Y-axis slightly so that the interaction between the tyrosine (position 229 in PH20, position 227 in bee venom hyaluronidase and position 247 in Hyal-1) and the HA tetramer is visible.



159. In the figures below, I have hidden the Hyal-1 and bee venom structures so that only the PH20 model is visible, and I labeled the residues in the PH20 model:

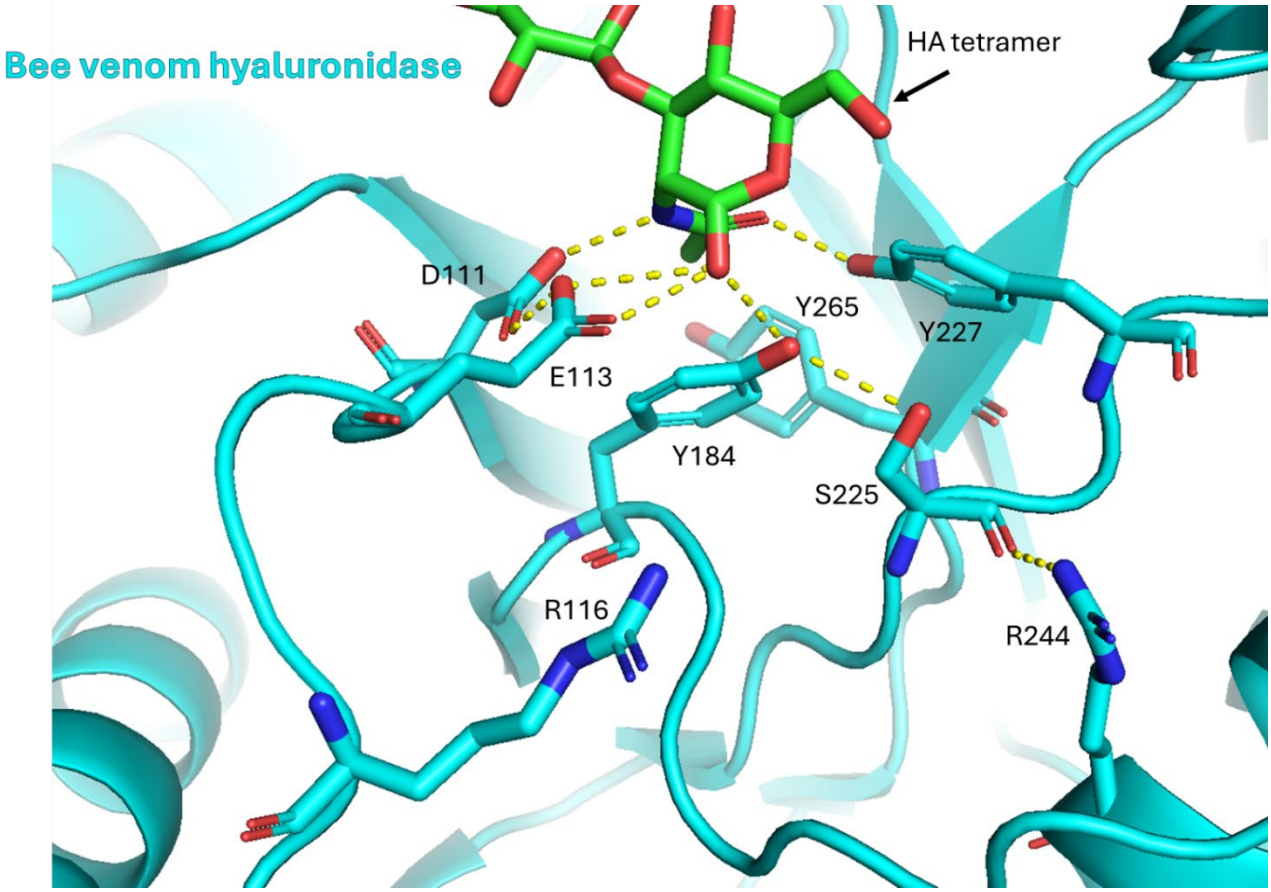


160. In the figure below, I have rotated the model clockwise around the Y-axis slightly so that the interaction between the Tyr229 and the HA tetramer is visible.



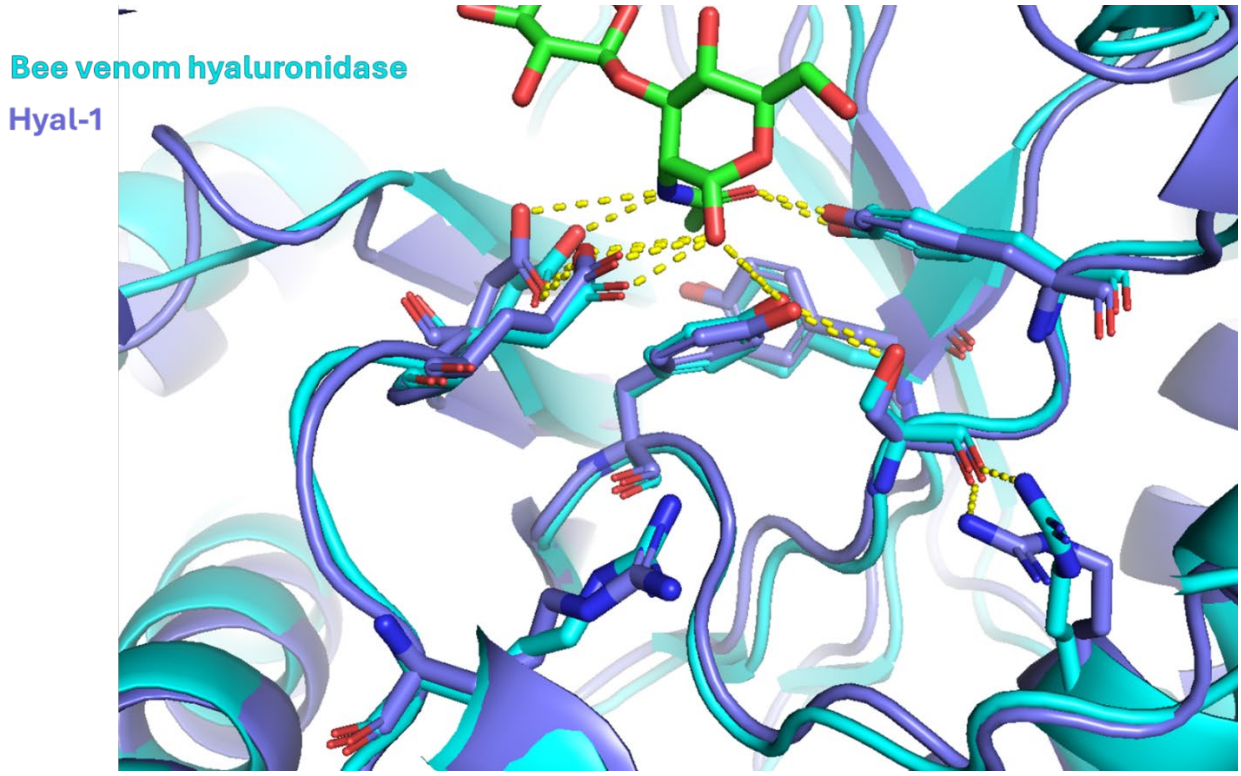
(b) Additional Active Site Residues

161. In the image below of the bee venom hyaluronidase structure, I have labeled additional residues that Marković-Housley identifies as being in the active site—Asp111, Glu113, Arg116, Arg244, Tyr184, Tyr227, Tyr265, and Ser225—and I've shown additional interactions as yellow dotted lines.

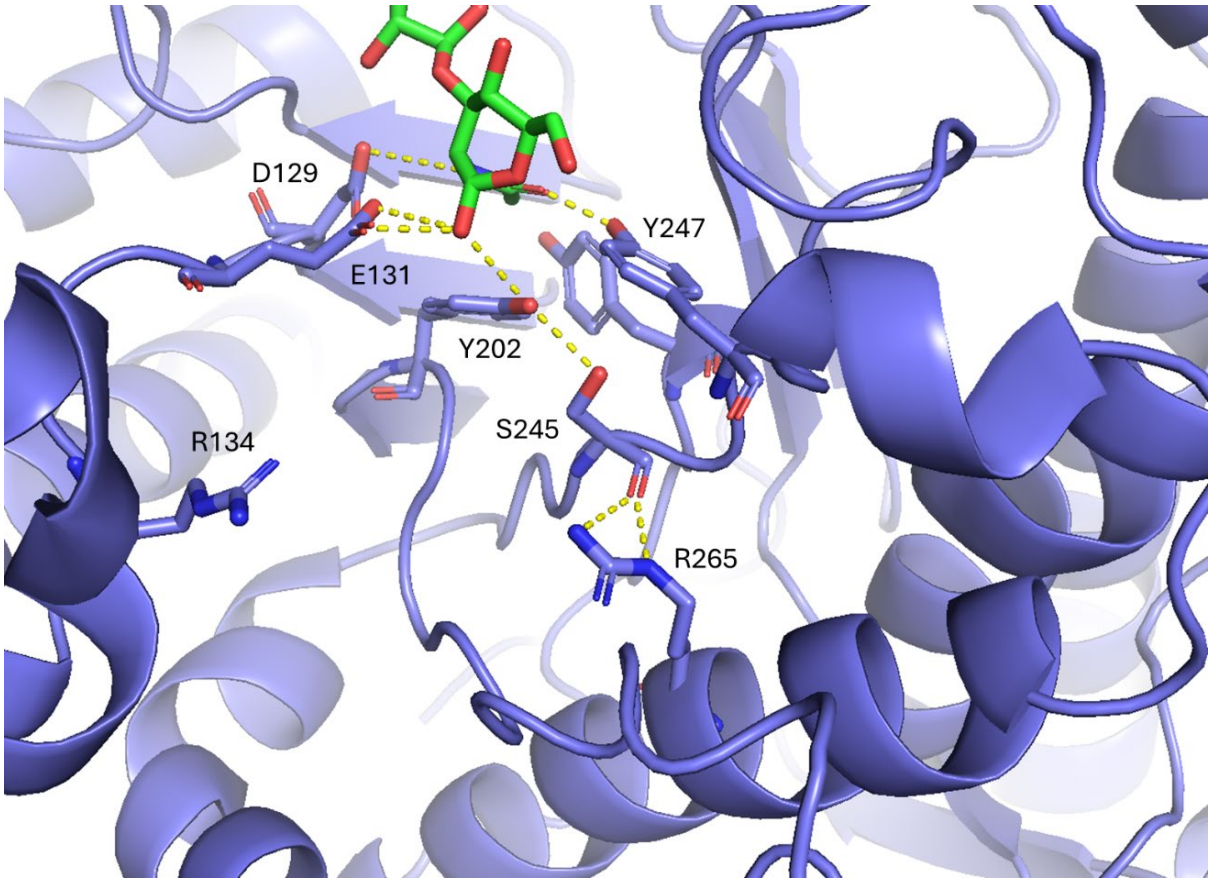


162. As I will show below, these residues and their positioning also are conserved in the Hyal-1 structure and the PH20 model.

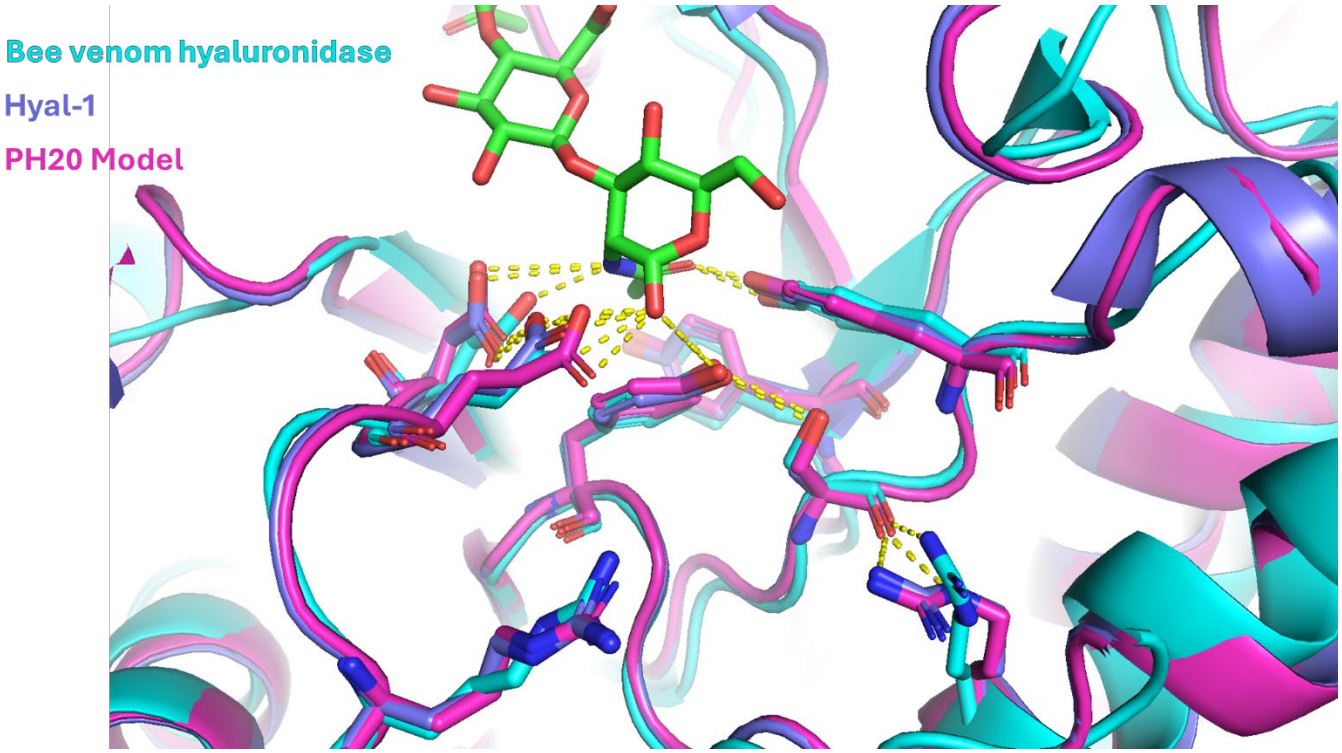
163. The figure below shows the bee venom hyaluronidase structure superimposed on the Hyal-1 structure. In the figure below, Asp111, Glu113, Arg116, Arg244, Tyr184, Tyr227, Tyr265, and Ser225 in the bee venom hyaluronidase structure and Asp129, Glu131, Arg134, Arg265, Tyr202, Tyr247, Tyr286 and Ser245 in the Hyal-1 structure are shown in stick format, with interactions shown as dashed yellow lines.



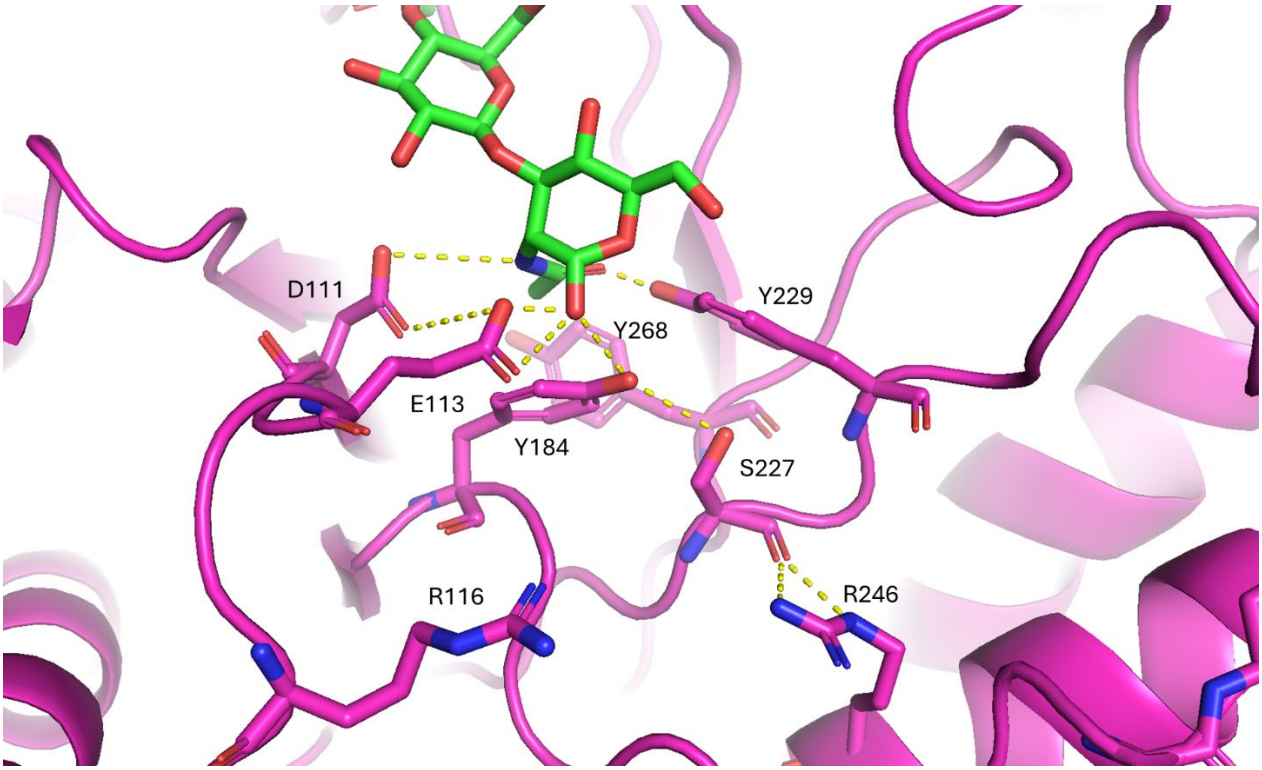
164. In the figure below, I have hidden the bee venom structure so that only the Hyal-1 structure is visible, and I have labeled the residues in the Hyal-1 structure.



165. To show that these residues also adopt similar positions and interactions in the PH20 model, I superimposed the bee venom hyaluronidase structure and the PH20 model onto the Hyal-1 structure in PyMOL. In the figure of these three superimposed structures below, Asp111, Glu113, Arg116, Arg244, Tyr184, Tyr227, Tyr265, and Ser225 in the bee venom hyaluronidase structure, Asp129, Glu131, Arg134, Arg265, Tyr202, Tyr247, Tyr286 and Ser245 in the Hyal-1 structure and Asp111, Glu113, Arg116, Arg246, Tyr184, Tyr229, Tyr268, and Ser227 in the PH20 structure are shown in stick format, with interactions shown as dashed yellow lines. This is a nice example of a common structure-function relationship in atomic detail.



166. In the figure below, I have hidden the bee venom and Hyal-1 structures such that only the PH20 model is visible, and I have labeled the residues in the PH20 model:



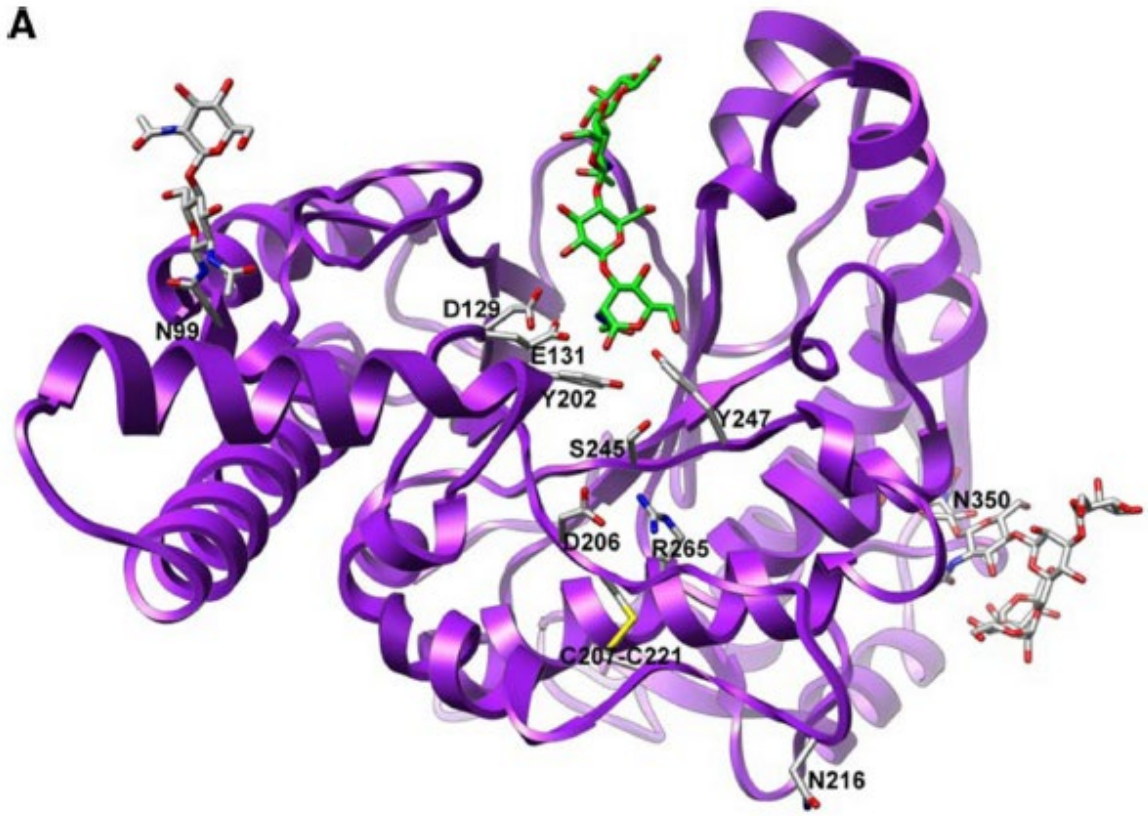
(c) Hyal-1 and PH20 Mutagenesis Data

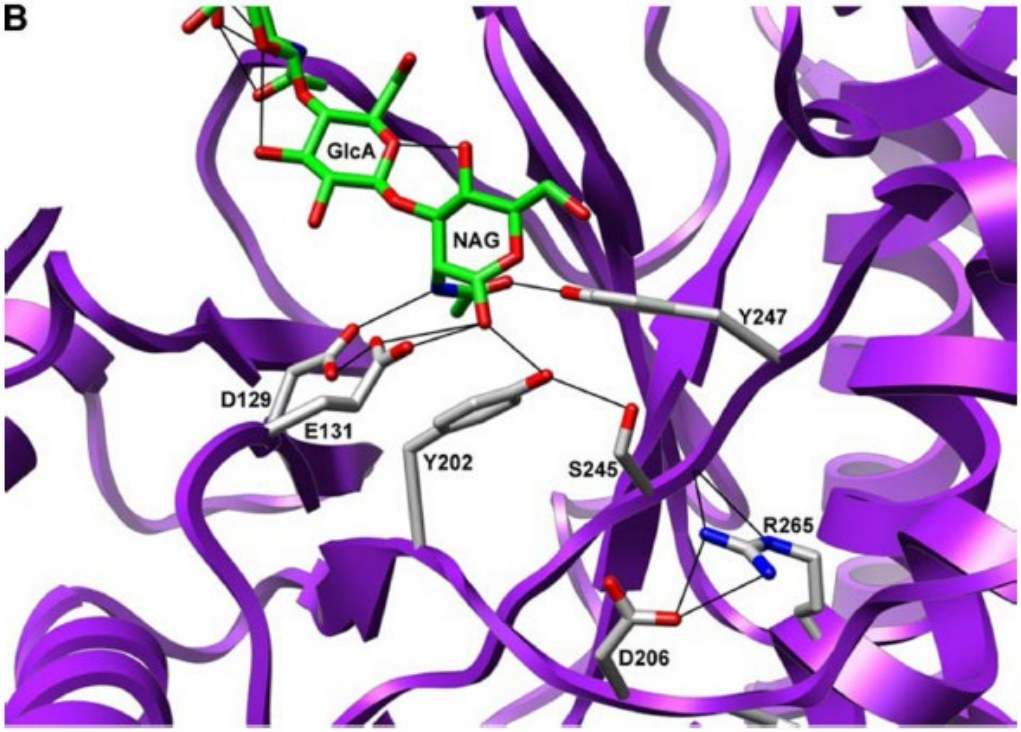
167. As of December 28, 2012, the importance of certain Hyal-1 and PH20 residues for enzymatic activity had been established through mutagenesis experiments. Specifically, Zhang, on which Dr. Simpson is an author, contains mutagenesis data and provides insight regarding Hyal-1 residues required for activity. Arming reports the results of mutagenesis experiments with PH20. Those data can be mapped onto the Hyal-1 structure and PH20 model (and the bee venom hyaluronidase structure) to provide further insight regarding common structural features shared by these three homologous proteins. And the POSA seeking to evaluate structure/function correlations and common structural features among

enzymatically active hyaluronidases in 2012 would have performed this analysis. I perform that analysis below, starting with Zhang.

168. Zhang used the Hyal-1 crystal structure (and the HA tetramer from the bee venom structure) to identify potential critical residues to mutate and test in a steady state kinetic assay. EX1010 (Zhang), 9434.

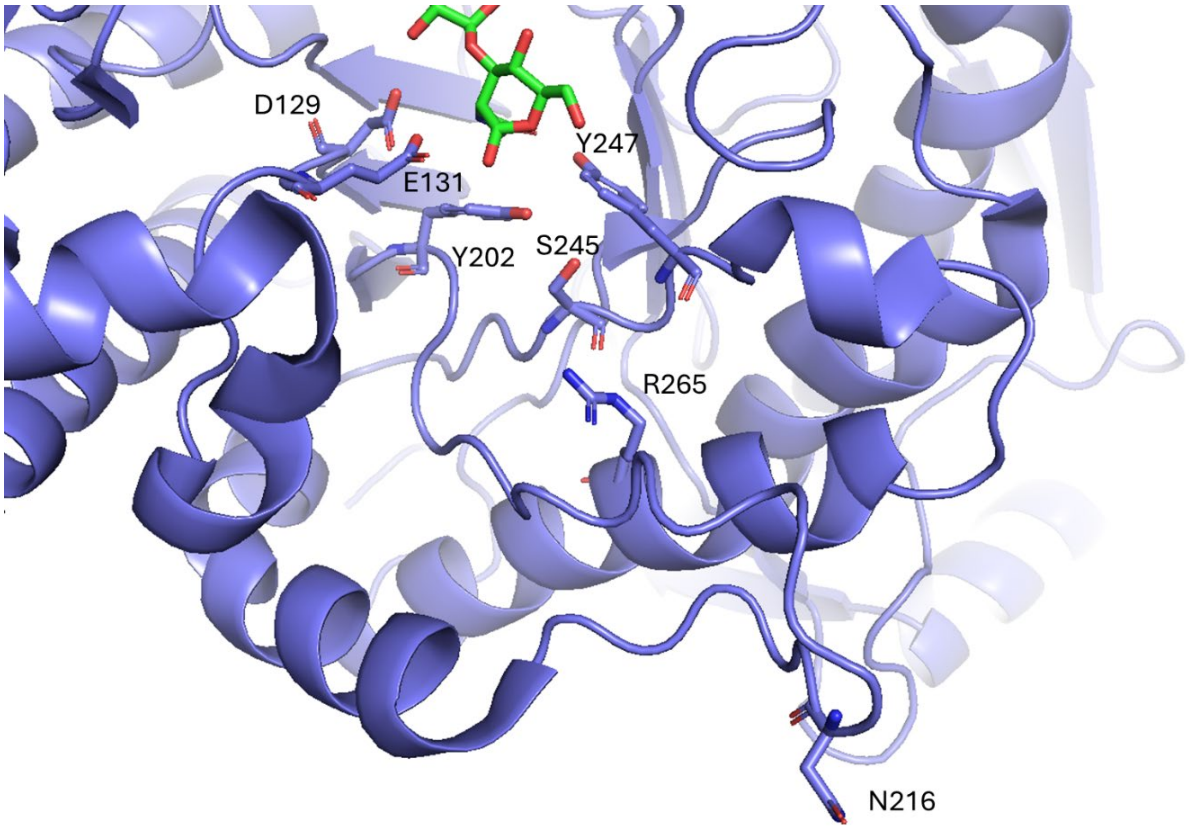
169. The residues that Zhang studied are shown in Figures 1A and 1B, which I have reproduced below.





170. Zhang explains that "point mutations were generated at residues Asp129, Glu131, Tyr202, Asn216, Tyr247, Ser245, and Arg265 to investigate their individual contributions to the enzyme mechanism." EX1010 (Zhang), 9435.

171. I note that residues Asp129, Glu131, Tyr202, Tyr247, Ser245, and Arg265 are shown in the bee venom/Hyal-1 images I generated above. As shown in the figures above, those residues and their interactions are conserved between bee venom hyaluronidase, Hyal-1 and PH20. The figures above did not include Asn216. The figure below of the Hyal-1 structure shows Asp129, Glu131, Tyr202, Asn216, Tyr247, Ser245, and Arg265 in stick format:



172. The residues that Zhang tested and the results are shown in the Table below.

TABLE 1
Summary of Hyal1 wild-type (WT) and mutant kinetic constants

Enzyme	K_m	V_{max}	% WT activity at 50 μM HA
	μM	*μmol/min/mg*	
Hyal1 wild-type	38.1 ± 4.8	12.5 ± 0.7	100.0 ± 1.8
Catalytic mutants			
E131Q	NA[a]	NA	0.08 ± 0.01
Y247F	NA	NA	0.04 ± 0.01
D129N	181 ± 19[b]	1.9 ± 0.1[b]	5.10 ± 0.09
Substrate binding mutants			
Y202F	367 ± 37[b]	6.7 ± 0.5[b]	11.1 ± 0.0
S245A	110 ± 19	10.7 ± 1.1	41.1 ± 0.4
Putative structural mutants			
R265L	NS[c]	NS	4.18 ± 0.09
N216A	103 ± 14	10.0 ± 0.8	44.6 ± 1.3
N350A	NS	NS	0.12 ± 0.01
N350tr	NS	NS	2.49 ± 0.17[d]
L356tr	NS	NS	6.29 ± 1.50[d]

[a] NA indicates no measurable activity at any HA concentration.
[b] Indicates extrapolated value from saturable curve fit.
[c] NS, not saturable, indicates data do not fit a saturation curve.
[d] Values measured at 125 μM HA.

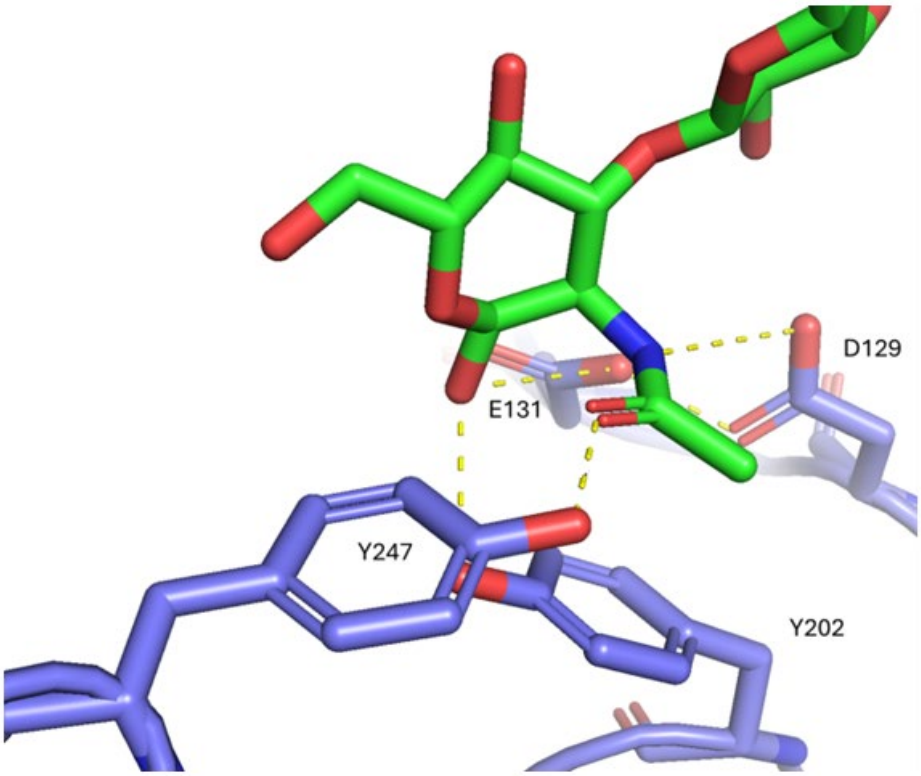
EX1010 (Zhang), 9438.

173. Zhang's results provide the K_m, V_{max} and %WT activity at 50 μM HA for each variant. K_m is roughly related to the affinity of the enzyme for its substrate, and V_{max} is roughly related to the efficiency of the chemical catalysis; when these values are not saturable, it often means the K_m has become so weak that the substrate concentrations used in the experiment are not nearly high enough to occupy all of the enzyme molecules in solution.

174. Based on these data, Zhang drew conclusions regarding the role of the tested residues. Some, like E131Q and Y247F essentially eliminated activity (0.08% and 0.04% activity was observed, respectively). EX1010 (Zhang), 9437-

38. Those residues were deemed critical to the catalytic mechanism of action. *Id.*, 9437.

175. As previously explained, E131 acts as the proton donor during the catalytic reaction. Zhang explained that based on its position in the structure, Tyr247's "role is to coordinate and stabilize the oxyanion during transition state formation: the hydroxyl of Tyr247 is optimally located for hydrogen bond donation to the carbonyl oxygen. An inert role in binding is less likely, because the Y247F mutation, which is inactive, preserves the hydrophobic character of the residue that is thought to be important for association with sugar rings in the substrate." EX1010 (Zhang), 9440. As shown in the figure below, hydroxyl of Tyr247 forms an interaction with the carbonyl oxygen of the product.



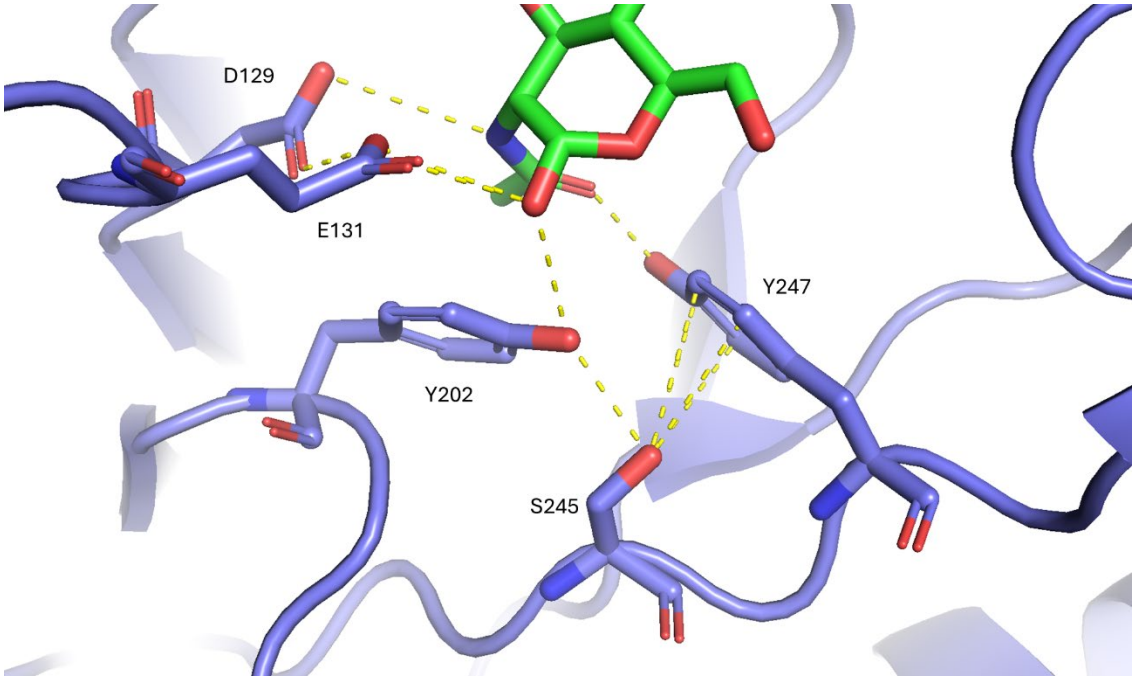
176. Consistent with Zhang's observations for Hyal-1, Arming discloses a mutagenesis experiment involving PH20 in which E113 (which corresponds to E131 in Hyal-1) was mutated to glutamine. EX1011 (Arming), 810. Arming found that the resulting protein lacked any enzymatic activity. *Id.*, 810, 812.

177. Zhang concluded that D129N is "not essential" to catalytic activity because some activity (specifically, 5% of wild type activity at 50 μM HA) was retained. EX1010 (Zhang), 9437-38. In other words, Hyal-1 is able to hydrolyze HA with an asparagine in position 129, but activity is substantially reduced.

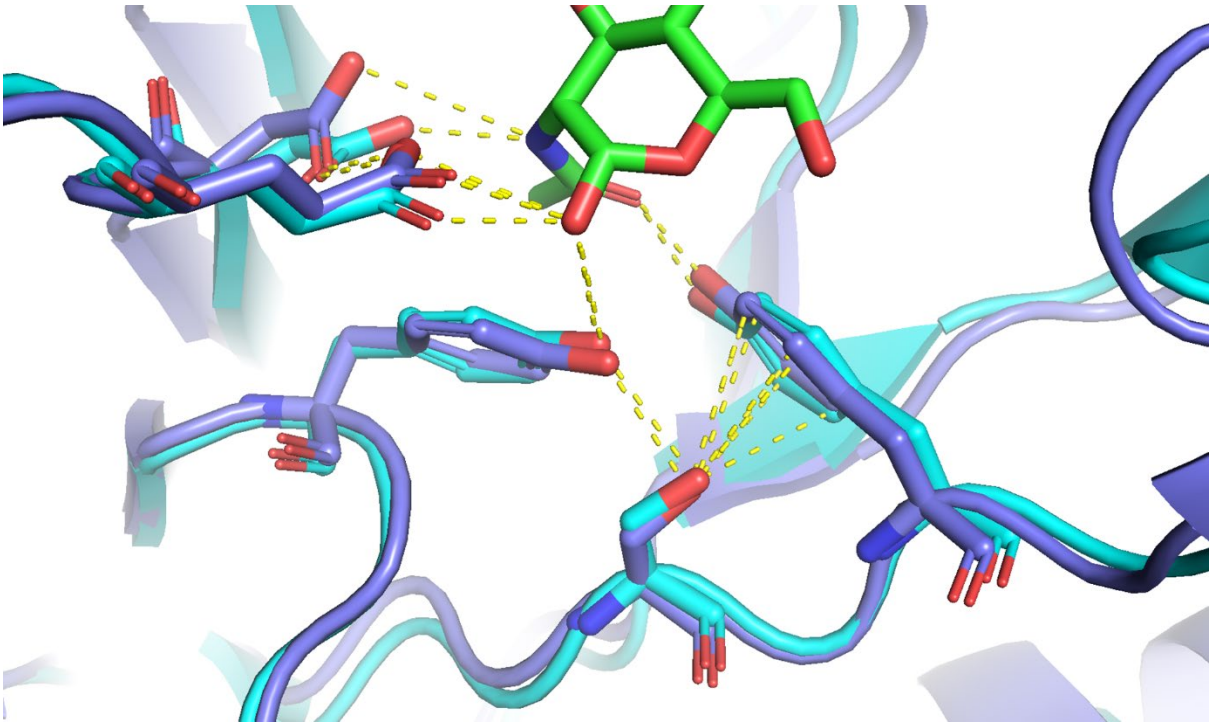
178. Arming obtained a similar result. Arming mutated Asp111 (Asp129 in Hyal-1) to asparagine in PH20 and found that it significantly reduced activity (to

a few percent or less of WT) but did not completely eliminate activity. EX1011 (Arming), 813.

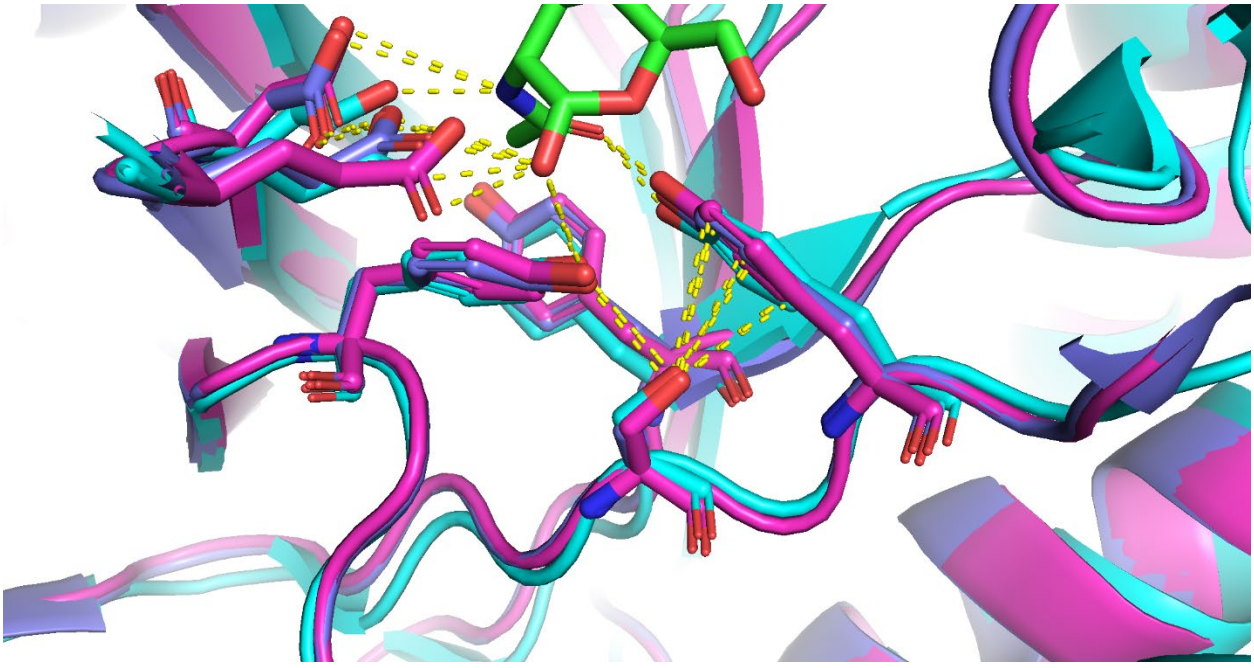
179. With respect to Y202F and S245A, Zhang concluded that both positions play a role in HA binding in view of increased K_m. EX1010 (Zhang), 9437. Zhang explained that although Ser245 "does not contact the substrate directly," "the side chain hydroxyl would be an essential linchpin between Tyr202 and Tyr247, forming a hydrogen bond with the hydroxyl of Tyr202 and the *pi* cloud of Tyr247 and thereby positioning them both." EX1010 (Zhang), 9440. This statement is not precisely correct. The pi cloud of an aromatic ring is negatively polarized, as is the oxygen atom of a serine side chain. Those will not interact favorably. As the figure below shows, it is actually the edge carbon atoms of the aromatic ring, whose hydrogens are positively polarized, that are making the favorable interaction with the serine oxygen. I have illustrated this in the figure below.



180. To show that these interactions are also formed in the bee venom hyaluronidase structure and the PH20 model, I have again superimposed the bee venom structure and the PH20 model on the Hyal-1 structure. The figure below shows the bee venom hyaluronidase structure superimposed on the Hyal-1 structure:



181. The figure below shows the bee venom hyaluronidase structure and the PH20 model superimposed on the Hyal-1 structure:

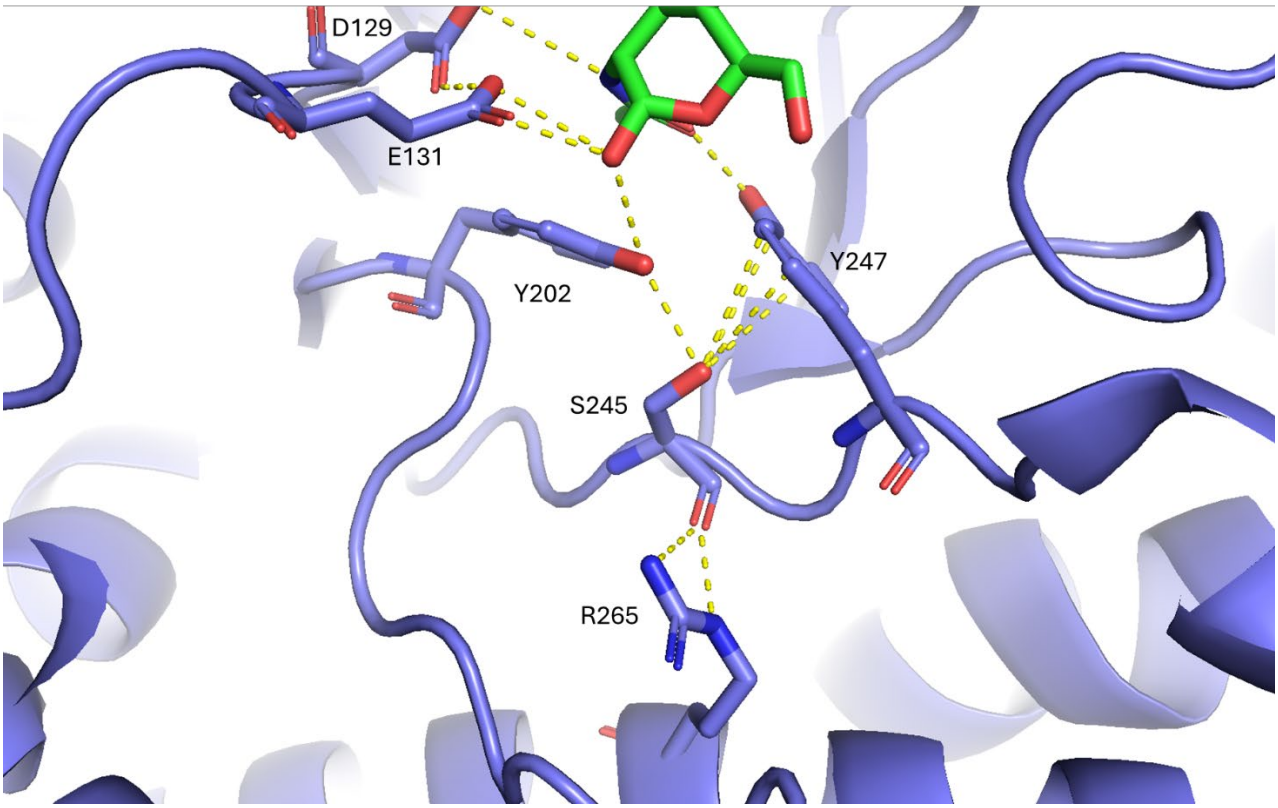


182. The fact that the critical catalytic residues in the bee venom hyaluronidase and Hyal-1 structures and the PH20 model adopt similar positions and form similar interactions shows that bee venom hyaluronidase, Hyal-1 and PH20 share a common structure/function relationship. The structure of the residues—their orientation and chemistry—results in the function of hydrolyzing HA through the same mechanism of action.

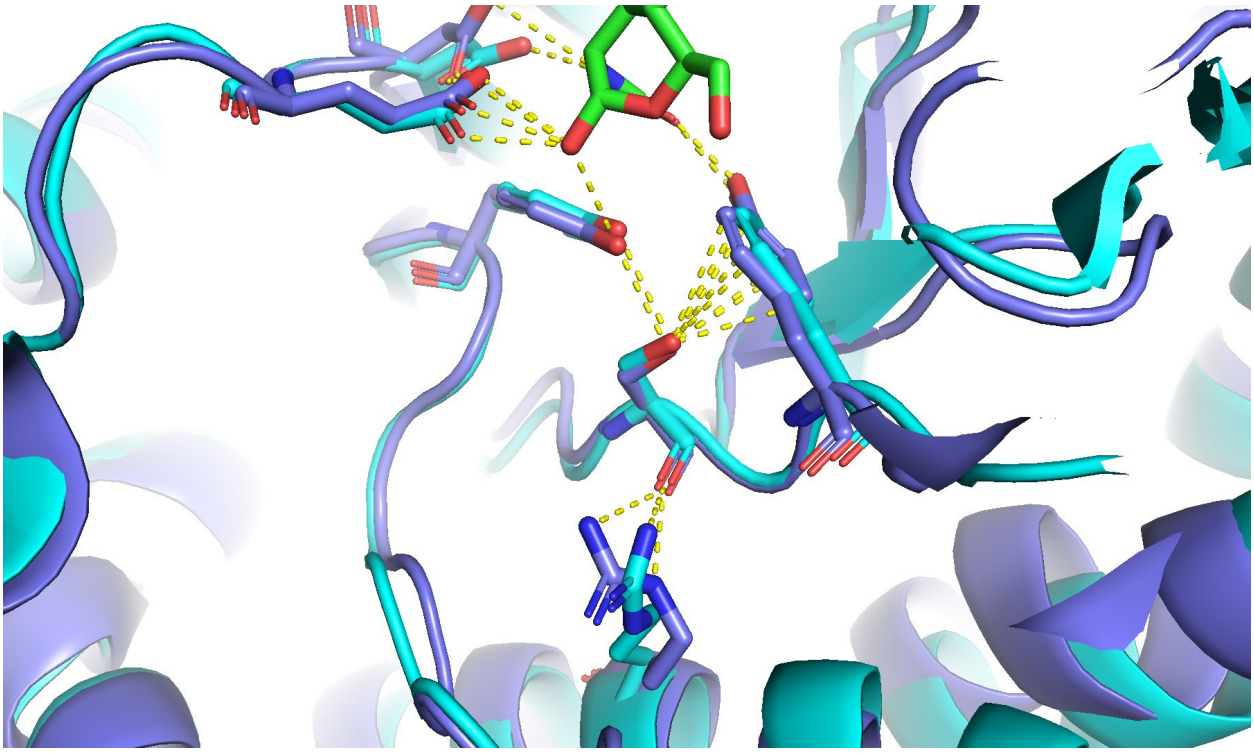
183. Although R265 is not directly involved in the catalytic reaction, the R265L mutation had a substantial impact on activity. Zhang reports that "this mutation was as disruptive as D129N, retaining only ~4% of wildtype activity at 50 μM HA." EX1010 (Zhang), 9437. Activity increased linearly over the range tested and did not reach saturation, so it was not possible to calculate kinetic constants for this mutation. *Id.*, 9437-38. At the highest concentration of HA tested, activity "was 7.3% of the wild-type enzyme activity at this amount." *Id.*, 9438.

184. To explain the substantial impact on activity, Zhang noted that the R265 residue "orients Ser245 and the essential Tyr247, exact placement of which is critical for transition state formation." EX1010 (Zhang), 9440. (It is probably better if the authors had said "transition state stabilization" here, since that is the actual assumed function of these residues in the mechanism.) This relationship is shown in the figure below. Zhang also explained that the "magnitude of its effect

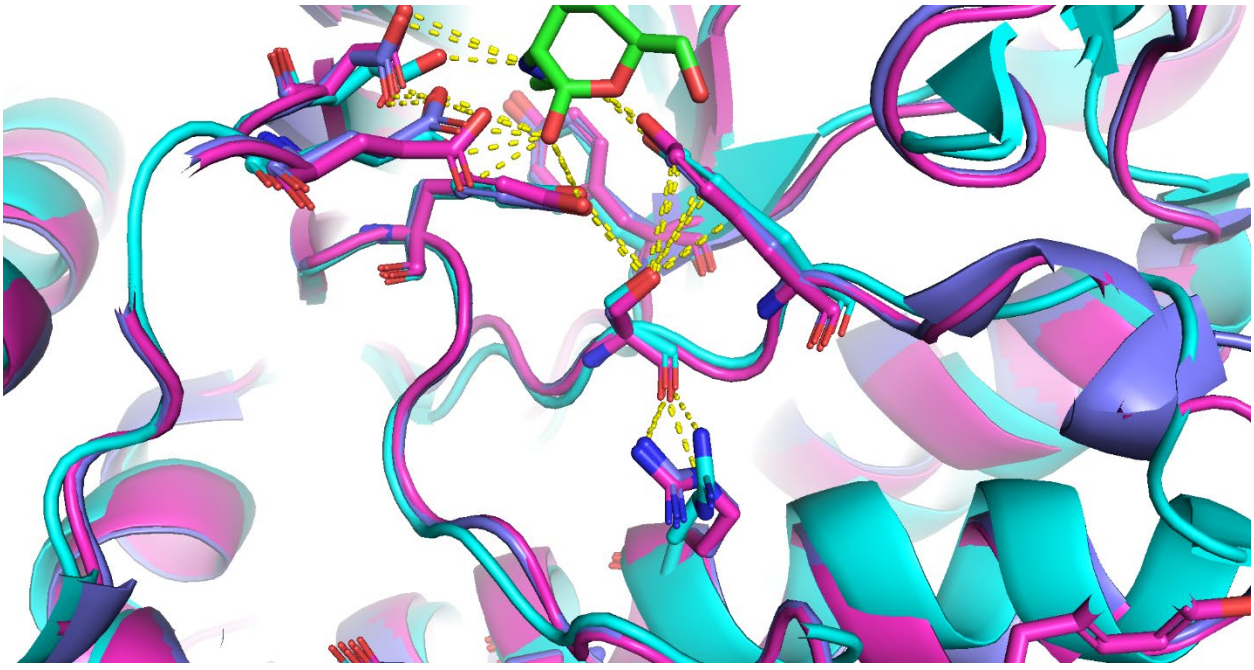
on catalysis also suggests the shape and rigidity of the active site cavity is critical to its function, regardless of the presence of specific catalytic residues." *Id.* Observations like this, which have also been made for other enzymes, point to the importance of proximity and orientation in the structural basis for enzyme catalytic power.



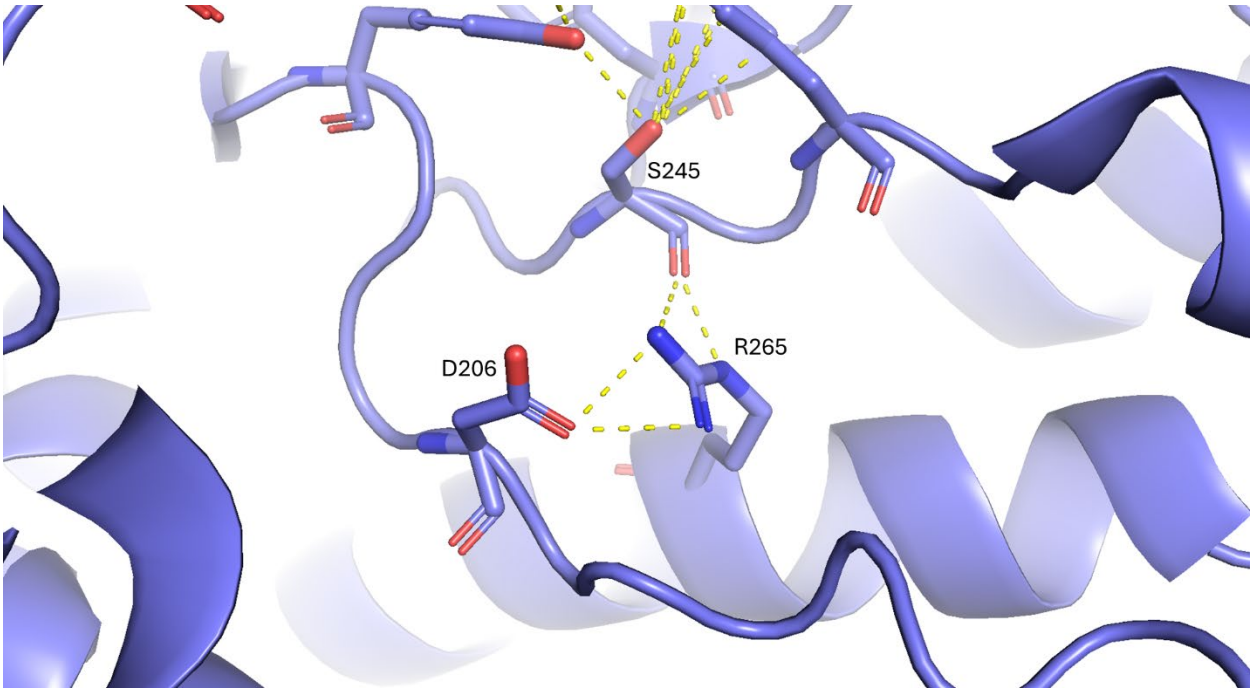
185. And, as shown in the figures below, these interactions are also formed in the bee venom hyaluronidase structure and the PH20 model. The figure below shows the bee venom hyaluronidase structure superimposed on the Hyal-1 structure:



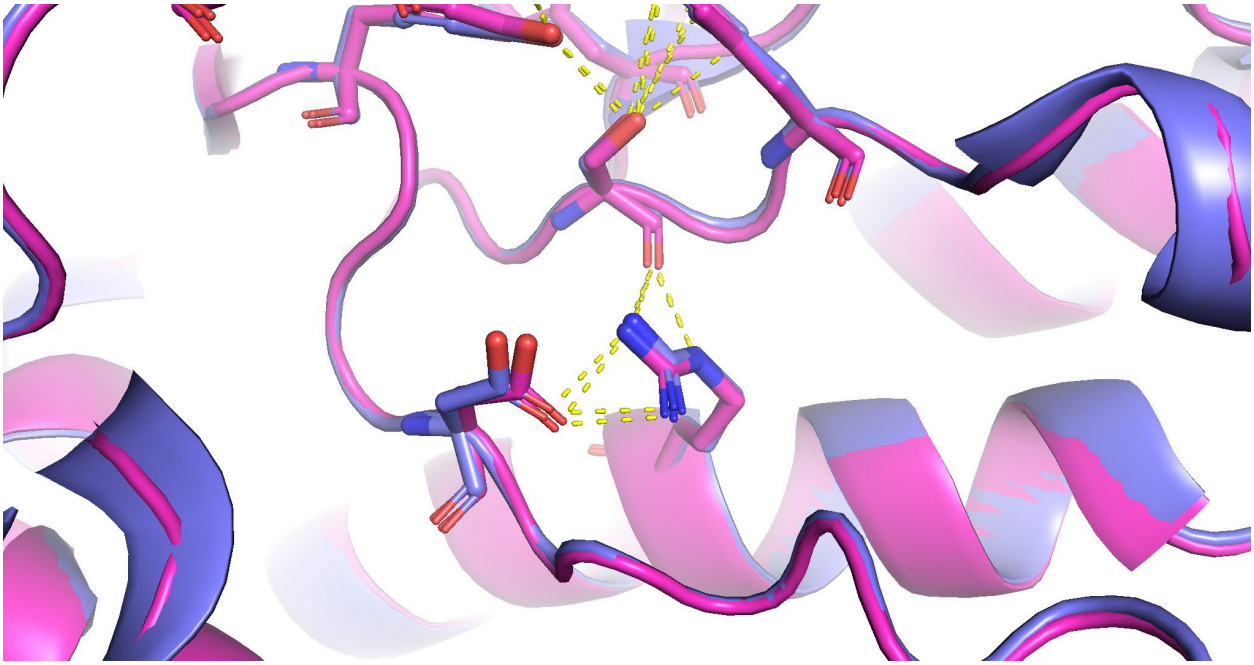
186. The figure below shows the bee venom hyaluronidase structure and the PH20 model superimposed on the Hyal-1 structure:



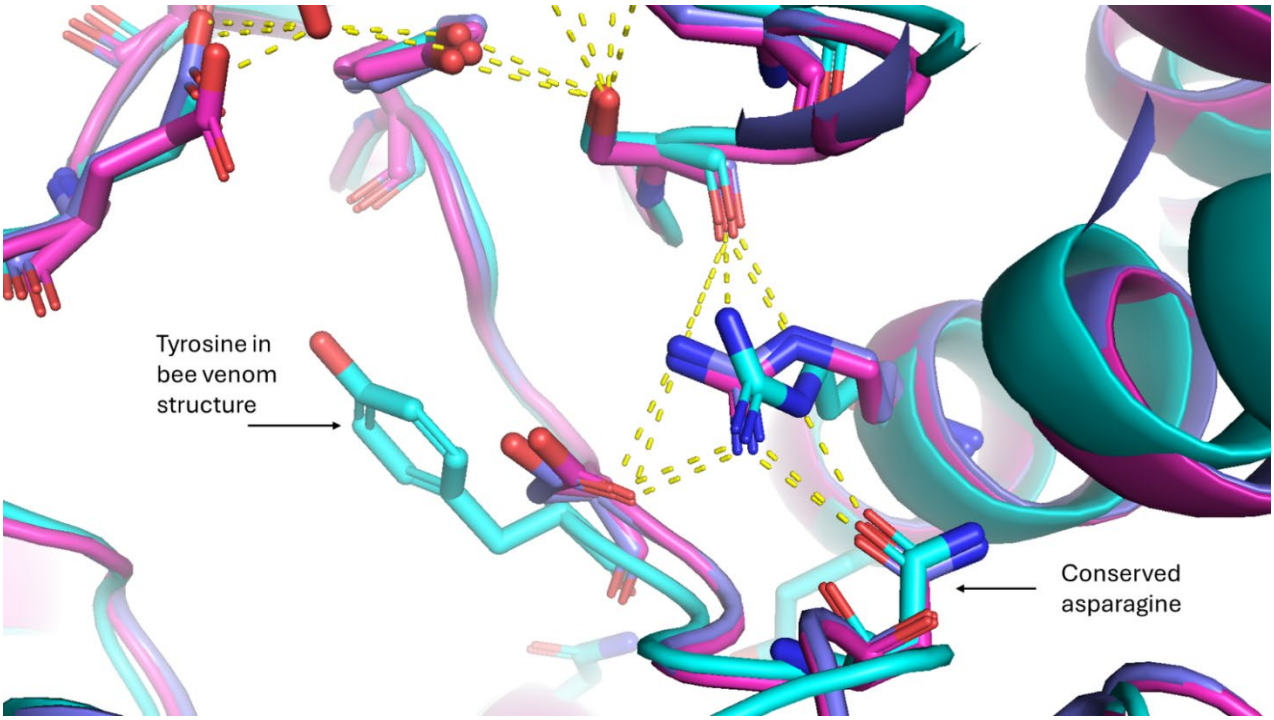
187. Zhang also notes that the "Arg265 guanidino forms a salt bridge with the side chain carboxylate of Asp206 in the crystal structure of human Hyal1." EX1010 (Zhang), 9440. As shown in the figure below, this interaction is involved in orienting Arg265.



188. This interaction also appears in the PH20 model, as shown in the figure below.



189. The bee venom hyaluronidase sequence has a tyrosine at that position instead of an aspartate, so this interaction does not appear in the bee venom hyaluronidase structure. However, in both structures and in the PH20 model, a conserved asparagine also interacts with this arginine, as shown below:



190. Other residues further from the active site also were found to be critical to protein stability, and as a result, function.

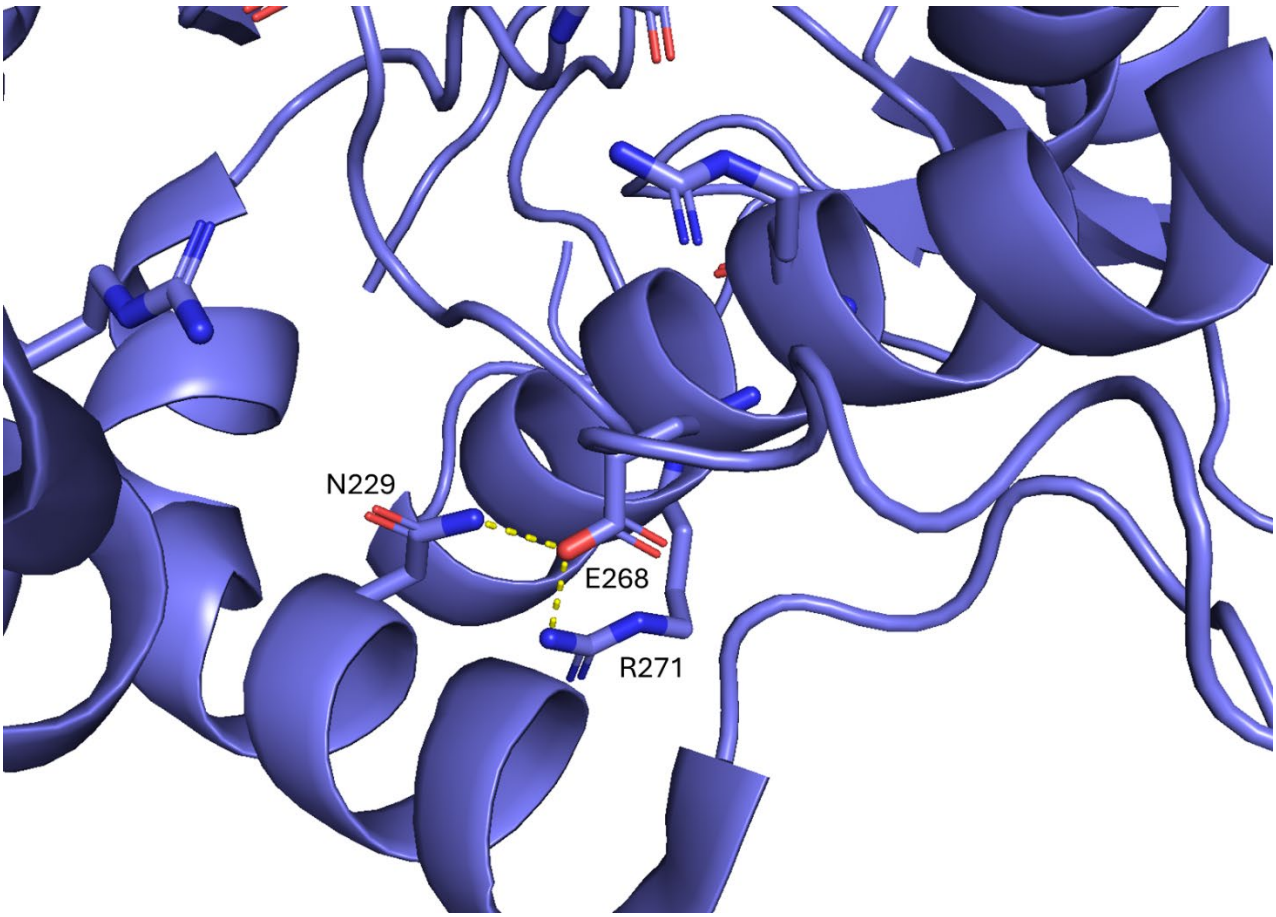
191. Arming mutated glutamate 249 to glutamine and found that the resulting protein lacked activity. Chao addressed the glutamate mutation result (which corresponds to Glu268 in Hyal-1) and postulated that the loss of activity is due to a loss of stability:

> Glu268 is located remotely from the active site cleft (Figure 4), yet this residue is conserved in all human hyaluronidases. Its side chain is partially buried, forming a hydrogen bond with Asn229 (located on [R]5) and a salt bridge with Arg271 (located on [R]6), both invariant residues in the human hyaluronidases. Replacement of this glutamic acid by a glutamine residue in hPH-20 led to loss of activity (*41*). The deleterious effect of a mutation in this position is expected to arise from

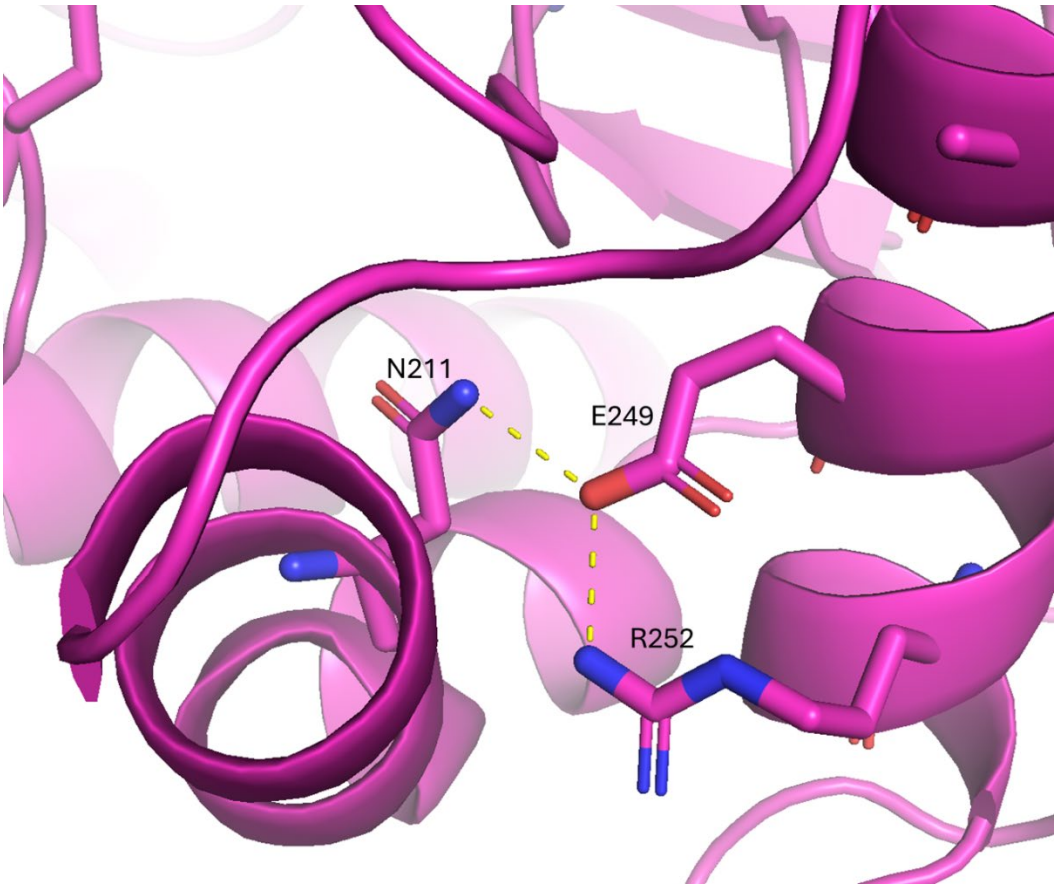
> reduced protein stability due to removal of the electrostatic interaction between [R]5 and [R]6.

EX1006 (Chao), 6914.

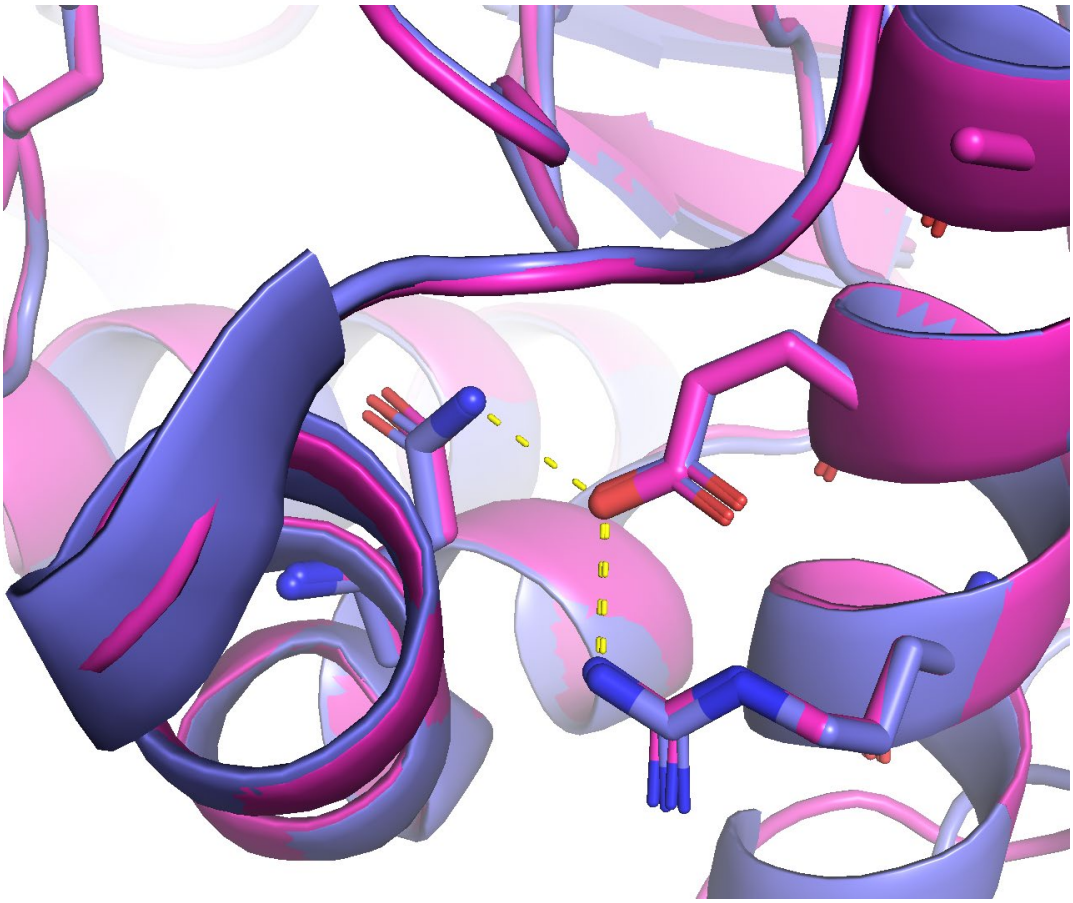
192. In the image below, I show Glu268, Arg271 and Asn229 in the Hyal-1 structure in stick form along with the interactions between the three residues.



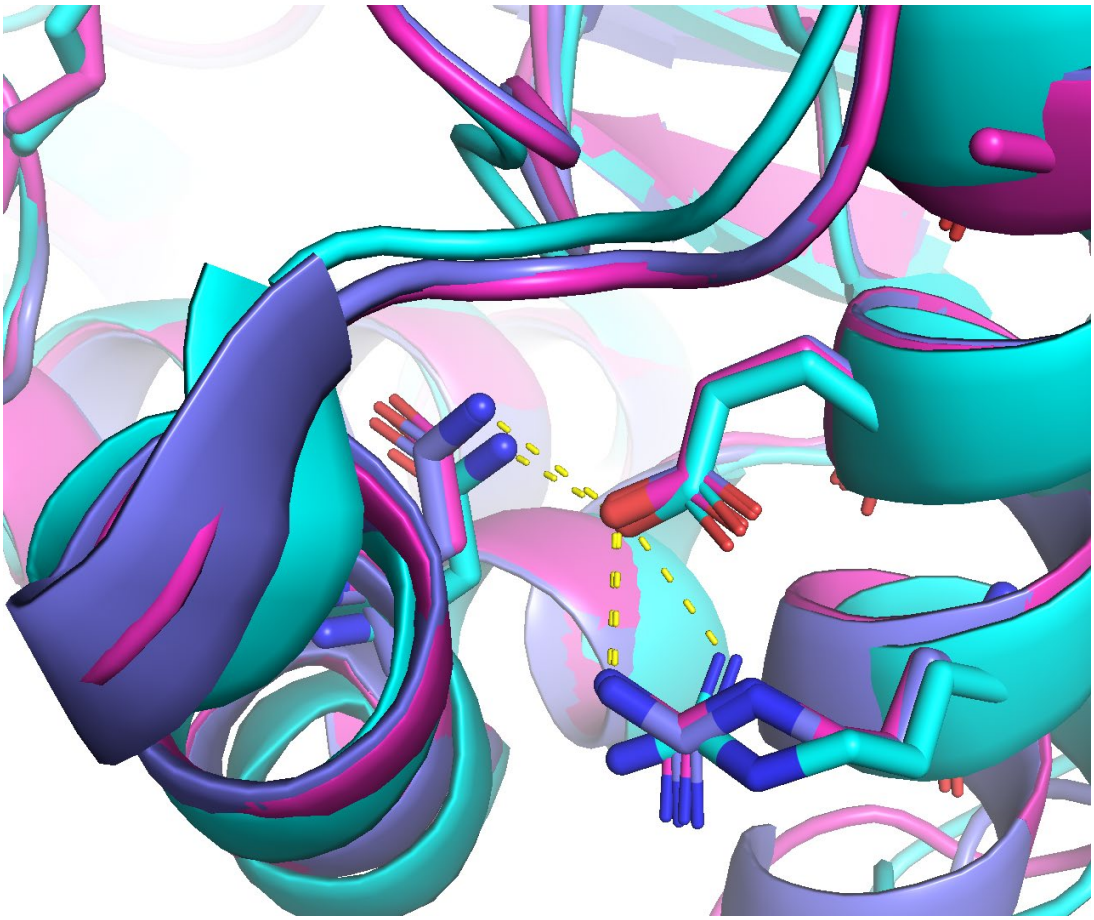
193. In the figure below, I show the interactions in the PH20 model between Glu249, Arg252 and Asn211. Arming teaches that changing Arg252 (Arg271 in Hyal-1) to threonine similarly abrogated activity. EX1011 (Arming), 813.



194. The figure below shows the similar placement of residues and interactions in the PH20 model and the Hyal-1 structure:



195. As shown in figures below, these three resides and their interactions are conserved in the bee venom hyaluronidase structure:



196. Like Chao, Marković-Housley concluded that these residues and interactions are critical for stability:

> Glu249 and Arg252 correspond to Glu247 and Arg250 in Hya (Figure 1), where they form an intrahelical (helix G) salt bridge that is partly buried by the adjacent helix E. Although this salt bridge is too far away from the putative active site for direct involvement in catalysis (Figure 6), its disruption may destabilize the entire structure.

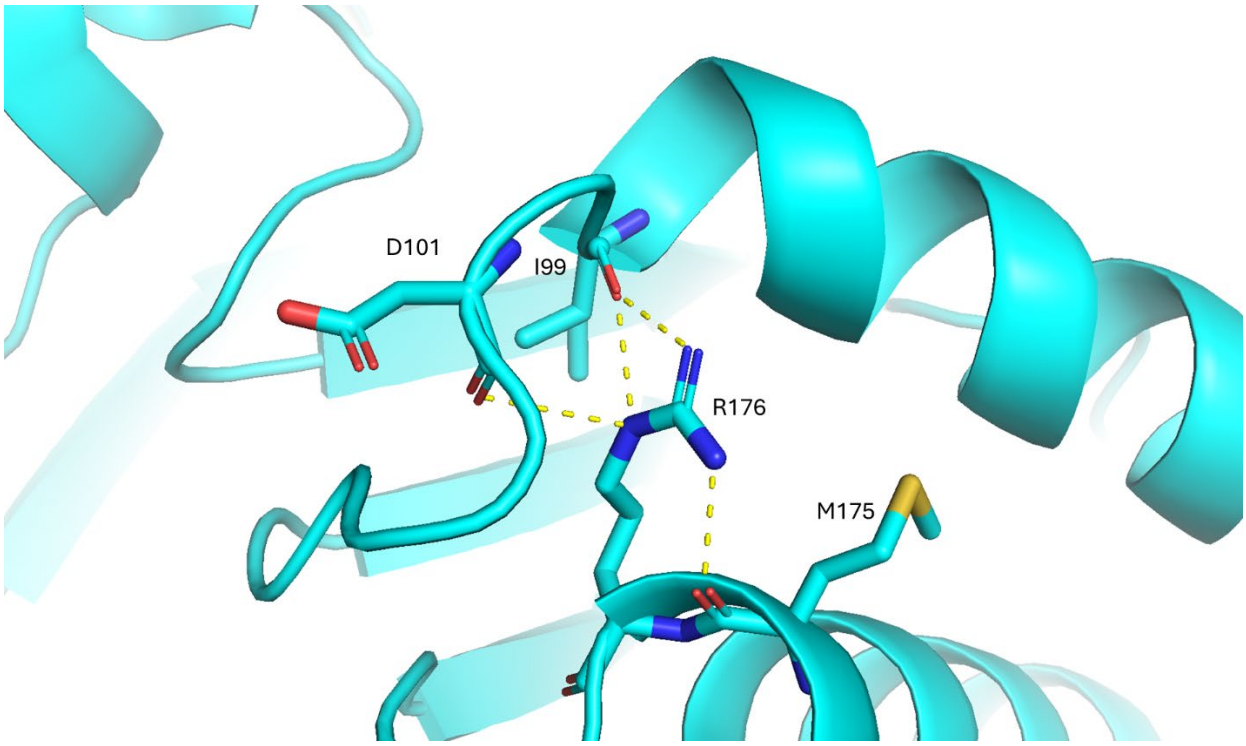
EX1033 (Marković-Housley), 1029.

197. Finally, Arming found that mutating Arg176 to glycine in PH20 reduced activity to a few percent or less of WT. EX1011 (Arming), 813. Marković-Housley provided the following rationale for this result:

> The other mutated residue, Arg176 (the equivalent of Arg176 in PH-20), is also not part of the binding groove, but is buried between helices B and D with its guanidinium moiety bound to mainchain carbonyl groups of both helices.

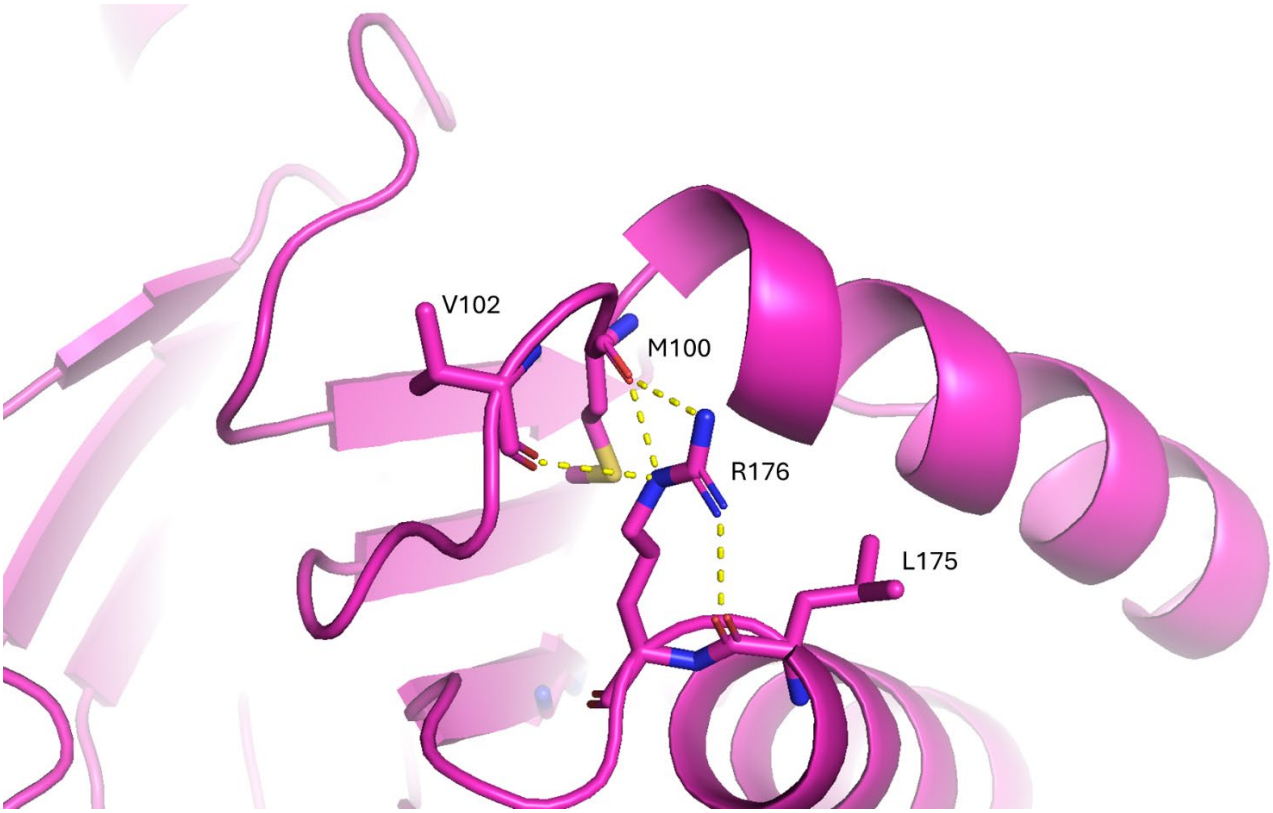
EX1033 (Marković-Housley), 1029.

198. The interactions involving Arg176 that Marković-Housley describes above are shown in the figure below. Arg176 forms interactions with the backbone carbonyls of three residues—Asp101, Ile99 and Met175.



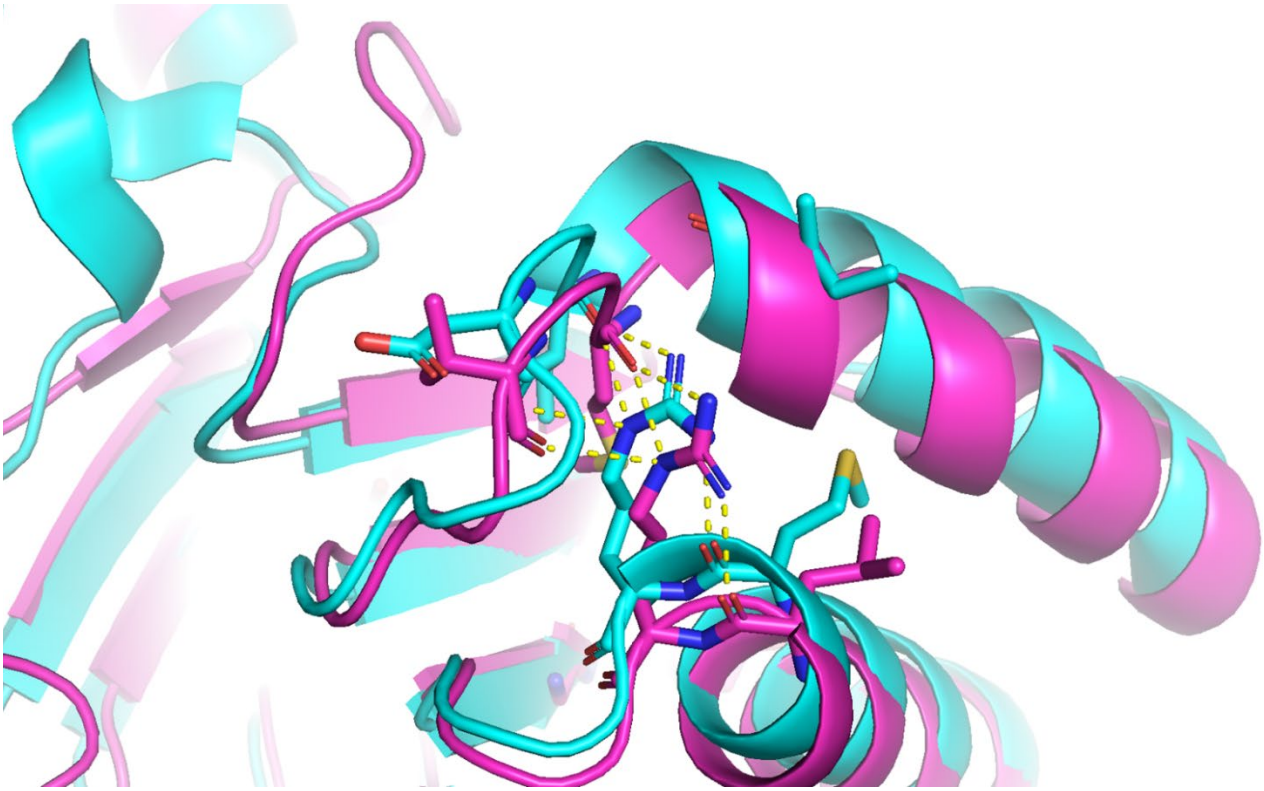
199. Notably, both PH20 and Hyal-1 have an arginine in this position that forms interactions with the backbone carbonyls of three residues in similar positions, although the identity of the residues that interact with the arginine varies across the three proteins.

200. As shown in the figure below, Arg176 in PH20 forms interactions with the backbone carbonyls of M100, V102 and L175.

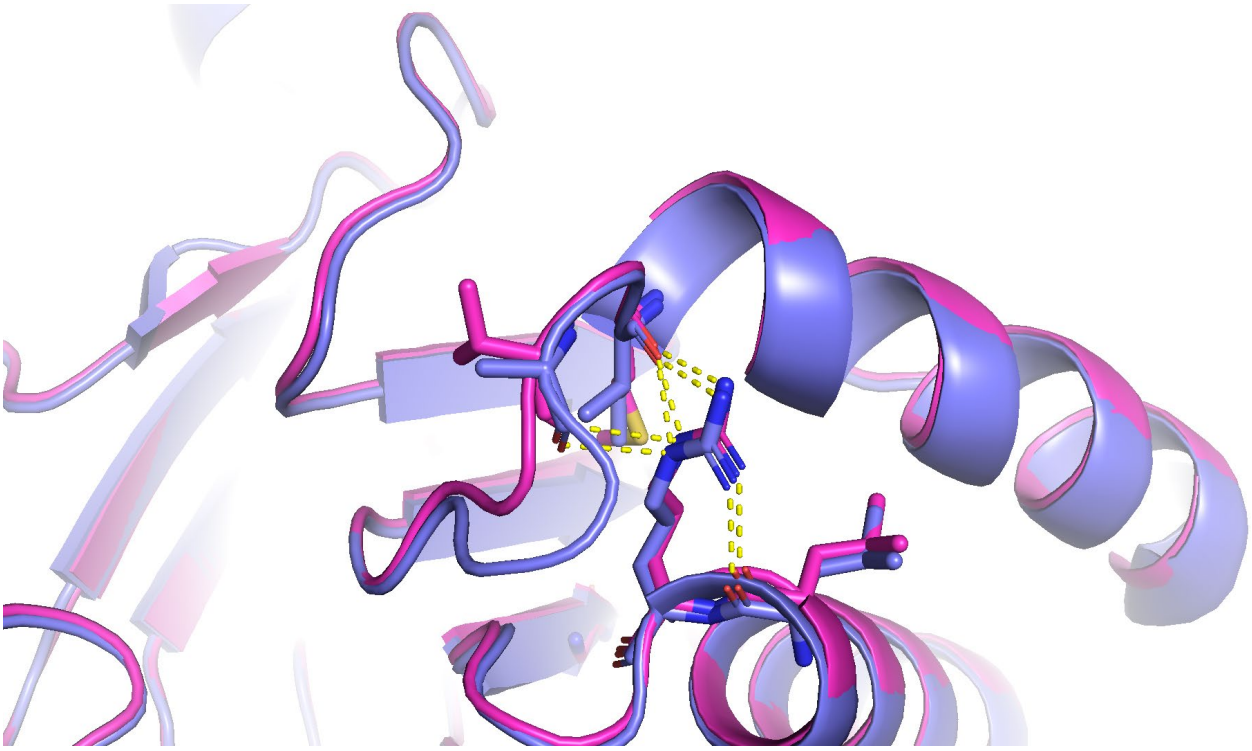


201. Notably, Arg176 caps the C-terminal ends of two parallel alpha helices. The C-terminal end of an alpha helix has a partial negative charge, so a positively charged arginine is a favorable residue for this position.

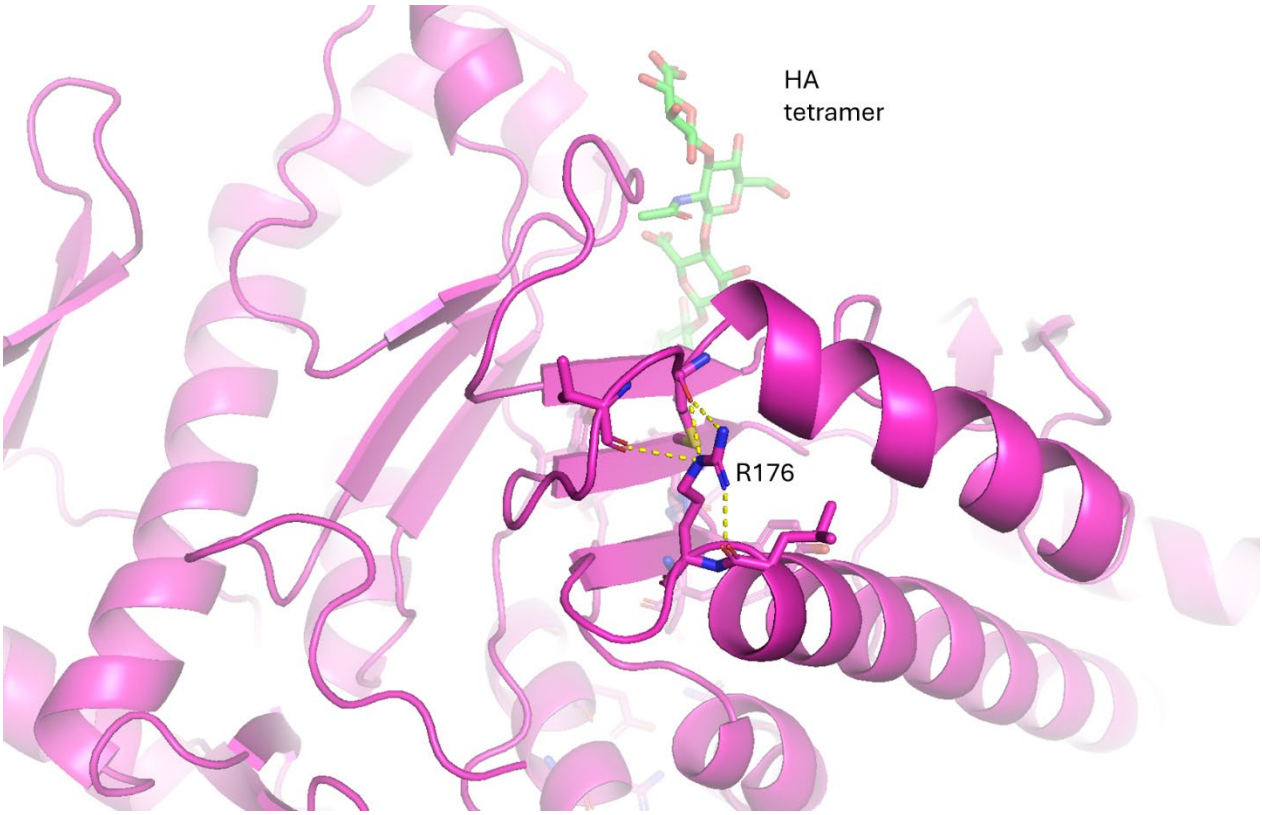
202. The figure below shows PH20 and the bee venom hyaluronidase structure. As noted above, the arginine and its interactions are conserved:



203. The figure below shows PH20 and the Hyal-1 structure. As noted above, the arginine and its interactions are conserved:



204. As shown in the figure below, R176 is part of the supportive structure of the protein. It is not directly part of the active site.



205. In view of its location in the protein and the interactions it forms, Arg176 appears important for protein stability.

206. The analysis above illustrates the basic, fundamental principle that critical residues in homologous proteins are conserved in terms of their identity, positioning in the structure and interactions. That principle is why it is possible to generate reliable homology models using an appropriate template. POSAs expect that homologous proteins with high sequence similarity adopt the same fold because they share critical residues that adopt essentially the same positions and interactions (which in turn is what allows them to adopt the same fold). Because

this principle holds for 40% identical homologous (but different) hyaluronidases, it certainly applies for 95% identical (properly folded) PH20 variants.

D. Hyal-1 and PH20 Both Have An EGF Motif That Is Required For Enzymatic Activity

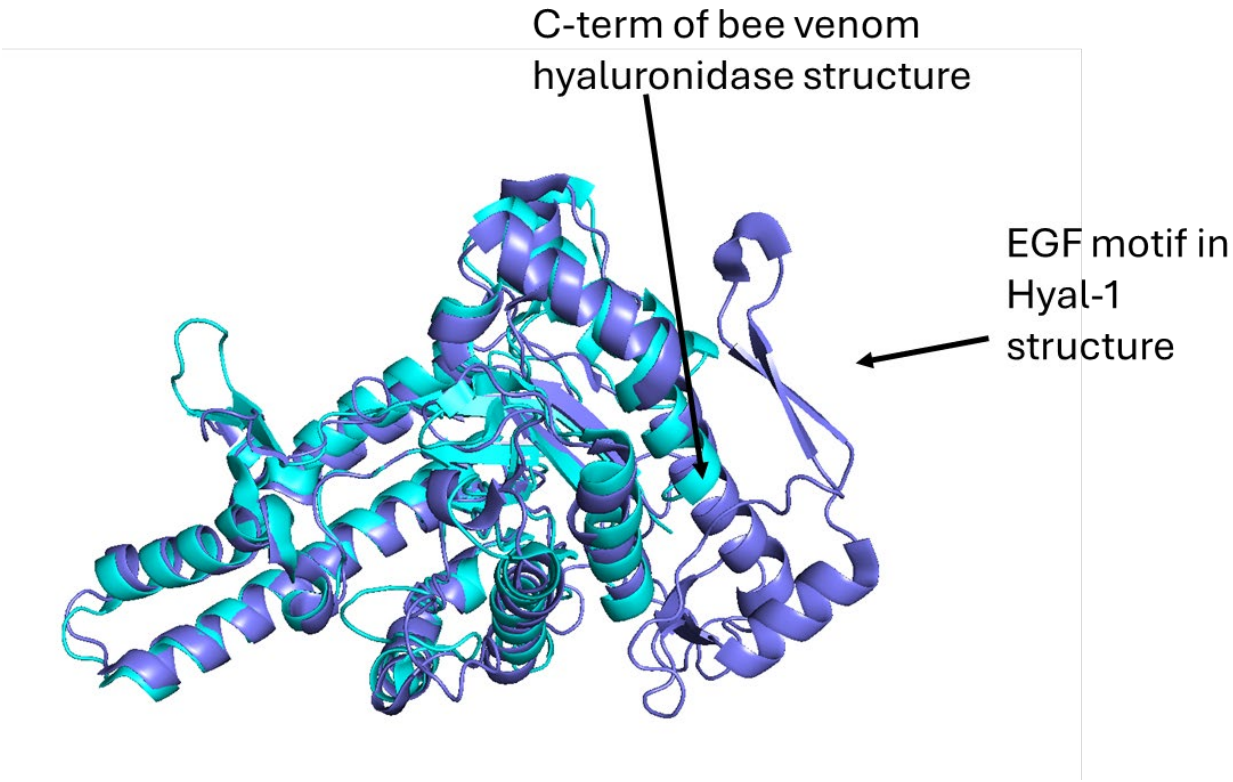
207. Another common structural feature shared by Hyal-1 and PH20 is the EGF motif. As explained below, the EGF region is required for enzymatic activity in both proteins. Zhang and Arming both report mutagenesis data relevant to this analysis that I describe below.

208. Zhang investigated the importance of the EGF domain in Hyal-1. Zhang tested Hyal-1 truncated after position 349, truncated at position 350, which "which deleted 86 amino acids, including the Asn350 glycosylation site" and Hyal-1 truncated after position 355 "which deleted the 80-amino acid EGF-like domain but retained Asn350." EX1010 (Zhang), 9439.

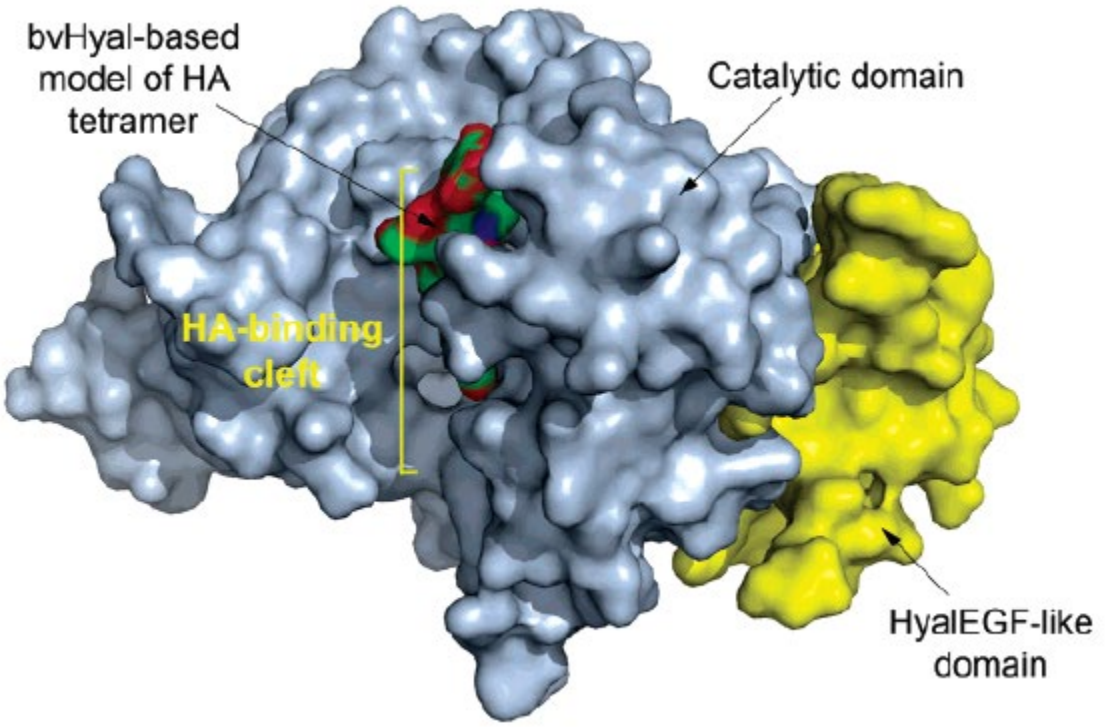
209. Zhang found that "[n]either deletion mutant was secreted to the extracellular space but both were expressed well in the soluble lysate." EX1010 (Zhang), 9439. Zhang confirmed via Western blot that the 356 truncated mutant was glycosylated. *Id*. Zhang found that "[t]he lysates showed ≈2.5 and ≈6.3% of wild-type activity for N350tr and L356tr, respectively, at the 125 μM HA amount." *Id*. Zhang concluded based on these results that "loss of this domain is deleterious to both secretion and activity of Hyal1, although the residual activity suggests the effect is indirect." *Id*. In other words, deleting the EGF domain had a significant

impact on activity, but because some activity was still observed at high HA concentrations, Zhang concluded that the EGF domain is not absolutely required for the catalytic mechanism.

210. Notably, bee venom hyaluronidase does not include an EGF domain at all, as shown in the figure below, consistent with this conclusion:



211. Zhang's conclusion that the EGF domain is not itself involved in catalysis is also consistent with Chao. Chao teaches that, "[i]n hHyal-1, the EGF-like domain is located remotely from the active site (over 30 Å away), and thus is not likely to be involved in substrate recognition (Figure 4B)." EX1006 (Chao), 6916. I have reproduced Chao's Figure 4B below:



212. Arming also tested whether "PH-20 polypeptides with deletions at the C-terminus had any enzymatic activity." EX1011 (Arming), 813. Arming found that deleting 134 amino acids from the end of the protein resulted in a protein "devoid of enzymatic activity" that was not secreted. *Id*. Thus, the Arming results are consistent with Zhang in that the EGF motif appears to be required for enzymatic activity for both Hyal-1 and PH20.

213. In general, EGF domains in proteins can have any of several functions, depending on the biological pathway of the protein, the precise sequence of the domain, and its position in the structure. One such function is to participate in protein-protein interactions. Given the position of PH20 on the surface of the sperm, it is possible that the EGF domain is involved in interactions with proteins

on the surface of the egg. But the substrate HA is also a large molecule and the possibility exists that the EGF domain may make interactions with the polymeric substrate at carbohydrate residues that are remote from the cleavage site.

E. Certain Glycosylation Sites Were Known To Be Required for PH20 Enzymatic Activity

214. U.S. Patent Publication No. 2010/0143457 ("the '457 publication") investigated the import of glycosylation sites in PH20. EX2165. Example 10 discloses site-directed mutagenesis of several N-linked glycosylation sites, including single mutations, double mutations and triple mutations. EX2165, ¶¶ 376-381. The results are described in Table 16, which I have reproduced below. *Id.*, ¶ 378. I note that Table 16 refers to residue positions in the precursor PH20 sequence, and the corresponding positions in the mature sequence are N47, N131, N200, N219, N333 and N358.

TABLE 16

Human PH20 Hyaluronidase Deglycosylation Mutants

Mutant	SEQ ID NO	Glycan Type	Secretion	Activity pH 5.5	Activity pH 7.4
PH20sHis (parent)	187	Both	YES	YES	YES
N82A	202	Complex	YES	YES	YES
N166A	203	Complex	YES	YES	YES
N235A	204	High Mannose (80%) Complex (20%)	YES	NO	NO
N254A	205	Complex	YES	YES	YES
N368A	188	High Mannose	NO	NO	NO
N393A	189	High Mannose	YES (WEAK)	NO	NO
N82A/N166A	206	Complex	YES	YES	YES
N82A/N254A	207	Complex	YES	YES	YES
N166A/N254A	208	Complex	YES	YES	YES
N82A/N166A/ N254A	209	Complex	YES	YES	YES
N368A/N393A	190	High Mannose	NO	NO	NO

215. The results showed that single point mutations of asparagine to alanine at positions 82, 166 and 254 (in the precursor sequence) were tolerated (corresponding to positions 47, 131 and 219 in the mature sequence), while mutations at positions 235, 368 and 393 (in the precursor sequence) inactivated the protein and, in some cases, impacted secretion (corresponding to positions 200, 333 and 358 in the mature sequence). Double mutants at precursor sequence positions 82/166, 82/254 and 166/254 (mature sequence positions 47/131, 47/219 and 166/219) also were tolerated, consistent with the result that single point mutations at those three positions were tolerated. The double mutant at precursor sequence positions 368/393 (mature sequence positions 333/358) inactivated the

protein, which also is consistent with the single point mutation results. Finally, the triple mutant at precursor sequence positions 82/166/254 (mature sequence positions 47/131/219) was active, which again is consistent with the single point mutation results and double point mutation results for those positions.

216. The above results indicate that N-linked glycosylation sites 235, 368 and 393 in PH20 (precursor sequence) (mature sequence positions 200, 333 and 358) may be required for activity, possibly because they affect the folding, solubility, secretion and/or stability of the protein. However, as I explain below, the ’791 patent teaches that certain substitutions are permitted at positions 200 and 358 (but not 333).

217. The specific function of glycosylation sites in proteins varies from protein to protein and even from site to site within the same protein. In general, it cannot be predicted from sequence or structure and must be established experimentally, as was done in the ’457 publication (and in the ’791 patent).

F. PH20 C-Terminal Truncation Results Show Where PH20 Can Be Truncated Without Abrogating Activity

218. U.S. Patent No. 7,767,429 (“the ’429 patent”) issued on August 3, 2010. EX1005.

219. The ’429 patent, the ’457 publication and a paper by Frost published in 2007 (EX1013) all disclose the results of assays testing C-terminal truncation mutants of PH20 to determine which are soluble and active.

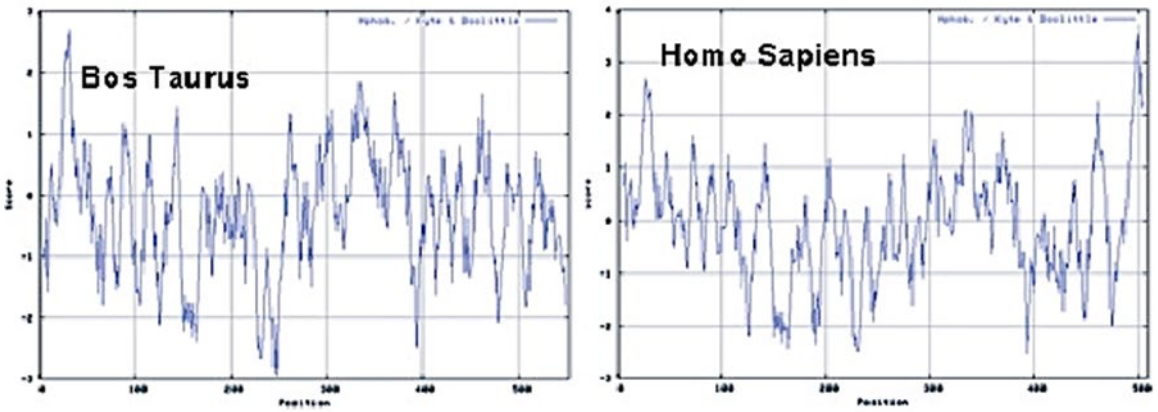
220. As I explain below, based on the results disclosed in the '429 patent, the '457 publication and Frost, as of December 2012, POSAs knew which C-terminal truncations of PH20 would result in an active protein, which would result in an inactive protein and which would result in a protein with reduced or little activity.

221. The '429 patent discloses, for the first time, soluble PH20 active at neutral pH. The '429 patent teaches that "[p]revious attempts to generate secreted and neutral active human sHASEGP were unsuccessful. It was concluded that truncations of the human sHASEGP polypeptide resulted in both a loss of neutral enzymatic activity, and an inability of cells to secrete the recombinant protein in mammalian expression systems" and cites to Arming. EX1005, 4:1-7.

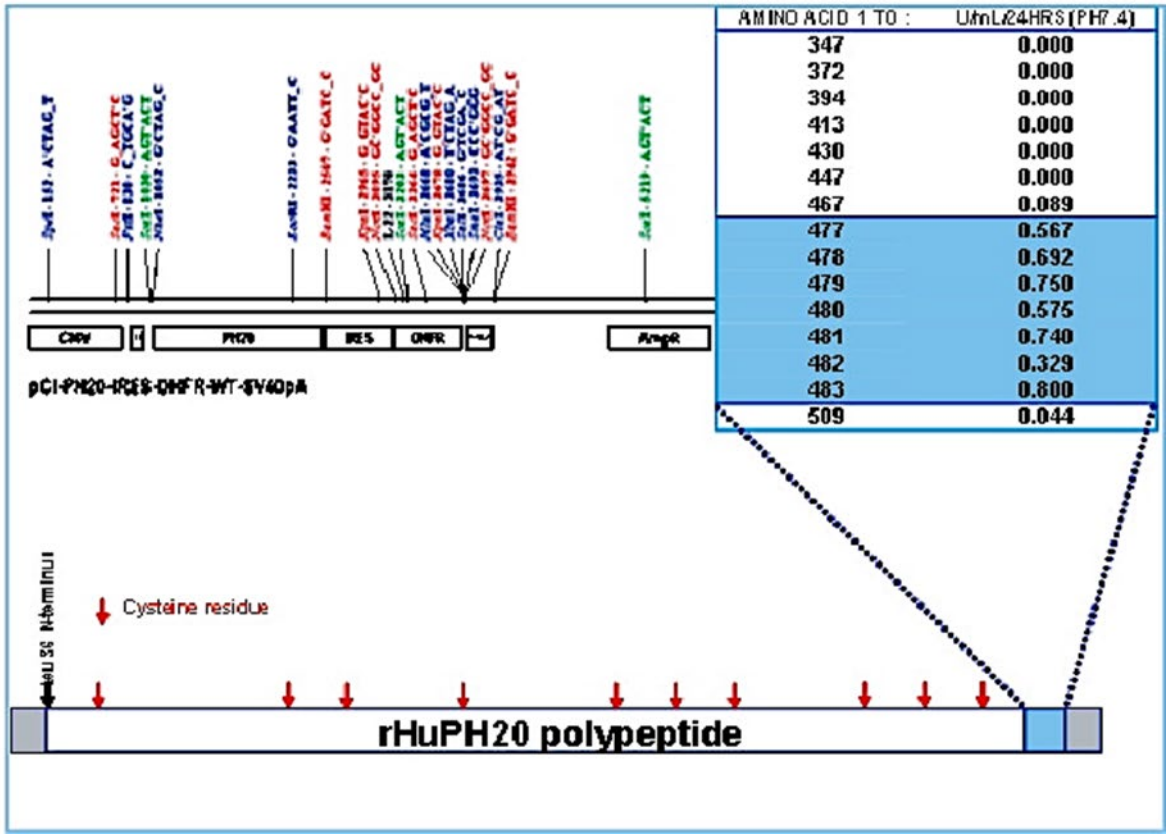
222. Frost teaches "soluble hyaluronidase activity could be recovered in the conditioned medium from deletion mutants terminating after amino acids 477 – 483." EX1013 (Frost), 432.

223. Frost provides the following figure summarizing the results of the truncation experiment:

B.



C.



224. Frost teaches that terminating PH20 at positions between residues 477-483 are soluble and active.

225. In the mature sequence without the signal peptide, those positions correspond to 442-448. So Frost teaches that PH20 polypeptides (mature sequence) ending at positions between residues 442-448 are soluble and active.

226. The '457 publication expands on the results disclosed in Frost and discloses the results obtained with additional C-terminal truncation mutants.

227. Example 5 provides the results of an experiment in which "human PH20 hyaluronidase carboxy-terminus deletion mutants were tested for their PH20 hyaluronidase activity … at both pH 7.4 and pH 5.5." EX2165 ('457 publication), ¶ 360. The disclosure teaches that "[h]uman PH20 hyaluronidase carboxy-terminus deletion mutants shorter than I430 … are inactive." *Id.*, ¶ 362. "Human PH20 hyaluronidase carboxy-terminus deletion mutants ending at I498 [ ], L499 [ ], F500 [ ], L501 [ ] and I502 [ ] have little detectable activity due to low expression level." *Id.* Finally, "[a]ll other human PH20 hyaluronidase carboxy-terminus deletion mutants … are active hyaluronidases at both pH 7.4 and pH 5.5." *Id.* The data are provided in Tables 6 and 6A, which I've combined into the chart below:

Deletion Mutant	Precursor (AA)	Mature (AA)	pH 7.4 Activity (Units/ml)	pH 5.5 Activity (Units/ml)
VASL	509	474	1.8045	0.891
VASL + PLC	509	474	3.96	2.313
SPAM1-SSVA	507	472	1.4715	1.125
SPAM1-SSVA	507	472	1.782	1.256

Deletion Mutant	Precursor (AA)	Mature (AA)	pH 7.4 Activity (Units/ml)	pH 5.5 Activity (Units/ml)
SPAM1-IISS	505	470	1.458	0.837
SPAM1-IISS	505	470	1.863	0.932
SPAM1-FLII	503	468	0.9405	0.6345
SPAM1-FLII	503	468	1.094	0.648
SPAM1-LFLI	502	467	0.608	0.324
SPAM1-ILFL	501	466	0.0405	0.0405
SPAM1-ILFL	501	466	0.446	0.122
SPAM1-SILF	500	465	0.365	0.162
SPAM1-VSIL	499	464	0.02025	0.045
SPAM1-VSIL	499	464	0.486	0.122
SPAM1-IVSI	498	463	0.527	0.203
SPAM1-FIVS	497	462	0.1755	0.216
SPAM1-FIVS	497	462	0.365	0.162
SPAM1-TMFI	495	460	0.45	0.612
SPAM1-TMFI	495	460	0.689	0.77
SPAM1-SATM	493	458	0.5715	0.7335
SPAM1-SATM	493	458	0.689	0.851
SPAM1-TLSA	491	456	0.3645	0.5625
SPAM1-TLSA	491	456	0.851	0.729
SPAM1-STLS	490	455	0.819	1.2375
SPAM1-PSTL	489	454	1.557	1.089
SPAM1-PSTL	489	454	1.985	3.321
SPAM1-ASPS	487	452	1.017	0.9225
SPAM1-ASPS	487	452	1.134	1.58
SPAM1-YNAS	485	450	1.8765	1.74825
SPAM1-FYNA	484	449	1.4985	1.26225
SPAM1-IFYN	483	448	2.45025	2.3085
SPAM1-QIFY	482	447	2.03175	1.647
SPAM1-PQIF	481	446	1.818	1.701
SPAM1-EPQI	480	445	2.1825	1.6425
SPAM1-EEPQ	479	444	1.917	2.0745
SPAM1-TEEP	478	443	1.764	1.584
SPAM1-ETEE	477	442	2.088	2.0475

Deletion Mutant	Precursor (AA)	Mature (AA)	pH 7.4 Activity (Units/ml)	pH 5.5 Activity (Units/ml)
SPAM1-METE	476	441	1.332	1.278
SPAM1-PMET	475	440	2.223	2.0925
SPAM1-PPME	474	439	1.2105	1.341
SPAM1-KPPM	473	438	0.8595	0.91575
SPAM1-LKPP	472	437	0.5445	0.9
SPAM1-FLKP	471	436	3.321	2.79
SPAM1-AFLK	470	435	3.204	2.925
SPAM1-DAFL	469	434	2.3895	2.2365
SPAM1-IDAF	468	433	0.5625	0.62775
SPAM1-CIDA	467	432	0.5535	0.4725
SPAM1-VCID	466	431	0	0.2115
SPAM1-GVCI	465	430	0.441	0.468
SPAM1-DGVC	464	429	0	0.045
SPAM1-IADG	462	427	0	0.00225
SPAM1-VCIA	460	425	0	0.0135
SPAM1-VDVC	458	423	0.0495	0.0585
SPAM1-DAVD	456	421	0	0.0675
SPAM1-DTDA	454	419	0	0.054
SPAM1-VKDT	452	417	0.054	0.0225
SPAM1-ADVK	450	415	0.063	0.0405
HZ24-PH20	482	447	0.499	0.726188
CHO-S	n/a	n/a	0	0.012375

228. I have been asked to evaluate whether in view of the above data, a POSA as of December 28, 2012 would have expected PH20 polypeptides ending at positions 465 to 500 in the precursor sequence (positions 430 to 465 in the mature sequence) to be active.

229. The ’457 publication confirms that PH20s ending at positions 477 to 483 in the precursor sequence are active. Activity appears to decrease at 468 to

467 and then drop off at 466. Based on these data, a POSA would have expected a polypeptide terminating at position 466 or 465 (mature sequence positions 431 or 430) to be inactive. Polypeptides terminating at 468 or 467 (mature sequence positions 433 and 432) may have reduced activity as compared to, for example, polypeptides terminating at positions 469 to 489 (mature positions 434 to 454). A POSA would have expected polypeptides terminating from 469 to 489 (mature positions 434 to 454) to be active. Activity then appears to begin to decline at position 490 (mature position 455). The disclosure teaches that polypeptides terminating at positions 498-500 in the precursor sequence have little activity due to low expression levels. Accordingly, a POSA would have expected that polypeptides ending at positions 490-497 (mature sequence 455-462) to have reduced activity, with polypeptides ending at positions 498-500 (mature sequence 463-465) having little to no activity.

230. I have summarized this in the chart below:

Precursor Sequence C-terminal position	**Mature Sequence C-terminal position**	**Expected Activity**
465	430	Inactive
466	431	Inactive
467	432	Reduced activity
468	433	Reduced activity
469	434	Active
470	435	Active
471	436	Active
472	437	Active
473	438	Active

Precursor Sequence C-terminal position	Mature Sequence C-terminal position	Expected Activity
474	439	Active
475	440	Active
476	441	Active
477	442	Active
478	443	Active
479	444	Active
480	445	Active
481	446	Active
482	447	Active
483	448	Active
484	449	Active
485	450	Active
486	451	Active
487	452	Active
488	453	Active
489	454	Active
490	455	Reduced activity
491	456	Reduced activity
492	457	Reduced activity
493	458	Reduced activity
494	459	Reduced activity
495	460	Reduced activity
496	461	Reduced activity
497	462	Reduced activity
498	463	Inactive/little activity
499	464	Inactive/little activity
500	465	Inactive/little activity

IX. U.S. PATENT NO. 12,077,791

231. The ’791 patent issued on September 3, 2024 to Ge Wei, H. Michael Shepard, Qiping Zhao and Robert James Connor. The ’791 patent is entitled “PH20 Polypeptide Variants with a Modification at Position 309 of the PH20 Polypeptide and a Method of Making Thereof.” EX1001.

232. The ’791 patent is related to a patent application filed on December 28, 2012 and also is related to provisional applications filed on December 30, 2011, and November 1, 2012.

A. Modified PH20 Polypeptides

233. The ’791 patent states that it provides “[d]etailed structure/function of virtually each amino acid in a PH20 polypeptide” along with “the identification of residues and loci that contribute to alteration of a property, such as stability in particular conditions.” EX1001, 3:19-23. Specifically, the ’791 patent provides the results of a mutagenesis experiment in which every single residue in SEQ ID No. 3 was mutated to up to 18 different amino acids and then tested for hyaluronidase activity in duplicate. The results show the extent to which each position tolerates mutation, as well as the types of substitutions tolerated at each position. Some residues are extremely intolerant to change, while others are quite permissive and appear to tolerate any residue irrespective of size or chemical properties.

234. This level of detailed structure/function mapping was virtually unheard of in 2012, and still is, especially in the public domain. I am not aware of a comprehensive mutagenesis study like this for any other human protein of comparable size. These data are extraordinarily useful for the POSA seeking to practice the claims.

235. Based on this structure/function mapping, the '791 patent teaches "modified PH20 polypeptides" that "exhibit altered activities or properties compared to a wildtype, native or reference PH20 polypeptide." EX1001, 73:62-64. The patent teaches that the modified PH20 polypeptides disclosed include those that are active variants, inactive variants, and variants with increased stability. *Id.*, 73:64-74:8.

236. The '791 patent explains that modifications "can be a single amino acid modification, such as single amino acid replacements (substitutions), insertions or deletions, or multiple amino acid modifications, such as multiple amino acid replacements, insertions or deletions. Exemplary modifications are amino acid replacements, including single or multiple amino acid replacements." EX1001, 74:9-15. Substitutions can be "conservative" or "non-conservative." *Id.*, 74:15-17.

237. In addition to the mutagenesis study I referenced above, in which the variants were incubated for 90 minutes at 37 degrees C, the '791 patent also provides the results of hyaluronidase activity testing at 4 degrees C compared to activity following 4 hours incubation at 37 degrees C and in the presence of preservative m-cresol for some of the variants.[15] EX1001, Tables 11 and 12.

[15] The patent teaches that PH20 "rapidly loses activity in the presence of preservatives, likely due to unfolding of the protein and subsequent aggregate formation." EX1001, 107:57-60. "For example, following incubation with 0.4%

238. Before analyzing the patent data I first address the patent claims.

B. '791 Patent Claims

239. The '791 patent as issued included 10 claims.

240. I understand that claims 4-5 have been disclaimed, leaving a total of 8 claims.

241. The '791 patent includes one independent claim (claim 1), and the rest are dependent claims.

242. I understand that dependent claims depend from independent claims and are narrower than independent claims.

243. I have reproduced claim 1 below:

> A modified PH20 polypeptide comprising an amino acid sequence, wherein: (a) at least 95% of the residues of the amino acid sequence of the modified PH20 polypeptide are identical to the residues in the amino acid sequence of SEQ ID NO: 35 when the sequence of the modified PH20 polypeptide is aligned at positions corresponding to the sequence set forth in the amino acid sequence of SEQ ID NO: 35 to maximize identical residues, and terminal gaps are treated as non-identical; (b) the amino acid sequence of the modified PH20 polypeptide comprises an amino acid modification at a position corresponding to position 309 with reference to amino acid positions set forth in the amino acid sequence of SEQ ID NO: 3; and (c) the modification at position 309 is a replacement selected from among E, G, H, L, M,

m-cresol for 4 hours, PH20 (e.g., rHuPH20) retains only about 10% of its activity (see e.g., Example 5)." *Id*., 107:64-66.

> N, Q, R, S, and T, and wherein the modified PH20 polypeptide exhibits increased hyaluronidase activity compared to the hyaluronidase activity of the polypeptide of SEQ ID NO: 3, measured under identical conditions.

244. Claim 1 is directed to a modified PH20 polypeptide.

245. In claim 1, the modified PH20 polypeptide must have (1) at least 95% sequence identity to SEQ ID No. 35,(2) a modification at position 309 to E, G, H, L, M, N, Q, R, S, and T, and (3) increased hyaluronidase activity.

246.

C. Summary of Known Important Residues

247. The ’791 patent summarizes the knowledge in the art, much of which I described above, regarding the residues in PH20 known to be important for activity as of the priority date.

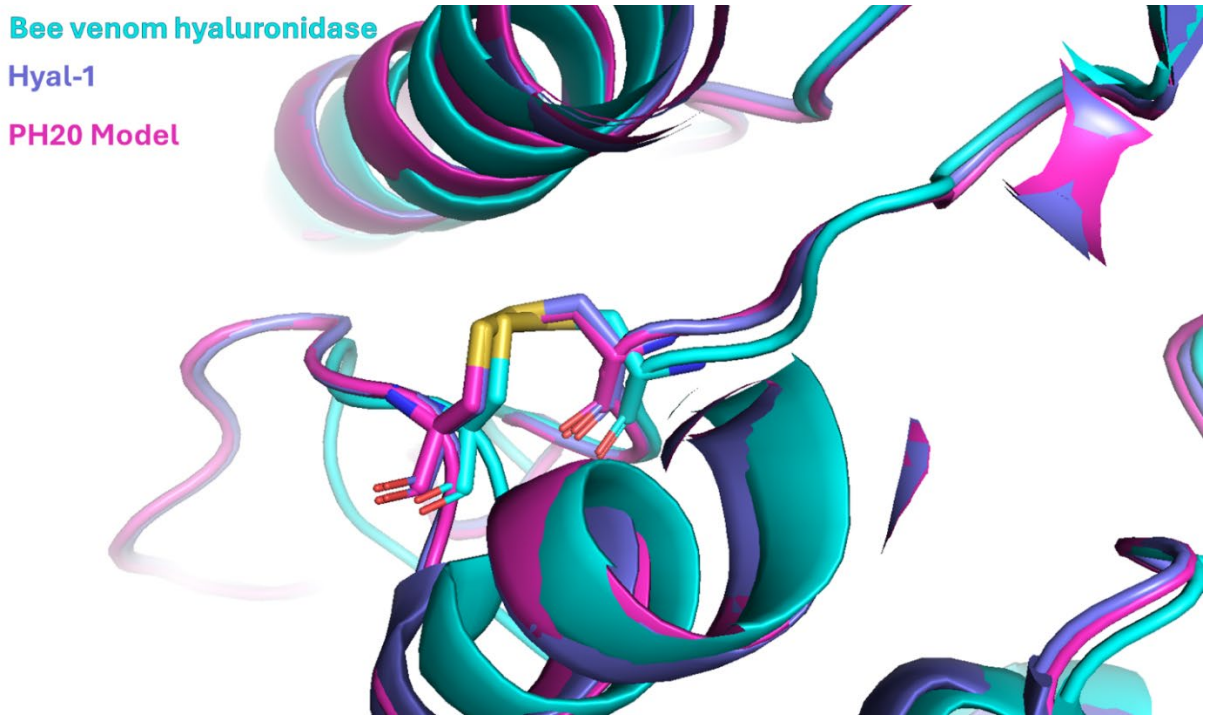
248. The ’791 patent also describes conserved cysteine residues, which I did not address above. As I explain in detail below, the ’791 patent then proceeds far beyond what was known, to disclose detailed information regarding which residues tolerate substitutions—and to what extent—and which do not.

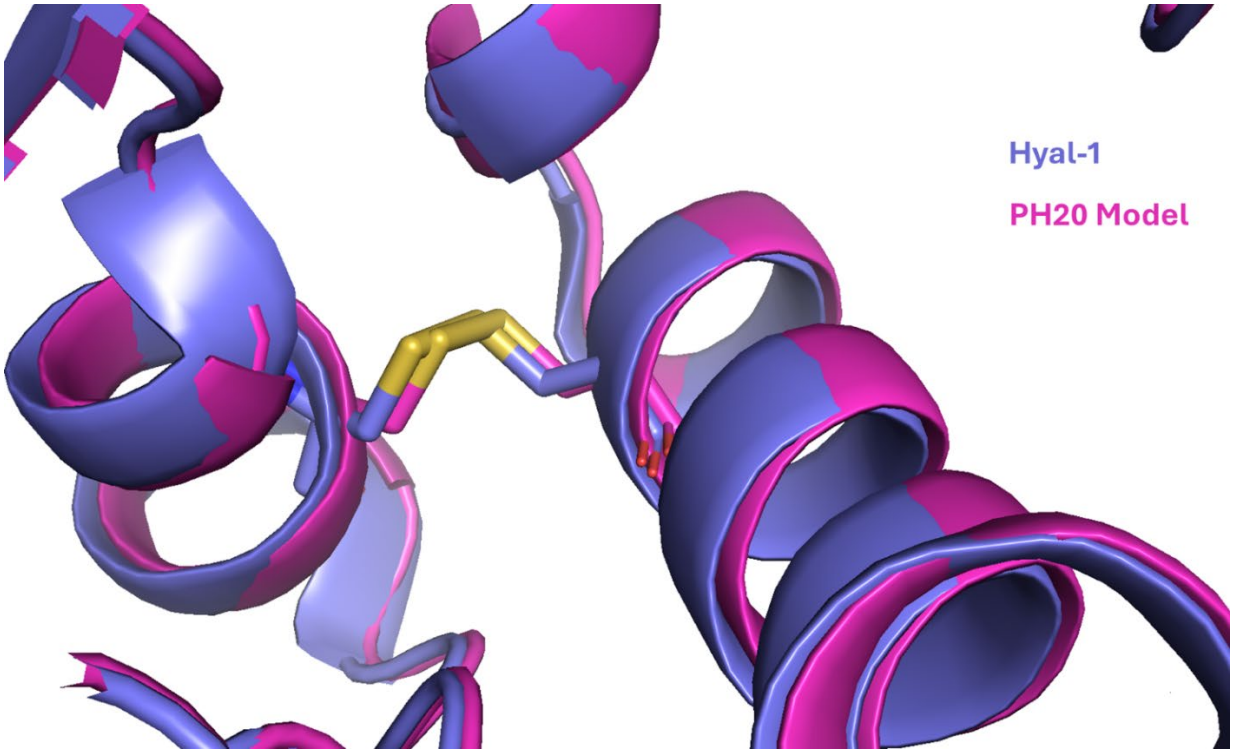
249. The ’791 patent recognizes that “sequence and structure of PH20 polypeptides is highly conserved,” and specifically “[s]equence identity between and among PH20 proteins from various species is about 50% to 90%.” EX1001, 68:7-9. There is a “common core hyaluronidase domain region of about 340

amino acids in length that corresponds to amino acid residues 38-374 of the precursor human PH20 sequence set forth in SEQ ID NO:6." *Id.*, 68:12-16. The patent cites several articles[16] for the proposition that "[w]ithin the common hyaluronidase domain region, at least 57 amino acids are conserved between and among species." *Id.*, 68:26-27.

250. The conserved residues include 12 conserved cysteines that form disulfide bonds. In the mature sequence, those 12 conserved cysteines are at positions 25, 189, 203, 316, 341, 346, 352, 400, 402, 408, 423 and 429. *Id.*, 68:31-38. Disulfide bonds are formed between cysteines at positions C25 and C316, positions C189 and C203, positions C341 and C352, positions C346 and C400, positions, C402 and C408 and positions C423 and C429 of the mature polypeptide. *Id.*, 68:38-50. Images showing some of the conserved cysteines (in stick format) in all three structures and in the Hyal-1 and PH20 structure are shown below:

[16] Arming et al. (1997) Eur. J. Biochem., 247:810-814; ten Have et al. (1998) Reprod. Fertil. Dev., 10:165-72; Chowpongpang et al. (2004) Biotechnology Letters, 26:1247-1252).





251. The ’791 patent teaches that “[a]mino acid residues corresponding to amino acid residue D111, E113 and E249 of the sequence of amino acids set forth

in SEQ ID NO: 3 or 7 are acidic residues [that are] part of the enzyme active site and are conserved between and among PH20 species," and "[a]mino acid residues R176, R246, R252 of the sequence of amino acids set forth in SEQ ID NO: 3 or 7 are also conserved between and among species and contribute to substrate binding and/or hyaluronidase activity." *Id.*, 68:51-58. I discussed the teaching in the art regarding those six residues above. The patent also cites the Arming results that "[a]mino acid mutations D111N, E113Q, R176G, E249N and R252T result in enzymes that have no detectable enzymatic activity or residual enzymatic activity." *Id.*, 68:58-61.

252. The patent also recognizes that "[g]lycosylation also is required for PH20 hyaluronidase activity," and, in particular, "at least N-linked glycosylation sites corresponding to amino acid residues N200, N333 and N358 are required for secretion and/or activity of the enzyme." *Id.*, 69:7-8, 14-17. "For example,… amino acid mutations N200A, N333A, N358A or N333A/N393A" produce "inactive proteins." *Id.*, 69:18-20. By contrast, "[s]ingle mutations of glycosylation sites N47A, N131A, N219A, N47A/N131A, N47A/N219A, N131A/N291A retain activity." *Id.*, 69:20-22. That is consistent with the teaching of the '457 publication described above. I note that the '791 patent incorporates the '457 publication by reference, which I understand means that the text of the '457 publication disclosure is considered to be part of the text of the '791

patent disclosure. However, the patent also goes above and beyond the teachings of the '457 publication in that it teaches that some limited changes can be made to positions 200 and 358 while maintaining some (albeit reduced) activity (while 333 is invariant).

253. As explained below, the '791 patent confirms the importance of the critical PH20 residues and further expands the list of critical residues that, when individually mutated, inactivate the protein. The patent also discloses positions that can be mutated to improve activity or stability and that can be changed without impacting activity.

D. Hyaluronidase Activity Testing In Examples 2-4

254. As noted above, the '791 patent discloses a mutagenesis experiment involving systematically changing each of the 447 positions in SEQ ID NO:3 "to one of about 15 amino acid residues." EX1001, 200:12-16. Each modified polypeptide "contained a single amino change." *Id*., 200:16-17. That mutagenesis experiment and the results are described in Examples 2-4.

255. Example 2 discloses the generation of the PH20 variant library used in the experiment. Specifically, a library of variants was produced containing "6753 variant members," and "[e]ach member was expressed and screened for hyaluronidase activity as described below." *Id*., 200:17-18, 32-34. The 6753 variants are listed in Table 8.

256. The test method for the hyaluronidase assay is described in Example 3.

257. The results of the hyaluronidase activity testing are provided in Example 4 and in Tables 9 and 10.

258. Table 9 discloses "[a]ctive mutants" for which "at least one duplicate sample exhibited greater than 40% of wildtype activity when normalized to SEAP activity." *Id*., 232:50-52. Based on this disclosure, I understand that for each of the variants listed in Table 9, at least one of the two samples tested exhibited greater than 40% of wildtype activity.[17] Table 9 provides "the average hyaluronidase activity of tested duplicates normalized by SEAP values compared to average of wildtype PH20 activities in each plate, which were also normalized by their own SEAP values." *Id*., 232:57-61. "For example, a value of 0.40 indicates that the variant exhibits 40% of the hyaluronidase activity of wildtype PH20, a value of 1 indicates that the variant exhibits a similar hyaluronidase activity of wildtype and a value of 3.00 indicates that the variant exhibits 300% of the hyaluronidase activity of wildtype PH20 or 3-fold increased activity compared to wildtype." *Id*., 232:61-67.

[17] I note that there are variants in Table 9 with activity values less than 40% of wild type. I understand that for those variants, one of two samples exhibited an activity greater than 40% of wild type, and the value presented is the average obtained for the two samples.

259. "The results in Table 9 show that over 600 tested mutants exhibit activity that is increased compared to wildtype. For example, about 536 mutants exhibit 120% or greater than 120% of the hyaluronidase activity of wildtype PH20 and about 75 of the mutants exhibit 300% or greater than 300% of the hyaluronidase activity of wildtype PH20." *Id*., 233:1-6.

260. Mutants that "exhibited less than [20%] hyaluronidase activity of wildtype PH-20, in at least one of the duplicates, were rescreened to confirm that the dead mutants are inactive." *Id*., 255:43-46. This experiment also is described in Example 4. The patent teaches that this "assay is intended to detect PH20 activities below 0.2 U/mL." *Id*., 255:50-51. Table 10 provides the "reconfirmed inactive mutants," which again are those that achieved less than 20% of WT activity. *Id*., 256:54-55.

261. Additionally, the patent lists the residues at each position that resulted in active PH20s (greater than 40% activity) in Table 3 and that resulted in less than 20% activity in Table 5.

262. These data are extremely useful because, as I illustrate below, they show the tolerability of each position to substitution. They also show which amino acids (and which types of amino acids) are tolerated at each position. With the '791 patent data in hand, the POSA seeking to generate PH20 variants now knows which positions, if changed, are likely to abrogate activity and which are

not. As I explain below, the POSA could use this information to generate single and multiple mutants with desired properties.

X. PATENT DATA ANALYSIS

A. Spreadsheet Analysis

263. To facilitate my analysis, I imported the data from Tables 3, 5, 9 and 10[18] into Excel so that I could color code and sort the data. The spreadsheet is EX2166.

1. Tabulating Positions

264. I tabulated the number of positions (and percentage of positions) for which all tested[19] substitutions retain activity. There were a minimum of 2 substitutions tested per position and a maximum of 18 substitutions tested per position. On average, 13 substitutions were tested per position, and the median of substitutions tested per position was 14. I calculated that 40 positions out of 447 (9%) tolerated all tested substitutions.

[18] To the extent there were discrepancies between substitutions listed in Tables 3, 5, 9 and 10, I used the characterization in Tables 9 and 10 in the spreadsheet. For example, there were some substitutions identified as "active" in Table 9 but not listed in Table 3. Because they were included in Table 9, I characterized those substitutions as "active" in the spreadsheet.

[19] I use the term "tested" here to refer to substitutions that are identified as active or inactive in Tables 9 and 10. I understand that there are additional substitutions in Table 8 that are not identified in Table 9 or 10. The POSA would not draw any conclusions about those residues. That is because it is not possible to tell why they were not included in either Table. Accordingly, I do not consider them in my analysis.

265. I also tabulated the number of positions (and percentage of positions) for which none of the tested substitutions retain activity. For 95 positions of the 447 (21%), every single mutation inactivated the protein.

266. 312 positions (70%) tolerated some, but not all, substitutions. For those 312 positions, I calculated the percentage of tested substitutions that were tolerated.

267. 158 positions (35%) tolerated 60% or more of the tested substitutions. Accordingly, about 35% of the positions in PH20 are generally tolerant of substitutions.

268. 214 positions (48%) tolerate 30% or fewer of the tested substitutions. Accordingly, about 48% of the positions in PH20 are generally intolerant of substitution.

269. I color coded the spreadsheet based on the tolerability of each position.

270. Those positions for which all substitutions are tolerated are colored green, and those for which no substitutions were tolerated are colored red.

271. Positions for which 60% or more of the substitutions tested were tolerated are colored light green.

272. Positions for which 30% or less of the substitutions were tolerated are colored orange.

273. Residues not colored were those that tolerated more than 30% but less than 60% of the tested substitutions.

2. Color-Coded Positions

274. In the figures below, I show the coloring for all 447 mutated residues in PH20.

Position	WT	30	D	61	L	92	A	123	W	154	K	185	L
1	L	31	E	62	G	93	K	124	K	155	Q	186	F
2	N	32	P	63	Y	94	K	125	P	156	E	187	P
3	F	33	L	64	Y	95	D	126	K	157	F	188	D
4	R	34	D	65	P	96	I	127	D	158	E	189	C
5	A	35	M	66	Y	97	T	128	V	159	K	190	Y
6	P	36	S	67	I	98	F	129	Y	160	A	191	N
7	P	37	L	68	D	99	Y	130	K	161	G	192	H
8	V	38	F	69	S	100	M	131	N	162	K	193	H
9	I	39	S	70	I	101	P	132	R	163	D	194	Y
10	P	40	F	71	T	102	V	133	S	164	F	195	K
11	N	41	I	72	G	103	D	134	I	165	L	196	K
12	V	42	G	73	V	104	N	135	E	166	V	197	P
13	P	43	S	74	T	105	L	136	L	167	E	198	G
14	F	44	P	75	V	106	G	137	V	168	T	199	Y
15	L	45	R	76	N	107	M	138	Q	169	I	200	N
16	W	46	I	77	G	108	A	139	Q	170	K	201	G
17	A	47	N	78	G	109	V	140	Q	171	L	202	S
18	W	48	A	79	I	110	I	141	N	172	G	203	C
19	N	49	T	80	P	111	D	142	V	173	K	204	F
20	A	50	G	81	Q	112	W	143	Q	174	L	205	N
21	P	51	Q	82	K	113	E	144	L	175	L	206	V
22	S	52	G	83	I	114	E	145	S	176	R	207	E
23	E	53	V	84	S	115	W	146	L	177	P	208	I
24	F	54	T	85	L	116	R	147	T	178	N	209	K
25	C	55	I	86	Q	117	P	148	E	179	H	210	R
26	L	56	F	87	D	118	T	149	A	180	L	211	N
27	G	57	Y	88	H	119	W	150	T	181	W	212	D
28	K	58	V	89	L	120	A	151	E	182	G	213	D
29	F	59	D	90	D	121	R	152	K	183	Y	214	L
		60	R	91	K	122	N	153	A	184	Y	215	S

216	W	247	V	278	L	309	I	340	M	371	A	402	C	433	F
217	L	248	R	279	K	310	M	341	C	372	I	403	Y	434	L
218	W	249	E	280	F	311	R	342	S	373	Q	404	S	435	K
219	N	250	A	281	L	312	S	343	Q	374	L	405	T	436	P
220	E	251	I	282	S	313	M	344	V	375	E	406	L	437	P
221	S	252	R	283	Q	314	K	345	L	376	K	407	S	438	M
222	T	253	V	284	D	315	S	346	C	377	G	408	C	439	E
223	A	254	S	285	E	316	C	347	Q	378	G	409	K	440	T
224	L	255	K	286	L	317	L	348	E	379	K	410	E	441	E
225	Y	256	I	287	V	318	L	349	Q	380	F	411	K	442	E
226	P	257	P	288	Y	319	L	350	G	381	T	412	A	443	P
227	S	258	D	289	T	320	D	351	V	382	V	413	D	444	Q
228	I	259	A	290	F	321	N	352	C	383	R	414	V	445	I
229	Y	260	K	291	G	322	Y	353	I	384	G	415	K	446	F
230	L	261	S	292	E	323	M	354	R	385	K	416	D	447	Y
231	N	262	P	293	T	324	E	355	K	386	P	417	T		
232	T	263	L	294	V	325	T	356	N	387	T	418	D		
233	Q	264	P	295	A	326	I	357	W	388	L	419	A		
234	Q	265	V	296	L	327	L	358	N	389	E	420	V		
235	S	266	F	297	G	328	N	359	S	390	D	421	D		
236	P	267	A	298	A	329	P	360	S	391	L	422	V		
237	V	268	Y	299	S	330	Y	361	D	392	E	423	C		
238	A	269	T	300	G	331	I	362	Y	393	Q	424	I		
239	A	270	R	301	I	332	I	363	L	394	F	425	A		
240	T	271	I	302	V	333	N	364	H	395	S	426	D		
241	L	272	V	303	I	334	V	365	L	396	E	427	G		
242	Y	273	F	304	W	335	T	366	N	397	K	428	V		
243	V	274	T	305	G	336	L	367	P	398	F	429	C		
244	R	275	D	306	T	337	A	368	D	399	Y	430	I		
245	N	276	Q	307	L	338	A	369	N	400	C	431	D		
246	R	277	V	308	S	339	K	370	F	401	S	432	A		

3. Patent Data Are Consistent With Literature Regarding Known Important Residues

275. The patent teaches that the data "confirm the requirement of [mature] PH20 amino acid residues corresponding to positions 25, 111, 113, 176, 189, 203, 246, 249, 252, 316, 341, 346, 352, 400, 402, 408, 423 and 429 … for hyaluronidase activity, since mutagenesis of these residues results in an enzyme that is not active (e.g., it is not expressed or is inactive when expressed, see e.g.,

Tables 5 and 10)." EX1001, 68:63-69:4. However, "[t]he exception is that amino acid replacement corresponding to R176K and C316D resulted in mutants that generated some residual hyaluronidase activity." *Id.*, 69:4-6. As shown in the figures above (and in the spreadsheet), those residues are colored red or orange, indicating that no substitutions or less than 30% of substitutions were tolerated. And as noted above, residues 25, 189, 203, 316, 341, 346, 352, 400, 402, 408, 423 and 429 are conserved cysteines and residues 111, 113, 176, 246, 249 and 252 were taught to be important to activity in the references described above.

4. Positions That Permit All Substitutions

276. A POSA would recognize that positions for which activity is retained for all mutations are likely very tolerant to change. For example, a position for which activity is maintained when the residue is large, small, hydrophobic, hydrophilic or charged may in fact tolerate any residue in that position. I've shown a few examples of such residues in the chart below:

Position	WT residue	Retained Activity
26	L	AEGHIKMPQRSTVWY
28	K	ADEFILMNPRSTVW
29	F	AEGHIKLMPRSTVW
30	D	AFGHKLMPQRSTVW
31	E	ACGHIKLPRSTVWY

32	P	ACFGHKLMNQRSTVWY
140	Q	ACDFGHIKLMRVWY
141	N	ADEFGHLMQRSTVWY
142	V	CDEGHIKLMNPQRST

277. For example, position 26 is leucine in WT PH20. Leucine is a hydrophobic residue. The data show that hydrophobic, hydrophilic, large, small, positively charged and negatively charged residues were all tolerated in this position without abrogating activity.

278. Similarly, position 29 is phenylalanine in WT PH20. Phenylalanine is a large hydrophobic residue. The data show that mutations to a broad spectrum of residues, including small residues, hydrophilic residues and positively and negatively charged residues were tolerated at this position.

5. Positions That Permit Some Substitutions

279. For those positions that tolerated some but not all substitutions, the POSA can derive information about the characteristics of residues that were not tolerated at that position.

280. I've shown a couple of examples in the chart below:

Position	WT residue	Inactivated	Retained activity
179	H	W	ACEGIKLMNPRSTV

257	P	D	ACGIKLMNQRTV

281. For example, at position 179, the WT residue is histidine. The only tested residue not tolerated at that position is tryptophan, which is a much larger and primarily hydrophobic residue. It may be that a tryptophan is too bulky to fit in that position and could destabilize the protein. Examining the PH20 model at this position could provide further insight.

282. With respect to position 257, the only tested residue that inactivated the protein is aspartate, which is negatively charged. I note that there are no data provided for glutamate at this position. I also note that glutamine and asparagine were both tolerated, which indicates that the problem likely is the negative charge. Residue 258 is aspartate, so having two negative charges next to each other in this position appears to be unfavorable.

6. Improved Activity

283. In Table 9, the patent provides the average normalized activity of tested duplicates compared to the average normalized wild type activity for that plate. The data in Table 9 shows that some substitutions substantially increased activity—by more than 2-fold, more than 3-fold, more than 5-fold or even more than 10-fold.

284. The ’791 patent teaches 173 modifications at 83 positions that improved activity more than 200%.

285. The ’791 patent teaches 73 modifications at 36 positions that improved activity more than 300%. It is interesting to see positions where more than one substitution increased activity by more than 300%. A POSA may be interested in making changes at that position because the data suggest there is room for improvement at that position.

286. The ’791 patent teaches 24 modifications at 12 positions that improved activity more than 500%. Those 12 positions are: 69, 70, 75, 84, 165, 166, 271, 278, 317, 320, 325 and 326.

287. The ’791 patent teaches 6 modifications that improved activity more than 1000%. Those modifications are at 3 positions: 69, 70 and 271.

288. These positions may be of interest to the POSA seeking to increase activity. Alternatively, those positions may be of interest to the POSA as a means of compensating for a potential decrease in activity resulting from another desired change.

7. Substitution Tolerability Is Not Randomly Distributed Across the Sequence

289. Dr. Hecht states in paragraph 143 of his declaration that the patent discloses a “random mutagenesis study” and that “the individual substitutions did not yield PH20 mutants with consistently observed effects; rather, the effects observed were random and unexplained among the tested mutants.” EX1003 (Declaration of Michael Hecht, Ph. D.), ¶ 150. To the extent Dr. Hecht intends to

suggest that tolerance for substitution is randomly distributed throughout the sequence, I disagree.

290. The data show that substitution tolerance is not random. If it were random, then one would expect to see red, orange and green residues scattered randomly throughout the sequence with no rhyme or reason to their placement. That is not observed, nor is it what the POSA would expect to see. Amino acid sequences of naturally-occurring proteins are not random, nor is protein structure. I note that POSAs, like Dr. Park, use multiple sequence alignments to identify conserved residues precisely because tolerance to mutation is not random.

291. As shown in the figure above (and the spreadsheet), in many cases there are clusters of residues that tolerate substitutions and clusters that do not. For example, positions 26-32 tolerate 60% or more of the tested substitutions, and within that cluster positions 26 and 28-32 tolerate all tested substitutions. Positions 181-192 tolerate 30% or fewer of the tested substitutions, and within that cluster, positions 185 and 187-191 did not tolerate any of the tested substitutions. Similarly, positions 329-346 tolerate 30% or fewer of the tested substitutions.

8. Percentage of Intolerant Positions and Impact On Predictability

292. As of December 28, 2012, POSA would have expected that PH20, like many other hydrolase enzymes (which would include hyaluronidases), was likely to be relatively robust in structure. That is, in part, because it adopts an

alpha-beta barrel fold, and that is known to be a generally robust fold that is tolerant to substitution. Based on this general knowledge, hydrolase enzymes would typically be thought to be relatively tolerant of substitutions outside of the active site. But the data in the '791 patent shows that it is not. As I will explain in detail below, those data are extremely valuable to the POSA seeking to make variant PH20 polypeptides because the data show the POSA which positions are likely to tolerate substitutions (and what types of substitutions) and which are not.

293. This does not mean PH20 is unpredictable or that making and using the claimed modified polypeptides is unpredictable. It means PH20 is generally intolerant to mutations unless those mutations are made in certain places, and the '791 patent teaches where to make mutations to likely retain activity. The '791 patent renders modifying PH20 predictable by identifying these areas that are particularly intolerant to mutation, and those that are tolerant.

294. The POSA would not necessarily have expected PH20 to be so sensitive to change. As I explain below, there are regions on the surface of the protein that the POSA would have expected to tolerate change and do not, and there are positions and substitutions that Dr. Park incorrectly predicted would be tolerated (without the benefit of the '791 patent's data). But the '791 patent provides the missing information. With the '791 patent data in hand, the POSA can identify which regions are likely to tolerate change and which are not.

295. A POSA would have understood from the data that substituting residues identified as intolerant to change—particularly those with no functional activity across all or nearly all substitutions—is likely to abolish enzymatic activity.

296. As discussed above, modified PH20 polypeptides may contain mutations that result in a loss of enzymatic activity. As I will explain below, a POSA would have understood that modified PH20 polypeptides may contain a further mutation which, under appropriate circumstances, can restore activity in the otherwise enzymatically inactive polypeptide (*i.e.,* a "rescue mutation"). I will provide examples of how a POSA could use the patent data to identify potential rescue mutations below. However, as a general matter, a POSA looking to retain activity would likely not select an invariant residue for mutation unless there is a specific reason for wanting to change that particular residue.

B. PH20 Model Illustrates Patent Functional Results

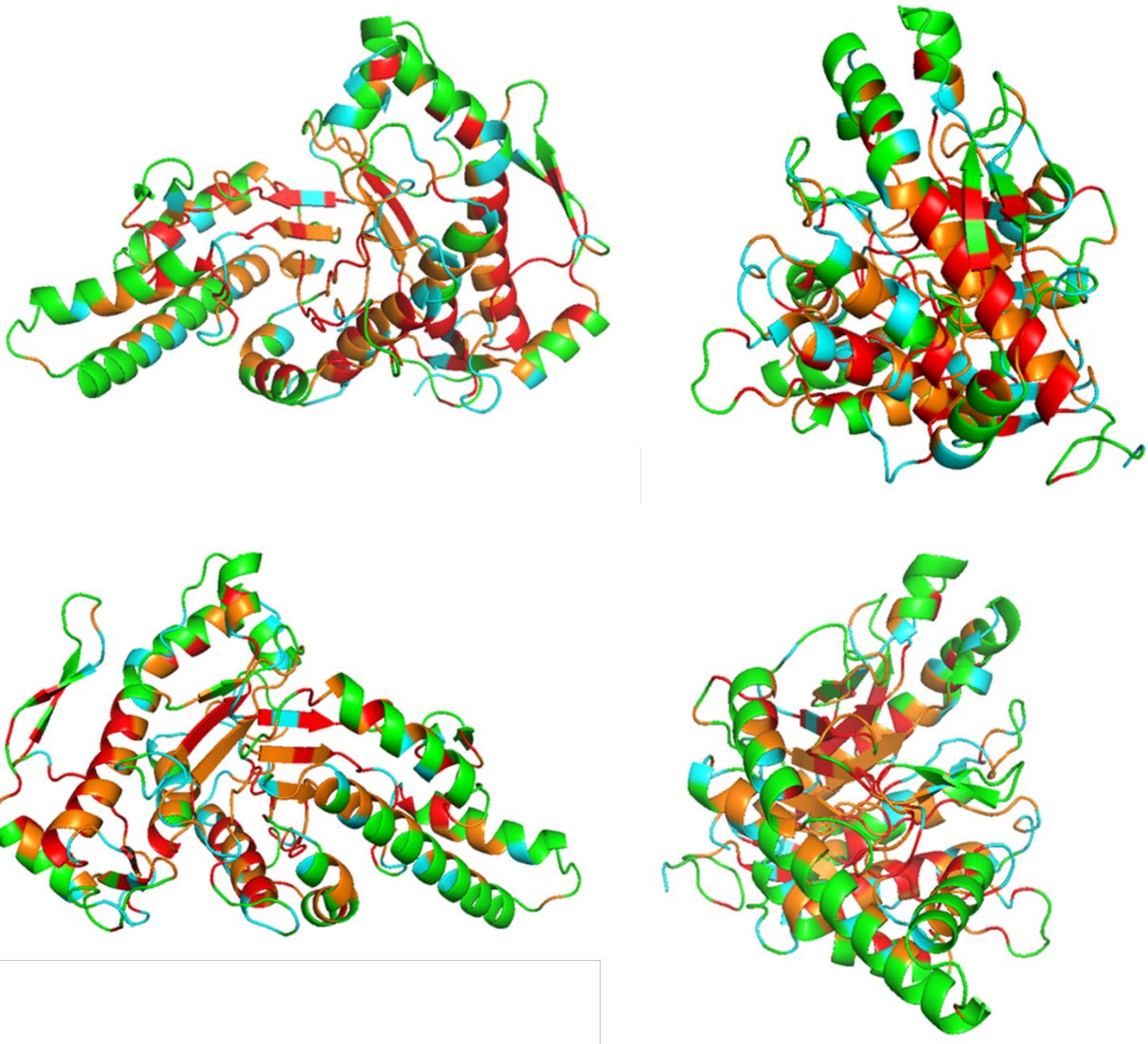
297. A POSA analyzing the specification would have considered the activity data in the context of the PH20 homology model to help determine which positions are amenable to change and to identify additional variants.

1. Color-Coded PH20 Model

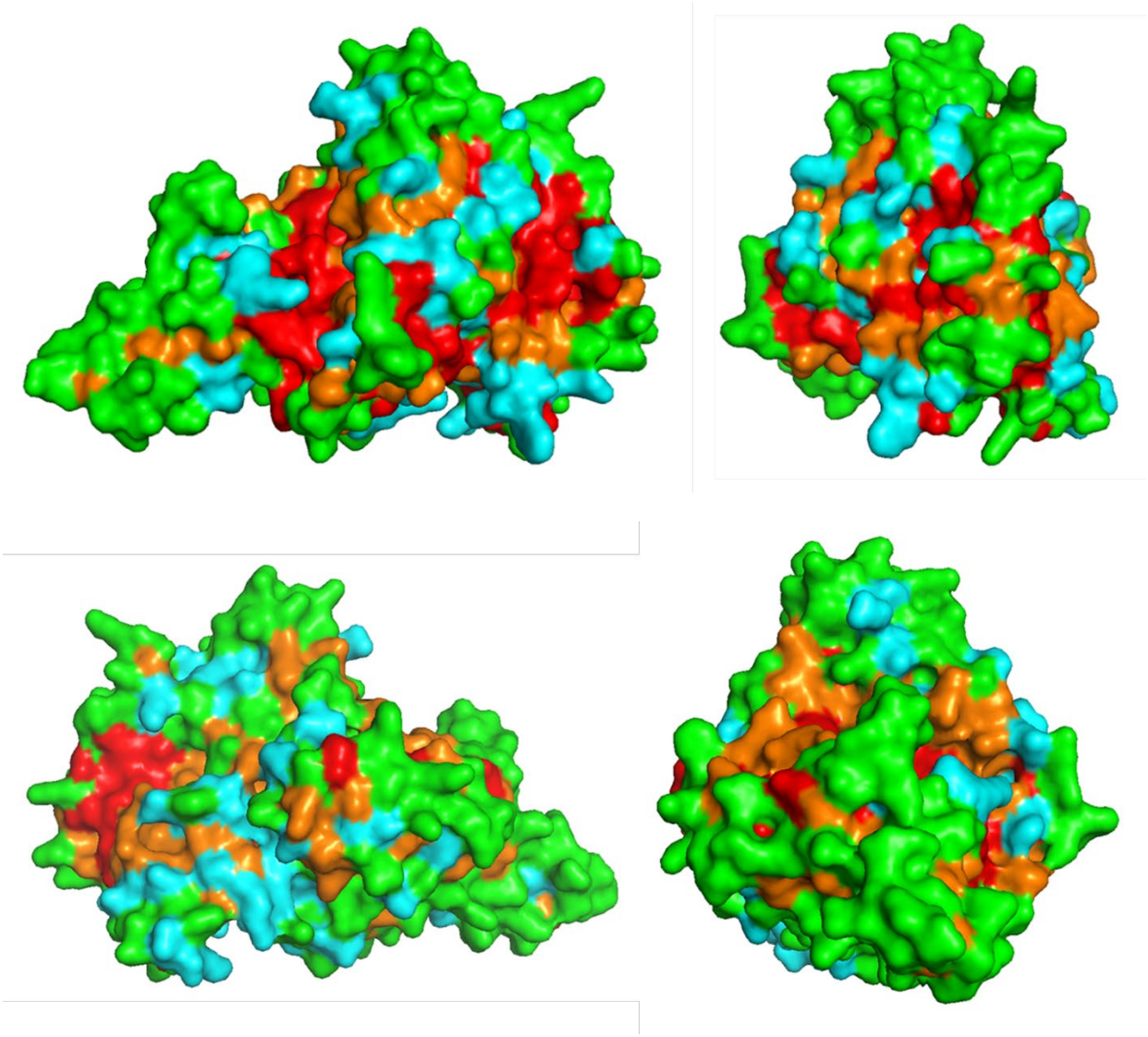
298. I highlighted the PH20 homology model I generated to correspond with the spreadsheet. For example, I colored red residues for which no tested

substitutions were tolerated. I colored orange residues for which 30% or fewer of the reported changes were tolerated. I colored green residues for which 60% or more of the reported substitutions were tolerated.

299. In the images below, I show the highlighted PH20 model rotated around the Y-axis in 90 degree increments. In the first set of images, which is shown in "cartoon" format in PyMOL, the secondary structures are visible:



300. In the second set of images, the highlighted PH20 model is rotated around the Y-axis, and the solvent-accessible surface is shown in the same coloring:



301. In general, the figures above show that, as expected, red, orange and green residues are not randomly distributed throughout the protein. Many of the "red" residues are part of the active site or are buried. Many appear to be at interfaces between secondary structure features and appear to be involved in

orienting the secondary structures. The POSA would expect these positions to be generally intolerant to change.

302. There are places where patches of orange surround the red residues, possibly to hold the red residues in place. Orange residues also appear to form interfaces between secondary structures and appear to be involved in orienting the secondary structures.

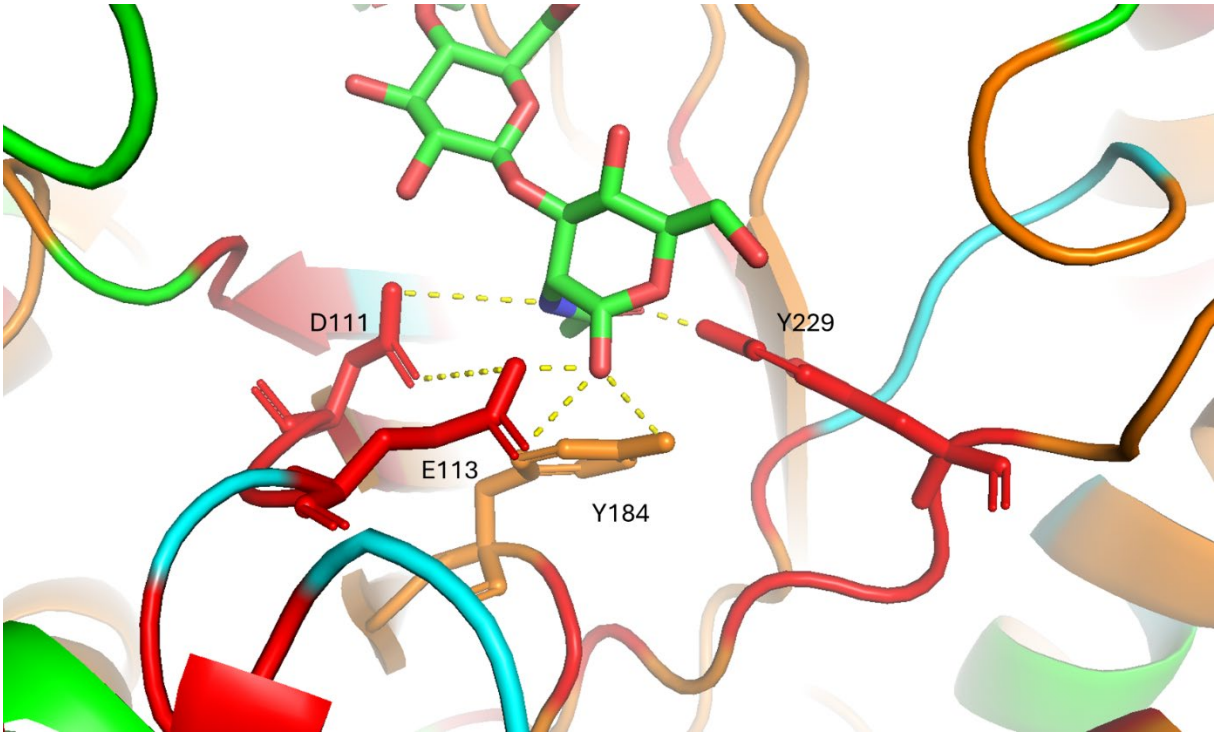
303. Green residues typically occur on the surface of the protein. That is what a POSA would expect. Typically, surface residues not involved in binding or activity are expected to be generally tolerant to mutation.

304. It is striking how much of the surface of the protein comprises red and orange residues—there are large orange and red patches across the surface. That is unexpected, and I address this issue further below.

2. Patent Data Consistent With Literature Regarding Known Important Residues

305. Above, I described the disclosure in the art regarding residues known to be important for hyaluronidase activity in bee venom hyaluronidase, Hyal-1 and PH20. Below, I show that the patent data are consistent with the teachings in the art regarding these residues. At the same time, the patent provides further insight regarding these residues and potential substitutions that may be made at these positions without completely abrogating activity.

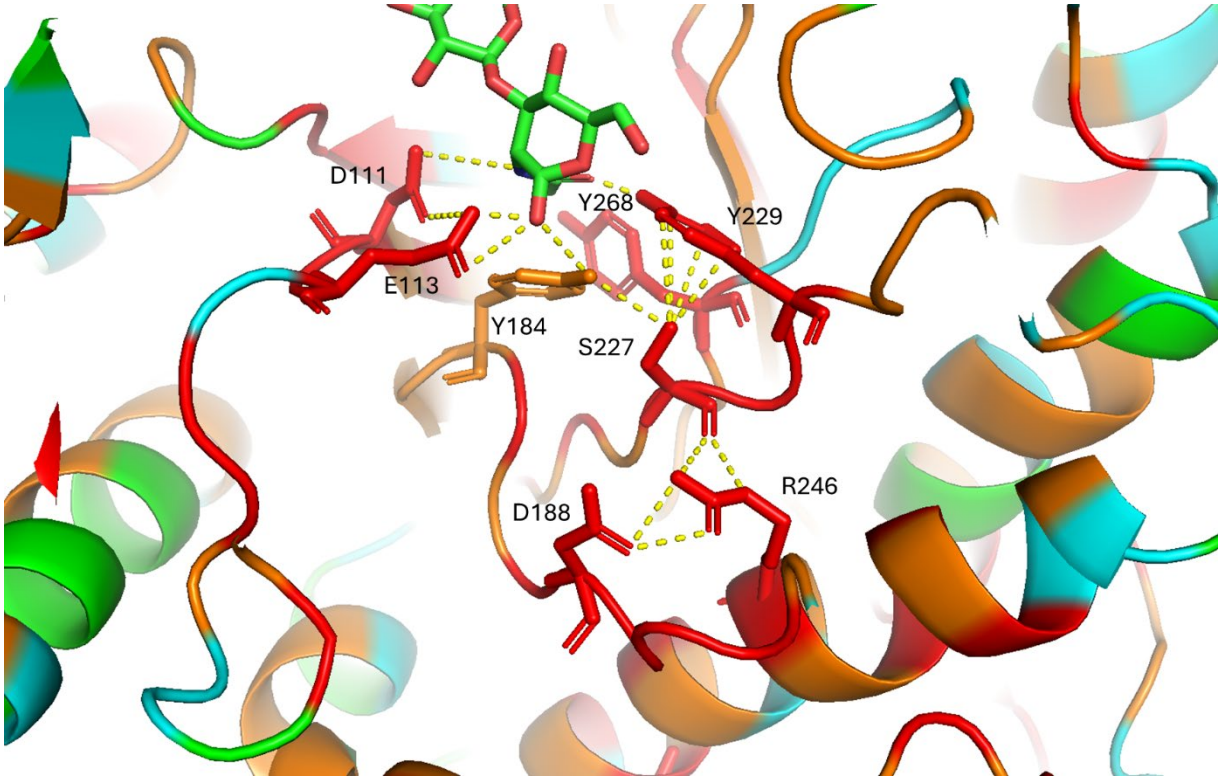
306. In the figure below, I show four key residues disclosed in the art as important to hyaluronidase activity (all of which I addressed above) in stick form in the color-coded PH20 model, with interactions shown as dotted lines:



307. Three of the four residues are red, showing that they did not tolerate any substitutions. Y184 is orange because Y184W achieved 39% activity. None of the other mutations at this position were tolerated.

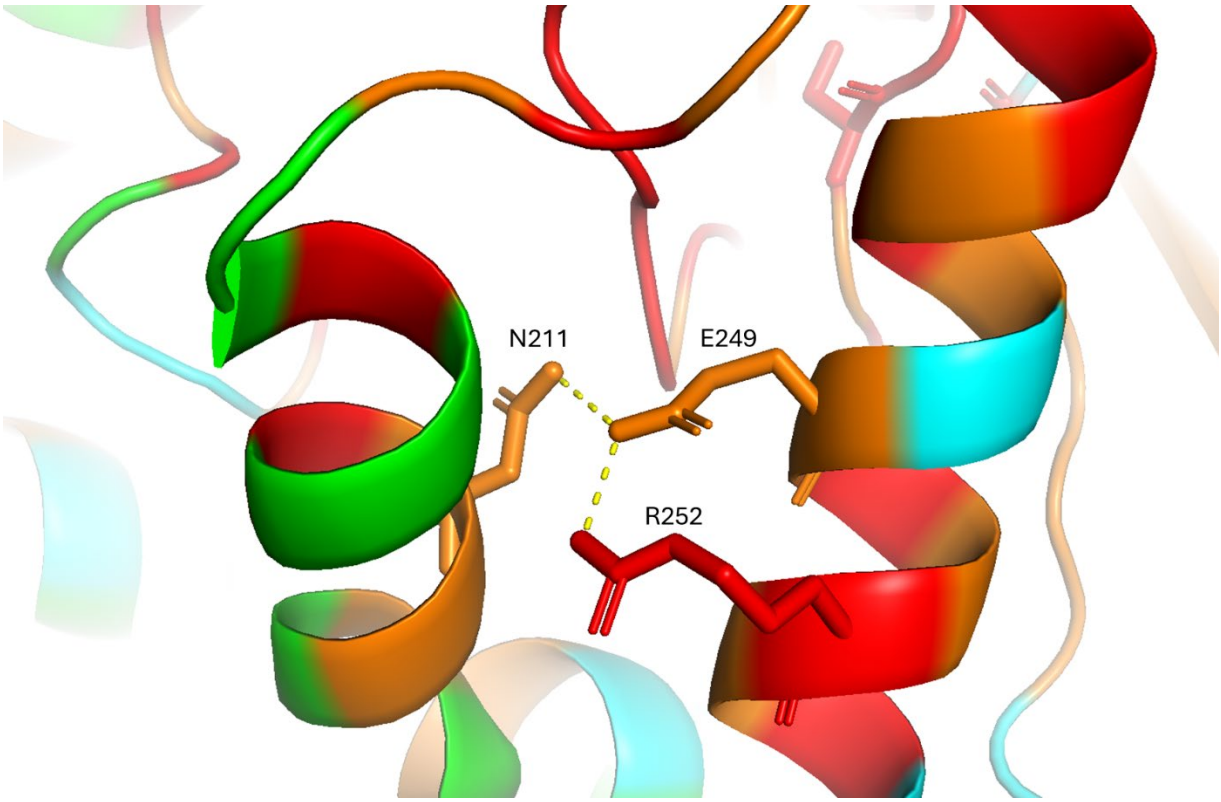
308. I note that the spreadsheet Dr. Park prepared with his analysis identifies Y184 as an essential residue that is conserved 100% of the time. The information in the patent that Y184W yields a protein with some activity is new, useful information that would not have been predicted by the POSA.

309. In the figure below, I show additional residues disclosed in the art and discussed above in stick form, with interactions shown as dotted lines:



310. These residues (except Y184) are all are colored red, so no substitutions were tolerated in these positions. That is consistent with what was known regarding those residues in the art.

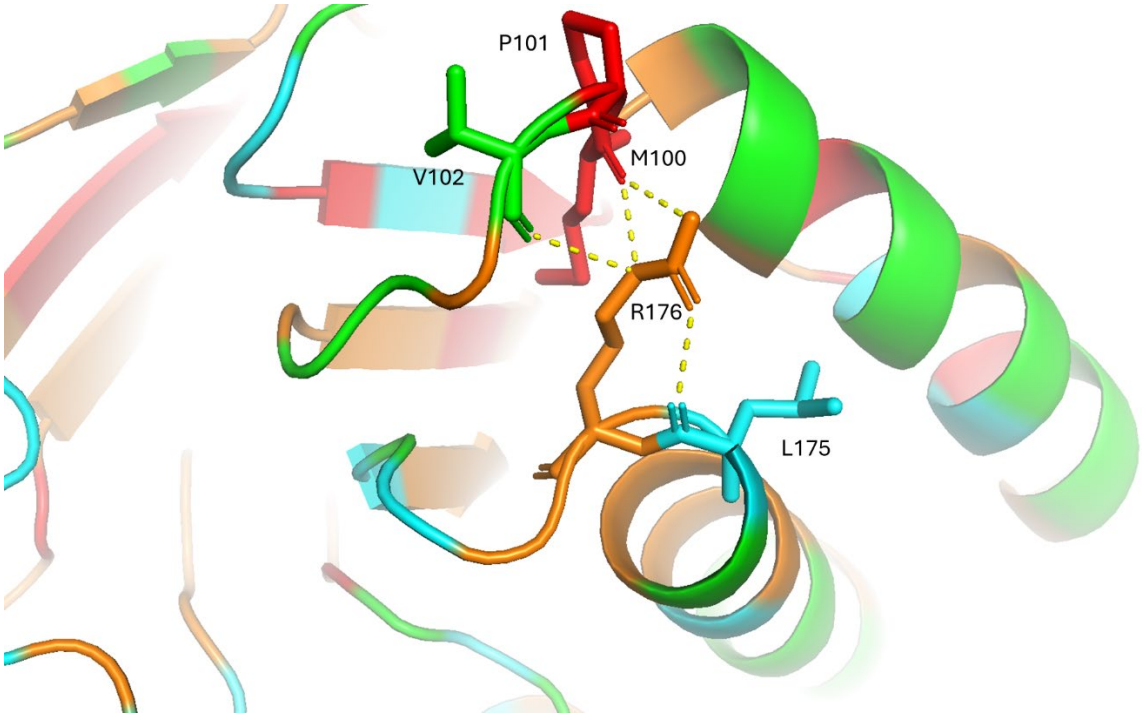
311. Below I show residues E249, N211 and R252 in stick form with their interactions shown as dotted lines. These residues also were disclosed in the art and were discussed above.



312. As shown in the figure above, R252 is colored red, meaning that it did not tolerate any substitutions. However, E249 and N211 are orange, so they did tolerate limited substitution. Specifically, N211L exhibited 41% activity, and N211W exhibited 51% activity. And E249V appears to have retained at least some activity in that it is listed in Table 9, although the activity value is not provided.

313. N211 also is identified as "essential" in Dr. Park's spreadsheet and is 100% conserved across Dr. Park's alignment. However, the patent data show that there is some limited capacity for substitution at this position while retaining a significant level of activity. The information in the patent that N211W or L yields a protein with some activity is new, useful information that would not have been predicted by the POSA.

314. Below, I show residues R176, M100, V102 and L175 in stick form with their interactions shown as dotted lines. R176 was mutated in Arming and discussed above.



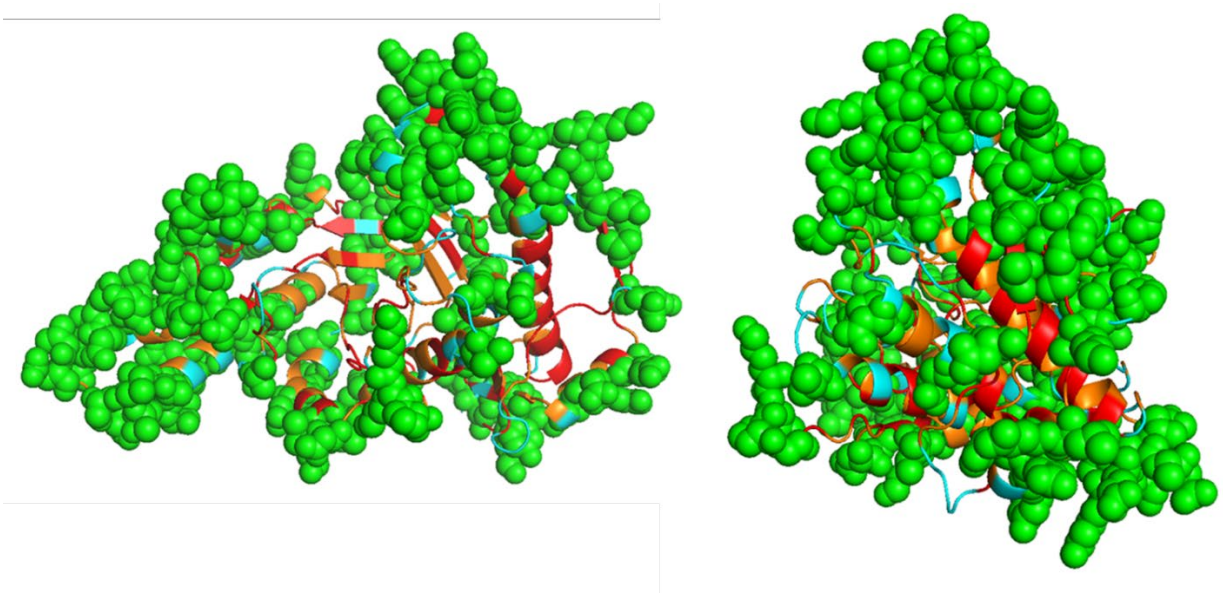
315. As shown in the figure above, R176 is colored orange, meaning that it did tolerate limited substitutions. Specifically, R176K exhibited 67% activity, and R176L exhibited 40% activity.

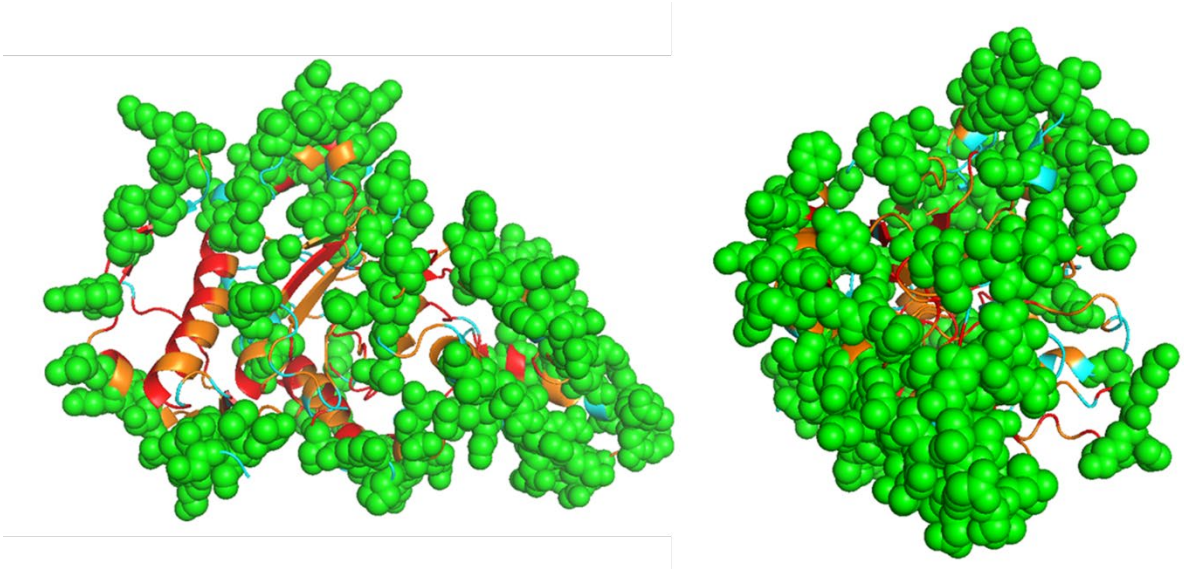
316. R176 is identified as "essential" in Dr. Park's spreadsheet and is 95% conserved across Dr. Park's alignment. However, the patent data show that there is some limited capacity for substitution at this position while retaining significant activity. The information in the patent that R176K and R176L yields a protein

with some activity is new, useful information that would not have been predicted by the POSA.

3. Positions Relatively Tolerant to Change

317. As I noted above, the residues that are relatively tolerant to change are generally on the surface of PH20. This is easier to see in the figures below, in which the PH20 model is shown in cartoon format and the green residues (those that tolerate 60% or more substitutions) are shown in "sphere" format.

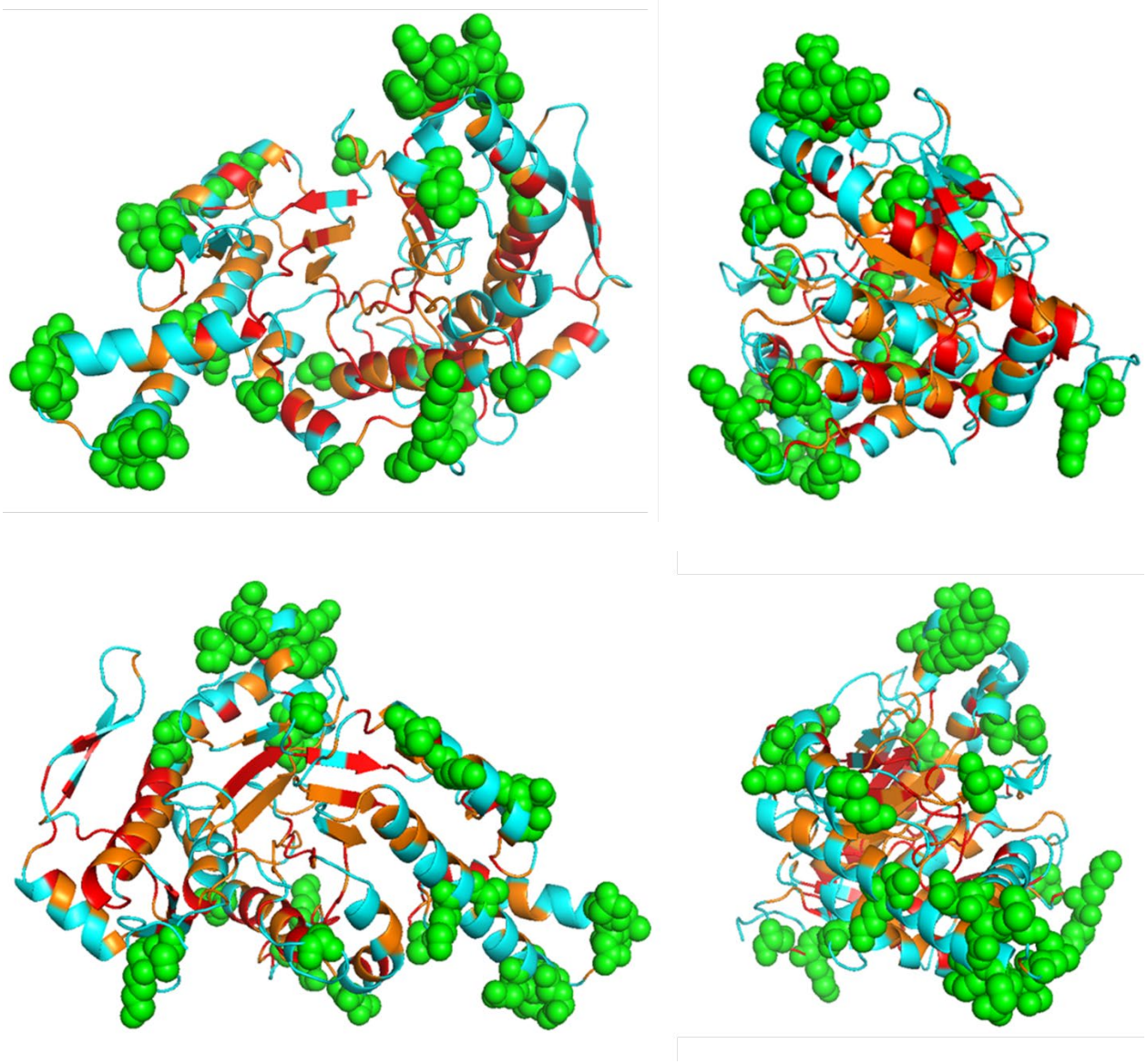




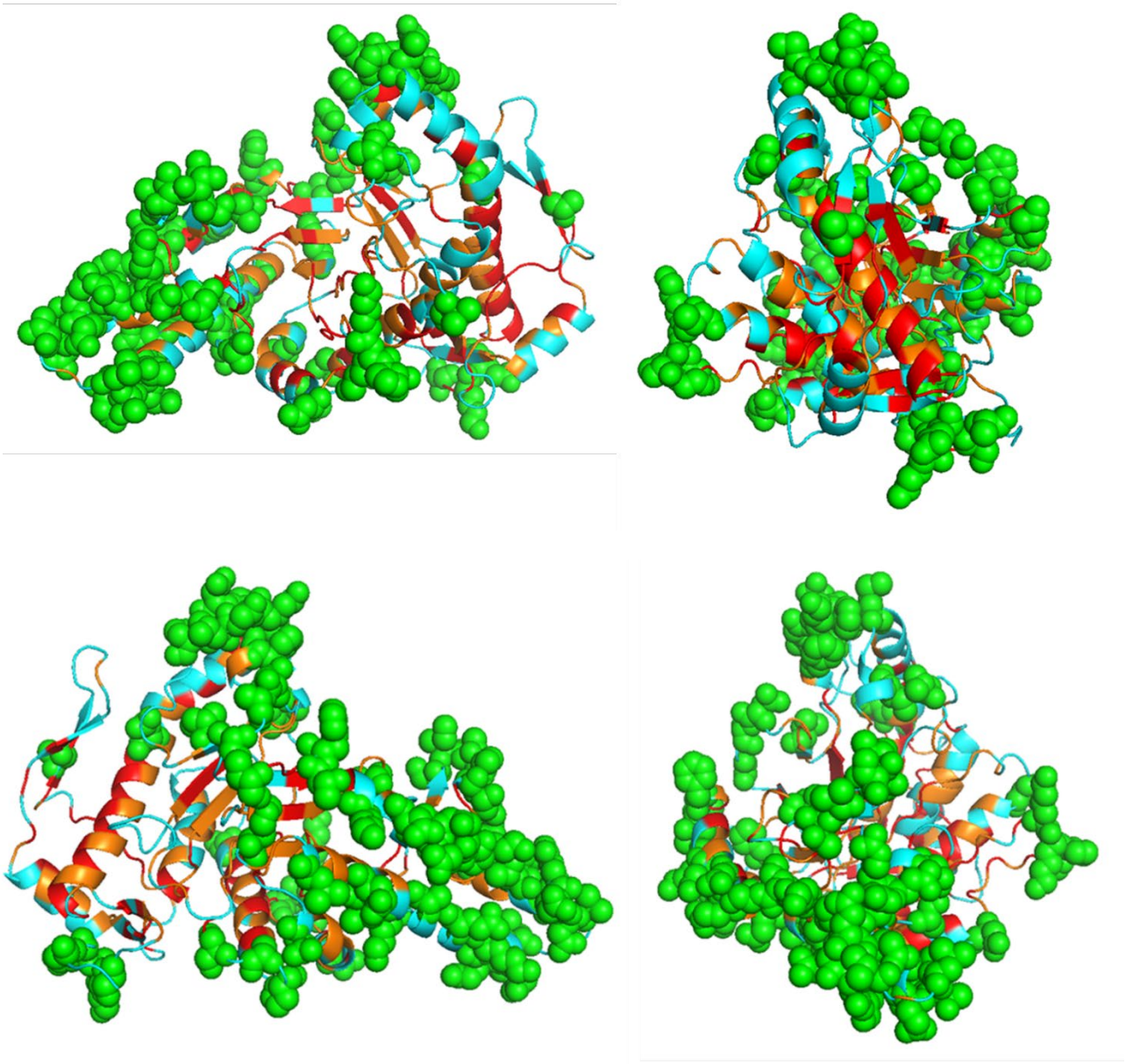
318. The figures above show that there are residues that are reasonably tolerant to change across the surface of PH20. The POSA seeking to generate modified PH20s within the scope of the claim would have understood that, in general, these residues are potential options for modification to alter some other property (e.g., solubility) while maintaining activity. The POSA could make more informed decisions regarding specific positions using the data in the patent regarding which substitutions were tolerated and the resulting activity.

319. The POSA wishing to be more conservative in selecting residues to mutate could also readily visualize those residues that are most tolerant to substitution by highlighting those for which all tested substitutions were tolerated, or for which 90% or more of the substitutions were tolerated. I provide those figures below.

320. The figures below show those positions that tolerate all of the tested changes in green in "sphere" format:



321. The figures below show those residues that tolerate 90% or more of the changes in green in sphere format:



322. Taken together, these figures show that there are many positions that are very tolerant of substitution on the surface of PH20 that can be targeted by the POSA seeking to generate a modified PH20 that retains hyaluronidase activity. The POSA would reasonably expect that changing one or multiple of these residues would produce a protein with hyaluronidase activity.

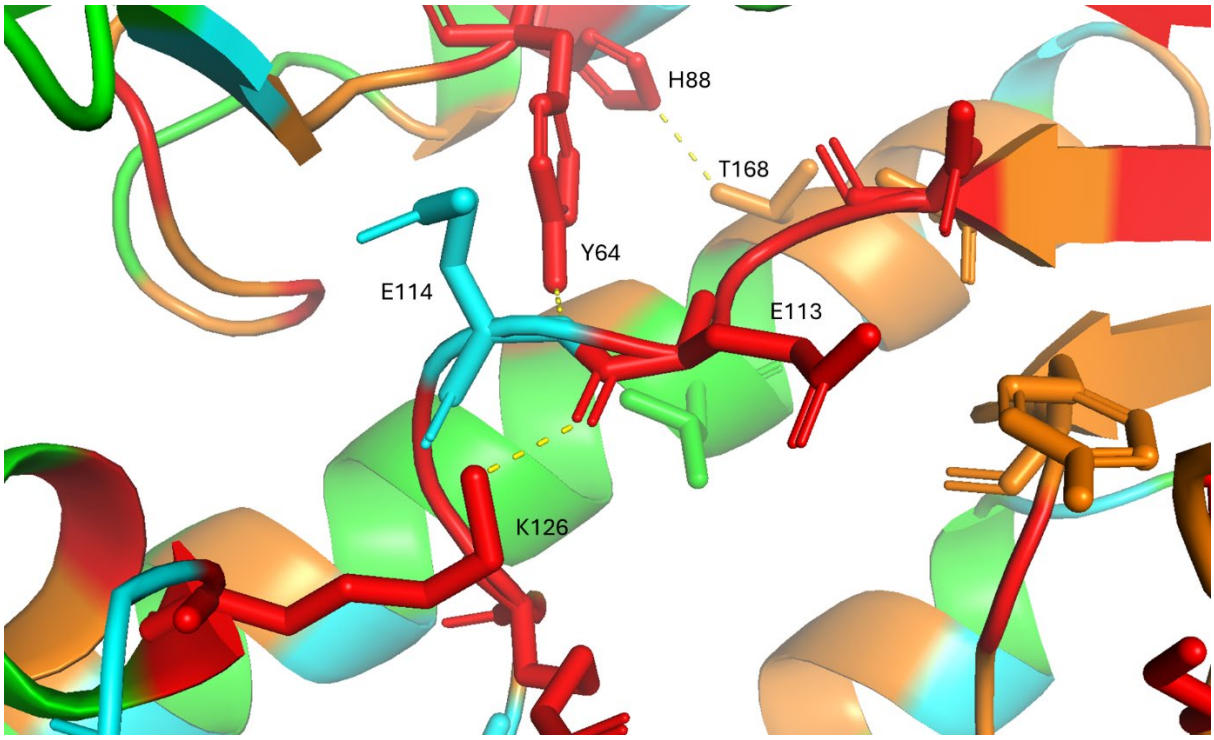
323. For example, a POSA would expect that, if substitutions are made at these residues in different parts of the protein, they would be independent of each other. A POSA would generally expect multiple independent changes to be tolerated.

324. The POSA also would be able to make informed decisions about making several changes close together on the protein using the data in the '791 patent regarding which substitutions were tested and the results for each, combined with an assessment of the local environment of the residues in question in the protein. The POSA would be able to evaluate whether the desired changes are likely to preserve the interactions formed by the WT residues or whether the desired substitutions are likely to have an adverse impact on, for example, packing.

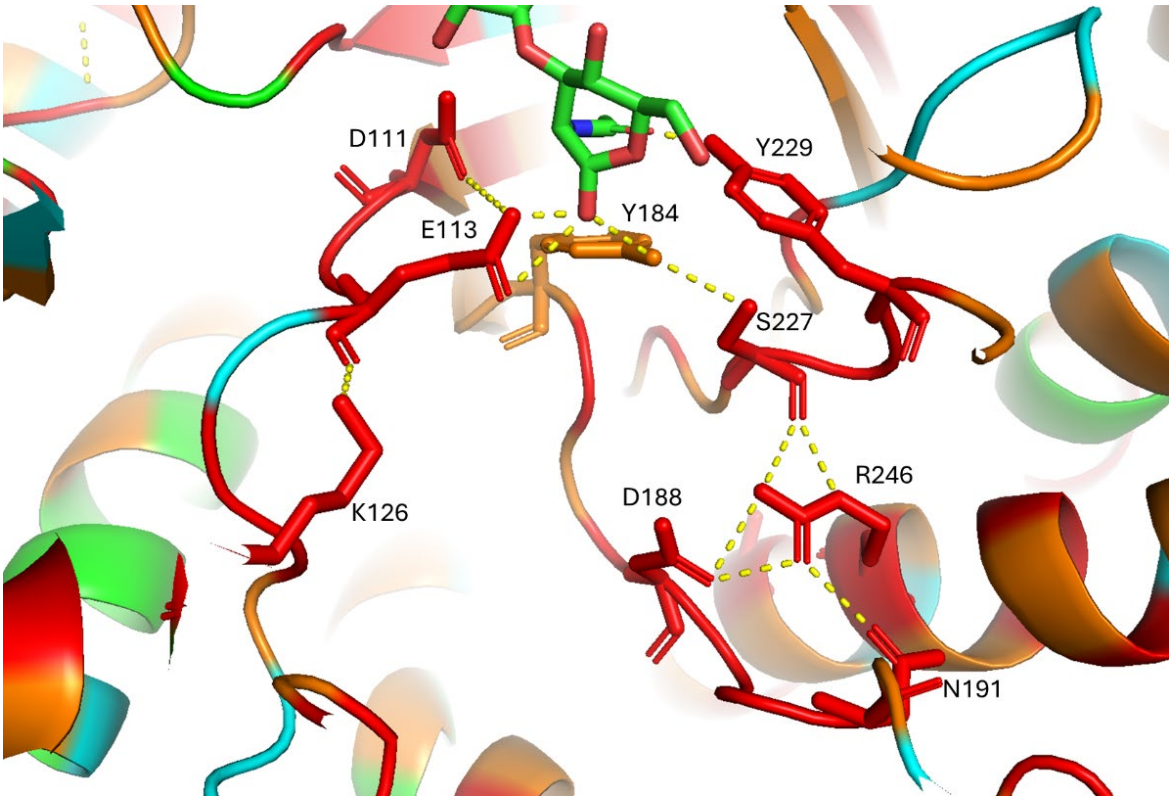
4. Positions Intolerant to Change

325. As noted above, the positions intolerant to change include residues in the active site, residues that position the active site residues, hydrophobic and buried residues, and residues that orient the secondary structures of the protein. I provide some examples below.

326. In the figure below, the nitrogen on K126 interacts with the main chain oxygen of critical catalytic residue E113. The hydroxyl of Y64 interacts with the main chain nitrogen of E114. These interactions likely are important for orienting the catalytic groups in the active site, including critical residue E113.



327. In the figure below, additional interactions that are important for orienting the active site are shown. Some of these residues and interactions were discussed above in the context of the knowledge in the art.



328. For example, as shown above, K126 and D111 interact with and orient the key catalytic residue E113. R246 forms interactions that orient the loop containing key catalytic residues Y229 and S227, which in turn interacts with Y184. D188 and N191 interact with and orient R246. With the exception of Y184, which I discussed elsewhere, none of these residues tolerated substitution.

329. It may be possible to change these residues in sets designed to replicate their interactions. For example, it may be possible to change D188, N191 and R246 at the same time to three other residues while attempting to maintain the interactions that they form and hold the active site in place. But the '791 patent data show that changing these residues one at a time abrogates activity. And the POSA would expect that, as a general matter, ***random*** changes to these positions

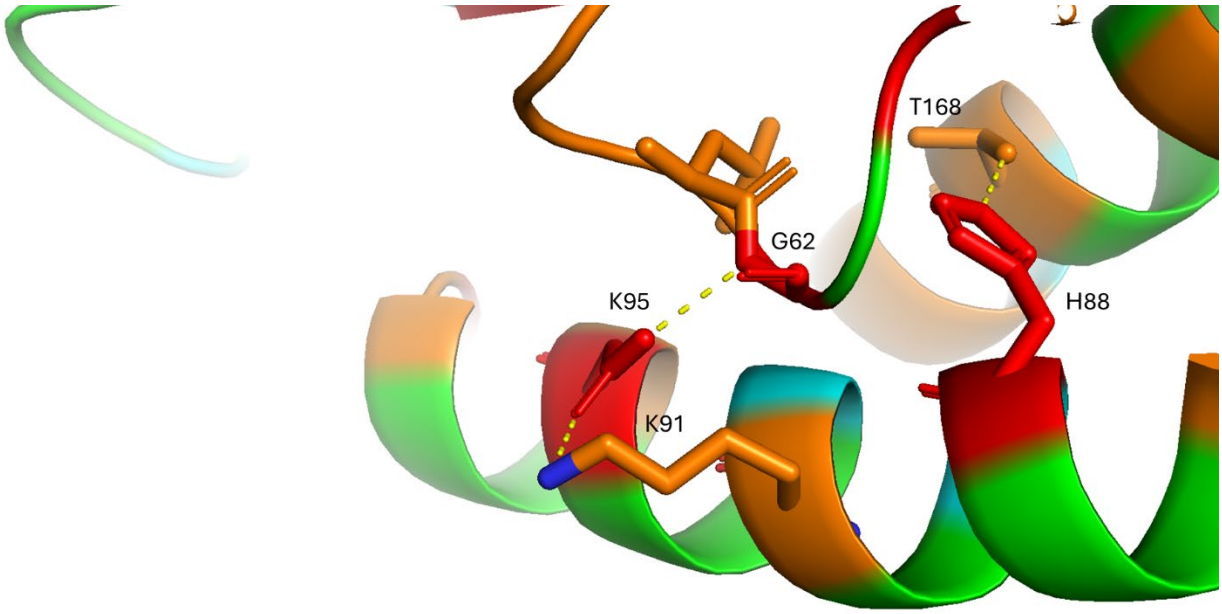
would similarly kill activity, whether made as single mutations or multiple mutations. Dr. Hecht explained why a POSA would expect this result with multiple mutations in his deposition:

> If you break something, it no longer performs its function or its function is diminished. If you break it simultaneously in five places, then its function is more dramatically diminished. If you break a bone in your leg, you can maybe hobble along. If you break five bones, you won't. Sorry for the negative graphic, but yeah.

EX2076 (Hecht Tr.), 228:2-9.

330. There is a structure/function correlation between the structure of the active site (and the residues that form the active site and orient the groups in the active site) and the function of cleaving HA. This structure/function correlation is shared by all PH20s that cleave HA. That is because all PH20s that cleave HA do so in the same way, through the same hydrolysis reaction mechanism.

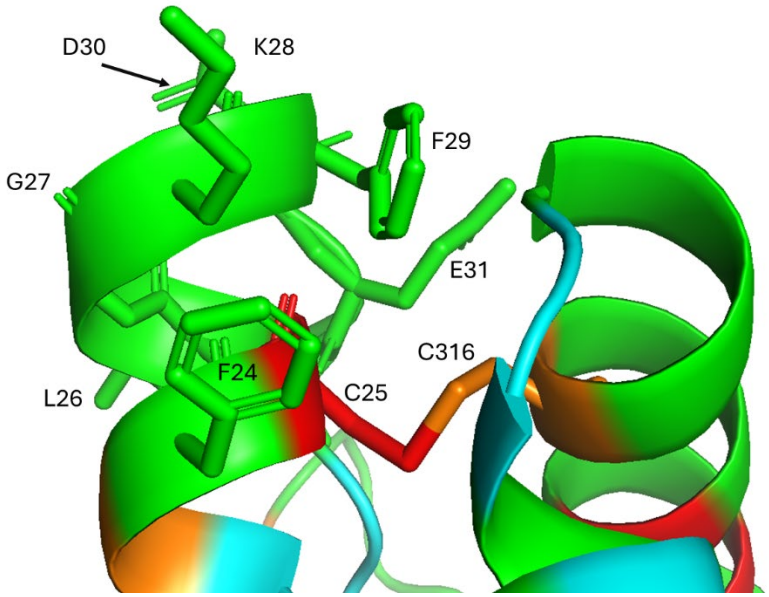
331. In the figure below, I provide an example of intolerant residues involved in orienting the secondary structure elements. In the figure below, for example, residues K95, G62 and H88 did not tolerate any substitutions, and K91 and T168 tolerated minimal substitutions. These residues are involved in orienting and stabilizing the packing of specific secondary structure elements (here alpha helices and loops) with respect to one another.



332. Notably, the solvent exposed residues in the alpha helix (on the bottom of the alpha helix in this figure) are green. That is consistent with what the POSA would expect; those residues are not involved in packing or secondary structure orientation, so they are more amenable to substitution.

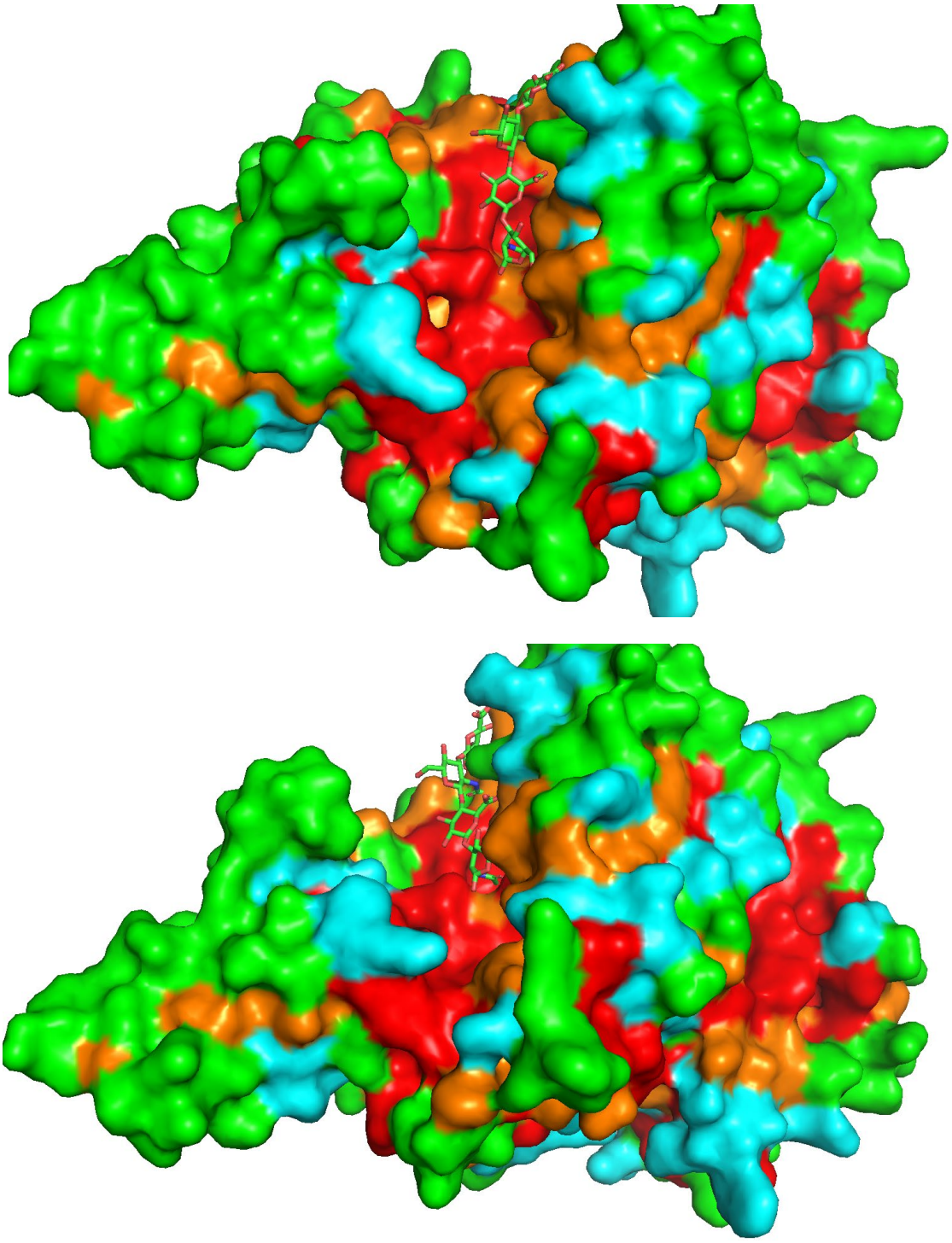
333. In the figure below, two alpha-helices are held together by a disulfide bond between C25 and C316. C316 is colored orange because some activity was retained when aspartate was substituted at this position (that is not something one would ordinarily predict without the data in the patent). However, the residues at the C-terminal end of the alpha-helix on the left are extremely tolerant of substitution. Residues 26 and 28-32 tolerated all tested substitutions. As a result, a

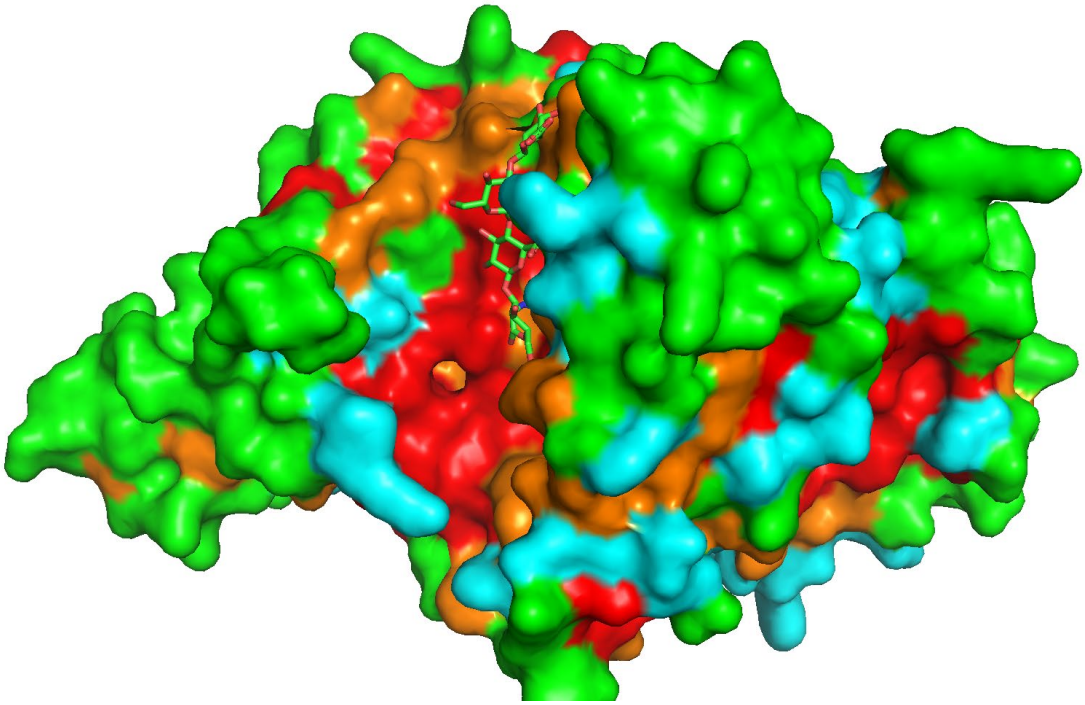
POSA would expect that changes made to those positions generally would be tolerated.



5. Surface Residues Intolerant to Change

334. As shown in the figures below, and as noted above, there are numerous stretches of solvent exposed residues that are not part of the active site groove but that appear to be intolerant or largely intolerant of substitution. That is contrary to what the POSA would expect for a protein like this.

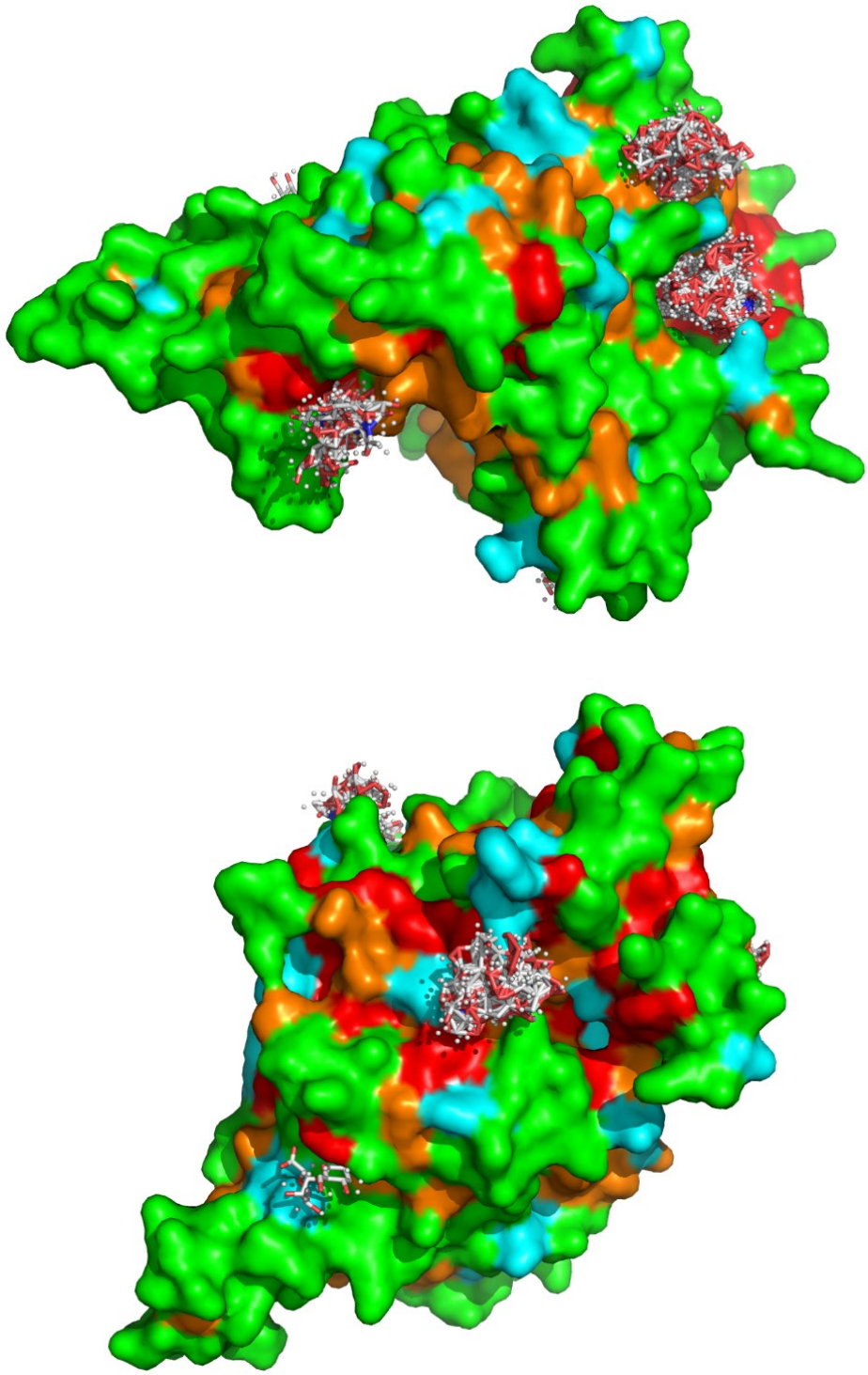




335. HA is a large polysaccharide. It is conceivable that residues in HA bind to PH20 outside of the active site groove, and that such additional interactions with HA may assist in orienting HA in the active site groove for catalysis, or, interestingly, in retaining PH20 in the vicinity of its substrate after a cleavage event, since the enzyme does not process along the HA chain. As explained above, PH20 releases HA following catalysis and then rebinds before cleaving again. If HA is able to interact with other portions of the PH20 surface, those interactions may facilitate rebinding for the next round of cleavage. To investigate this, I evaluated whether the red-colored surface-exposed regions are predicted to be HA binding sites, which is an analysis a POSA with the patent data in hand could have performed as of December 28, 2012.

336. I took the dimer of hyaluronic acid—the one structure that is the most reliable, as found in, *e.g.*, pdb file 8SMN (EX2181)—and used AutoDock Vina, which was available in 2011 to dock it to the PH20 model. I let the carbohydrate explore the entire structure (excluding the active site, where we already know it binds).

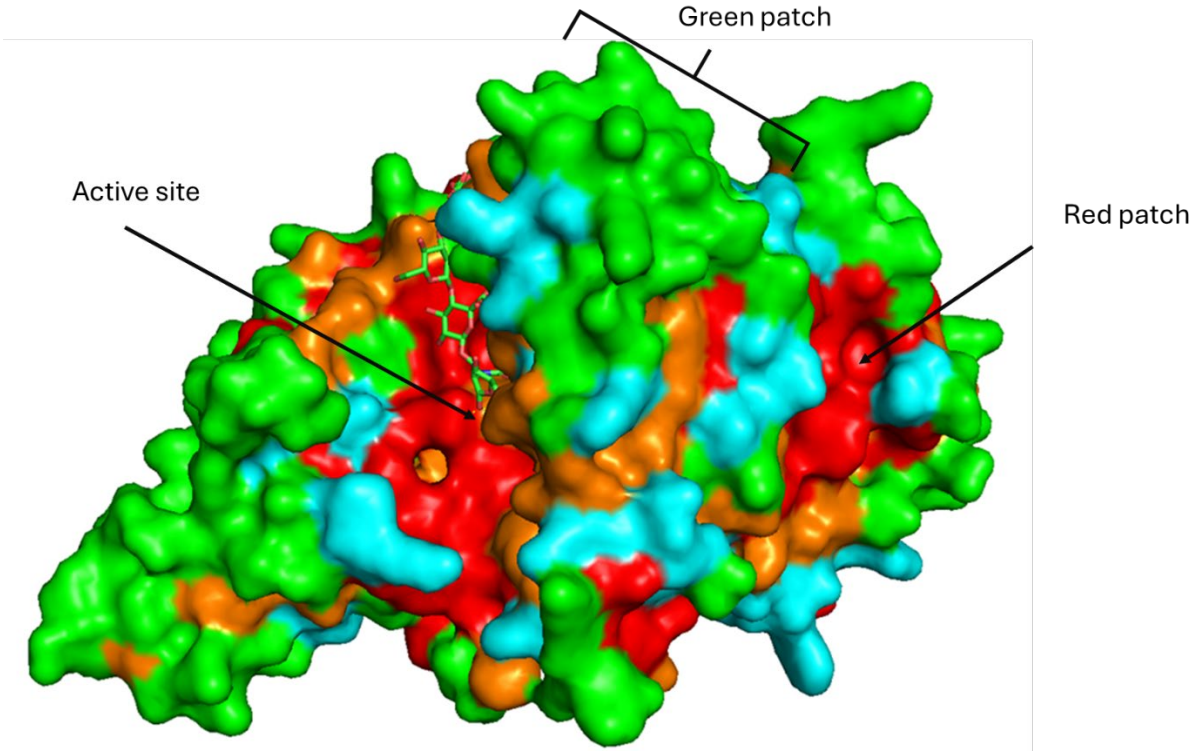
337. I found six strongly predicted binding regions, shown as clusters in the figure below. They overlap perfectly with red invariable positions disclosed in the ’791 patent. From this we can conclude that the patent information enables a person skilled in the art to predict the possible extended binding sites for the polymeric substrate.



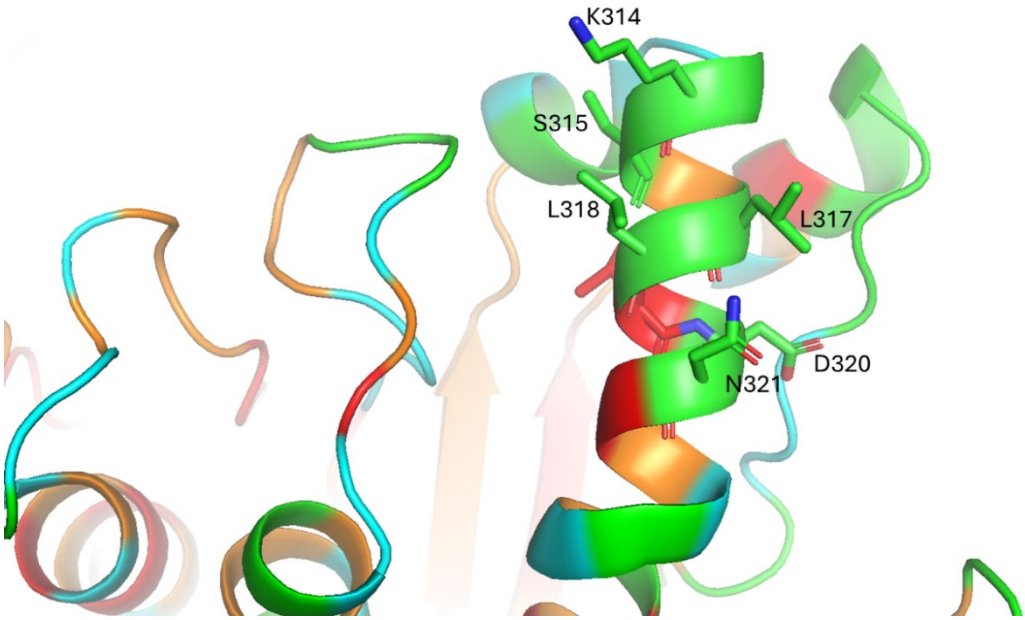
338. I note that the patent data were critical in this analysis, because without the patent data the POSA would not know (or expect) such a large number

of surface-exposed residues to be invariant, and thus would not have been able to formulate a hypothesis as to their function.

339. This HA binding hypothesis drew my attention to the green patch of tolerant residues in between the active site and the invariant region shown in the figure below. If the red patch highlighted below is in fact a site where HA binds PH20, then HA could potentially wrap over the green patch I've highlighted below. I examined those residues in the structure.



340. In the figure below, I show some of the residues in the green patch above in stick form:



341. Because HA is negatively charged, I hypothesized that introducing substitutions to positively charged residues in this region may improve activity. I then examined the data in the specification to see what results were obtained for these residues. Sure enough, L317K and L317R resulted in increased activity, with L317K achieving a five-fold increase in activity and L317R nearly doubling activity. L318K and L318R also increased activity, but by less than two fold. D320K increased activity more than six-fold, and D320R more than tripled activity.

342. None of this would have been anticipated by the POSA because nothing about this stretch of residues would have stood out to the POSA absent the data in the ’791 patent. Consistent with this, Dr. Park did not even consider the L317K mutation.

XI. WAYS IN WHICH A POSA COULD USE PATENT DATA TO MAKE AND USE MODIFIED PH20 POLYPEPTIDES

343. I was asked to describe exemplary ways in which a POSA could use the teachings of the ’791 patent to make modified PH20 polypeptides that are at least 95% identical to, for example SEQ ID NO: 3. In my analysis of the patent data above, I alluded to ways in which the POSA could use the ’791 patent data to identify locations to introduce substitutions. I further elaborate on those concepts below.

344. Before explaining how a POSA could use the patent disclosure, in response to points raised by Dr. Hecht, I first wish to address how POSAs approach making modified proteins, like modified PH20 polypeptides, that are 95% identical to a reference sequence.

345. POSAs understand that PH20 variants with such a small number of substitutions will usually adopt the same general tertiary structure (*i.e.*, the alpha-beta barrel fold) as WT PH20, in the same way that PH20 has a tertiary structure homologous to Hyal-1. POSAs think of PH20 as a folded protein, and POSAs think of 95% identical variants as proteins typically with the same tertiary structure but with 5% variability in the residues in that tertiary structure. POSAs do not think of modified PH20s as 10^60 strings of text that happen to be 95% identical to one another.

346. POSAs modify existing proteins for a purpose. This is not a situation where a POSA is seeking a new type of molecule or protein that performs a specific function and must identify that protein or molecule by screening. Here, a POSA is starting from a known protein sequence that is known to have the desired properties.

347. A POSA seeking to make a modified PH20 with the '791 patent in hand would not make a randomly generated combinatorial library of billions of sequences that happen to be at least 95% identical to SEQ ID NO: 3 and then screen it for activity, because it makes no sense to do so. It is difficult to imagine why anyone would do such a thing. If the sequences truly are random, then it is likely that a substantial number would include substantial modifications to residues known to be invariant because they are involved in catalysis, the active site, or in protein structure. A POSA would not make a library with billions of sequences that the POSA expects are not going to fold into PH20 proteins because it makes no sense to do that, and, thanks to the '791 patent, the POSA has a much more sensible strategy.

348. With the '791 patent in hand, the POSA knows what positions tolerate substitution and which do not. The POSA would use that information to obtain modified PH20 polypeptides with the desired properties. The POSA would then make and test the desired polypeptide. With the desired protein in hand, the POSA

would test it to characterize the protein and confirm desired properties. That is routine; it is what POSAs do. Making a desired protein and testing it to confirm activity is not a "research program." It is routine experimentation.

349. The POSA would not ignore the information in the patent, generate an enormous library, and then screen billions of sequences as Dr. Hecht suggests. Proceeding in that manner effectively ignores the patent disclosure instead of using the patent disclosure.

350. Nor would the POSA attempt to generate a matrix of all potential strings of amino acids that are 95% identical to the recited sequences and then somehow attempt to extract useful proteins from the resulting morass of sequences. That is not how POSAs make proteins, at least not in 2011 or 2012, and certainly not when they are seeking to make 95% identical variants of a known protein with a known function.

351. The POSA would, as I have explained, use the patent data in conjunction with the PH20 homology model to evaluate potential substitutions and make variants with the desired properties.

352. In his deposition, Dr. Hecht provided a helpful explanation of why a POSA would evaluate potential substitutions using the PH20 homology model (instead of in the abstract):

> Location, location, location. I mean, it's basically -- one could make a mutation in the abstract of this amino acid

> to that amino acid. If you do that in the abstract, it's pretty difficult to say anything intelligent about that. However, if you look at it in the context of its physical chemical environment in the protein and in the context of its homologous environment sequence space, then you're better able to come up with an assessment of the properties of a potential mutation. And the location of the position of interest matters, and the location of other amino acids in that neighborhood matters.

EX2076 (Hecht Tr.), 72:15-73:6. Similarly, Dr. Hecht testified as follows:

> A The core issues in this case deal with making changes in a sequence. And to understand the impact of changes in the sequence, it's wise to look at the sequence and look at the structure to have a more informed -- to be able to make a more informed analysis of what would the impact be of such changes.

EX2076 (Hecht Tr.), 75:10-17. I agree that it is wise to consider the PH20 homology model to make a more informed assessment of potential mutations.

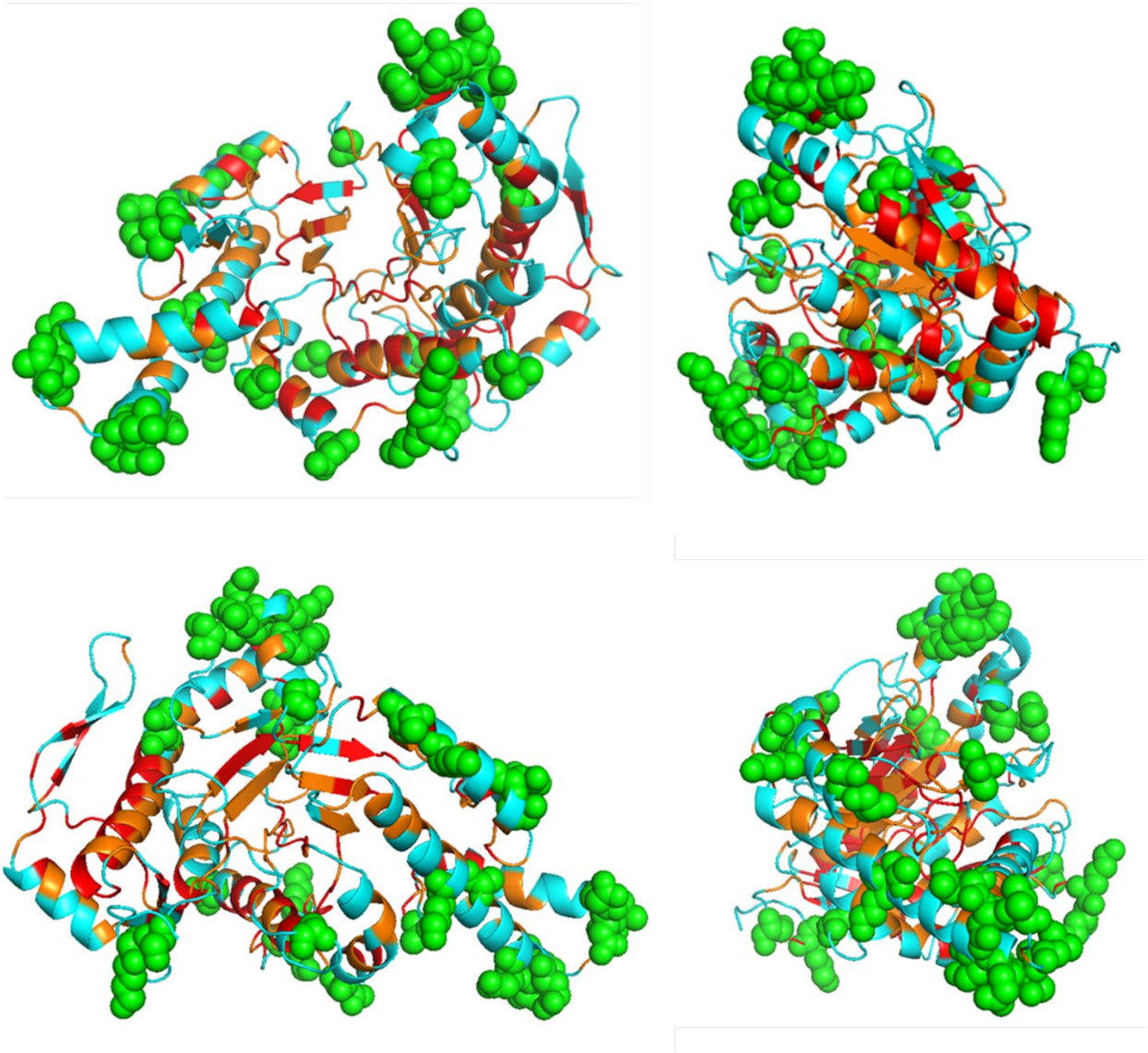
A. Maintain Activity

353. As I explained above, a POSA seeking to make a modified PH20 that simply maintains WT activity has a large spectrum of options based on the disclosure of the patent.

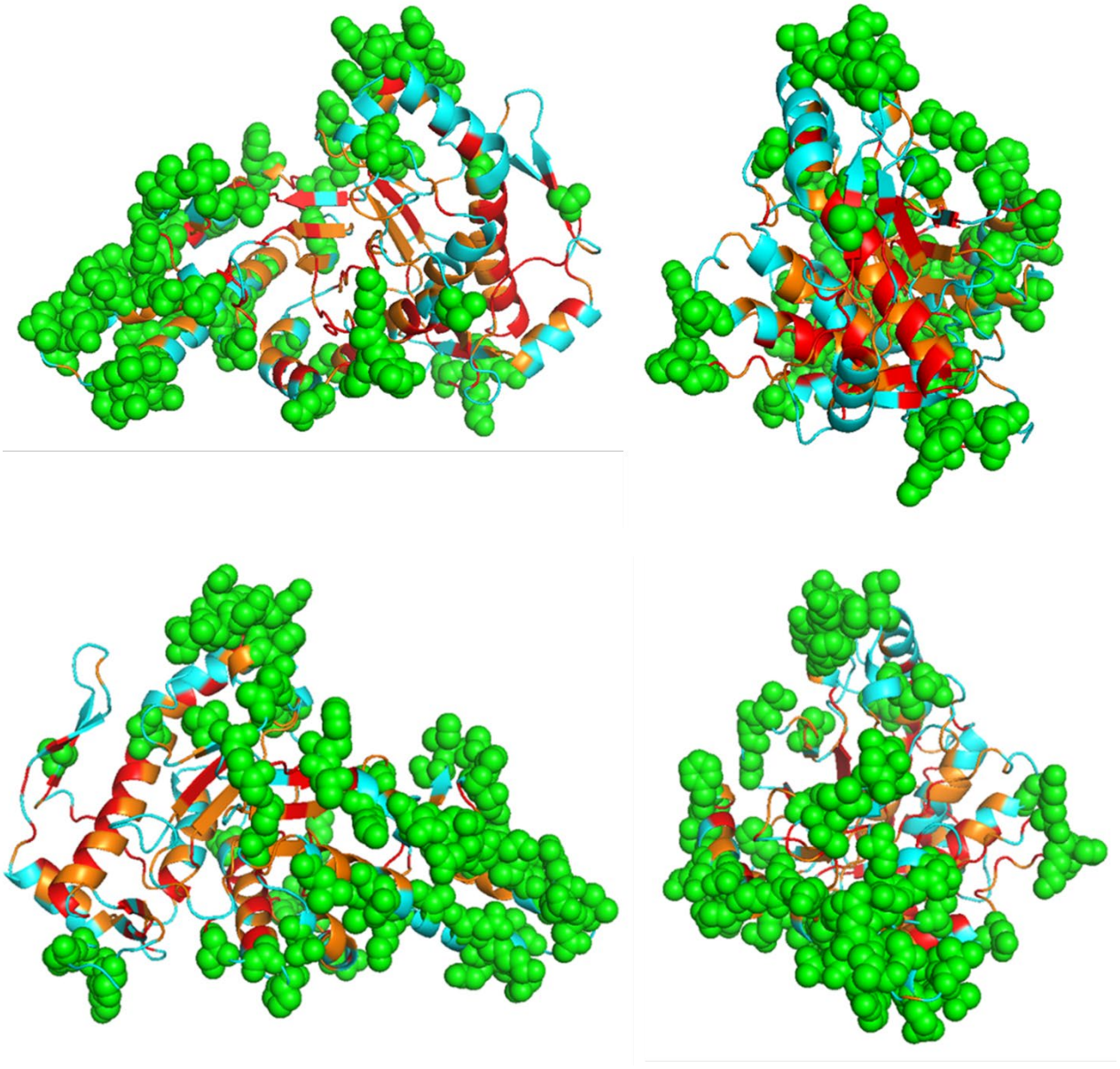
354. First, the POSA could generate any of the active mutants disclosed in the patent.

355. Second, the POSA wishing to make multiple modifications but retain activity could select from those positions that best tolerate substitution. The POSA seeking to be conservative in this regard could select positions that tolerated all

tested substitutions, as shown in the figures below. A POSA choosing this route has many potential substitution options to consider; as shown below, residues that tolerate all tested substitutions are scattered across the surface of the protein. A POSA would reasonably expect that introducing multiple substitutions at these positions would also not abrogate activity.

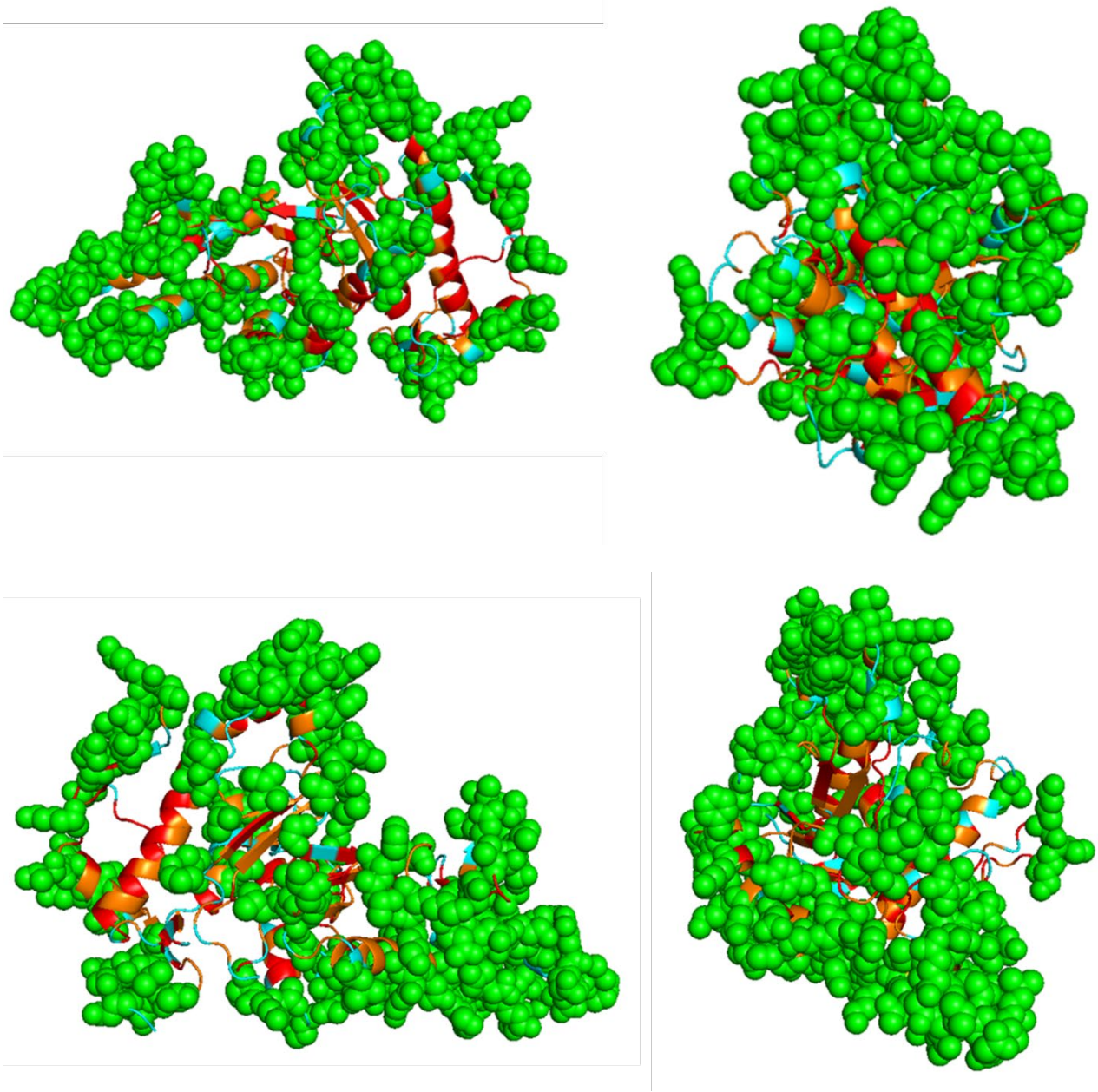


356. If a POSA wished to expand the set of mutated residues, the POSA could select from those that tolerated 90% or more of the tested substitutions, as shown below.



357. Again, the POSA would have a very large number of potential mutants to choose from, and the POSA would expect those variants to maintain activity.

358. The POSA also could select positions that tolerate 60% or more of the tested substitutions and still have a reasonable expectation of success in maintaining activity.



359. A POSA could select 23 residues to change and reasonably expect to obtain an active PH20 as those changes were based on the data in the patent. For

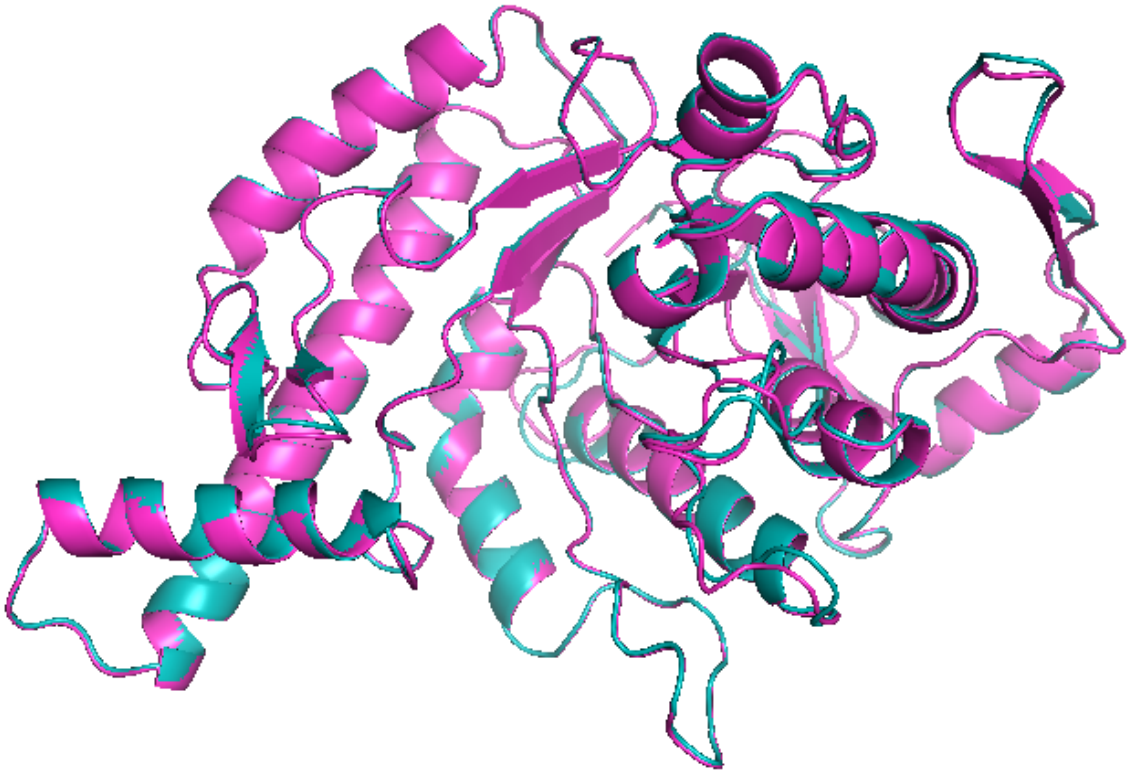
example, there are 40 positions that tolerated all tested substitutions, 87 that tolerated 90% or more and 158 that tolerated 60% or more. A POSA could select sets of 22 residues within this set based on the highlighted homology model that I described above and reasonably anticipate that most of the resulting proteins will be active.

360. The POSA seeking to make multiple mutations could generate a model using, for example, Swiss Model to evaluate the results of the anticipated modifications.

361. One way in which a POSA could obtain a modified PH20 and be quite confident of its activity is to take another primate PH20 and introduce the substitution at position 309 recited in the claims. This approach is disclosed in the patent at EX1001, 76:9-55. For example, the chimpanzee PH20 sequence disclosed in the patent has five modifications compared to human. EX1001, FIG. 2A. Introducing the I309N modification yields a modified PH20 with six substitutions compared to SEQ ID NO. 3.

362. Alternatively, the POSA could introduce the I309N mutation into gibbon PH20, the version of which disclosed in the patent has 16 mutations as compared to SEQ ID NO: 3. EX1001, FIG. 2J. Introducing the I309N substitution results in a protein with 17 substitutions as compared to SEQ ID NO: 3.

363. I used Swiss Model to generate a model of I309N gibbon PH20 using the gibbon PH20 sequence in the patent. I then aligned the resulting model to the PH20 model I generated in PyMOL. The figures below show that the two proteins are expected to have extremely similar structures, despite their 4% variation in sequence:





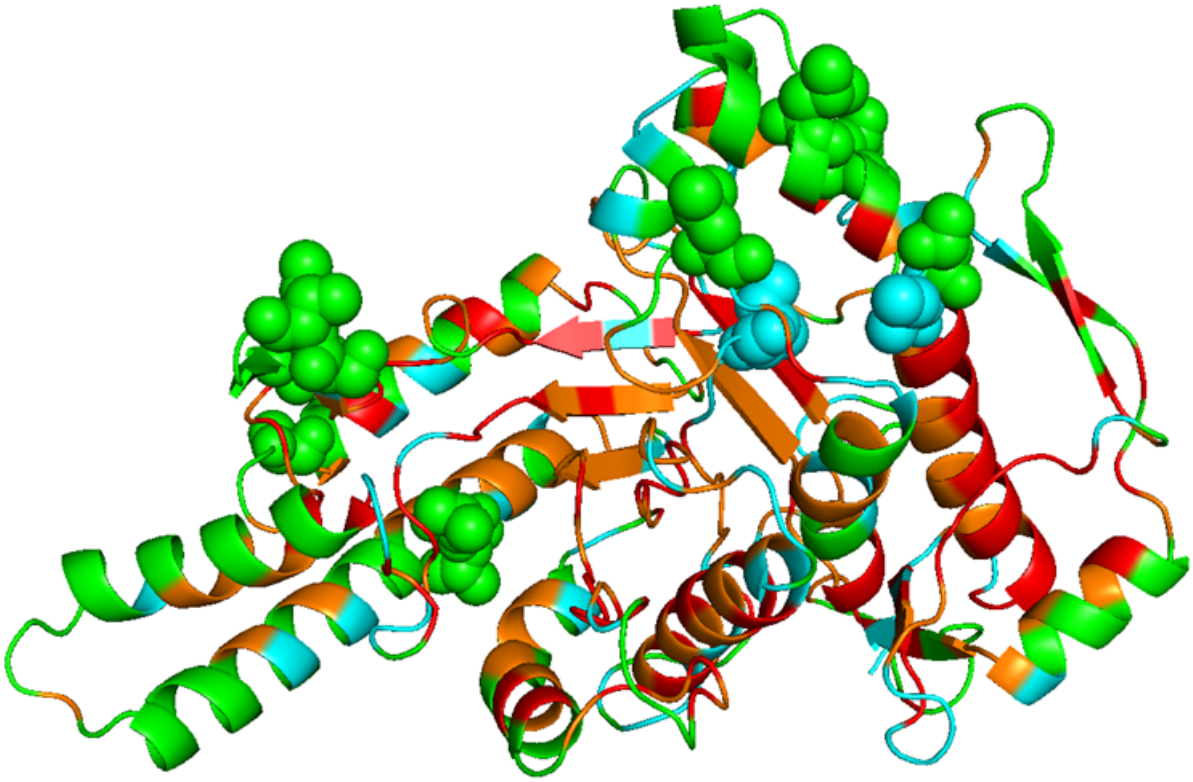
364. The POSA also could use the orangutan PH20 sequence disclosed in the patent as the starting point. Comparing the first 447 residues of orangutan PH20 to the 447 residues of SEQ ID NO: 3 shows that orangutan PH20 has 13 modifications as compared to SEQ ID NO: 3. EX1001, FIG. 2L. Introducing the I309N mutation into that protein provides another means of generating a 97% identical polypeptide that the POSA expects to be active.

365. If the POSA wished to make deletions or insertions and retain activity, there are ways to do that as well. For example, a POSA would expect that hairpin loops comprising tolerant residues will likely tolerate an insertion or deletion, and the ’791 patent discloses C-terminal truncations that are active as well.

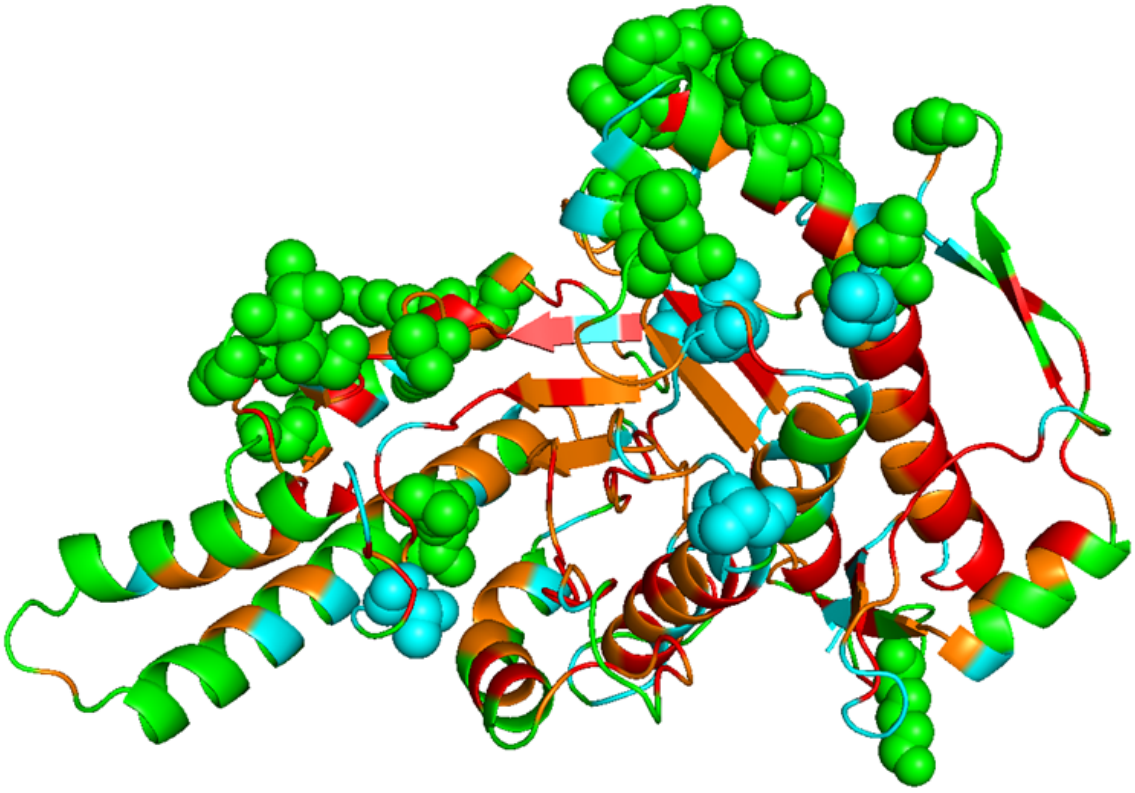
B. Increase Activity

366. A POSA seeking to increase activity has many potential avenues to explore with the ’791 patent in hand. As shown in the figures below, the patent teaches multiple clusters of positions where substitutions increased activity by more than 2-fold, more than 3-fold or more than 5-fold. Some substitutions increased activity by more than 10-fold.

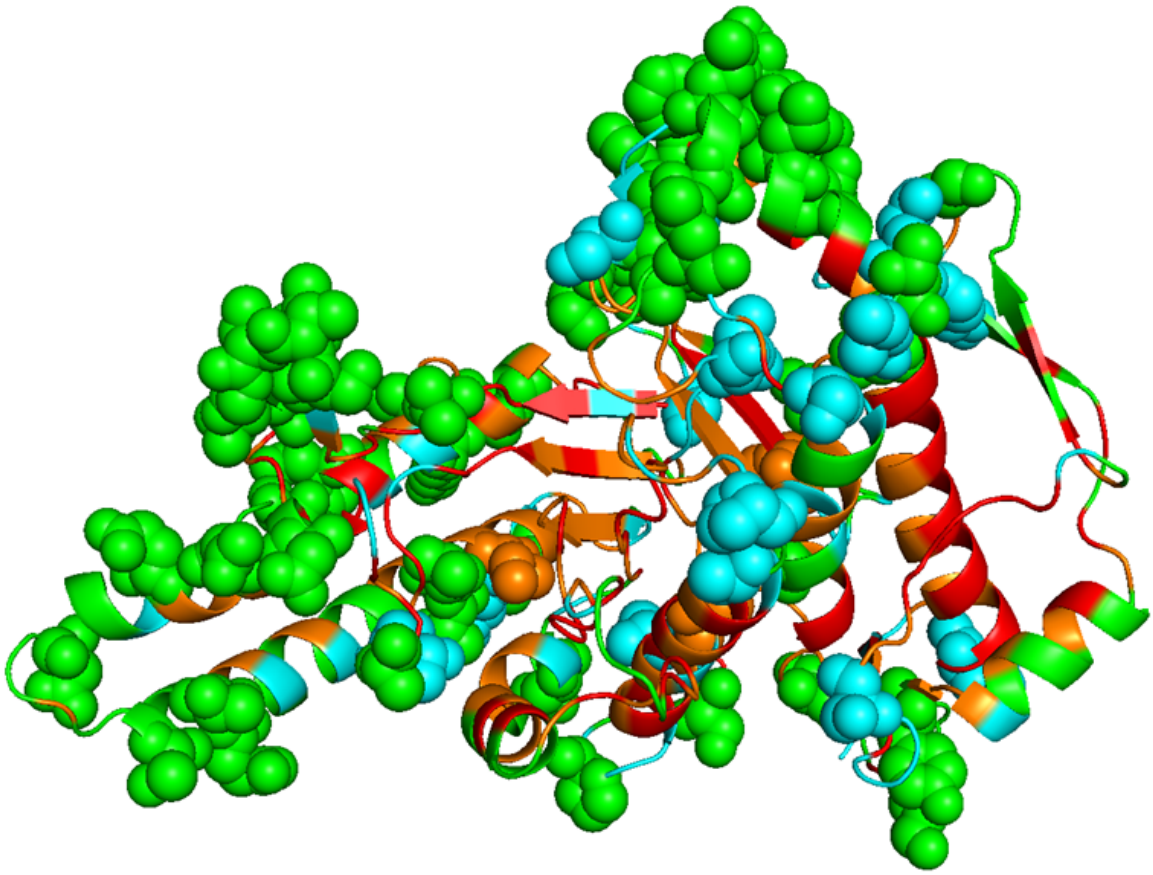
367. The figure below shows those positions in which substitution improved activity more than five-fold in sphere format:



368. The figure below shows those positions in which substitution improved activity more than three-fold in sphere format:



369. The figure below shows those positions in which substitution improved activity more than two-fold in sphere format:



370. A POSA seeking to improve HA activity could consider all of those potential changes, both individually and potentially in tandem.

371. Interestingly, as I noted above, some positions have multiple substitutions that substantially increased activity. A POSA may look at those positions with particular interest as it appears that there is potential for improvement at that residue, and that combining such substitutions with those at one or more other positions of similar behavior might give additive improvement.

372. I also note that there are three orange residues that more than doubled activity. The POSA may also have interest in those residues because, despite their low tolerance for change, they do have the potential to increase activity.

373. The POSA also may consider making one or more changes that were found to increase activity for other reasons. For example, a POSA concerned with stability issues resulting from deamidation of asparagines may seek to remove asparagines from the sequence, and in an effort to counterbalance an impact on activity the POSA may introduce one or more substitutions that increased activity. The patent shows which asparagines can safely be altered, and also warns not to touch those involved in essential glycosylation modifications.

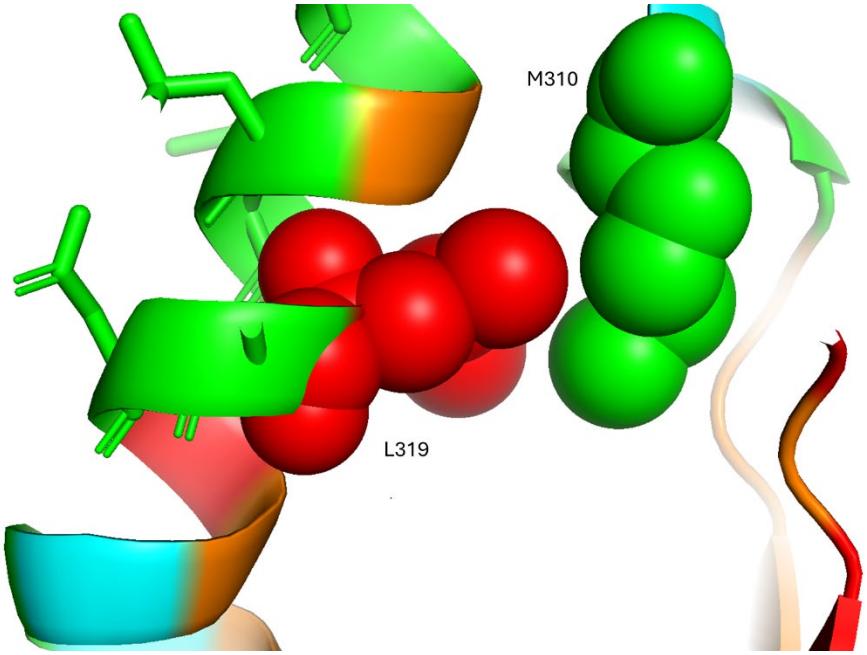
C. Rescue Mutations

374. The patent provides valuable data for identifying potential rescue mutations. To determine likely candidates for rescue mutations, a POSA would have examined the PH20 model to determine why activity was lost for a particular substitution. If the interactions that were lost can be replaced by simultaneously making another substitution (*i.e.*, a "rescue mutation"), then activity may be restored. This is something that frequently seems to happen in evolution. Examples that a POSA would have found particularly interesting are positions that never tolerate substitution when singly altered. Based on the patent data, two residues that normally interact with each other, and, when altered individually, were found not to tolerate any substitutions, are good candidates for rescue mutations. The POSA could have identified the interactions that these two

residues make with each other and surrounding residues and made simultaneous substitutions intended to replicate those interactions to restore enzymatic activity.

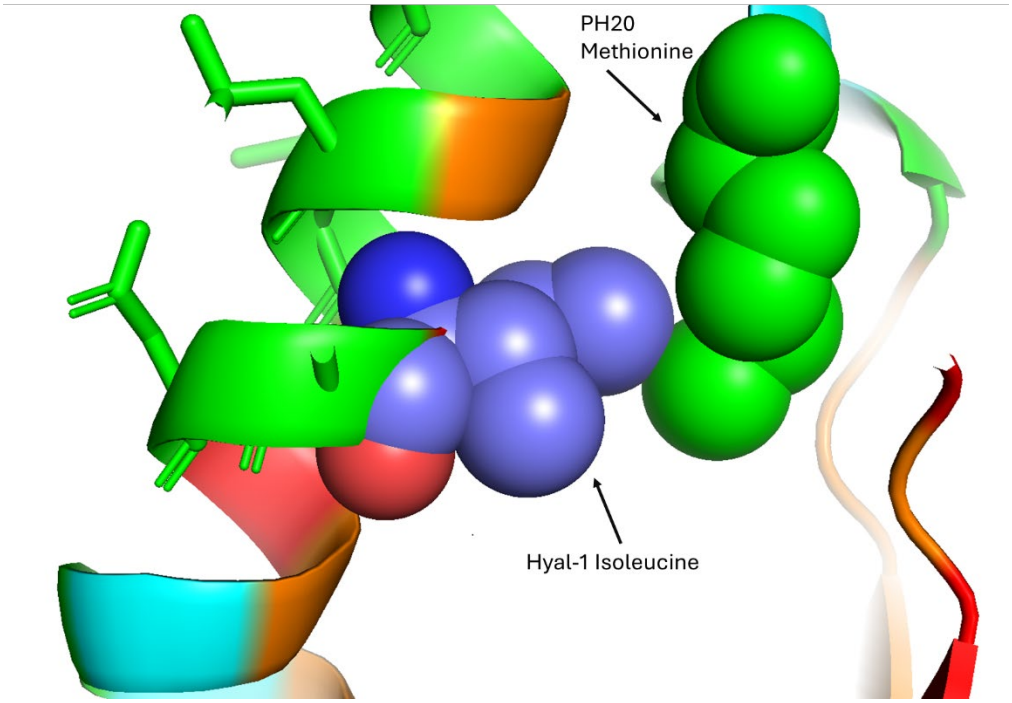
375. One example of how a POSA could have identified a potential rescue mutation using the patent data is described below.

376. The figure below shows M310 and L319 in the PH20 model, with those two residues shown in sphere format.



377. The ’791 patent teaches that all substitutions tested at position 319 inactivated the protein, including L319I (leucine to isoleucine), which is typically considered to be a conservative substitution. The PH20 model indicates that mutation was not tolerated because it interferes with the packing in this region of the protein. In fact, it is possible to illustrate this concept using the Hyal-1 structure (which as I described above was aligned to the PH20 model in PyMOL).

The Hyal-1 structure has an isoleucine instead of a leucine at that position. In the figure below, the leucine at position 319 in the PH20 model is "hidden," and the isoleucine at that position in Hyal-1 is shown in sphere format. As the figure shows, the isoleucine and methionine clash.



378. The figure above illustrates the concept that isoleucine at position 319 may not be compatible with methionine at position 310 because of packing issues.[20]

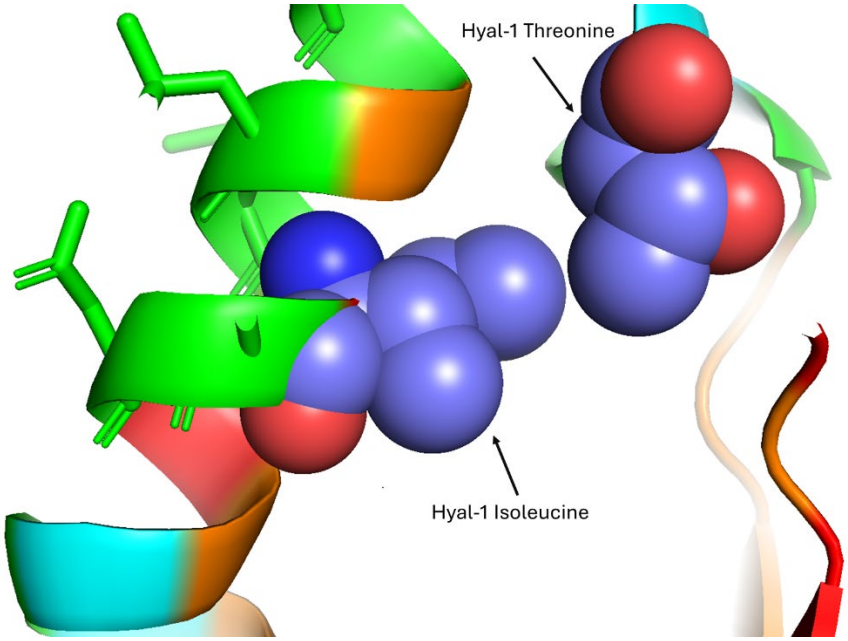
379. The POSA seeking to change L319 to, for example, isoleucine, would investigate replacing the methionine at position 310. And the '791 patent discloses

[20] The figure above shows residues in the Hyal-1 structure superimposed on the PH20 structure and is meant for illustrative purposes. I am not attempting to model what would happen if leucine in position 319 in PH20 is replaced with isoleucine.

residues that were shown to retain (or even improve) activity in position 310. Notably, changes to alanine, glycine and serine improved activity, with the glycine substitution improving activity by 273%.

380. These data suggest that a smaller residue (smaller than methionine) at position 310 would rescue the L319I mutation.

381. Again, the Hyal-1 structure provides insight. Hyal-1 has a threonine instead of a methionine at that position. In the figure below, the leucine and methionine in PH20 are hidden and the threonine and isoleucine in Hyal-1 are shown in sphere format. The threonine and isoleucine do not clash. This analysis supports the concept that the packing issues resulting from introducing isoleucine at position 319 could be ameliorated by changing the methionine at position 310 to a smaller residue.



382. In view of the above, the POSA would have expected that changing the methionine to glycine at position 310 would rescue the leucine to isoleucine substitution at position 319. And the POSA would expect an increase in activity by mutating methionine 310 to glycine.

383. Because changing methionine to serine was shown to improve activity and because Hyal-1 has a threonine at that position, which is compatible with an isoleucine at position 319, the POSA would have reasonably expected changing leucine 319 to isoleucine while at the same time changing methionine 310 to threonine would have resulted in an active modified PH20.

D. C-Terminal Truncation Sequences Recited in the Claims

384. I have been asked to consider the extent to which the results disclosed in the specification for a given substitution in SEQ ID NO: 3 would apply if that substitution were made in one of the other recited sequences.

385. I explained above which sequences I expect to be active, which I expect to have reduced activity, and which I expect to be inactive. I have categorized the recited sequences accordingly in the chart below:

SEQ ID NO.	C term Position (Precursor sequence)	C term Position (Mature sequence)	Expected Activity
32-33	465-466	430-431	Inactive
34-35	467-468	432-433	Reduced activity

36-55 & 3	469-489	434-454	Active
56-63	490-497	455-462	Reduced activity
64-66	498-500	463-465	Little to no activity

386. The POSA would expect that substitutions will have essentially the same effect across all active polypeptides.

387. The POSA would expect that substitutions will have essentially the same effect across all inactive polypeptides (meaning that the resulting polypeptide will still be inactive). That is because the lack of activity is due to the C terminus of the protein, and the POSA would not expect to fix the C-terminal issues by making changes elsewhere in the sequence.

388. The POSA would expect that making substitutions in polypeptides with C-terminal truncations that result in reduced activity (*e.g.*, truncations such that the polypeptide ends at positions 433 or 432) would exhibit reduced activity as compared to, for example, the polypeptide of SEQ ID NO: 3 with those substitutions.

XII. COMMON STRUCTURAL FEATURES

389. As explained above, claim 1, which I understand is the broadest claim, is directed to modified PH20 polypeptides with 95% identity to one of the recited sequences and the recited change at position 309 (from isoleucine to either

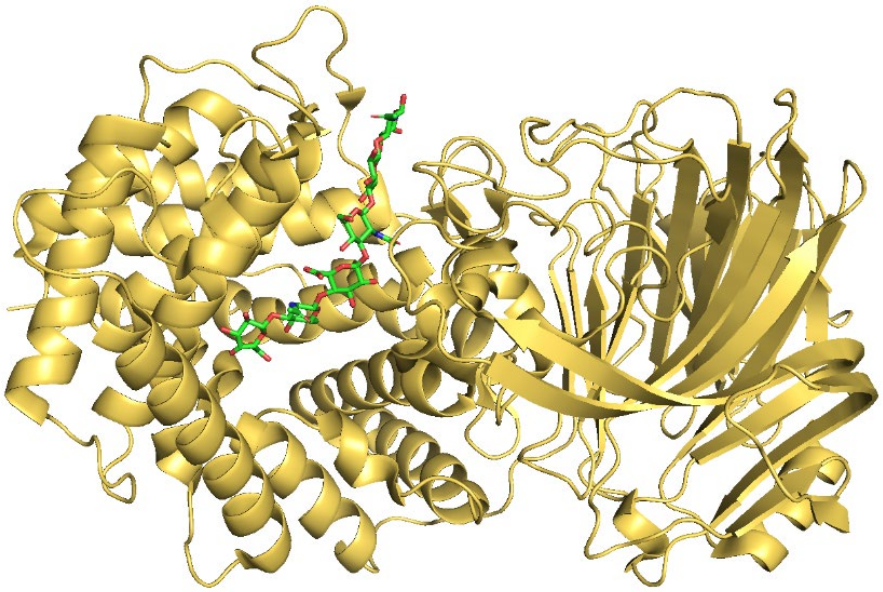
glutamic acid, glycine, histidine, leucine, methionine, asparagine, glutamine, arginine, serine or threonine).

390. POSAs understand that the set of claimed modified PH20 polypeptides that satisfy the claims share many common structural features, as I explain below.

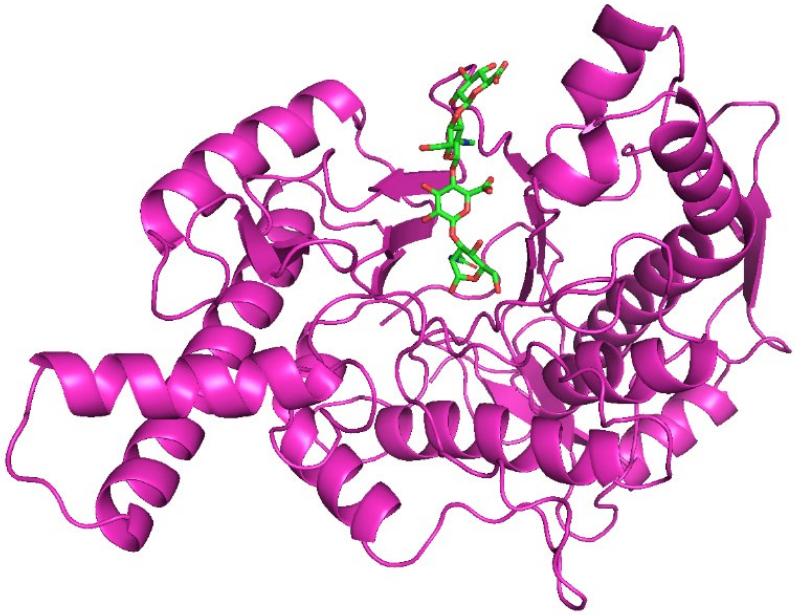
391. POSAs think of the class of modified PH20 proteins sharing 95% of their sequence (including the recited change at position 309) as an extremely limited set of proteins. Those that fold are a limited set of structurally homogeneous proteins.

392. To put this in perspective, consider a different type of hyaluronidase. *Streptococcus pneumoniae* hyaluronate lyase has a different tertiary structure, as shown in the figure below.[21] This enzyme also has a different mechanism of action—it is a lyase, not hydrolase.

[21] The figure shows the structure of *Streptococcus pneumoniae* Hyaluronate Lyase in Complex with Hexasaccharide Hyaluronan Substrate (pdb entry number 1LOH) (EX2178).



393. The bacterial hyaluronidase looks very different from PH20 (and Hyal-1). For reference, the image below shows the PH20 model with the HA substrate.



394. PH20 and the bacterial hyaluronidase shown above are not homologous. They share a common function in that they cleave HA, but they do so in different ways.

395. For additional perspective, consider PH20 and Hyal-1, which both cleave HA through hydrolysis and have a common fold. Not only that, they share common structural features down to the amino acid level. In some cases there are common features at the atom level in that, in some positions, the same atoms are making essentially the same interactions in all three proteins. That is the case even though more than half of their amino acids (and thus more than half of their primary structure) are different. The critical residues that enable them to fold and function are conserved.

396. The claimed 95% identical PH20s are significantly more similar to each other than PH20 is to Hyal-1. They share a substitution at position 309. And enzymatically active PH20s will adopt the same alpha-beta barrel fold. They will cleave HA through the same mechanism of action using the same chemistry carried out by the same active site residues oriented in about the same way. And they share more than the critical residues necessary for folding and function: they share at least 95% of a common primary structure.

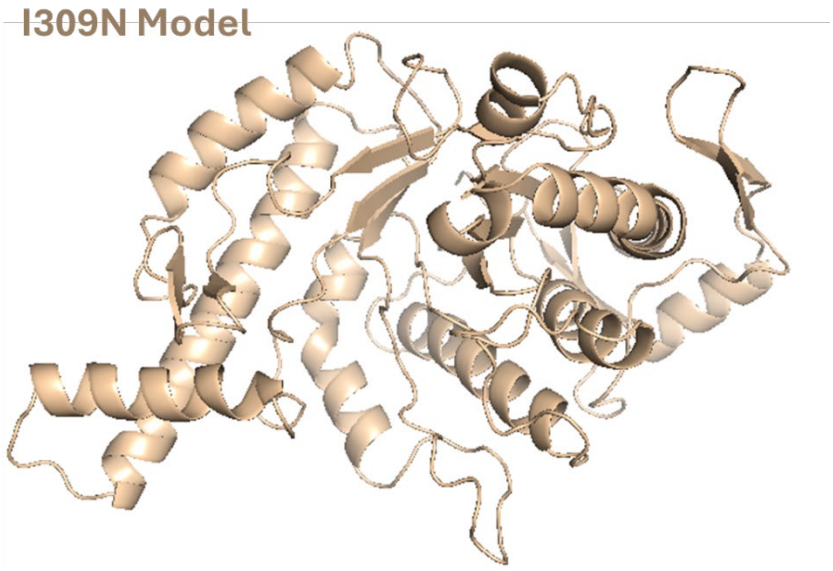
397. The claimed modified PH20 polypeptides (covered by claim 1) share the common structure of a modification at position 309 to either glutamic acid,

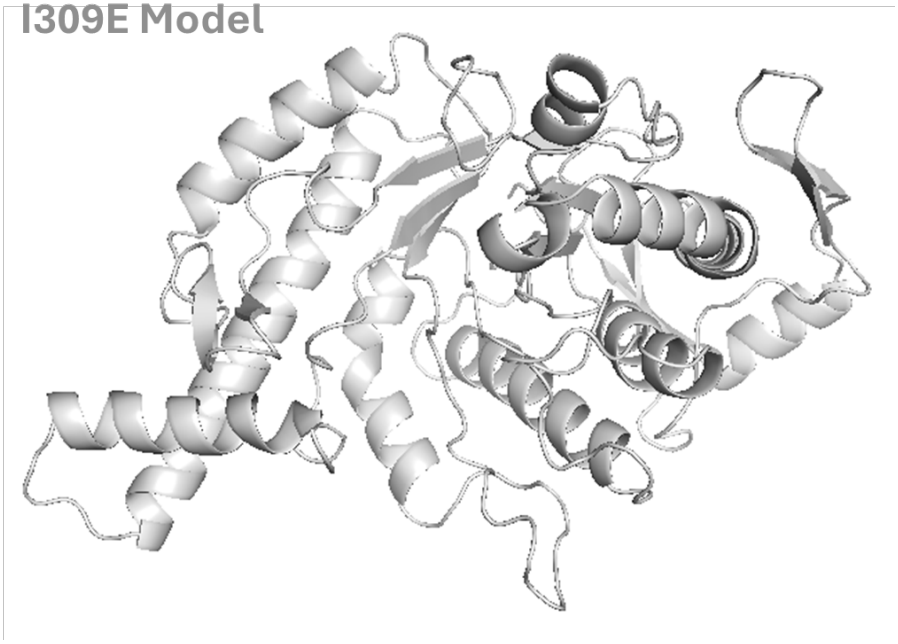
glycine, histidine, leucine, methionine, asparagine, glutamine, arginine, serine or threonine. To illustrate this, I generated homology models of the I309E, I309G, I309H, I309L, I309M, I309N, I309Q, I309R, I309S and I309T variants using Swiss Model.

398. To generate the I309N PH20 model, I used SEQ ID NO: 3 disclosed in the patent. I converted the sequence in the sequence text file into FASTA format and then introduced the I309N mutation into the text file. I then generated a homology model using Swiss Model. I used 2PE4 as the template just as I did for the WT PH20 model. I examined the resulting model, downloaded the model, opened it in PyMOL and aligned it to the 2PE4 structure in PyMOL. I then proceeded with my analysis.

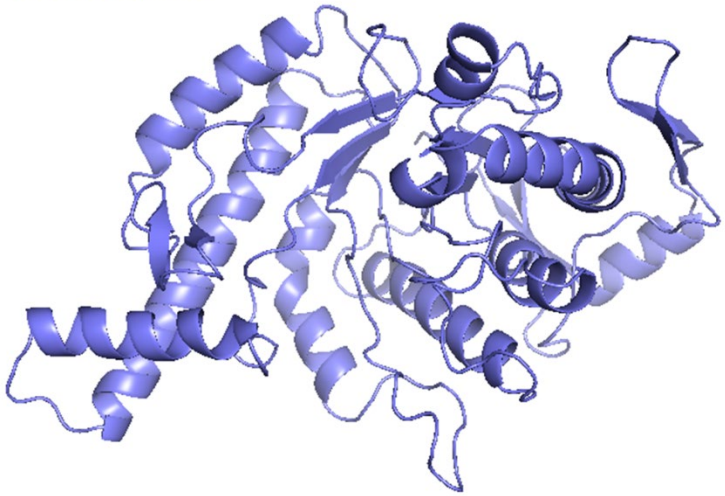
399. I followed the same procedure (making the appropriate mutation in the sequence for each model in the text file) to generate homology models for I309E, I309G, I309H, I309L, I309M, I309Q, I309R, I309S and I309T.

400. In the figures below, I show that all ten PH20 variants share the same common alpha-beta barrel fold as WT PH20.

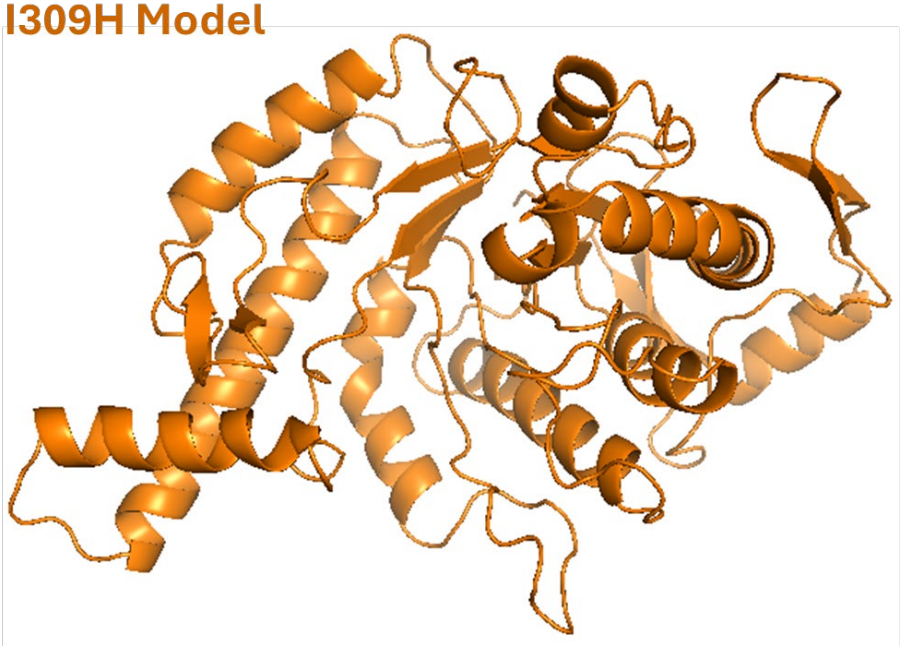

I309N Model


I309E Model

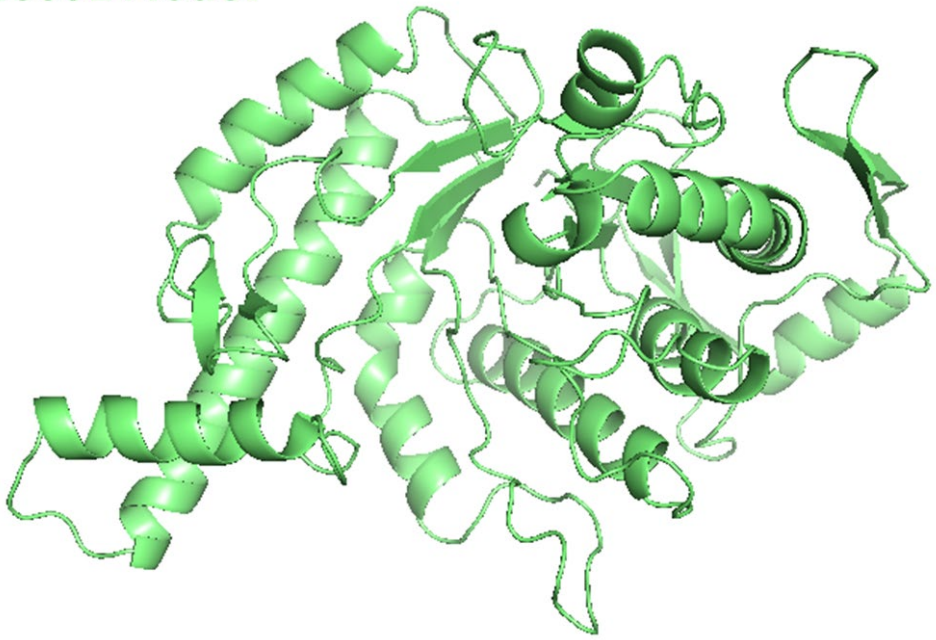
I309G Model



I309H Model



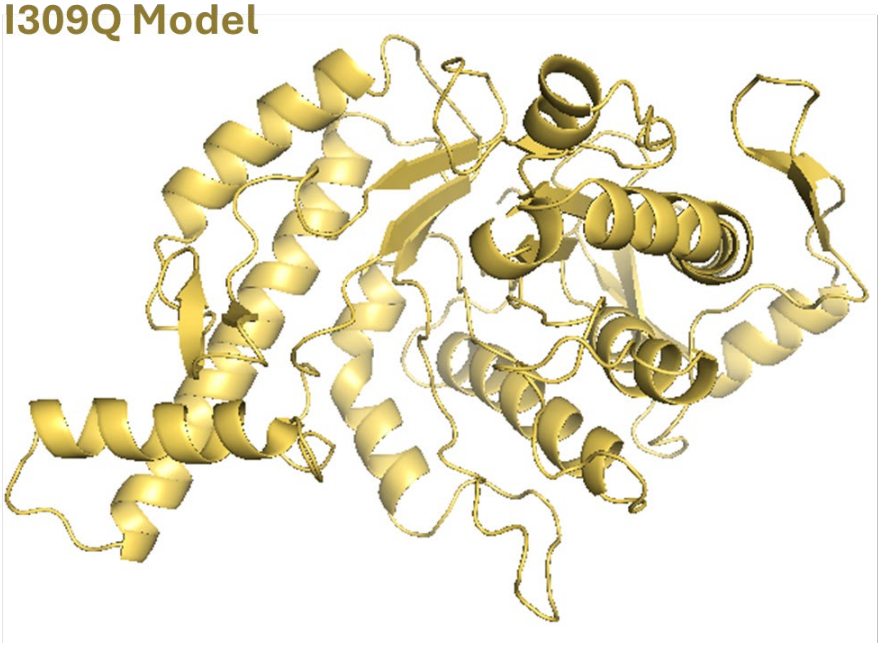
I309L Model



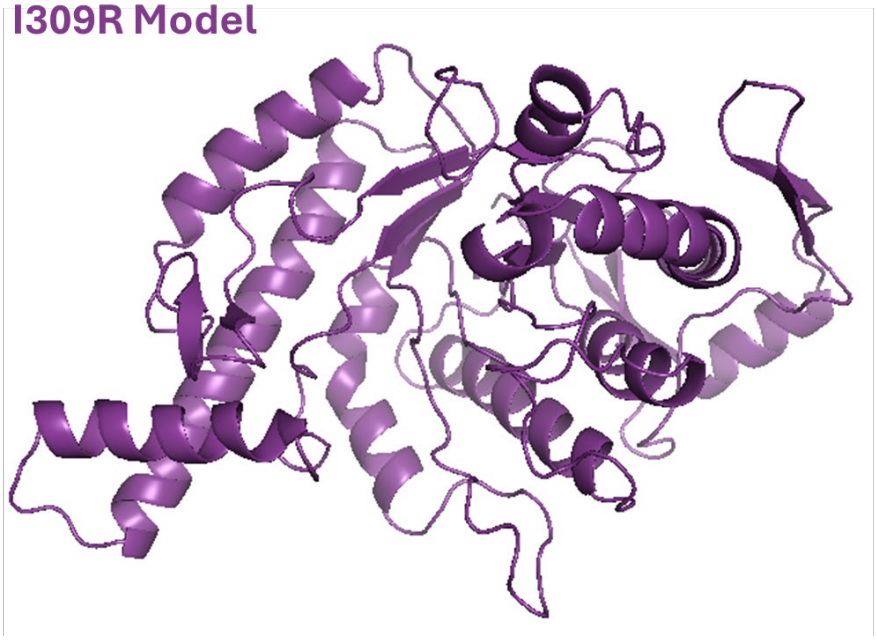
I309M Model

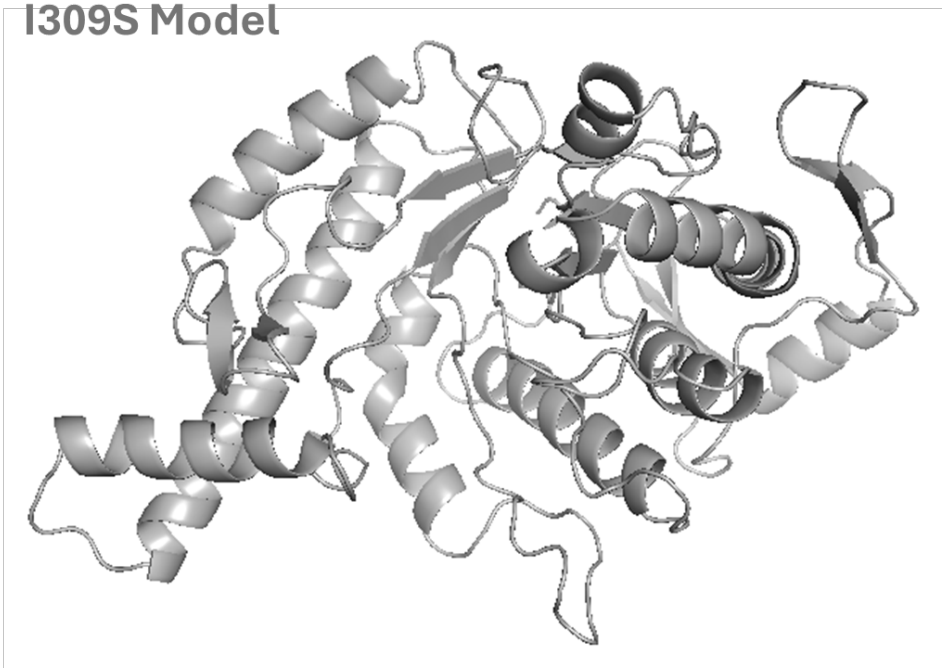


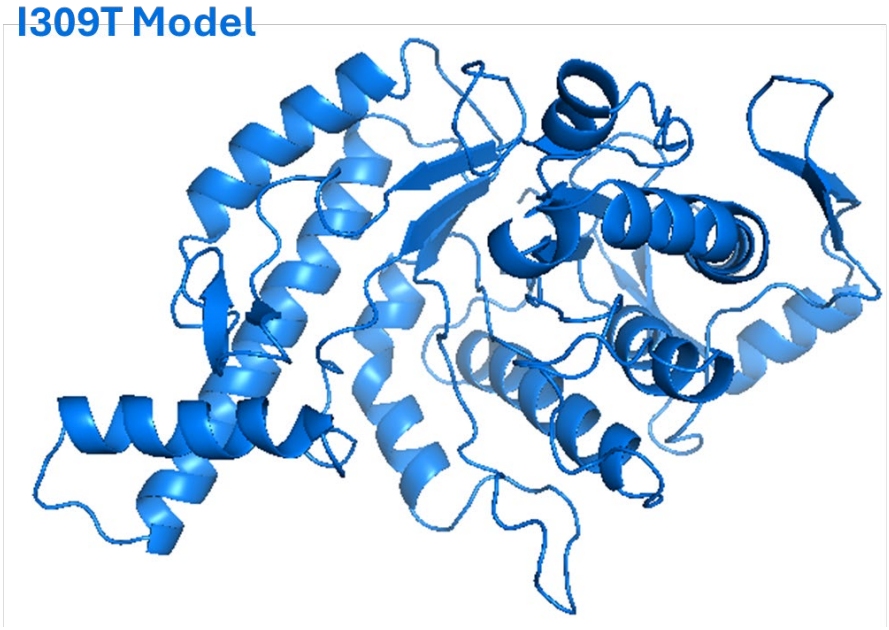
I309Q Model



I309R Model

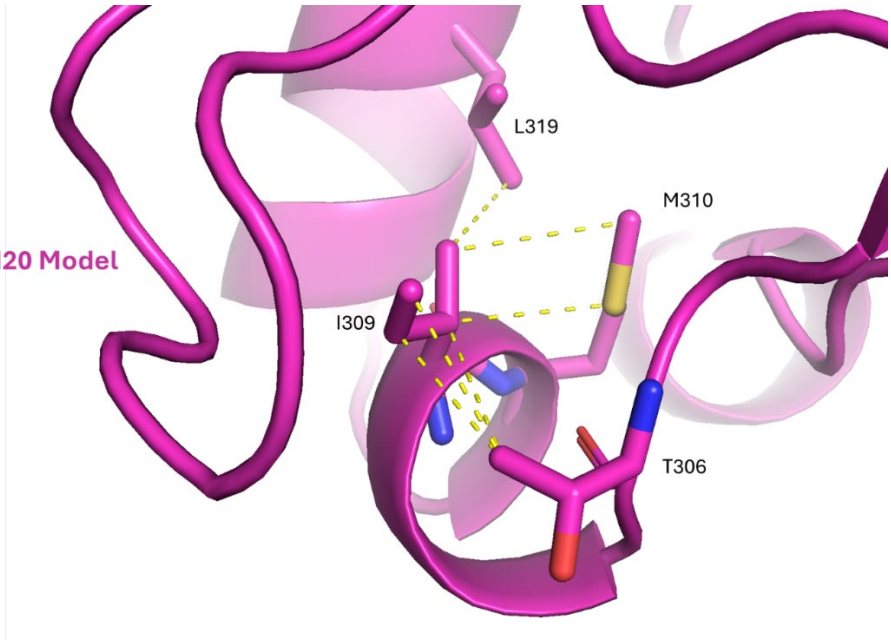






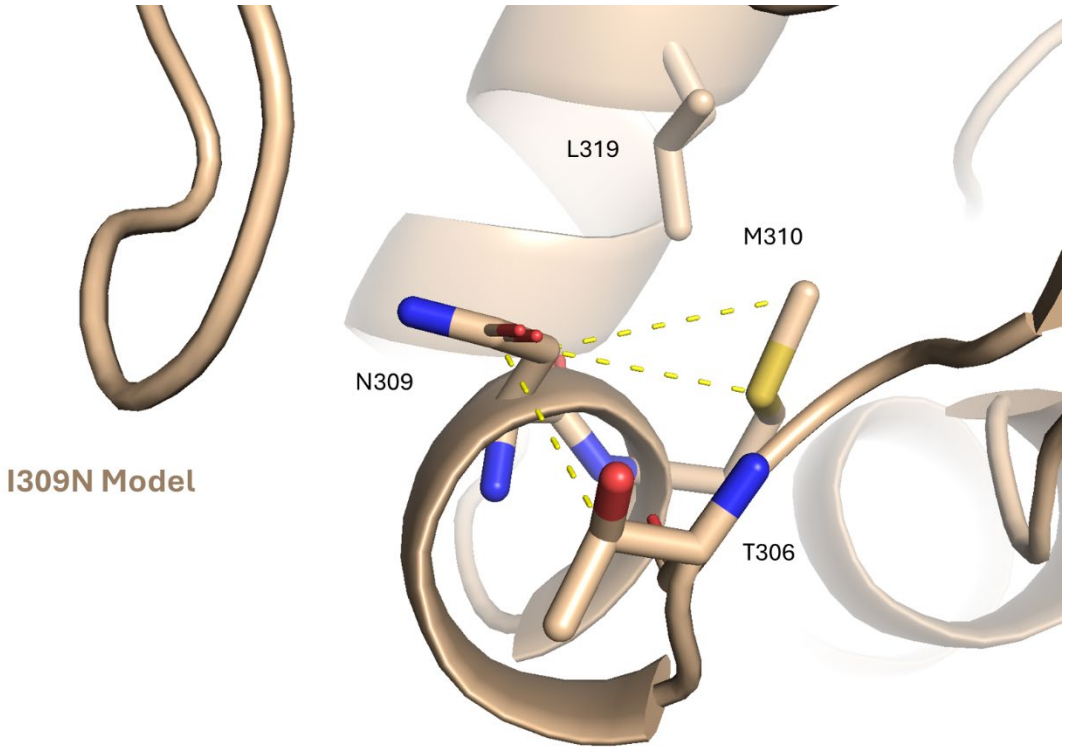
401. In the figures below, I show how the residue at position 309 and the surrounding residues are oriented in each of the variant models.

402. In the figure below, , I show I309, T306, M310 and L319 in the WT PH20 model.

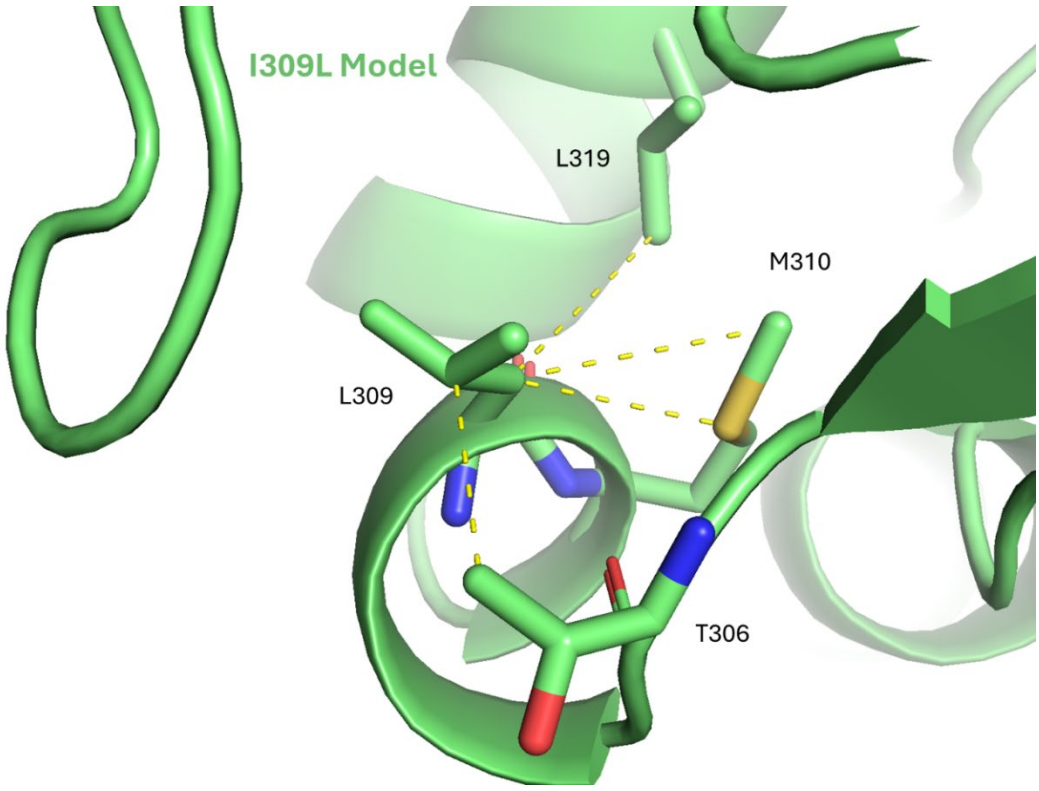


403. As shown in the figure above, isoleucine at position 309 is forming van der Waals interactions with T306, M310 and L319.

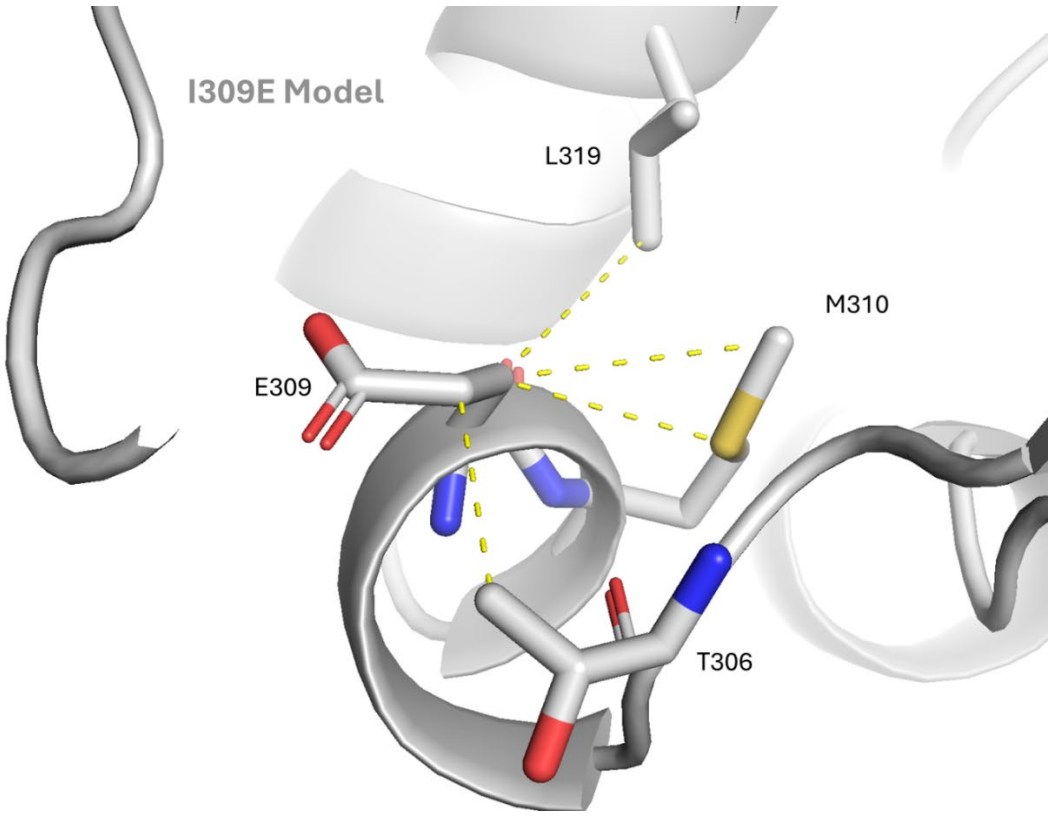
404. As shown in the figure below, for the I309N model, asparagine at position 309 is forming van der Waals interactions with T306 and M310.



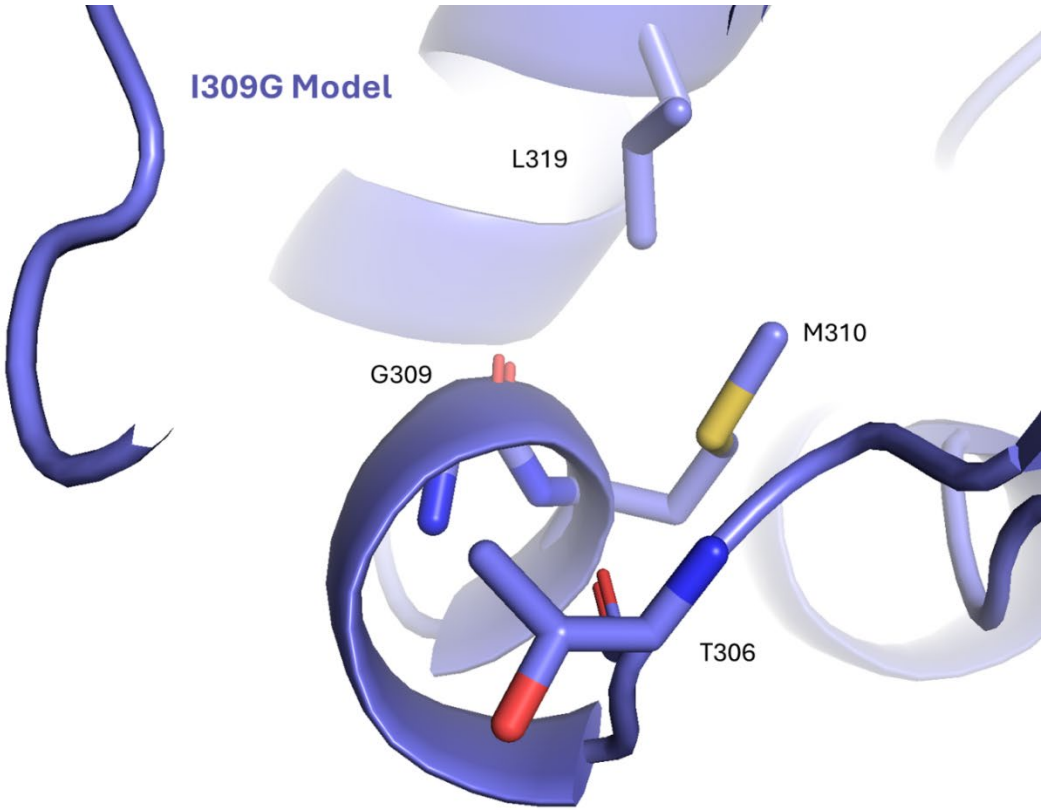
405. As shown in the figure below, for the I309L model, leucine at position 309 is forming van der Waals interactions with T306, M310 and L319.



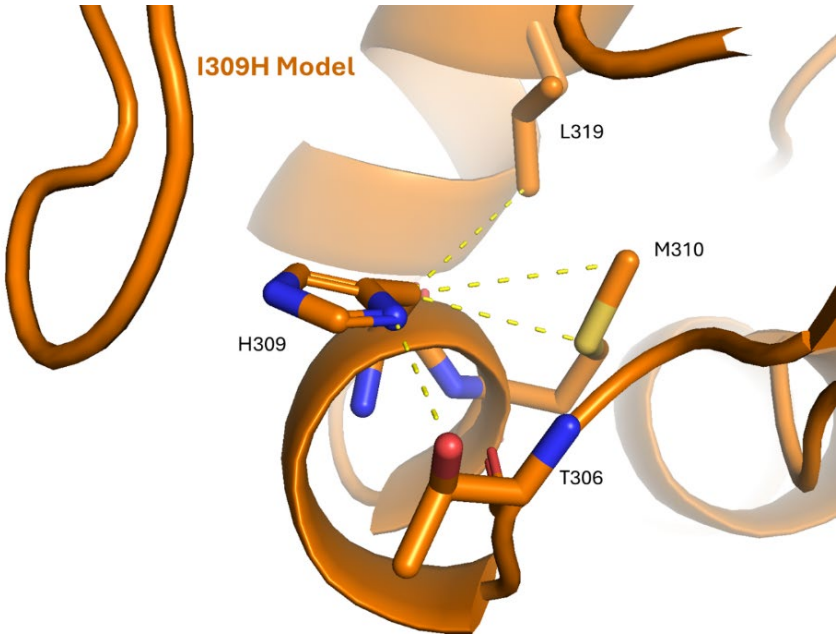
406. As shown in the figure below, for the I309E model, glutamate at position 309 is forming van der Waals interactions with T306, M310 and L319. .



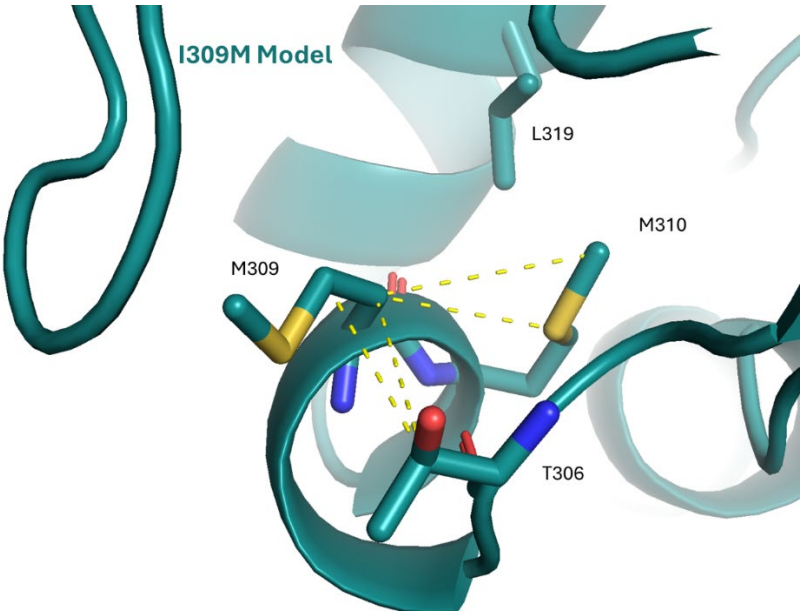
407. In the figure below, in the I309G model, glycine at position 309 does not make the interactions that the isoleucine made. .



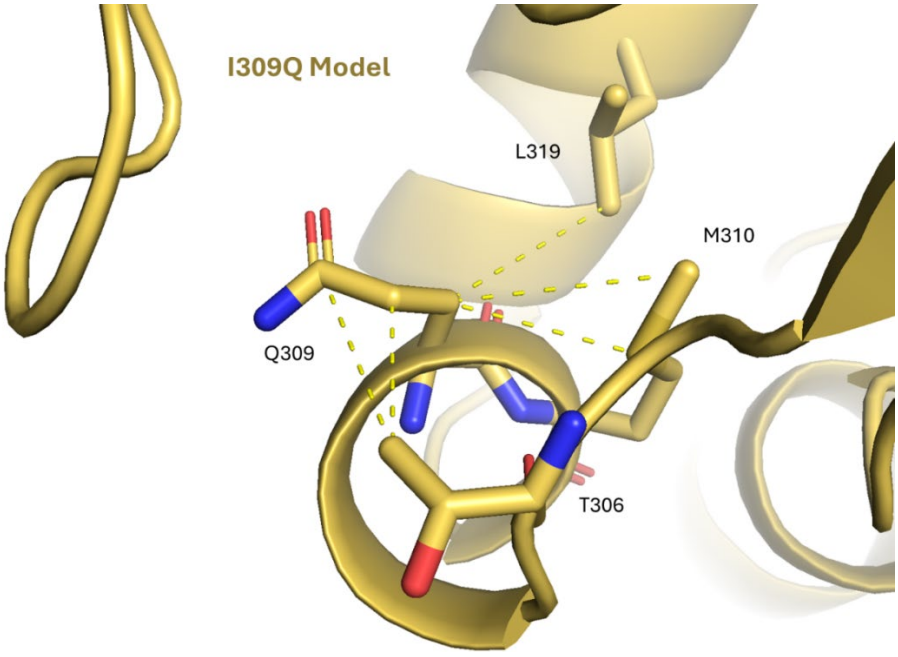
408. In the figure below, in the I309H model, the histidine at position 309 is forming van der Waals interactions with M310 and L319 and is forming an interaction with T306.



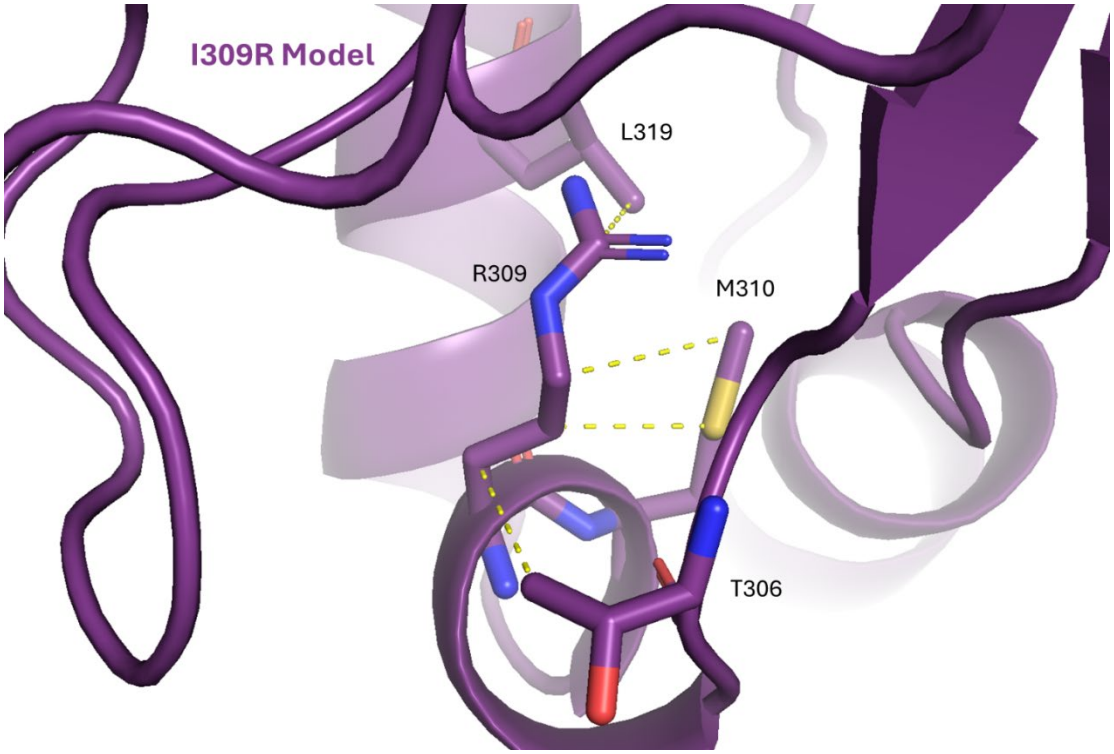
409. In the figure below, in the I309M model, the methionine at position 309 is forming van der Waals interactions with T306 and M310.



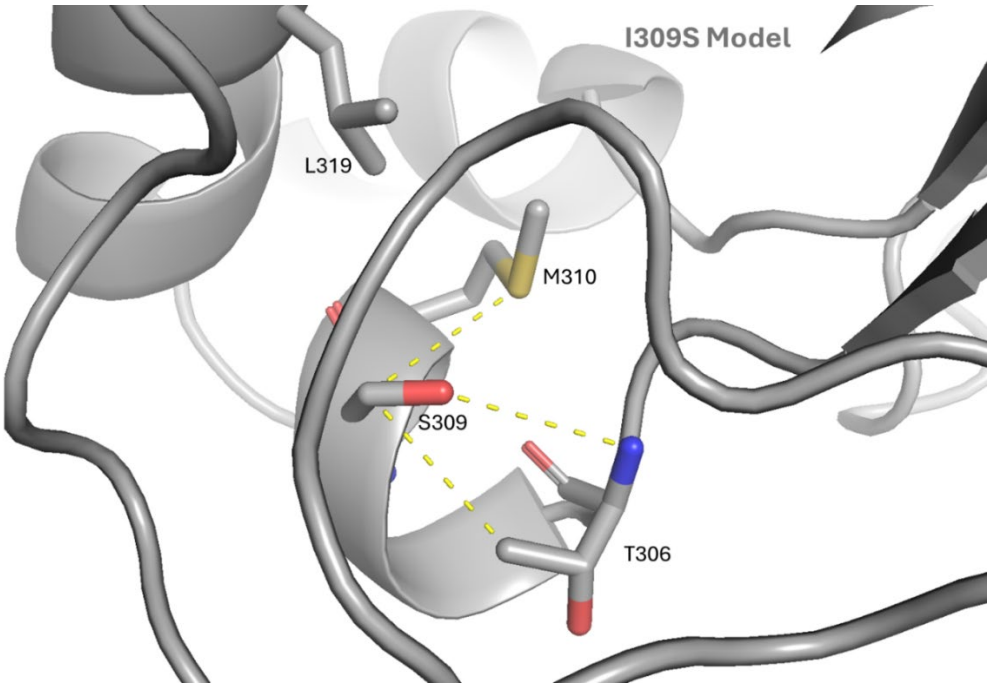
410. In the figure below, in the I309Q model, the glutamine at position 309 is forming van der Waals interactions with T306, M310 and L319.



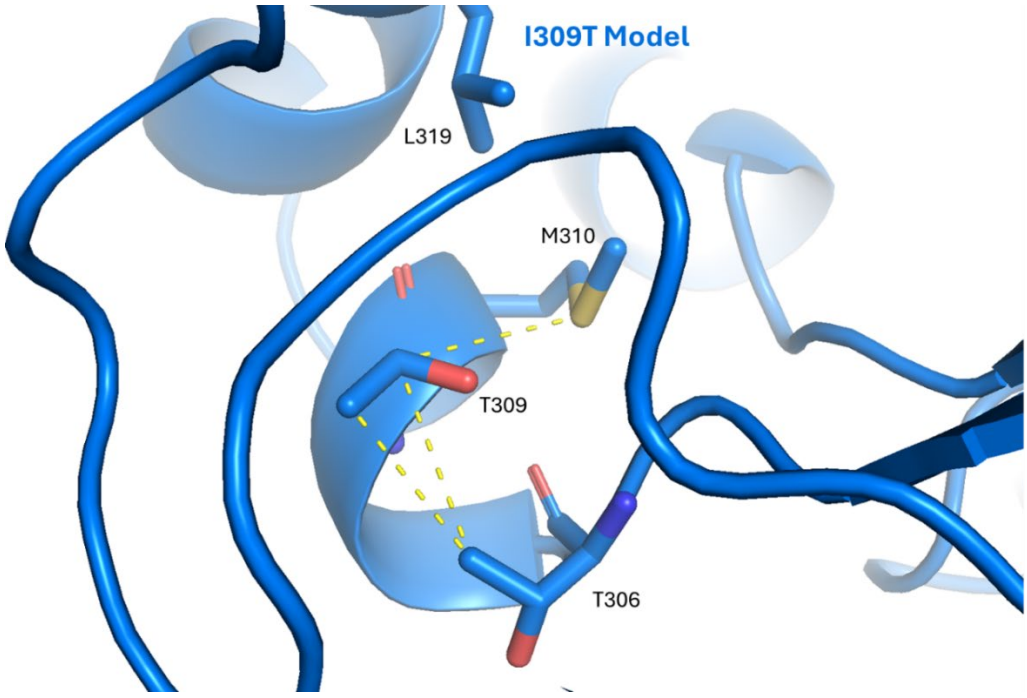
411. In the figure below, in the I309R model, the arginine at position 309 is forming van der Waals interactions with T306, M310 and L319.



412. In the figure below, in the I309S model, the serine at position 309 is forming interactions with T306 and M310.

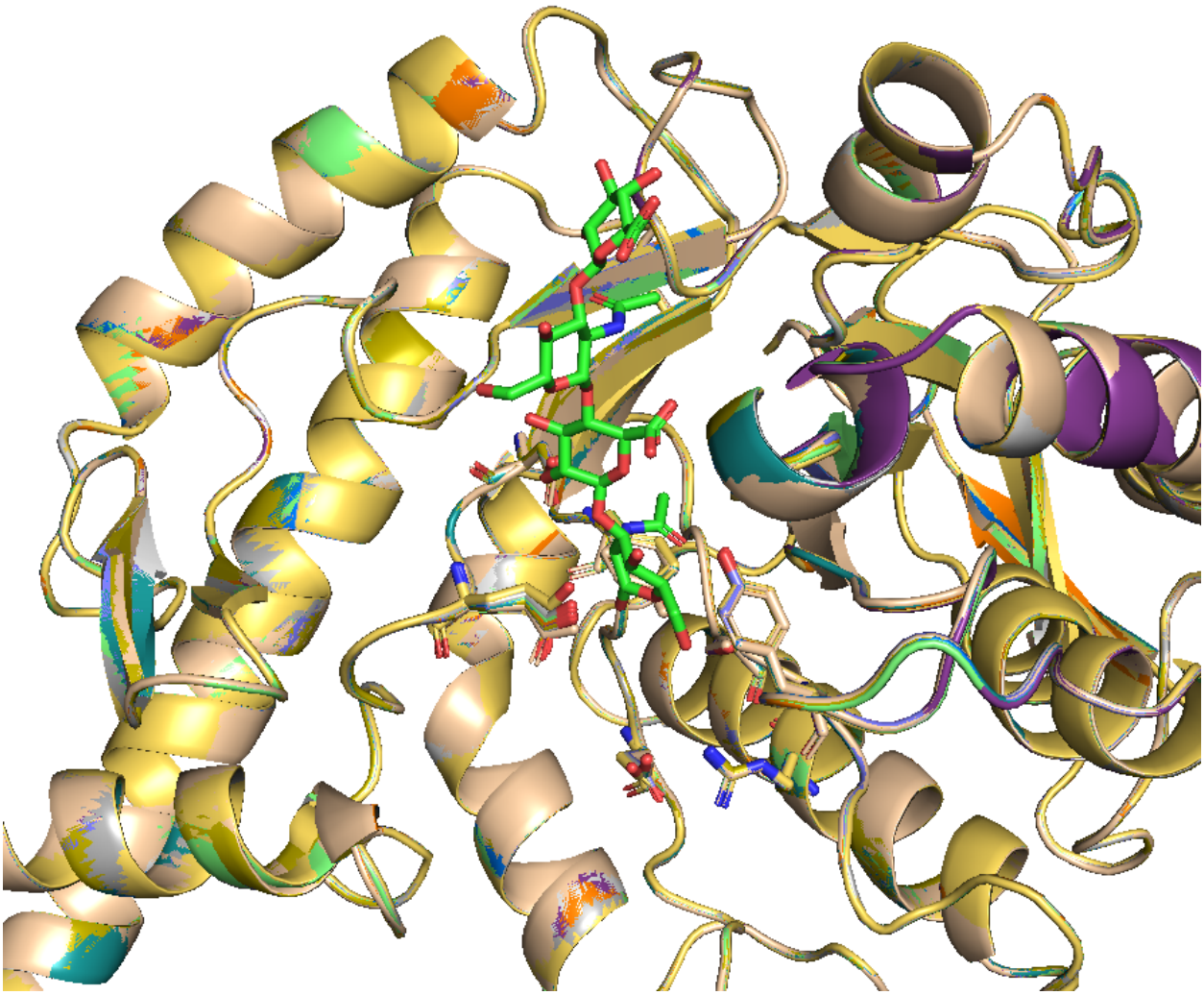


413. In the figure below, in the I309T model, the threonine at position 309 is forming van der Waals interactions with T306 and M310.



414. As shown above, the I309E, I309G, I309H, I309L, I309M, I309N, I309Q, I309R, I309S and I309T variants share a common structural feature in that they have a substitution at position 309.

415. In the figure below, all ten I309 variant models are shown on top of each other. The figure shows that the key active region residues I described above adopt essentially the same position in all ten variant models, illustrating how the variants adopt a common structure in the active site:



416. The figures above show that the claimed I309 variants share common structural features in terms of their primary, secondary and tertiary structures.

417. To further illustrate the common structural features shared by the claimed polypeptides, I generated models of several I309N variant PH20s that satisfy the sequence identity requirements of claim 1. Specifically, I generated models for the following multiply modified PH20s:

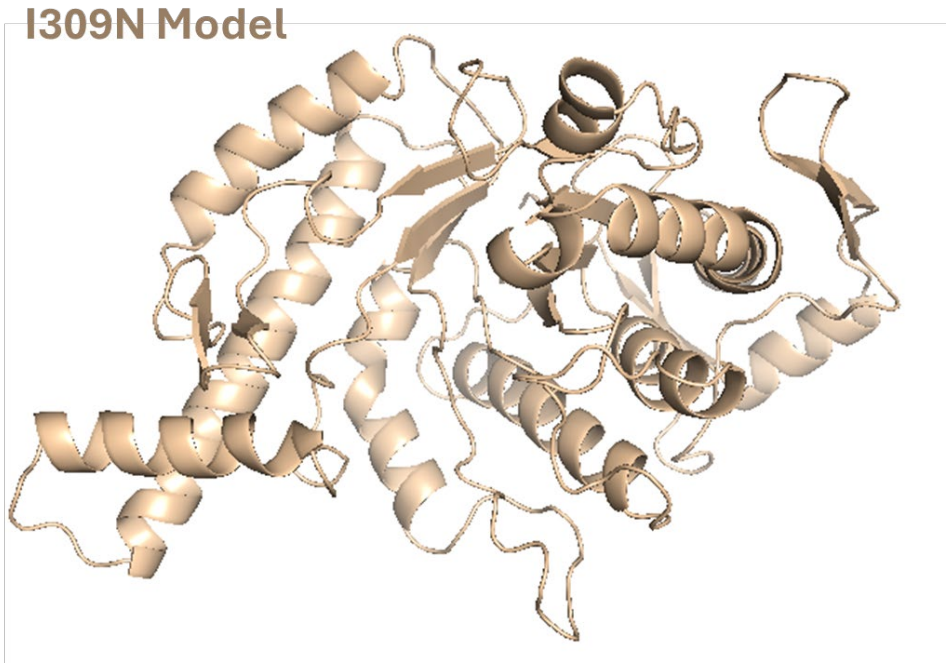
- I309N Chimpanzee PH20
- I309N Gibbon PH20
- I309N Orangutan PH20
- N47A/N131A/I309N PH20
- N47A/N219A/I309N PH20
- N131A/N219A/I309N PH20
- N47A/N219A/N131A/I309N PH20

418. To generate the I309N chimpanzee PH20 model, I used SEQ ID NO: 10 disclosed in the patent. I converted the sequence in the sequence text file into FASTA format and then introduced the I309N mutation into the text file. I then generated a homology model using Swiss Model. I used 2PE4 as the template just as I did for the WT PH20 model. I examined the resulting model, downloaded the model, opened it in PyMOL and aligned it to the 2PE4 structure in PyMOL. I then proceeded with my analysis.

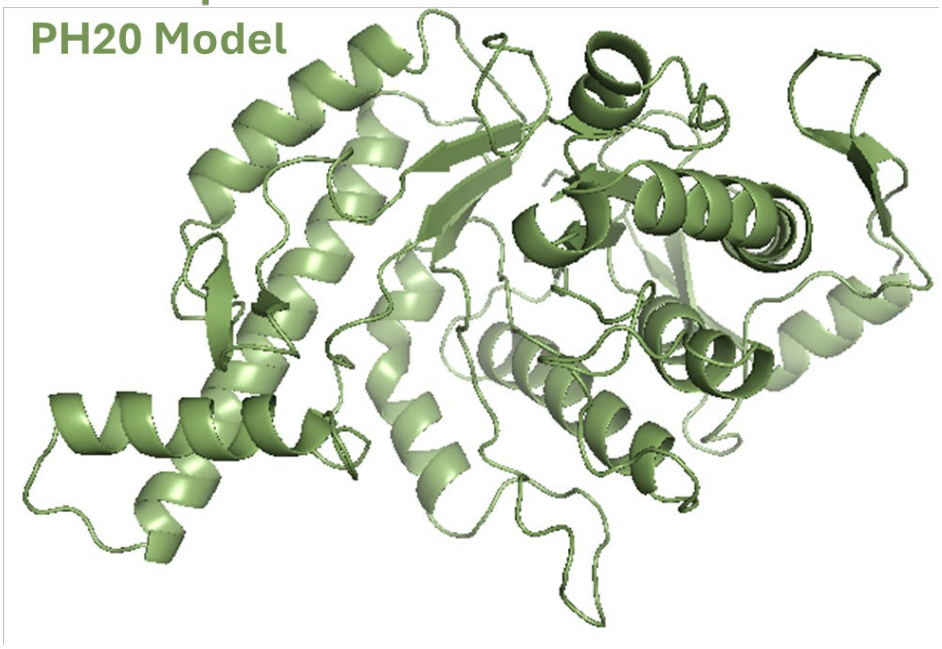
419. I performed this same procedure to generate homology models for I309N gibbon PH20, and I309N orangutan PH20. For the I309N gibbon model, I used SEQ ID NO: 857 and introduced the I309N mutation into the text file as I did for the chimpanzee model. I did the same to generate the I309N orangutan model (using SEQ ID NO: 861). In each case, I examined the resulting model, downloaded the model, opened the file in PyMOL, aligned it to the 2PE4 structure and then proceeded with my analysis.

420. For the N47A/N131A/I309N model, I used SEQ ID NO: 3 and introduced the N47A/N131A/I309N mutations into the text file. I performed the same procedure, making the appropriate mutations in SEQ ID NO: 3, for the N47A/N219A/I309N, N131A/N219A/I309N, and N47A/N131A/N219A/I309N models. In each case, I examined the model, downloaded the model, opened the file in PyMOL, aligned it to the 2PE4 structure and then proceeded with my analysis.

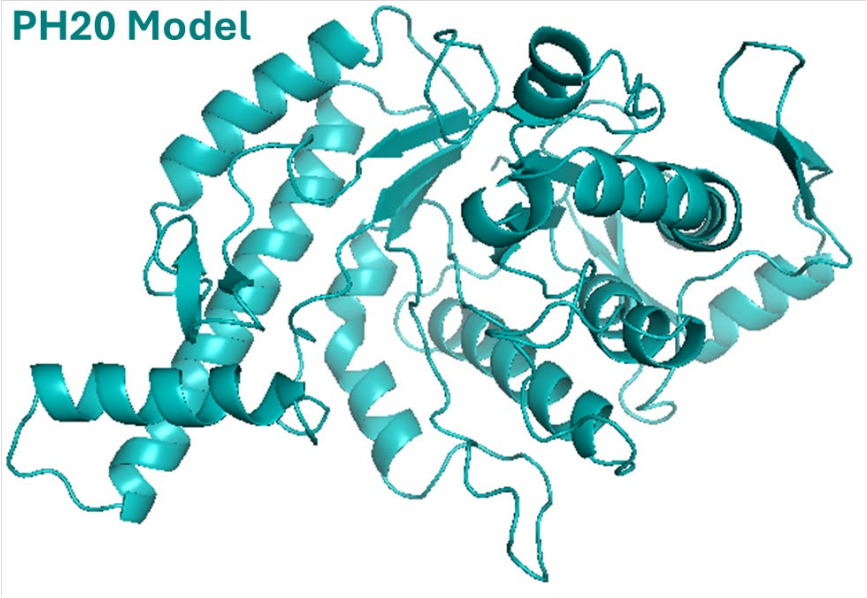
421. As shown below, each of the I309N models has the PH20 alpha-beta barrel fold:



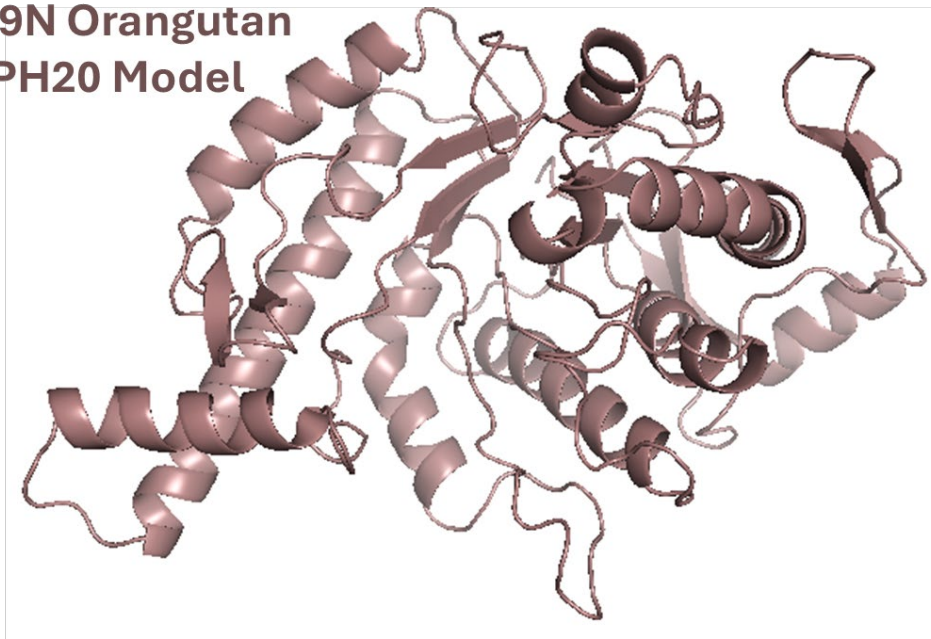
I309N Chimpanzee



I309N Gibbon
PH20 Model



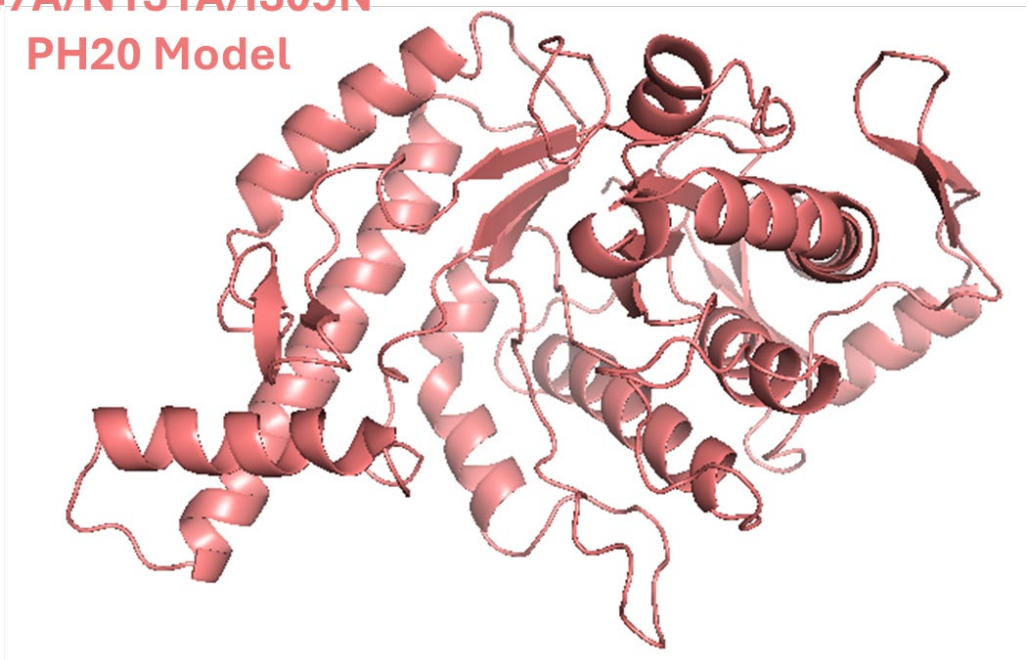
I309N Orangutan
PH20 Model



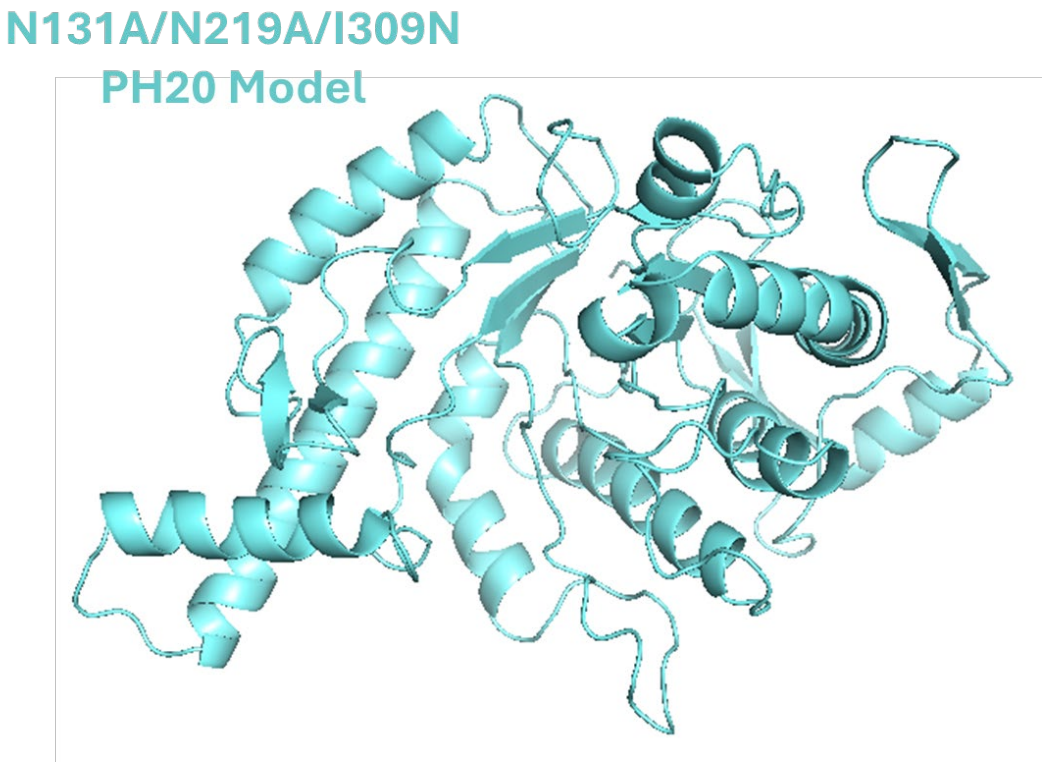
N47A/N131A/N219A/I309N
PH20 Model



N47A/N131A/I309N
PH20 Model

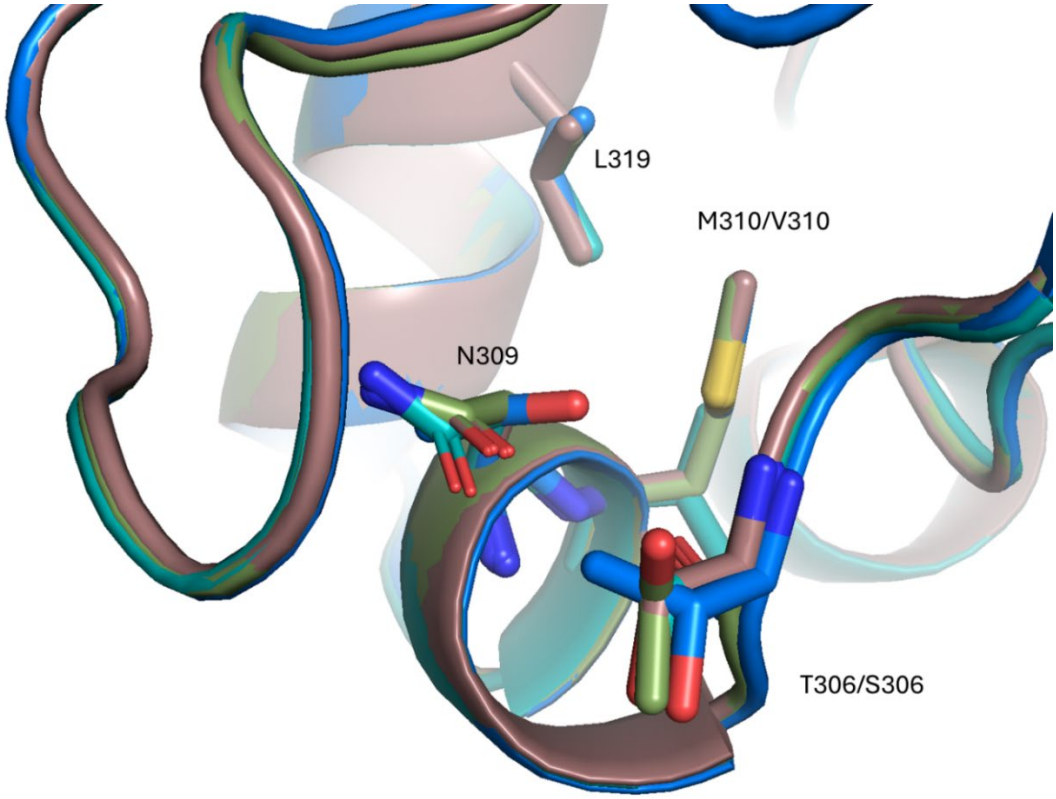




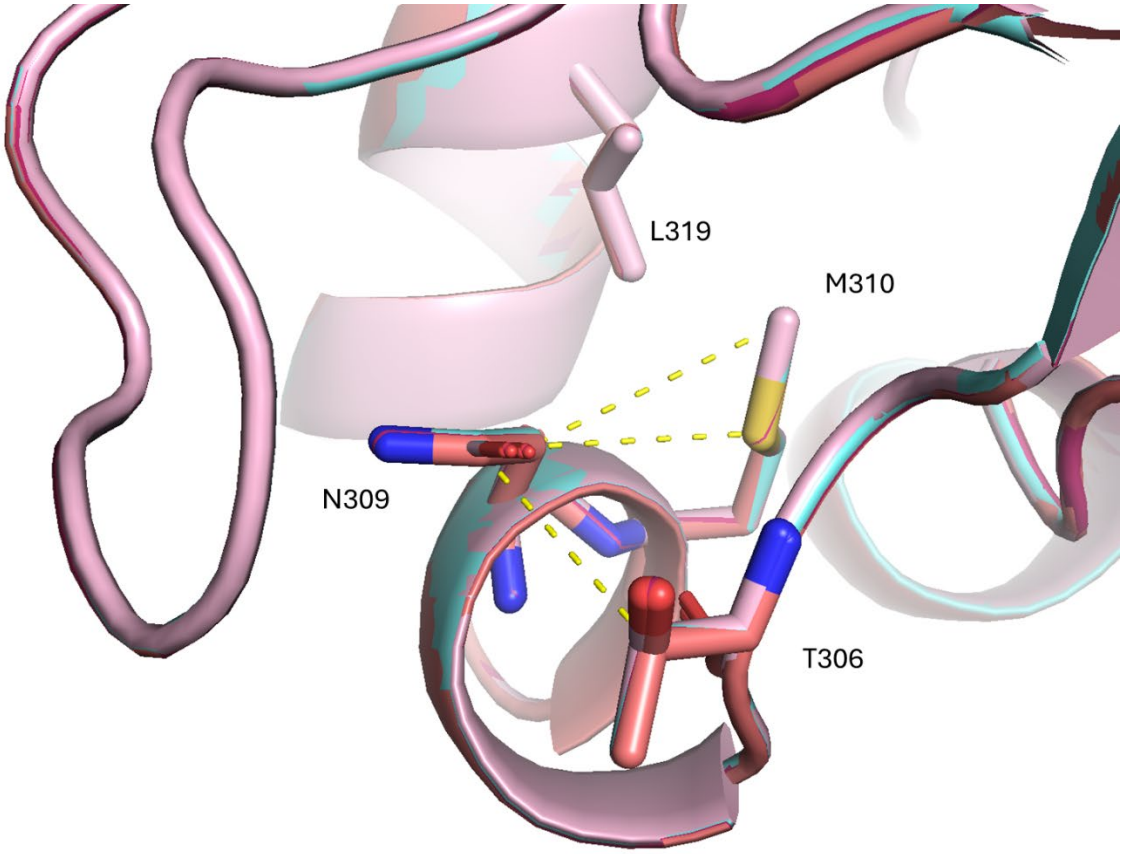


422. The figure below shows the human, chimpanzee, gibbon and orangutan I309N modified PH20 models all superimposed, with N309, T306 (or

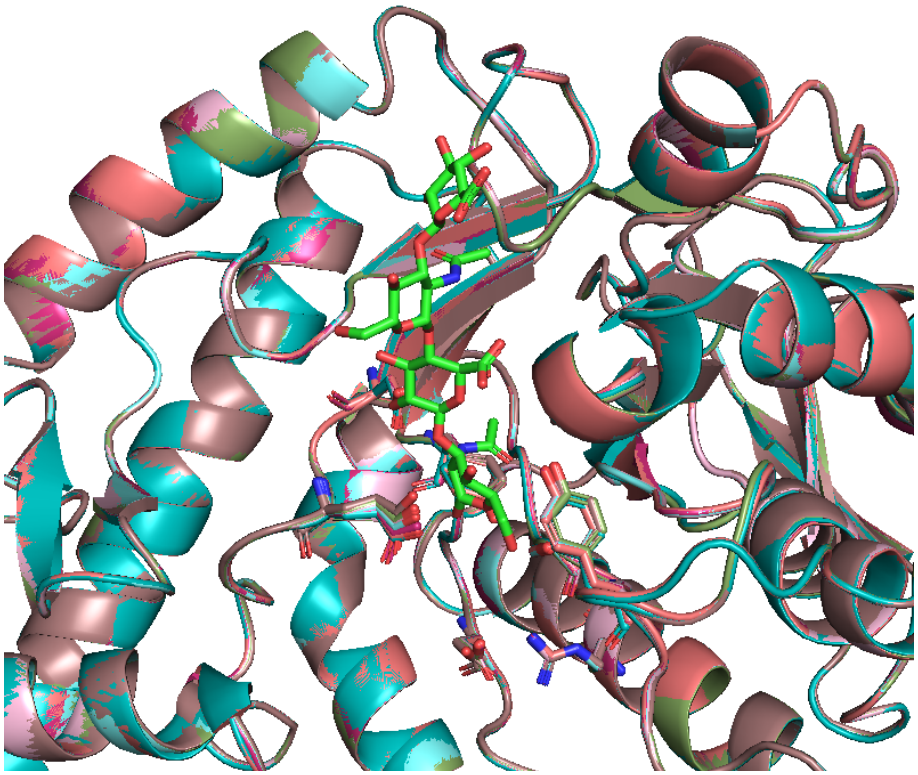
S306 in the gibbon and orangutan models), M310 (or V310 in the gibbon model) and L319 shown in sticks. As the figure shows, in each modified PH20, those residues are oriented in about the same position.

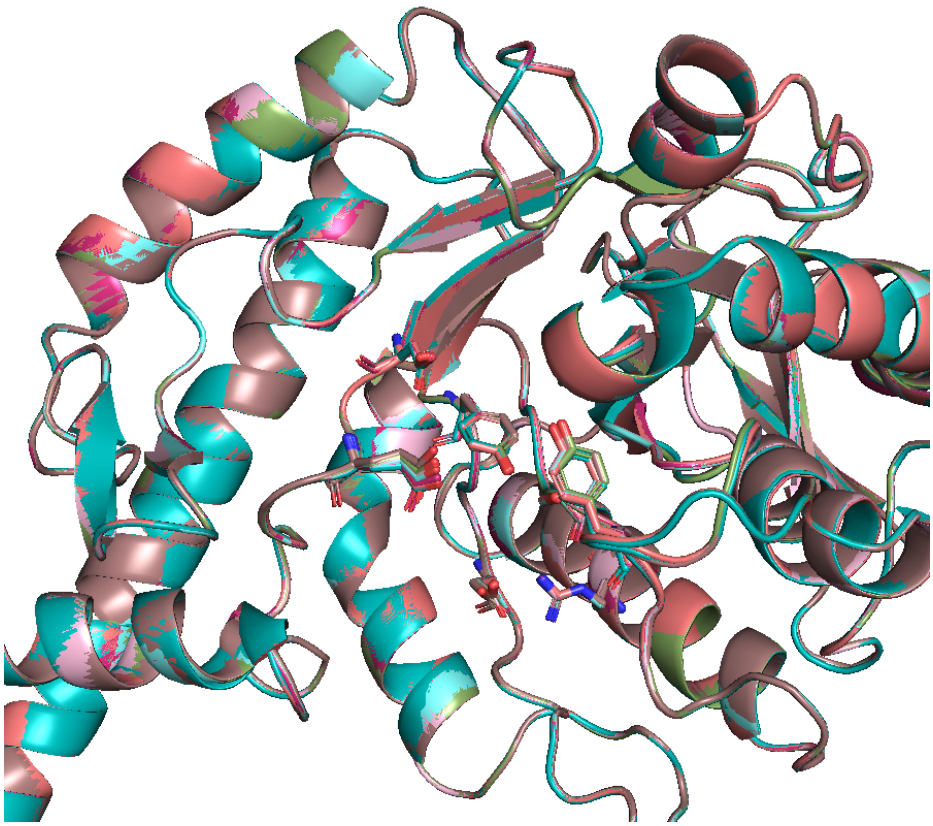


423. The figure below shows the N47A/N131A/I309N, N47A/N219A/I309N, N131A/N219A/I309N and N47A/N131A/N219A/I309N modified PH20 models all superimposed, with N309 and T306, M310 and L319 shown in sticks in all models. The interactions between N309 and T306 and betweeen N309 and M310 are shown as dotted lines. As the figure shows, in each modified PH20, N309 and T306 and N309 and M310 are oriented in about the same position and form an interaction.



424. In the figures below, the human I309N, chimpanzee I309N, gibbon I309N, orangutan I309N, N47A/N131A/I309N, N47A/N219A/I309N, N131A/N219A/I309N and N47A/N131A/N219A/I309N variant models are shown on top of each other. The figures shows that the key active region residues I described above are predicted to adopt essentially the same position in all variant models, illustrating how the variants adopt a common structure in the active site:





425. My above analysis shows that when the I309N mutation is introduced into the various multiply-modified polypeptides for which proof of principle is provided in the patent, the resulting model shows a common alpha-beta barrel fold, a common structure and interactions at position 309, as well as common active site residues in similar positions. In other words, all share common structural features, reflecting a common structure-function relationship.

426. The claims also encompass minor variations to primary structure (up to 5% variation). POSAs would use the patent disclosure to identify substitutions likely to maintain the PH20 tertiary structure and activity or likely to abrogate activity, depending on the POSA's preference.

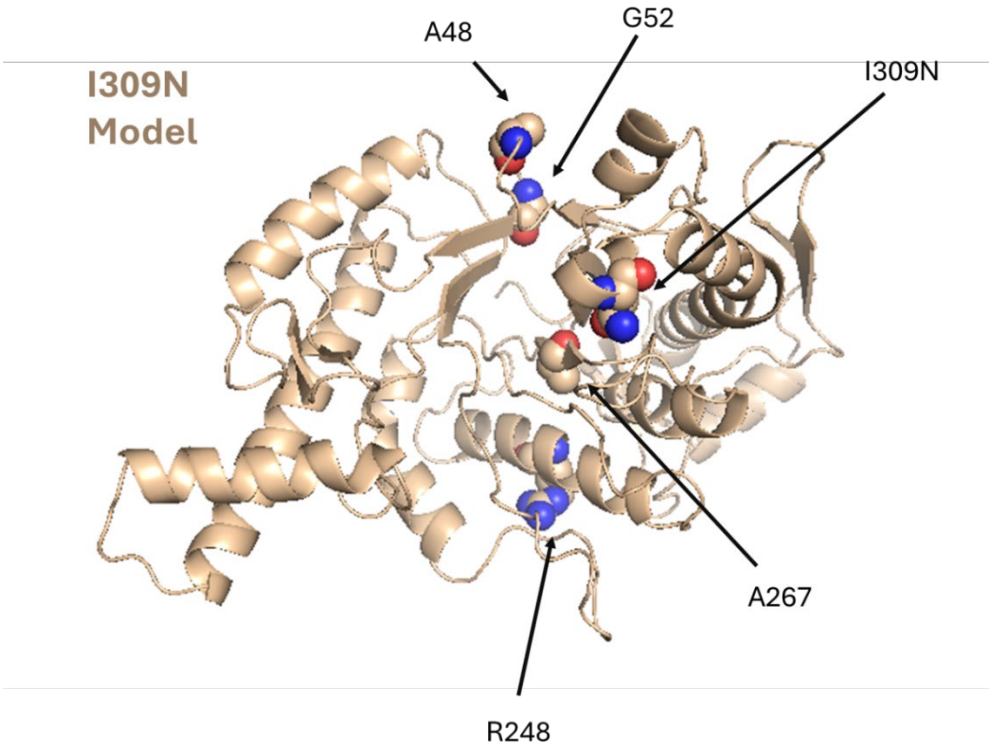
XIII. REPRESENTATIVE SPECIES

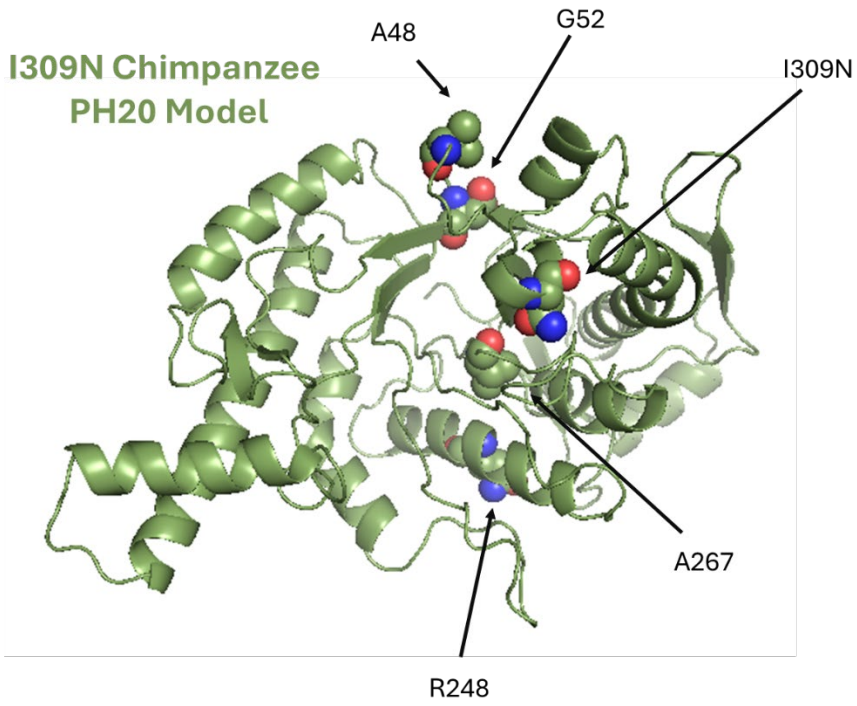
427. As I explained above, a POSA would view the claims as directed to a narrow set of PH20 variants with common structural features including 95% sequence identity and a defined substitution at position 309. The claims encompass enzymatically active PH20s with the same tertiary structure—the PH20 alpha-beta barrel fold. And all enzymatically active claimed PH20s cleave HA in the same way, using the same mechanism of action and the same chemistry and residues.

428. In view of the narrow scope of the claims and the homogeneity of the claimed polypeptides, a POSA would consider the disclosed I309E, I309G, I309H,

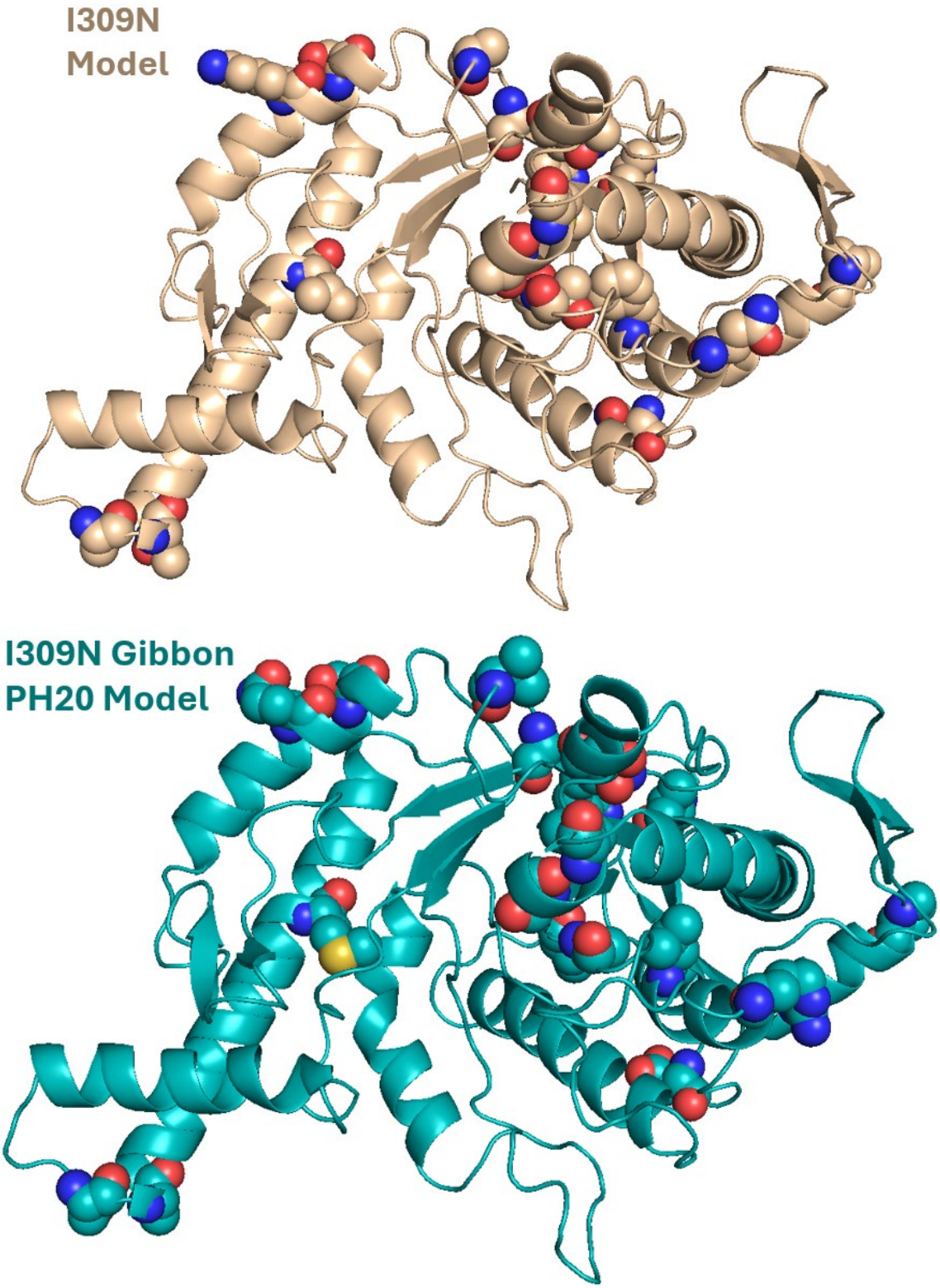
I309L, I309M, I309N, I309Q, I309R, I309S and I309T examples to be representative of the scope of the claims.

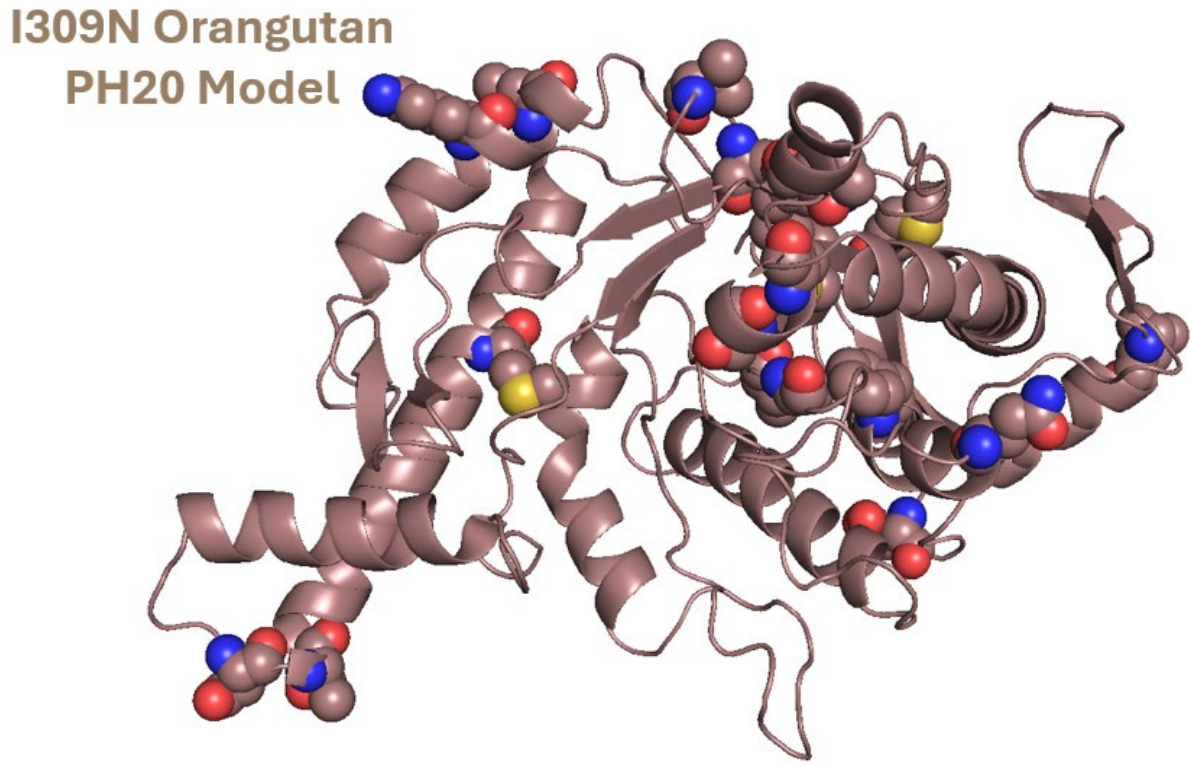
429. I309N is representative of modified PH20 polypeptides with this substitution that vary by no more than 5% of their amino acid sequence. As explained above, this is a structurally limited set of proteins. To the extent that the proteins fold, they adopt the same fold. To the extent the proteins are enzymatically active, they function in the same way. To illustrate the structural similarities and differences within the set of claimed proteins, I generated the figures below. These figures show that multiple mutated I309N PH20s that meet claim 1 have the same alpha-beta barrel fold, but they have minor variations in amino acids at various positions in the structure.



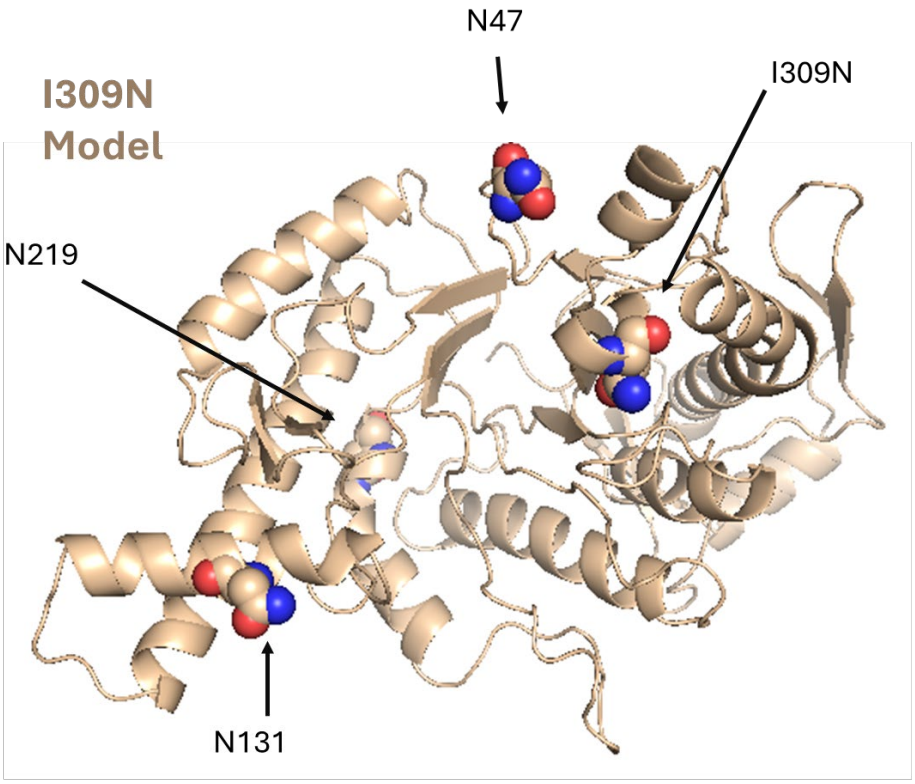


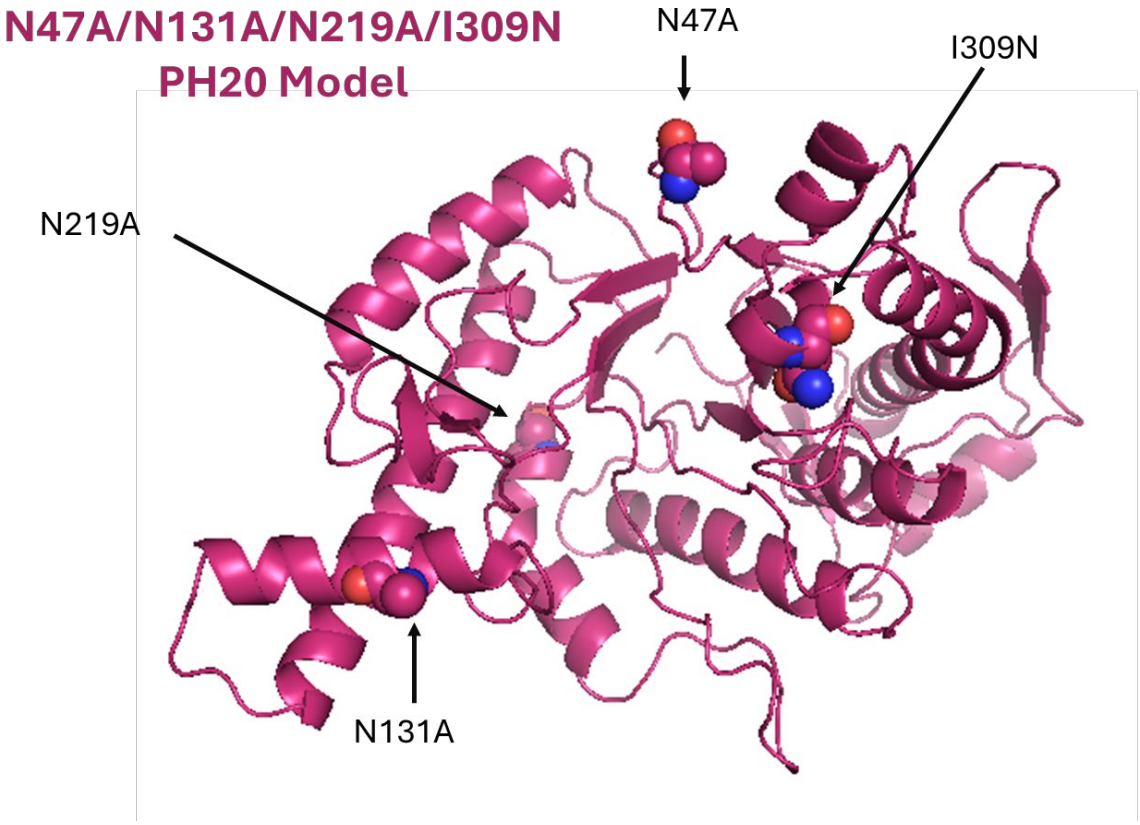
430. In the figures below, I have shown in sphere format the residues that vary between human, gibbon and orangutan PH20:

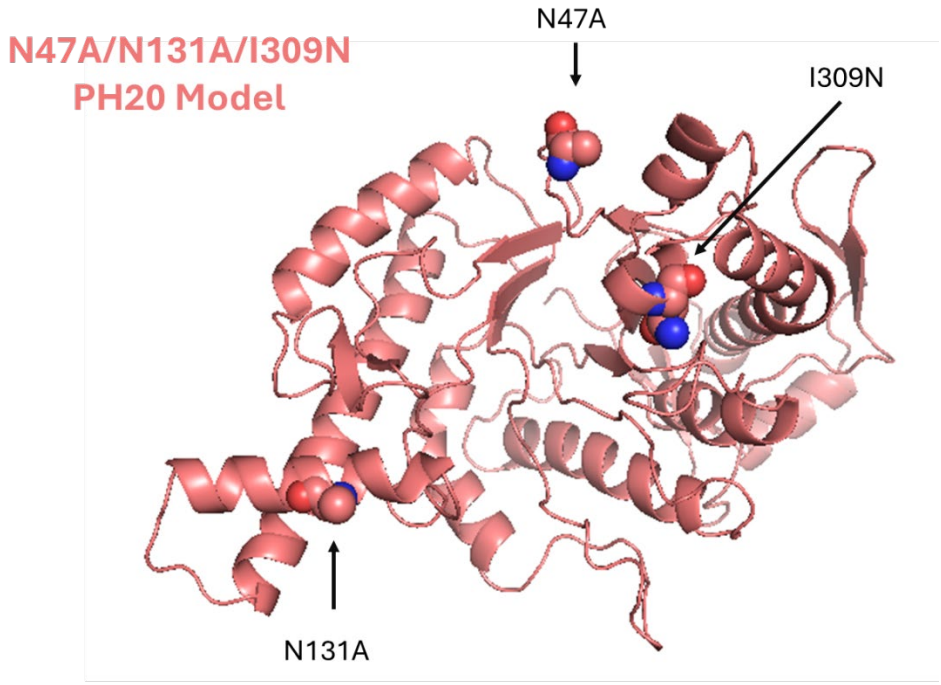

I309N
Model
I309N Gibbon
PH20 Model

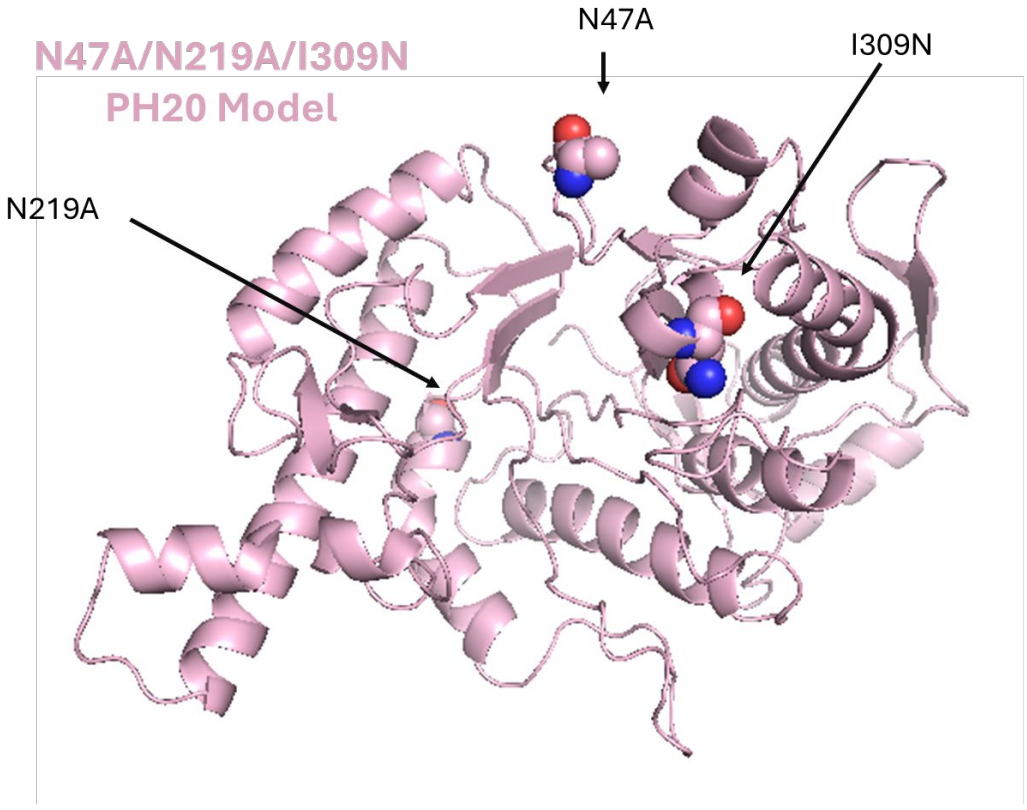


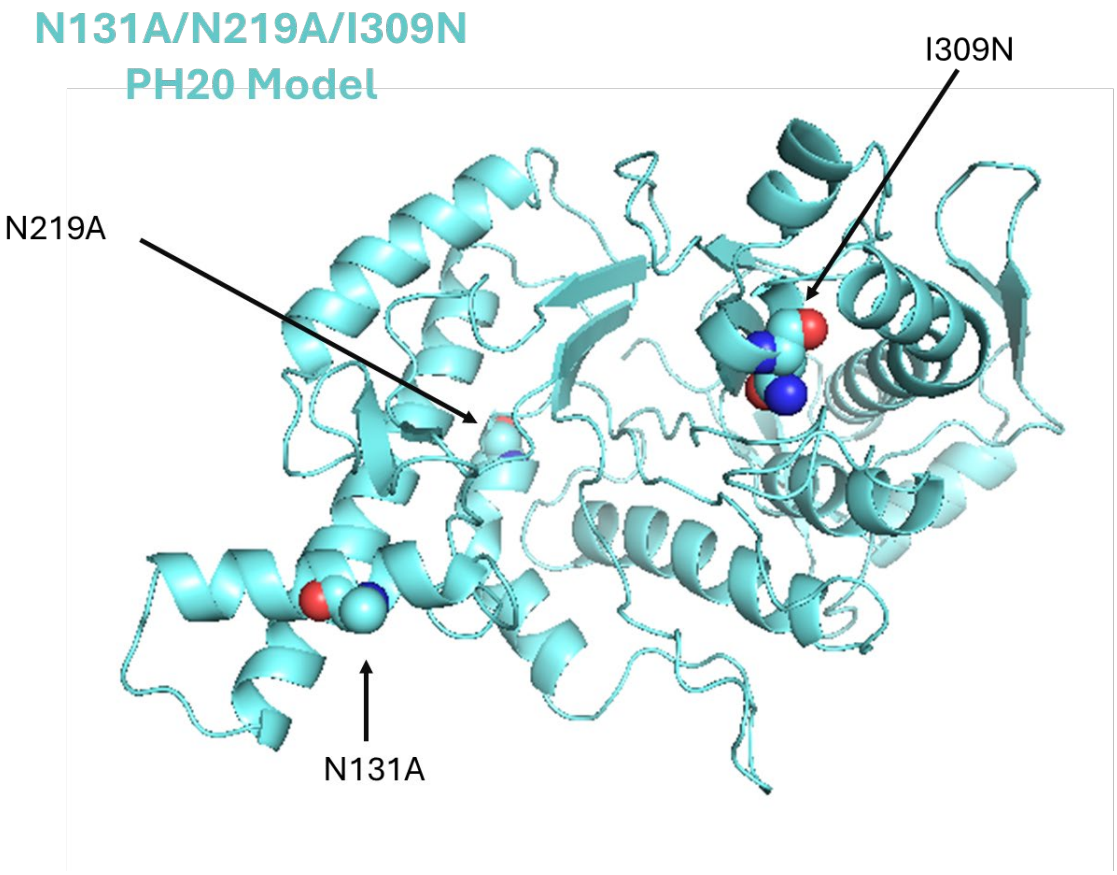
431. In the figures below, I have shown in sphere format the residues that vary between the N47A/N131A/I309N, N47A/N219A/I309N, N131A/N219A/I309N, N47A/N131A/N219A/I309N, and I309N variants.


I309N
Model
N47
I309N
N219
N131


N47A/N131A/N219A/I309N
PH20 Model
N47A
I309N
N219A
N131A


N47A/N131A/I309N
PH20 Model
N47A
I309N
N131A


N47A/N219A/I309N
PH20 Model
N47A
I309N
N219A



432. The figures and analysis above show that, while there is minor variation in the claimed polypeptides, they share significant common structural features. As explained above, the claims cover a structurally limited set of proteins. To the extent that the proteins fold, they adopt the same fold. To the extent the proteins are enzymatically active, they function in the same way. Additional modifications throughout the sequence may yield variations in, for example, stability (*e.g.*, storage stability or stability in the presence of various excipients) or other properties. As a general matter, though I309N is representative of the minor variation across the 95% identical modified PH20 I309N variants. For the same reasons, the I309E PH20 is representative of the

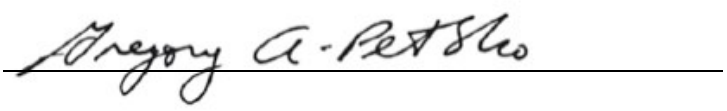
minor variation across the 95% identical modified PH20 I309E variants. The same is true for the I309G, I309H, I309L, I309M, I309Q, I309R, I309S and I309T modified PH20s.

433. Finally, while the large number of variants without the 309 modification that were tested do not meet the claims, they still provide extremely useful information to the POSA in terms of the additional variation in the structure that will be tolerated, where it will be tolerated, and where it will not be tolerated. The data for the other substitutions direct the POSA as to which changes to make to obtain the desired result, including a folded active modified PH20 polypeptide.

434. I also note that, as I explained above, the POSA would have understood that the results achieved for I309E, I309G, I309H, I309L, I309M, I309N, I309Q, I309R, I309S and I309T substitutions in SEQ ID NO: 3 apply to the other active C-terminal truncation polypeptides (SEQ ID Nos. 36-55). And the POSA would have expected that SEQ ID Nos. 32-33 would have been inactive and SEQ ID Nos. 34-35, 56-63 and 64-66 would have had reduced activity (as compared to, for example, SEQ ID NO: 3) or little to no activity irrespective of any changes at position 309. Thus, the examples disclosed in the specification are representative of the set of claimed polypeptides with the specified modification at position 309.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

Executed on this _16th__ day of January, 2026.



26221451.1

TABLE OF EXHIBITS

Exhibit No.	Description
1001	U.S. Patent No. 12,077,791
1003	Declaration of Dr. Michael Hecht
1004	Declaration of Dr. Sheldon Park
1005	U.S. Patent No. 7,767,429
1006	Chao et al., "Structure of Human Hyaluronidase-1, a Hyaluronan Hydrolyzing Enzyme Involved in Tumor Growth and Angiogenesis," Biochemistry, 46:6911-6920 (2007)
1008	Stern et al., "The Hyaluronidases: Their Genomics, Structures, and Mechanisms of Action," Chem. Rev. 106:818-839 (2006)
1010	Zhang et al., "Hyaluronidase Activity of Human Hyall Requires Active Site Acidic and Tyrosine Residues," J. Biol. Chem., 284(14):9433-9442 (2009)
1011	Arming et al., "In vitro mutagenesis of PH-20 hyaluronidase from human sperm," Eur. J. Biochem., 247:810-814 (1997)
1012	Bordoli et al., "Protein structure homology modeling using SWISSMODEL workspace," Nature Protocols, 4(1):1-13 (2008)
1013	Frost, "Recombinant human hyaluronidase (rHuPH20): an enabling platform for subcutaneous drug and fluid administration," Expert Opinion on Drug Delivery, 4(4):427-440 (2007)
1014	Brandon & Tooze, "Introduction to Protein Structure," Second Ed., Chapters 1-6, 11-12, 17-18 (1999)
1016	Steipe, "Consensus-Based Engineering of Protein Stability: From Intrabodies to Thermostable Enzymes," Methods in Enzymology, 388:176-186 (2004)

Exhibit No.	Description
1017	Green, "Computer Graphics, Homology Modeling, and Bioinformatics," Protein Eng'g & Design, Ch. 10, 223-237 (2010)
1018	Chica et al., "Semi-rational approaches to engineering enzyme activity: combining the benefits of directed evolution and rational design," Curr. Opin. Biotechnol., (4):378-384 (2005)
1029	Gmachl et al., "The human sperm protein PH-20 has hyaluronidase activity," FEBS Letters, 3:545-548 (1993)
1031	Yue et al., "Loss of Protein Structure Stability as a Major Causative Factor in Monogenic Disease," J. Mol. Biol., 353:459-473 (2005)
1032	Wang & Moult, "SNPs, Protein Structure, and Disease," Hum. Mutation, 17:263-270 (2001)
1033	Markovic-Housley et al., "Crystal Structure of Hyaluronidase, a Major Allergen of Bee Venom," Structure, 8:1025-1035 (2000)
1035	Lins et al., "Analysis of Accessible Surface of Residues in Proteins," Protein Sci., 12:1406-1417 (2003)
1037	Benkert et al., "Toward the Estimation of the Absolute Quality of Individual Protein Structure Models," Bioinformatics, 27:343-350 (2010)
1038	Schwede et al., "SWISS-MODEL: An Automated Protein Homology-Modeling Server," Nucleic Acids Rsch., 31:3381-3385 (20
1039	Alberts, "Molecular Biology of the Cell," Fifth Edition, Chapter 3 (2007).
1043	Sievers et al., "Fast, Scalable Generation of High-Quality Protein Multiple Sequence Alignments Using Clustal Omega," Molecular Sys. Biology, 7.1 (2011)

Exhibit No.	Description
1044	Mihel, "PSAIA – Protein Structure and Interaction Analyzer," BMC Structural Biology, 8:21 (2008)
1053	Hom_pre2011
1054	Hom_pre2011_header
1055	Hom_pre2011_header_clean
1056	Hom_pre2011.fasta
1057	Ph20_pre2011.aln-clustal_num
1058	Ph20_pre2011 Alignment html
1064	Collection of BLAST Webpages from the Internet Archive, navigable from: https://web.archive.org/web/20111022151531/http://www.clustal.org/omega/
1065	Collection of Clustal Omega Webpages from the Internet Archive, navigable from: https://web.archive.org/web/20111022151531/http://www.clustal.org/omega/
1066	Collection of SWISS-MODEL Webpages from the Internet Archive, navigable from: https://web.archive.org/web/20110519141121/http://swissmodel.expasy.org/?pid=smh01&uid=&token=
1067	Collection of PyMol Webpages from the Internet Archive, navigable from: https://web.archive.org/web/20110701072314/http://pymol.org
1069	Swiss Model Printout of PH20 Model
1070	Swiss Model Printout of PH20 Model with D320K Mutation
1071	Swiss Model Printout of PH20 Model with D320H Mutation

Exhibit No.	Description
1072	Swiss Model Printout of PH20 Model with D320R Mutation
1073	Swiss Model Printout of PH20 Model with D320S Mutation
2071	*Curriculum Vitae* of Gregory A. Petsko, Ph.D.
2076	Transcript of the Deposition of Michael Hecht, Ph.D., August 26, 2025
2077	Transcript of the Deposition of Dr. Sheldon Park, August 7, 2025
2091	Davies, G. et al., "Structures and mechanisms of glycosyl hydrolases," *Current Biology*, 3:853-859 (1995)
2163	National Medal of Science in Medicine Citation for Gregory Petsko, 2022 (accessible at https://nationalmedals.org/laureate/gregory-petsko/?amp=1, last accessed September 19, 2025)
2164	Petsko, G., et al., *Protein Structure and Function*, New Science Press (2004)
2165	U.S. Patent Application Publication No. 2010/0143457 A1 to Wei et al., published June 10, 2010
2166	Petsko Patent Analysis Data
2168	Profile of Gregory A. Petsko, American Academy of Arts and Sciences (accessible at https://www.amacad.org/person/gregory-petsko, last accessed September 22, 2025)
2169	"BLAST Topics," National Institutes of Health (accessible at https://blast.ncbi.nlm.nih.gov/doc/blast-topics, last accessed September 22, 2025)
2178	1LOH pdb file
2179	1FCV pdb file
2180	2PE4 pdb file

Exhibit No.	Description
2181	8SMN pdb file
2182	I309L Swiss Model
2183	I309N Swiss Model
2184	I309E Swiss Model
2185	I309G Swiss Model
2186	I309H Swiss Model
2187	I309M Swiss Model
2188	I309Q Swiss Model
2189	I309R Swiss Model
2190	I309S Swiss Model
2191	PH20 Swiss Model
2192	*Intentionally Left Blank*
2193	N47A_N219A_I309N Swiss Model
2194	N47A_N131A_I309N Swiss Model
2195	N47A_N131A_N219A_I309N Swiss Model
2196	N131A_N219A_I309N Swiss Model
2197	Orangutan_I309N Swiss Model
2198	Chimpanzee_I309N Swiss Model
2199	Gibbon_I309N Swiss Model
2200	I309T Swiss Model