Homology Models Cannot Predict Effects of Many Changes

Position	WT	V	S	T	W
33	L	-	0.48	0.45	1.58
165	L	1.22	1.31	-	1.14
175	L	0.94	-	1.43	

EX1133 (Naismith), ¶ 273; Reply, 29

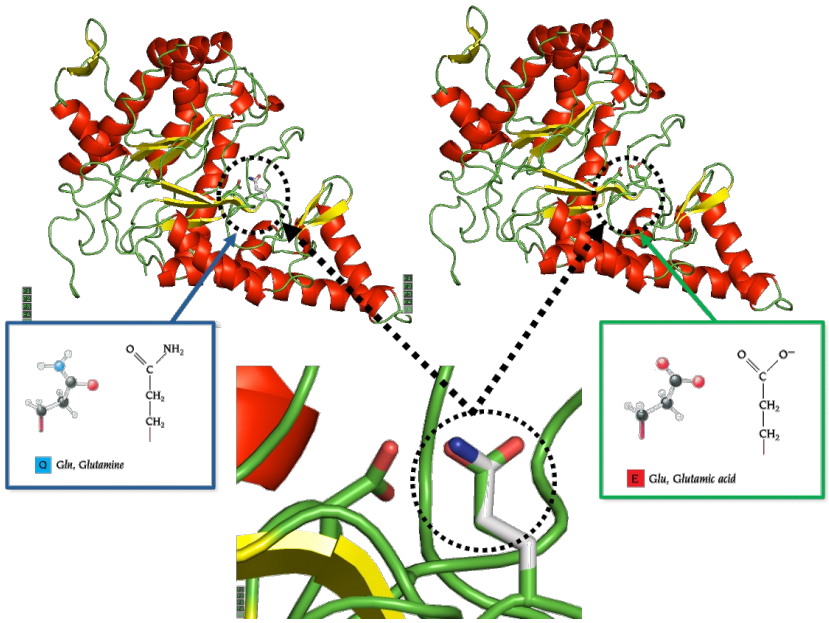
Position	WT	V	A	L	S	T	F
47	N	-	0.48	ND	0.85	1.49	1.32
291	G	1.63	-	ND	0.45	-	-
315	S	-	0.85	0.42		0.97	ND

EX1133 (Naismith), ¶ 274; Reply, 29

273. Much of the missing substitution data in the common disclosure cannot be reliably predicted using a homology model. This is because the disclosure reported different effects depending on sequence position, even where there are barely perceptible spatial differences in atoms. For example, Val and Thr are similarly sized residues; the difference is in Thr there is an OH in the side chain rather than a CH3. When compared to Ser which also has an OH, Thr has an additional CH3. As reflected in the table below, substitutions among these closely related residues at L175, L165, and L33—each located in hydrophobic environments—produce inconsistent and position-specific effects on activity, revealing no clear structural or chemical pattern that a POSA could have predicted based on residue size or chemistry alone. The common disclosure does not explain this pattern of results, which a POSA in 2011-2012 could not have predicted from homology modeling or residue size and chemistry alone. (Grey denotes not reported, pink inactive, green active).

EX1133 (Naismith), ¶ 273; Reply, 29

Homology Models Cannot Identify Effects of Many Changes



282. Second, depending on the degree of sequence identity, the accuracy of a model generated by Swiss-Model can vary significantly from reality. If the modeled protein has at least 50% sequence identity to the protein used as the template, the positioning of the backbone α-carbon atoms typically deviate by ~1 Å RMSD from an experimentally determined structure, with side chains and loop structures deviating more.[303] PH20 and Hyal-1 have approximately ~36% overall sequence identity, with about 41% sequence identity in the overlapping portion of the PH20 sequence. This suggests that structural deviations less than around 1-2 Å will not be reliably detectable in the homology model. Epistasis (in multiple mutants) and changes in amino acids can alter the positioning of atoms by less than 1-2 Å. Yet enzyme catalysis and substrate recognition are exquisitely sensitive to much smaller positional and electrostatic differences. Indeed, as shown in the literature, the conservative Glu131→Gln substitution abolishes activity.[304] This is because the Glu residue is essential for catalysis, emphasizing that it is the precise structure-function relationship that is key to enzyme activity; not simply the fold of the protein.

Methods Require Impossible Scale of Experimentation

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Iterative Mutagenesis Method in the ’731 Application

347. A POSA seeking to make a modified PH20 with the ’600 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 ’600 patent, the POSA has a much more sensible strategy.

EX2070 (Petsko Dec.), ¶ 347; Reply, 35

4. Iterative Methods

The method provided herein also is iterative. In one example, after the method is performed, any modified hyaluronan-degrading enzymes identified as exhibiting stability, such as increased stability, under a denaturation condition can be modified or further modified to increase or optimize the stability. A secondary library can be created by introducing additional modifications in a first identified modified hyaluronan-degrading enzyme. For example, modifications that were identified as conferring stability, such as increasing stability, can be combined to generate a combinatorial library. The secondary library can be tested using the assays and methods described herein.

In another example of an iterative aspect of the method, modified hyaluronan-degrading enzymes that are identified as not exhibiting stability such as increased stability (*e.g.*, such that they are not active or do not have increased activity under the a denaturation condition), can be further modified and retested for stability under a denaturation condition. The further modifications can be targeted near particular regions (*e.g.*, particular amino acid residues) associated with activity and/or stability of the molecule. For example, residues that are associated with activity and/or stability of the molecule generally are critical residues that are involved in the structural folding or other activities of the molecule. Hence, such residues are required for activity, generally under any condition. Critical residues can be identified because, when mutated, a normal activity of the protein is ablated or reduced. For example, critical residues can be identified that, when mutated in a hyaluronan-degrading enzyme, exhibit reduced or ablated hyaluronidase activity under a normal or control assay condition. A further library of modified proteins can be generated with amino acid mutations targeted at or near to the identified critical amino acid residues, such as adjacent to the identified critical amino acid residues. In some examples, the mutations can be amino acid replacement to any other of up to 19 other amino acid residues. The secondary library can be tested using the assays and methods described herein.

EX1026 (’731 Application), 150:1-26; Reply, 35

Limits of Screening Assays Well Below Scale Required

Table 1. Characteristics of general *in vivo* screening and selection strategies. It is the product of the enzymatic reaction or the enzymatic conversion itself that needs to be detected, making these strategies reaction dependent (in contrast to most reporter-based strategies). Requirements encompass everything except basic microbial techniques/reagents and substrate. m, metagenomic library; ev, enzyme variant library. Adapted from [3].

Strategy	Library type	Library size	Requirements	Advantages	Disadvantages
Selection	m/ev	~ 10^9	None	Only desired variants	Low activity variants might be missed
				Simple to operate	Difficult to quantify
					Higher false positive risk
Agar plate screening	m/ev	~ 10^5	Reagents[a]	Simple to operate	Low dynamic range
			Detection device[a]		Difficult to quantify
Microtiter plate screening	m/ev	~ 10^4	Detection device	Multiple analytical detection methods possible	
				Increased sensitivity by cell lysis	
				Large dynamic range	
Cell-in-droplets (screening)	m/ev	~ 10^9	Detection device	Pico/femto-litre scale (not much reagent needed)	Product should stay in the droplet
			Cell sorter		Technically challenging
Cells as micro-reactors (screening)	m/ev	~ 10^9	Detection device	Femto-litre scale (not much reagent needed)	Product should stay in the cell
			Cell sorter	No compartmentalization needed	Technically challenging
Cell surface display (screening)	ev	~ 10^9	Detection device	Substrate can stay extracellular	Product needs to attach to cell surface
			Cell sorter		Technically challenging
					Careful selection of host and anchoring motif needed
					Fusion can alter enzyme structure/activity

[a] In some cases.

EX1133 (Naismith), ¶ 124 (citing EX1147 (Van Rossum), 2991, Table 1); Reply, 8

Additional Mutations	Sequences	#
1	8,988	8.99E+03
2	40,306,696	4.03E+07
3	120,248,369,660	1.20E+11
4	268,484,748,936,790	2.68E+14
5	478,547,697,659,676,000	4.79E+17
6	709,288,341,231,375,000,000	7.09E+20
7	899,176,330,916,182,000,000,000	8.99E+23
8	995,277,575,700,743,000,000,000,000	9.95E+26
9	977,143,406,290,414,000,000,000,000,000	9.77E+29

EX1133 (Naismith), ¶ 127; Reply, 8

Limited Investigation Still Implicates Impossible Scale of Effort

Dr. Petsko described a similar methodology. In ¶ 359 of his declaration (below), he provides an example where the POSA inspects positions that tolerated between 60% and 100% of the tested changes using a homology model and selects sets of 22 substitutions from those positions. Dr. Petsko also suggests that "[t]the POSA seeking to make multiple mutations could generate a model using, for example, Swiss Model to evaluate the results of the anticipated modifications."[147] He also frequently mentions targeting surface residues. A POSA also would need to use a homology model to determine whether residues are surface residues or partially buried.[148] He proposes this methodology in several other paragraphs.

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.

EX1133 (Naismith), ¶ 131 (citing EX2070 (Petsko), ¶ 359); Reply, 9

132. A POSA could not have performed this homology model-guided selection and positional assessment methodology for even the tiniest fraction of the claim parameter sequence space. Assume that one worked with the shortest reference sequence (430 residues), allowed 1 position with 4 alternatives, avoided changing the 57 essential residues identified in the common disclosure (*see* ¶ 304-305) and only made changes at the ≥60% tolerated positions Dr. Petsko determined. Also assume that only 5 changes were made, and the options were restricted to the tested active mutants at those ≥60% positions (an average of 11.6 alternatives). For that "simple" investigation of only 5 substitutions per polypeptide, a POSA would have to individually model and assess 351,421,083,215,239 distinct sequences. Spending only 1 minute per assessment would translate into 240 billion days of working 24 hours a day. That is plainly impossible. As Dr. Hecht explained, in the 2011-2012 timeframe, computational tools for modeling protein structure were not capable of accurately predicting the effect of 5 or more mutations on a native protein sequence and its corresponding structure.[149]

EX1133 (Naismith), ¶ 132; Reply, 9

Problems with Dr. Petsko's Positional Tolerance Assessment

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Dr. Petsko's Positional Tolerance Table

Position	WT
1	L
2	N
3	F
4	R
5	A
6	P
7	P
8	V
9	I
10	P
11	N
12	V
13	P
14	F
15	L
16	W
17	A
18	W
19	N
20	A
21	P
22	S
23	E
24	F
25	C
26	L
27	G
28	K
29	F

30	31	32
D	E	P
L	E	P
L	Y	P
M	Y	P
S	I	F
L	F	S
D	P	Y
S	F	S
F	P	K
I	T	K
F	T	K
G	V	K
S	A	K
P	A	K
F	V	K
N	I	K
I	G	K
G	N	K
S	S	K
P	S	K
R	N	K
I	Q	K
G	K	K
N	E	K
Q	K	K
G	K	K
Q	K	K
I	K	K
G	K	K
D	K	K
R	K	K

61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91
L	G	Y	Y	P	Y	I	D	S	I	T	G	V	T	V	N	G	G	I	P	Q	K	I	S	L	Q	D	H	L	R	K

92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122
A	K	K	D	I	T	F	Y	M	P	V	D	N	L	G	N	A	V	I	D	W	E	E	W	R	P	T	W	A	R	N

123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153
W	K	P	K	D	V	Y	K	N	R	S	I	E	L	V	Q	Q	Q	N	V	Q	L	L	L	T	E	A	E	E	A

154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184
K	Q	E	F	E	K	A	G	K	D	F	L	V	E	T	I	K	L	G	K	L	L	R	P	N	H	L	W	G	Y	A

185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215
L	F	P	D	C	Y	N	H	H	Y	K	K	P	G	Y	N	G	S	G	F	N	V	I	I	K	R	N	D	L	S

216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246
W	L	W	N	E	S	T	A	L	Y	P	S	I	Y	L	N	T	Q	Q	S	P	V	A	A	T	L	Y	V	R	N	R

247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277
V	R	E	A	I	R	V	S	K	I	P	D	A	K	S	P	L	P	V	F	A	Y	T	R	I	V	F	T	D	Q	V

278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308
L	K	F	L	S	Q	D	E	L	V	Y	T	F	G	E	T	V	A	L	G	A	S	G	I	V	I	W	G	T	L	S

309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339
I	M	R	S	M	K	S	C	L	L	L	D	N	Y	M	E	T	I	L	N	P	Y	I	I	N	W	T	L	A	A	K

340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370
M	C	S	Q	V	L	C	Q	E	Q	G	V	C	I	R	K	N	W	N	S	S	D	Y	L	H	L	N	P	D	N	F

371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401
A	I	Q	L	E	K	G	G	K	F	T	V	R	G	K	P	T	L	E	D	L	E	Q	F	S	E	K	V	Y	C	S

402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432
C	Y	S	T	L	S	C	K	E	K	A	D	V	K	D	T	D	A	V	D	V	C	I	A	D	G	V	C	I	D	A

433	434	435	436	437	438	439	440	441	442	443	444	445	446	447
F	L	K	P	P	M	E	T	E	E	P	Q	I	F	Y

EX 2070 (Petsko), ¶ 274, Reply, 37

Inactive Mutant Results Cannot Provide Insights on PH20 Structure

246. Dr. Hecht expressed concerns over use of the inactive mutant data to draw conclusions about PH20 folding,[275] and I share those concerns. The primary reason for this conclusion is that the common disclosure does not provide information that permits a POSA to determine ***why*** any particular single amino acid substitution in the $PH20_{1-447}$ wild type sequence exhibited reduced (< 20 %) hyaluronidase activity. A POSA cannot tell from what is reported in the common disclosure whether an inactive mutant was classified as such because (i) it was not successfully synthesized by the cell, (ii) it was not secreted, (iii) it did not fold within the cell, (iv) it properly folded and was secreted but unfolded after secretion due to instability, (v) it was secreted in a misfolded or partially folded state, or (vi) it was properly folded but exhibited impaired or reduced enzymatic activity.

EX1133 (Naismith), ¶ 246; Reply, 21, 32

247. The possible outcomes of a single substitution in $PH20_{1-447}$ wild-type protein (*see* ¶ 48) have distinct implications for folding and positional tolerance. Outcomes (i)–(v) reflect defects in expression, secretion, correct folding, stability, or aggregation. It is not possible from the data provided in the common disclosure to ascertain which of these reasons (or experimental errors) caused the mutant to be reported having activity below the 20% threshold. Grouping these disparate outcomes under a single "inactive" classification obscures the cause of inactivity and prevents a POSA from drawing a meaningful structure-function inference about positional tolerance in PH20."

EX1133 (Naismith), ¶ 247; Reply, 20-21, 32

Disclosure's Experimental Assay Had Unusually High Variability

Experimental design flaws and incomplete reporting of results independently render Dr. Petsko's ratings unreliable:[113]

- inadequate numerical, structural context, and physicochemical interrogation of positions prevent reliable ratings of tolerance;
- no use of positive/negative controls in the primary assay, and no evidence of proteins in supernatants or their folded state;
- no activities reported for inactives, precluding differentiation of non-secreted mutants from those with residual activity;
- averaging of activities conceals inter-assay variability;
- unusually high (~40%) assay variability (ordinarily ~10-15%) render classifications unreliable.

Reply, 33

The results are reported in the table below:

Assay Condition	Number of WT Measurements (n)	Mean WT Activity (μ)	Standard Deviation (σ)	Coefficient of Variation (CV = σ/μ)
37 °C, No Preservative	17	3.756	1.488	40%
37 °C, + Preservative (m-cresol)	17	0.444	0.222	49%
Control Condition (4 °C baseline)	17	4.233	1.407	33%

EX1133 (Naismith), ¶ 294; Reply, 33

[0271] In another example, hyaluronidase activity is measured using a microtiter assay in which residual biotinylated hyaluronic acid is measured following incubation with hyaluronidase (see e.g. Frost and Stern (1997) *Anal. Biochem.* 251:263-269, U.S. Patent Publication No. 20050260186). In Example 4, the hyaluronidase activity of truncated human PH20 hyaluronidase is determined using biotinylated hyaluronic acid. The free carboxyl groups on the glucuronic acid residues of hyaluronic acid are biotinylated, and the biotinylated hyaluronic acid substrate is covalently coupled to a microtiter plate. Following incubation with hyaluronidase, the residual biotinylated hyaluronic acid substrate is detected using an avidin-peroxidase reaction, and compared to that obtained following reaction with hyaluronidase standards of known activity. As the substrate is covalently bound to the microtiter plate, artifacts such as pH-dependent displacement of the biotinylated substrate does not occur. The sensitivity permits rapid measurement of hyaluronidase activity from cultured cells and biological samples with an inter-assay variation of less than 10%.

EX1133 (Naismith), ¶ 297 (citing EX2165 (US-457), ¶ 0271); Reply, 33

Dr. Petsko's Tolerance Table Conflicts with Guidance in Disclosure

To retain hyaluronidase activity, modifications typically are not made at those positions that are less tolerant to change or required for hyaluronidase activity. For example, generally modifications are not made at a position corresponding to position 7, 16, 17, 18, 19, 21, 25, 53, 55, 56, 57, 62, 64, 76, 78, 80, 88, 95, 100, 101, 109, 111, 112, 113, 115, 116, 121, 123, 126, 129, 185, 187, 188, 189, 190, 191, 194, 199, 201, 203, 207, 210, 223, 225, 227, 228, 229, 241, 243, 244, 246, 249, 250, 252, 254, 262, 268, 282, 295, 296, 298, 299, 303, 319, 322, 329, 330, 332, 333, 336, 337, 340, 341, 344, 345, 346, 350, 352, 354, 355, 362, 363, 364, 365, 366, 370, 372, 382, 384, 386, 390, 400, 402, 408, 423, 424, 429, 430, 431, with reference to

EX1026 ('731 Application), 91:30-37; Reply, 37-38

Q. So if I understand your answer, you're saying that a POSA might change the residue of position 299, despite it being marked in red in the table?

A. I'm saying that a POSA might change that residue if their objective to -- is to retain hyaluronidase activity in careful examination with what additional residues might need to be also concurrently mutagenized to compensate for that, the effects of that mutation.

I'm saying a POSA could predict that by examining the area around that residue and considering what they were seeking to substitute it with.

EX1130 (Simpson Dep.), 195:12-25; Reply, 38

Contraceptive Vaccine Utility Issues

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Contents of Disclosure on Contraceptive Vaccines

C. MODIFIED PH20 POLYPEPTIDES

Provided herein are modified or variant PH20 polypeptides. The modified PH20 polypeptides provided herein exhibit altered activities or properties compared to a wildtype, native or reference PH20 polypeptide. Included among the modified PH20 polypeptides provided herein are PH20 polypeptide that are active mutants, whereby the polypeptides exhibit at least 40% of the hyaluronidase activity of the corresponding PH20 polypeptide not containing the amino acid modification (*e.g.*, amino acid replacement). In particular, provided herein are PH20 polypeptides that exhibit hyaluronidase activity and that exhibit increased stability compared to the PH20 not containing the amino acid modification. Also provided are modified PH20 polypeptides that are inactive, and that can be used, for example, as antigens in contraception vaccines.

EX1026, 99:13-22; Pet. 24 (cited as EX1001, 72:60-62)

Modified PH20 polypeptides provided herein can be used as vaccines in contraceptive applications. PH20 is present in the male reproductive tract, and is expressed in both the testis and epididymis and is present in sperm. PH20 plays a role in fertilization by facilitating entry of the sperm through the cumulus layer surrounding the unfertilized egg. PH20 also is able to bind to hyaluronic acid (HA) on the zona pellucida during early phases of fertilization. This binding also initiates intracellular signaling that aids in the acrosome reaction. Immunization with PH20 has been show to be an effective contraceptive in male guinea pigs (Primakoff et al. (1988) *Nature* 335:543-546, Tung et al. (1997) *Biol. Reprod.* 56:1133-1141). It also has been shown to be an effective contraceptive in female guinea pigs due to the generation of anti-PH20 antibodies that prevent sperm and egg binding. In examples herein, the modified PH20 polypeptides can be inactive enzymes, such as any described in Sections C.2. The polypeptides can be administered directly or can be administered as a recombinant virus to deliver the antigen.

EX1026 ('731 App.), 213:1-13; Pet., 80 (cited as EX1001, 194:54-195:6).

No Evidence of Any Modified PH20 Polypeptide Being Used in a Contraceptive Vaccine

Q. And you are not aware of studies before 2012 of successful contraception in humans using modified PH20 polypeptides as a vaccine?
A. I'm not aware, no.
Q. Are you aware of any evidence published after 2012 of successful use of a modified PH20 polypeptide as a vaccine?
MR. POWERS: Object to form.
THE DEPONENT: No, I am not.
Q. (By Ms. Wang) In humans or in any other mammals?
A. No, I am not.
Q. So is fair to say there's no evidence of modified PH20 polypeptide being used successfully for contraception in humans?
MR. POWERS: Objection. Form.
THE DEPONENT: Not that I'm aware of.

EX1129 (Cherr Dep.), 101:18–102:9; Reply, 40

Q. (By Ms. Wang) Are you aware of any evidence published before 2012 investigating the effect of human PH20 on fertility in nonhuman mammals?
MR. POWERS: Objection. Form.
THE DEPONENT: No, I am not aware.
Q. (By Ms. Wang) Are you aware of any evidence published before 2012 of using modified human PH20 polypeptide to see its effect on fertility in nonhuman mammal?
MR. POWERS: Same objection.
THE DEPONENT: No, I am not.

EX1129 (Cherr Dep.), 104:9-20; Reply, 40

Prior Art: PH-20 is Not a Useful Antigen for Immunocontraceptive Vaccines

Table 3
SPAM1 and Immunocontraception

Species	Immunization			Fertility	Response	Reference
	M	F	Form	Reduction		
Guinea pigs[a]	+	+	Aff. Purif.	+	(1) Caudal sperm depletion (2) Autoimmune orchitis	Primakoff et al. (1988), Tung et al. (1997)
Rabbit	+	+	rSPAM1	–	Restricted entry of IgG in reproductive tracts	Pomering et al. (2002)
Macaque	+	+	rSPAM1	–	”	Deng et al. (2002)
Mouse	+	+	rSpam1	–	”	Hardy et al. (2004)

M: male, F: female, Aff. Purif.: affinity purified, rSPAM1/Spam1: recombinant SPAM1.
[a] The guinea pig may be fundamentally different from other species in its autoimmune response.

Taken together, the data from non-primate and primate models (where there are no known redundant reproductive hyases) suggest that PH-20 is not a useful antigen for inclusion in immunocontraceptive vaccines. The absence of critical epitopes in recombinant PH-20 has been considered as an explanation for a lack of sterility after immunization in mice (Hardy et al., 2004). However, it is possible that the strong immunocontraceptive effect reported for affinity purified guinea pig PH-20 could reflect a fundamental difference in the autoimmune response in guinea pigs compared to other species.

EX1189 (Martin-DeLeon), 119; Reply 40

Usefulness According to Halozyme's Expert, Dr. Cherr

Q. And I am trying to understand how do you define "useful"?

A. Yes, so -- I define "useful" -- I mean, there's -- in the literature with experimental animals reducing litter size, people still use the term as an effective contraception. But in my mind, when it comes to human biology, it needs to be close to 100 percent.

Q. So from modified PH20 polypeptide to be a useful contraceptive in humans, it would need to cause 100 percent infertility?

MR. POWERS: Object to form.

THE DEPONENT: Or close to 100 percent, yes.

Q. (By Ms. Wang) For a modified PH20 polypeptide to be a useful contraceptive in nonhuman mammals, how much or what percentage of reduction in fertility would need to be achieved?

MR. POWERS: Objection. Form.

THE DEPONENT: It would be similar ideally.

Q. (By Ms. Wang) It would need to be 100 percent or close to it?

A. Similar to what hormone contraceptives are.

EX1129 (Cherr Dep.), 57:16-58:15; Reply 40

Extraordinarily Unlikely that Every Modified PH20 Polypeptide is Useful

52. Dr. Cherr stated that a modified PH20 polypeptide would need to cause close to 100 percent infertility to be useful as a contraceptive in humans. He also believes that a modified PH20 polypeptide would need to cause similar infertility to hormone contraceptives (which is typically near 100%) to be useful in nonhuman mammals.[66]

(EX1029 (Cherr Dep.), 57:16–58:15.). Dr. Cherr's opinions regarding the expected usefulness of modified PH20 polypeptides are irreconcilable with this high threshold, particularly in view of the experimental evidence available by the relevant timeframe. A POSA would have viewed it as extraordinarily unlikely that every possible modified PH20 polypeptide could elicit antibody responses that would provide near-100% effective contraception. To claim otherwise flouts well-established immunological principles.

EX1134 (Kelsoe) ¶ 52; *See also* Reply 40

Immunogens Must Retain Antigenic Determinants Relevant to Reproduction

105. A POSA would have expected that meaningful contraception (and certainly the near-100% contraceptive activity Dr. Cherr stated would be necessary for modified PH20 polypeptides to be useful antigens) could only be achieved with antibody responses that are specific to native epitopes implicated in reproductive function and that are sufficiently strong to carry out immune function.[162] A POSA would have expected that only a subset of modified PH20 polypeptides (if any) could elicit such a meaningful immune response. Determining which of the many possible modified PH20 polypeptides could reliably elicit contraceptive antibody responses could only be determined empirically.[163]

EX1134 (Kelsoe), ¶ 105; Reply 41-42

Modified PH20s Lacking Antigenic Determinants Relevant to Contraception Are Not Useful

93. I understand the modified PH20 polypeptides I've been asked to consider in my opinion can contain multiple amino acid modifications in addition to the required change at position 320. At least some of the possible multiple modifications would be expected to denature the native protein structure. This sort of structural change often results in misfolded proteins that cannot transit the endoplasmic reticulum (ER) and are targeted by ubiquitin for destruction in the proteosome.[142] A POSA would know methods of producing mammalian proteins in prokaryotes to bypass proteosomal degradation, but these proteins are recovered as insoluble inclusion bodies requiring solubilization and refolding.[143] These methods are inefficient and often produce non-native protein structures. Moreover, prokaryotic systems do not typically support glycosylation, which was known to be important to PH20 structure and function.[144] Thus, a POSA would have expected that it was highly likely that some, and perhaps a great many, of the possible modified PH20 polypeptides would be misfolded forms of the native structure, or would be unstable, causing them to unfold or denature before injection. Such forms would lack the epitopes present on native PH20, including those relevant to contraception. Immunization with such misfolded modified PH20 polypeptides would not be expected to elicit a quality polyclonal response against native PH20 because the modified antigen would be expected to have different structures from the native.

EX1134 (Kelsoe) ¶ 93; Reply 41

Prior Art Refutes Halozyme's Theory of Non-Specific Binding for Contraception

106. Contrary to Dr. Cherr's suggestion that binding location does not matter, researchers in 1999 sought to understand the nature of the immune response in guinea pigs that caused contraception.[164] These researchers recognized that "[m]ammalian gamate [sic] fusion involves multiple steps and is in most cases species specific," that PH20 has more than one functionality, and that "multiple isoforms of the protein exist in the sperms of a given mammalian species."[165] Though they sought to find linear epitopes on guinea pig PH20 (gpPH-20) the researchers reported results that "indicated the epitope sequence is conformation sensitive and likely to be discontinuous."[166] They also found two immunodominant and surface accessible regions that they hypothesized served different functions.[167] Importantly, they acknowledged that further research was required to dissect gpPH20 functions and that such experiments "are important to the ultimate definition of PH-20 ***structure*** as it relates to the development of a peptide-based contraceptive vaccine."[168]

EX1134 (Kelsoe), ¶ 106; Reply, 41.

In summary, we have defined two regions of sequences on gpPH-20 that are immunodominant and surface accessible in the native state. Since the anti-peptide sera are mono-specific with defined epitopes, they are valuable tools for future dissection of the mode of action of gpPH-20 in enzymatic function as well as the mediation of sperm-egg interaction. These experiments, together with the selection of mimitopes of conformation-dependent epitopes using phage-display technology, are important in the ultimate definition of PH-20 structure as it relates to the development of a peptide-based contraceptive vaccine.

EX2120 (Chan 1999), 1999; EX1134 (Kelsoe), ¶ 106; Reply, 41.

Prior Art Refutes Halozyme's Theory of Non-Specific Binding for Contraception

Like anti-PH-20 mAbs[10], the anti-PH-20 polyclonal sera from the infertile females blocked sperm binding to the egg *zona pellucida in vitro*. The relative inhibitory potency of the antiserum provided a second, perhaps more physiologically significant, measure of antiserum titres. In the *in vitro* sperm-*zona* binding assay[10], antisera from the infertile females inhibited sperm-*zona* binding by 90–94% at a 10^{-2} or 10^{-3} dilution. The extent of inhibition decreased at 10^{-4} dilution, but remained substantial even at 10^{-5} dilution (Table 2).

EX2010 (Primakoff 1988), 544; Reply, 40

Several factors may have contributed to the inability of the rMCMV-mPH20 or MBP-mPH20 to cause infertility in mice. In particular, native PH20 proteins may have antigenic epitopes with immunocontraceptive potential not present in either of the recombinant forms used in this study. For example, native PH20 is proteolytically cleaved late in sperm maturation during passage in the epididymis in rodents such as guinea pigs (Phelps *et al.* 1990), rats (Seaton *et al.* 2000) and mice (Deng *et al.* 1999). In addition, modifications to N-linked oligosaccharides on native mPH20 have also been reported during the passage of mouse sperm through the epididymis (Deng *et al.* 1999). Neither of these modifications is likely to have occurred to the recombinant proteins. It is therefore possible that such epididymis-specific modifications to PH20 are required to provide B-cell epitopes associated with the immunocontraceptive effects seen in guinea pigs with native gpPH20 isolated from sperm (Primakoff *et al.* 1988, 1997, Tung *et al.* 1997). In this respect, there is evidence

EX1019 (Hardy 2004), 332; Pet., 81, Reply, 40

Non-Specific Binding to PH20 Would Not Have Been Expected to Cause Infertility

109. I disagree that a POSA would have expected such non-PH20-specific mechanisms to play a significant role causing infertility. None of the papers Dr. Cherr cited for these mechanisms relate to PH20—they address anti-sperm antibody.[173] Anti-sperm antibodies are not necessarily specific to a particular sperm-protein (such as PH20) and may bind multiple and distinct targets on sperm, including in locations where PH20 is not expressed. Dr. Cherr did not explain why a POSA would have expected anti-PH20 antibodies to cause contraception through the same mechanisms as these non-specific interactions and did not identify any experimental evidence justifying such extrapolation.

EX1134 (Kelsoe), ¶¶ 109, 113; PO Sur-Reply 28

Immune Tolerance Applies to Misfolded Proteins

98. Finally, a POSA would have expected the issues of immunodominance and immune tolerance, as discussed above in Sections IV.B–C. and Paragraphs 85–91, to play a role in a polyclonal response to denatured or misfolded modified PH20 polypeptides as well. B cells reactive to such forms, even with limited T-cell help, would have been less likely to produce effective antibodies reactive with the native PH20 protein.

EX1134 (Kelsoe), ¶ 98; Reply 41

Sur-Reply – Dr. Kelsoe's Testimony on Claims

The claimed modified PH20 polypeptides are defined by their structure—a the D320 mutation and 95% sequence identity—not by their function. Petitioner's expert, Dr. Kelsoe, admitted as much…

PO Sur-Reply, 1

15. I understand that the '600 Patent at issue in these proceedings concerns "modified PH20 polypeptides" with certain required characteristics. I have been asked to develop my opinions based upon the following understanding of "modified PH20 polypeptides": polypeptides comprising an amino acid sequence that is at least 95% identical to the amino acid sequence of any one of SEQ ID NO: 3 and 32-66 in the '600 Patent, and includes at least one modification at position 320 such that D is replaced with H, K, R, or S.

EX1134 (Kelsoe), ¶ 15

Q Okay. And you reviewed this patent in association with preparation of your declarations, right?

A In the aspects that were relevant to my testimony, yes.

EX2302 (Kelsoe Dep.), 46:7-11; *see also* Sur-Reply, 1

Q And did you previously review the claims of this patent?

A Only those claims related to the use of PH20 variants as an immunocontraceptive.

Q Did you review claim 1 of this patent?

A If that -- I don't remember what claim 1 is, but if I -- whatever claim I reviewed only dealt with the contraceptive function of the potential contraceptive function of PH20 immunogen.

EX2302 (Kelsoe Dep.), 40:5-14

Obviousness

- **Halozyme's '429 patent motivates a POSA to make (and claims) single-substitution $PH20_{1-447}$ mutants in the scope of the claims**
- **Dr. Park used techniques that were conventional in 2011-2012**
- **Dr. Park's multi-sequence alignment identified positions and amino acids to consider as single-substitutions in non-essential regions of $PH20_{1-447}$ without hindsight**
- **Dr. Park's assessment of individual substitutions suggested by the multi-sequence alignment was objective and unbiased**

See e.g., Pet., 84-107; Reply, 42-47

’429 Patent Suggests Single Amino Acid Substitution in Non-Essential Regions of PH20

Thus, reference, for example, to “sHASEGP” encompasses all glycoproteins encoded by the sHASEGP gene family, including but not limited to: Human sHASEGP, mouse sHASEGP, or an equivalent molecule obtained from any other source or that has been prepared synthetically or that exhibits the same activity. Sequences of encoding nucleic acid molecules and the encoded amino acid sequences of exemplary sHASEGP’s and/or domains thereof are set forth, for example in SEQ ID NO: 4. The term also encompasses sHASEGP with amino acid substitutions that do not substantially alter activity of each member and also encompasses splice variants thereof. Suitable substitutions, including, although not necessarily, conservative substitutions of amino acids, are known to those of skill in this art and can be made without eliminating the biological activity, such as the catalytic activity, of the resulting molecule.

EX1005 (’429 Patent), 9:65-10:13; Pet. 87

Suitable conservative substitutions of amino acids are known to those of skill in this art and can be made generally without altering the biological activity, for example enzymatic activity, of the resulting molecule. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, e.g., Watson et al. Molecular

Nv Other substitutions are also permissible and can be determined empirically or in accord with known conservative substitutions.

EX1005 (’429 Patent), 16:14-20, 16:34-36; Pet., 96; Reply, 42

As used herein equivalent, when referring to two sequences of nucleic acids means that the two sequences in question encode the same sequence of amino acids or equivalent proteins. When equivalent is used in referring to two proteins or peptides, it means that the two proteins or peptides have substantially the same amino acid sequence with only amino acid substitutions (such, as but not limited to, conservative changes such as those set forth in Table 1, above) that do not substantially alter the activity or function of the protein or peptide. When equivalent refers to a property, the property

EX1005 (’429 Patent), 18:64-19:6; Pet. 87

'429 Patent Claims Modified $PH20_{1-447}$ with ≥1 Substitution

(10) **Patent No.: US 7,767,429 B2**
(45) **Date of Patent: Aug. 3, 2010**

(54) **SOLUBLE HYALURONIDASE GLYCOPROTEIN (SHASEGP), PROCESS FOR PREPARING THE SAME, USES AND PHARMACEUTICAL COMPOSITIONS COMPRISING THEREOF**

(75) Inventors: **Louis H. Bookbinder**, San Diego, CA (US); **Anirban Kundu**, San Diego, CA (US); **Gregory I. Frost**, Del Mar, CA (US)

(73) Assignee: **Halozyme, Inc.**, San Diego, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 1297 days.

(21) Appl. No.: **10/795,095**

(22) Filed: **Mar. 5, 2004**

1. A substantially purified hyaluronidase glycoprotein that consists of the sequence of amino acids set forth as amino acids 1-477, 36-477, 1-478, 36-478, 1-479, 36-479, 1-480, 36-480, 1-481, 36-481, 1-482, 36-482, 1-483 or 36-483 of SEQ ID NO: 1, or contains amino acid substitutions in the sequence of amino acids set forth as amino acids 1-477, 36-477, 1-478, 36-478, 1-479, 36-479, 1-480, 36-480, 1-481, 36-481, 1-482, 36-482, 1-483 or 36-483 of SEQ ID NO: 1, whereby the amino-acid substituted hyaluronidase glycoprotein consists of a sequence of amino acids that has at least 95% amino acid sequence identity with the sequence of amino acids set forth as amino acids 1-477, 36-477, 1-478, 36-478, 1-479, 36-479, 1-480, 36-480, 1-481, 36-481, 1-482, 36-482, 1-483 or 36-483 of SEQ ID NO: 1, wherein the glycoprotein contains at least one sugar moiety that is covalently attached to an asparagine (N) residue of the polypeptide.

EX1005 ('429 Patent), Claim 1; Pet. 99-100; Reply, 43

Dr. Park Identified Non-Essential Regions of PH20 Using MSA

25. In general, amino acids that do not vary at the same aligned positions within a set of structurally-related proteins are essential to the structure and functions of the protein.[6] When a residue does not vary in so many naturally occurring proteins, it is strong evidence that it is essential to the protein's structure and function and needs to be preserved.[7]

26. I investigated essential residues in hyaluronidase enzymes in two ways. First, I identified invariant residues by analyzing a multiple sequence alignment of a set of published hyaluronidase sequences that was available in December of 2011. Second, I reviewed scientific literature that identified important residues in hyaluronidase proteins or which reported experimental results showing that modifying single residues impaired or eliminated activity of the enzymes.[8] These two overlapped significantly and many residues that were experimentally shown to be essential for activity proved to be highly conserved based on sequence alignment. Below I address the first investigation.

EX1004 (Park), ¶¶ 25-26; Pet., 10, 14, 92; Reply, 44

2. Essential Residues in PH20

30. Using the alignment, I identified 68 largely invariant residues that a skilled artisan would have deemed "essential residues" in $PH20_{1-447}$ based on the sequence alignment (table below).[15] These are positions where the amino acid was conserved 95% of the time or more and non-identical amino acids appear in less than ~5% of the proteins in the data set[16].

3. Non-Essential Residues in PH20

31. The 379 positions in $PH20_{1-447}$ other than the 68 essential residues are positions at which there is evolutionary variation among the 88 protein sequences that I analyzed. The existence of evolutionary variation at these positions indicates that the homologous proteins have tolerated different amino acids at those positions. Amino acids at these positions in the different hyaluronidase proteins would be considered "non-essential" residues because the proteins presumably still exhibit hyaluronidase activity when they are mutated. The 379 non-essential positions in $PH20_{1-447}$ are compiled in Appendix D-2.

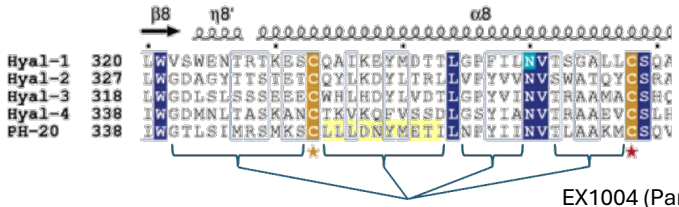
EX1004 (Park), ¶¶ 30-31, Pet., 92; Reply, 44

Dr. Park Identified Non-Essential Regions of PH20 Using MSA

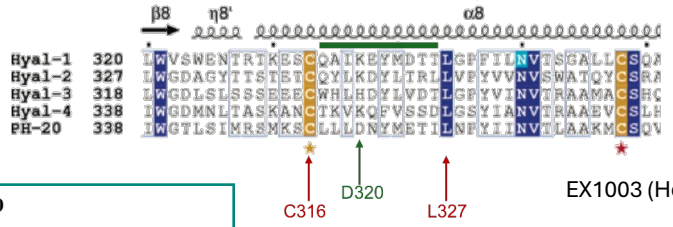
2. Essential Residues in PH20

Residue #	Mature Residue #	PH20 Residue	Residue %		Residue #	Mature Residue #	PH20 Residue	Residue %
49	14	F	95.5		222	187	P	100
53	18	W	100		224	189	C	100
60	25	C	100		226	191	N	98.9
91	56	F	98.9		234	199	Y	98.9
158	123	W	96.6		335	300	G	98.9
164	129	Y	100		339	304	W	98.9
168	133	S	100		351	316	C	100
188	153	A	100		362	327	L	96.6
192	157	F	100		368	333	N	97.7
203	168	T	98.9		369	334	V	96.6
211	176	R	95.5		376	341	C	100
212	177	P	97.7		377	342	S	96.6
215	180	L	95.5		381	346	C	100
216	181	W	100		385			
217	182	G	100		387			
219	184	Y	100		398			

EX1004 (Park), ¶ 30; Pet., 92; Reply, 44



EX1004 (Park), ¶ 32; Pet., 92; Reply, 44



EX1003 (Hecht), ¶ 213; Pet., 94

3. Non-Essential Residues in PH20

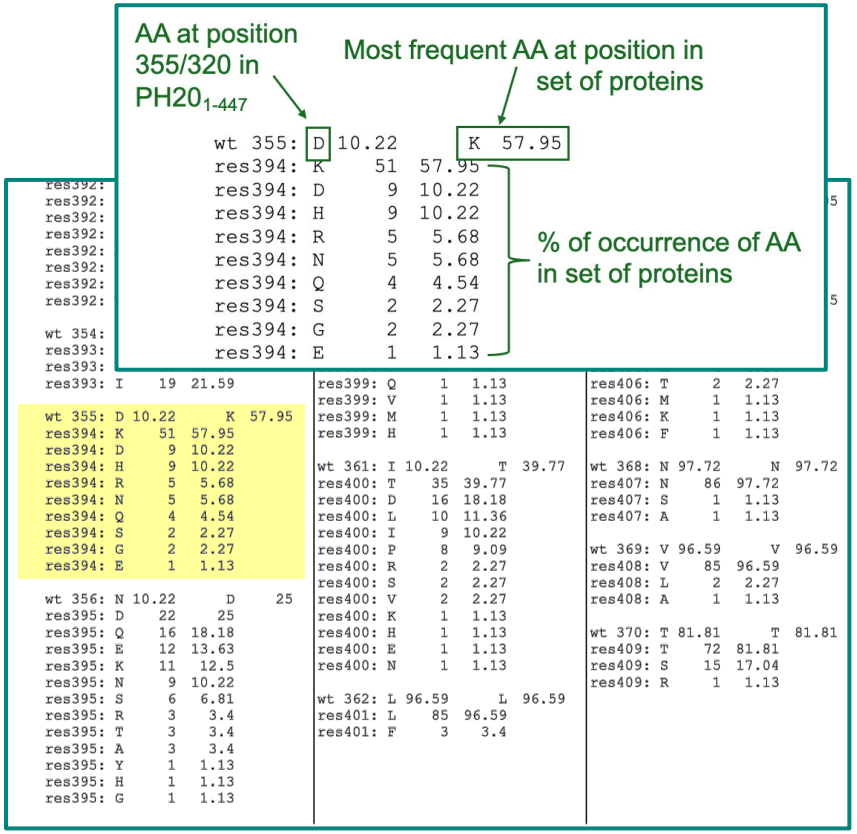
31. The 379 positions in $PH20_{1-447}$ other than the 68 essential residues are positions at which there is evolutionary variation among the 88 protein sequences that I analyzed. The existence of evolutionary variation at these positions indicates that the homologous proteins have tolerated different amino acids at those positions. Amino acids at these positions in the different hyaluronidase proteins would be considered "non-essential" residues because the proteins presumably still exhibit hyaluronidase activity when they are mutated. The 379 non-essential positions in $PH20_{1-447}$ are compiled in Appendix D-2.

EX1004 (Park), ¶ 31; Pet., 92; Reply, 44

Dr. Park Identified Substitutions to Assess Using MSA

42. Within the 88 homologous hyaluronidase proteins that I analyzed, there were varying numbers of amino acids that occurred at each non-essential position.[35] A higher frequency for one amino acid within a set of amino acids at a particular position indicates that a larger number of naturally occurring hyaluronidase proteins contain that amino acid at that position. For example, a 5% frequency of occurrence of an amino acid in a set of 88 related proteins means that ~4 naturally occurring hyaluronidase proteins have that amino acid at that position. In essence, evolution has "sampled" those substitutions and found they are tolerated in at least 4 isoforms of PH20.

43. An example of a set of amino acids from the MSA based on 88 sequences for position 320 (355 with the signal sequence) of $PH20_{1-447}$ is provided below.



EX1004 (Park), ¶¶ 42-43, App'x D-1; Pet., 95-96; Reply, 45

Dr. Park's Ratings for Amino Acid Substitutions

85. In my evaluation of whether single amino acid substitutions may be tolerated at a position, I considered all of the factors discussed above.[57] I balanced the type of impact of the substitutions based on the magnitude of each interaction may have on the protein's structure.

86. Based on my assessment, I assigned each substitution a score of 1, 2, or 3.

- A score of 3 indicates that I would expect the substitution to improve the overall stability of the protein.
- A score of 1 indicates that the mutation would likely reduce the protein stability and may even result in some loss of protein function.
- A score of 2 indicates that the substitution would have either no effect on the structure of the protein, or a slightly positive or slightly negative effect on the structure and function.

EX1004 (Park), ¶¶ 85-86; Reply, 46

87. Substitutions that I scored as a 2 or 3 would be expected to be tolerated by the PH20 protein. These types of changes would either be inconsequential to the protein structure or would cause a change that would likely be beneficial to the stability of the protein. The substitutions that I scored as a 1 would likely not be tolerated by the PH20 protein. These types of changes often significantly disrupt the protein structure at the location of the substitution, which significantly increases the probability that the substitution will adversely affect folding of the protein or structural features of the PH20 protein that are important to its biological activity (*e.g.*, catalysis, ligand binding). My scoring table is provided below.

Score	Expected Impact	Expected Toleration
1	Significantly Destabilized	Likely Not Tolerated
2	Neutral or Minor Impacts	Tolerated
3	Improved Stability	Tolerated

EX1004 (Park), ¶ 87; Reply, 46

Dr. Park Ensured His Methodology Was Unbiased

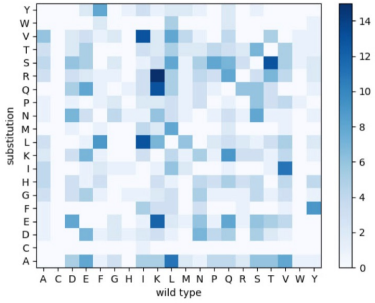
4. **Review of the Methodology Demonstrates Unbiased Evaluation**

102. To develop an unbiased scoring system, it is necessary to evaluate a lot of different types of substitutions. Therefore, it was important to me to evaluate and assign scores to many different substitutions because, through this process, I developed a consistent methodology to evaluate potential substitutions. As I went through the analysis, I would revisit the scores for each position multiple times to make sure that the way I assigned scores was consistent. Because I considered a broad range of situations, I believe my methodology provided an objective and unbiased evaluation of substitutions throughout the protein.

EX1004 (Park), ¶ 102; Pet., 102; Reply, 46

103. I conducted my analysis in a manner that did not focus on any particular position. For instance, I considered some substitutions involving similar amino acids (*e.g.*, both amino acids were hydrophobic, charged or polar) as well as those with dissimilar amino acids (*e.g.*, hydrophobic vs. charged, polar vs. hydrophobic, large vs. small). I also considered substitutions at buried positions as well as substitutions at solvent exposed positions. In this declaration, I have been asked by counsel to report my conclusions with respect to position 320.

EX1004 (Park), ¶ 103; Pet., 102; Reply, 46



EX2176 (Park Spreadsheet); Reply, 47

Q Why did you prepare this heat map?

A One of the things that I was aiming for as I was going through the substitution analysis was to develop a robust system, and I needed a way of knowing how robust my system was, and that I believed involved looking at many different kinds of substitutions; and therefore, I wanted to keep track of what sort of substitutions I was looking at.

Q Okay. So you wanted to make sure that you were evaluating substitutions across the spectrum of -- of the 20 amino acids?

A That's correct.

EX2078 (Park Dep.); Reply, 47

Dr. Park Recorded Observations and Later Explained Them in His Declaration

119. The long aliphatic side chain in lysine that is terminated by an amine group is compatible with the surrounding pocket at position 320. The aliphatic side chain of the lysine side chain will fit in and be compatible with the hydrophobic walls of the pocket, while the amine group will stick out at the top of the pocket into the solvent, similar to how the carboxyl group of the aspartic acid is positioned in the wild-type protein. Lysine is also generally classified as a hydrophilic residue, which means it can tolerate a solvent accessible environment.

120. Lysine also participates in hydrophobic interactions with nearby hydrophobic residues, including P32 and L317.[82] The terminal amine group extends out of the pocket, thus avoiding the energetic penalty of having a buried charge. Furthermore, the amine group can form both a salt bridge to E324 and a hydrogen bond to the main chain carbonyl of P32. The salt bridge to E324 is illustrated in the figure below by the dotted line labeled "2.6" and the hydrogen bond to P32 is illustrated by the dotted line labeled "3.0."[83]

121. Lysine at position 320 positions the positively charged amine group between two negatively charged residues. The introduction of a positive charge would avoid clustering of net negative charges and likely stabilize the structure of PH20.

EX1004 (Park), ¶¶ 119-121; Pet., 104

D	E	F	G	H	I	J	K	L	M	N
PH20 Mature Residue #	PH20 Residue	Alternative Residue	Residue %	Rating (1-3)	Comments	#neigh	fSASA	Factors		
								Hydrophobicity	Secondary Structure	Interactions
320	D	-	10.22			9	0.17			
320	-	K	57.95	3	gain: increased van der Waals, possible salt bridge, better H propensity	9	0.16	1		1
320	-	H	10.22	2	gain: increased van der Waals	9	0.16			1
320	-	R	5.68	3	gain: increased van der Waals, possible salt bridge, better H propensity	8	0.26		1	1
320	-	N	5.68	2	neutral	9	0.1			
320	-	Q	4.54	2	gain: higher H propensity, hbond	9	0.16		1	1
320	-	S	2.27	2	loss: lower H propensity, lost van der Waals	7	0.11		1	1
320	-	G	2.27	1	loss: Gly destabilizes helix	8	0.16		1	1
320	-	E	1.13	2	neutral	9	0.16			

EX1004 (Park),App'x C; Pet., 104

Q So why did you evaluate those substitutions?

A By that time, I had already completed all my analysis of all of positions, and provided a spreadsheet to counsel, who then asked me to elaborate on certain positions, including 320, 317, and so on. And since I'm being asked to provide detailed description of the substitutions and evaluation at those positions, I went back and finished the analysis with all the substitutions that were available at those positions.

EX2078 (Park Dep.),320:10-20; Reply, 46

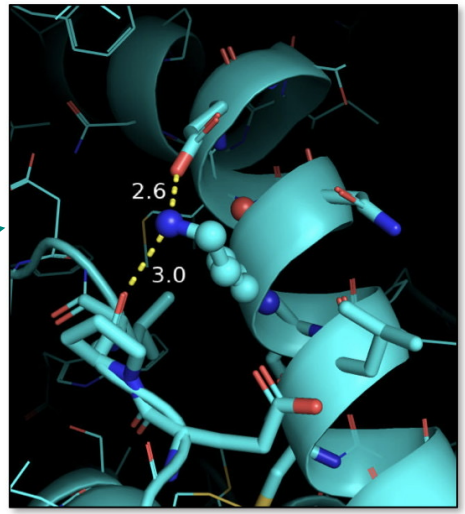
Dr. Park and Dr. Petsko Identified the Same Stabilizing Interaction

119. The long aliphatic side chain in lysine that is terminated by an amine group is compatible with the surrounding pocket at position 320. The aliphatic side chain of the lysine side chain will fit in and be compatible with the hydrophobic walls of the pocket, while the amine group will stick out at the top of the pocket into the solvent, similar to how the carboxyl group of the aspartic acid is positioned in the wild-type protein. Lysine is also generally classified as a hydrophilic residue, which means it can tolerate a solvent accessible environment.

120. Lysine also participates in hydrophobic interactions with nearby hydrophobic residues, including P32 and L317.[82] The terminal amine group extends out of the pocket, thus avoiding the energetic penalty of having a buried charge. Furthermore, the amine group can form both a salt bridge to E324 and a hydrogen bond to the main chain carbonyl of P32. The salt bridge to E324 is illustrated in the figure below by the dotted line labeled "2.6" and the hydrogen bond to P32 is illustrated by the dotted line labeled "3.0."[83]

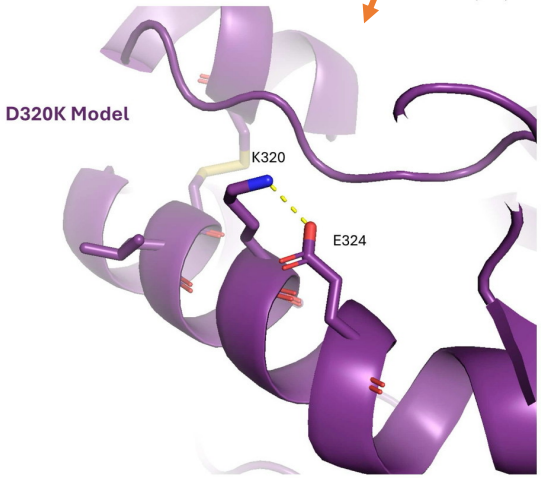
121. Lysine at position 320 positions the positively charged amine group between two negatively charged residues. The introduction of a positive charge would avoid clustering of net negative charges and likely stabilize the structure of PH20.

EX1004 (Park), ¶¶ 119-121; Pet., 104



EX2070 (Petsko), ¶ 403; Reply, 45

403. In the figure below, in the D320K model, K320 forms an interaction with E324.



The Positions and Numbers of Substitutions Dr. Park Evaluated

Position	WT	Position	WT	Position	WT	Position	WT	Position	WT	Position	WT	Position	WT	Position	WT	Position	WT	Position	WT	Position	WT
4	R	42	G	80	P	118	T	156	E	194	Y	232	T	270	R	308	S	346	C	384	G
5	A	43	S	81	Q	119	W	157	F	195	K	233	Q	271	I	309	I	347	Q	385	K
6	P	44	P	82	K	120	A	158	E	196	K	234	Q	272	V	310	M	348	E	386	P
7	P	45	R	83	I	121	R	159	K	197	P	235	S	273	F	311	R	349	Q	387	T
8	V	46	I	84	S	122	N	160	A	198	G	236	P	274	T	312	S	350	G	388	L
9	I	47	N	85	L	123	W	161	G	199	Y	237	V	275	D	313	M	351	V	389	E
10	P	48	A	86	Q	124	K	162	K	200	N	238	A	276	Q	314	K	352	C	390	D
11	N	49	T	87	D	125	P	163	D	201	G	239	A	277	V	315	S	353	I	391	L
12	V	50	G	88	H	126	K	164	F	202	S	240	T	278	L	316	C	354	R	392	E
13	P	51	Q	89	L	127	D	165	L	203	C	241	L	279	K	317	L	355	K	393	Q
14	F	52	G	90	D	128	V	166	V	204	F	242	Y	280	F	318	L	356	N	394	F
15	L	53	V	91	K	129	Y	167	E	205	N	243	V	281	L	319	L	357	W	395	S
16	W	54	T	92	A	130	K	168	T	206	V	244	R	282	S	320	D	358	N	396	E
17	A	55	I	93	K	131	N	169	I	207	E	245	N	283	Q	321	N	359	S	397	K
18	W	56	F	94	K	132	R	170	K	208	I	246	R	284	D	322	Y	360	S	398	F
19	N	57	Y	95	D	133	S	171	L	209	K	247	V	285	E	323	M	361	D	399	Y
20	A	58	V	96	I	134	I	172	G	210	R	248	R	286	L	324	E	362	Y	400	C
21	P	59	D	97	T	135	E	173	K	211	N	249	E	287	V	325	T	363	L	401	S
22	S	60	R	98	F	136	L	174	L	212	D	250	A	288	Y	326	I	364	H	402	C
23	E	61	L	99	Y	137	V	175	L	213	D	251	I	289	T	327	L	365	L	403	Y
24	F	62	G	100	M	138	Q	176	R	214	L	252	R	290	F	328	N	366	N		
25	C	63	Y	101	P	139	Q	177	P	215	S	253	V	291	G	329	P	367	P		
26	L	64	Y	102	V	140	Q	178	N	216	W	254	S	292	E	330	Y	368	D		
27	G	65	P	103	D	141	N	179	H	217	L	255	K	293	T	331	I	369	N		
28	K	66	Y	104	N	142	V	180	L	218	W	256	I	294	V	332	I	370	F		
29	F	67	I	105	L	143	Q	181	W	219	N	257	P	295	A	333	N	371	A		
30	D	68	D	106	G	144	L	182	G	220	E	258	D	296	L	334	V	372	I		
31	E	69	S	107	M	145	S	183	Y	221	S	259	A	297	G	335	T	373	Q		
32	P	70	I	108	A	146	L	184	Y	222	T	260	K	298	A	336	L	374	L		
33	L	71	T	109	V	147	T	185	L	223	A	261	S	299	S	337	A	375	E		
34	D	72	G	110	I	148	E	186	F	224	L	262	P	300	G	338	A	376	K		
35	M	73	V	111	D	149	A	187	P	225	Y	263	L	301	I	339	K	377	G		
36	S	74	T	112	W	150	T	188	D	226	P	264	P	302	V	340	M	378	G		
37	L	75	V	113	E	151	E	189	C	227	S	265	V	303	I	341	C	379	K		
38	F	76	N	114	E	152	K	190	Y	228	I	266	F	304	W	342	S	380	F		
39	S	77	G	115	W	153	A	191	N	229	Y	267	A	305	G	343	Q	381	T		
40	F	78	G	116	R	154	K	192	H	230	L	268	Y	306	T	344	V	382	V		
41	I	79	I	117	P	155	Q	193	H	231	N	269	T	307	L	345	L	383	R		

Note :
Red - essential residues
Dark Green - ≥ 66% MSA Options assessed
Green - 33% to 66% MSA options assessed
Light Green - < 33% MSA options assessed
Unshaded - No MSA options assessed

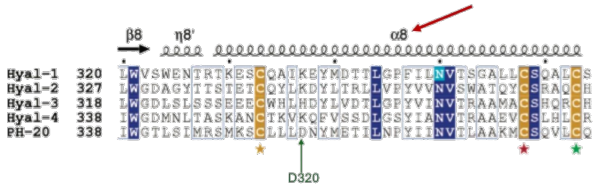
EX1133 (Naismith), ¶ 340 (citing EX2176 (Park Spreadsheet)), Reply, 47

Petition Explained Motivation to Assess D320K

A skilled artisan would also have had several specific reasons to make the single substitution of lysine for aspartic acid at position 320.

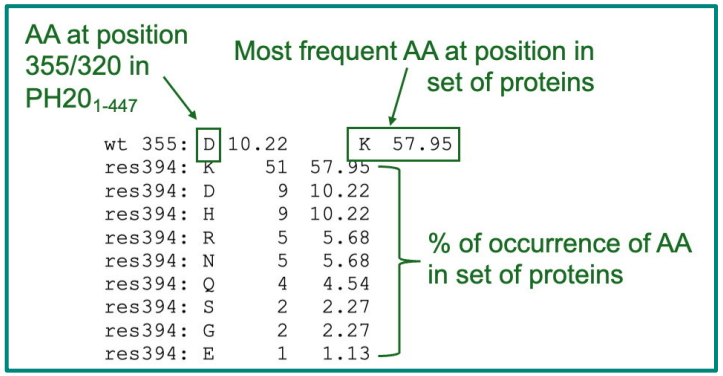
First, lysine is the most prevalent amino acid at this position in the set of homologous hyaluronidase enzymes—it occurs in nearly 60% of those proteins (51 different hyaluronidase enzymes) and in 3 of the 5 human hyaluronidases. The high frequency with which lysine occurs in this position makes it an obvious candidate for being incorporated into position 320 of PH20.[258]

Second, lysine has a high helix propensity, meaning it is more likely to be favored in sequences that form α-helix secondary structures.[259] Position 320 of PH20 is within the middle of a long α-helix sequence designated "α8" (below):[260]



The high propensity of lysine to favor (*i.e.*, support) α-helix structures would have made lysine a logical option to incorporate as a substitution for aspartic acid at position 320 in the α8 helix region of in $PH20_{1-447}$.[261]

Pet., 97-98



EX1004 (Park), ¶ 43; Pet., 96-97

Halozyme Representations in the ‘429 Patent Must Be Credited

In its ’429 Patent, Patentee stated:

> Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity.[264]

Patentee also secured claims in that patent to single amino acid substitutions in the wild-type sequence of $PH20_{1-447}$, even though it provided no examples of any PH20 proteins with any substitutions (*e.g.*, claim 1). Patentee, thus, made and relied on an affirmative statement that a skilled artisan would have expected ***any*** single amino acid substitution in ***any*** non-essential position of $PH20_{1-447}$ to not substantially affect the biological activity of the enzyme. Patentee should not be permitted now to dispute that a skilled artisan would have reasonably expected that making the D320K substitution in $PH20_{1-447}$ would yield an enzyme with substantially the same activity as unmodified $PH20_{1-447}$.

Pet., 99-100

Drs. Park and Hecht Reasonably Expected D320K to be Tolerated

Dr. Park assigned a score of 3 for the D320K substitution in $PH20_{1-447}$, indicating that the substitution would be expected to confer improved stability.[280] He observed that in the wild-type environment, there is a deep hydrophobic pocket around position 320 that limits solvent accessibility to the side chains, but that it is exposed to solvent at the top.[281] He also observed that there is a negative surface charge at 320 that creates electrostatic repulsion with the charged carboxyl group of D320.[282] When the lysine was substituted in position 320, Dr. Park observed that: (i) it introduces a stabilizing salt-bridge with E324 (left image), and a hydrogen bond to the main carbonyl group of P32, and (ii) the long aliphatic chain of lysine participates in hydrophobic interactions with P32 and L317 residues (right image).[283]

Pet., 104

Dr. Hecht reviewed Dr. Park's analysis and conclusions, and agreed with both.[287] Through his own assessment, he also observed that lysine substituted into position 320 would have a stabilizing effect due to (i) the compatibility of the shape of lysine with the solvent-exposed pocket at that location, and (ii) the formation of a salt bridge with E324.[288]

Pet., 106

Other Topics

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Dr. Simpson Mischaracterizes How the Disclosure Describes Certain C-terminal Truncation Mutants

216. The $PH20_{1-430}$ and $PH20_{1-431}$ mutants are described as being enzymatically active in US-457. Specifically, ¶ 0362 states that truncation mutants ending ***before*** position 430 (*i.e.*, $PH20_{1-415}$ through $PH20_{1-429}$) are inactive,[242] and separately notes that mutants terminating between positions 463 and 467 "have little detectable activity due to low expression level." US-457 then states that "***[a]ll other mutants*** (SEQ ID NO:55-57 and **60-87**) ***are active hyaluronidases*** at both pH 7.4 and pH 5.5." (below).[243] In US-457, SEQ ID NO:87 corresponds to $PH20_{1-430}$, while SEQ ID NO:86 corresponds to $PH20_{1-431}$.[244]

[0362] The results are shown in Tables 6 and 6A, below. Human PH20 hyaluronidase carboxy-terminus deletion mutants shorter than I430, corresponding to SPAM1-GDVC to SPAM1-ADVK (SEQ ID NOS:88-95), are inactive. Human PH20 hyaluronidase carboxy-terminus deletion mutants ending at 1498 (SEQ ID NO:101), L499 (SEQ ID NO:59), F500 (SEQ ID NO:100), L501 (SEQ ID NO:58) and I502 (SEQ ID NO:99) have little detectable activity due to low expression level. All other human PH20 hyaluronidase carboxy-terminus deletion mutants (SEQ ID NOS:55-57 and 60-87) are active hyaluronidases at both pH 7.4 and pH 5.5.

Deletion Mutant	Precursor (AA)	Mature (AA)
SPAM1-VCID (SEQ ID NO: 86)	466	431
SPAM1-GVCI (SEQ ID NO: 87)	465	430

Terminus (precursor)	Terminus (mature)	pH 7.4	pH 5.5	Normalized Activity (pH 7.4)	Normalized Activity (pH 5.5)	Simpson Classification	US-457 Classification	Table
482	447	2.03175	1.647	100%	100%	Active	Active	6
465	430	0.441	0.468	22%	28%	Inactive	Active	6
466	431	0.000	0.212	0%	13%	Inactive	Active	6
467	432	0.545	0.900	27%	55%	Reduced Activity	Active	6
468	433	0.860	0.916	42%	56%	Reduced Activity	Active	6
472	437	0.545	0.900	27%	55%	Active	Active	6
490	455	0.819	1.238	40%	75%	Reduced Activity	Active	6
491	456	0.365	0.563	18%	34%	Reduced Activity	Active	6
491	456	0.851	0.729	42%	44%	Reduced Activity	Active	6A
492	457	n/t	n/t	n/a	n/a	Reduced Activity	-	none
493	458	0.689	0.851	34%	52%	Reduced Activity	Active	6
494	459	n/t	n/t	n/a	n/a	Reduced Activity	-	none
495	460	0.689	0.770	34%	47%	Reduced Activity	Active	6
496	461	n/t	n/t	n/a	n/a	Reduced Activity	-	none
497	462	0.176	0.216	9%	13%	Reduced Activity	Active	6
497	462	0.365	0.162	18%	10%	Reduced Activity	Active	6A
498	463	0.527	0.203	26%	12%	Inactive/Little Activity	Low expression	6A
499	464	0.020	0.045	1%	3%	Inactive/Little Activity	Low expression	6
499	464	0.486	0.122	24%	7%	Inactive/Little Activity	Low expression	6A
500	465	0.365	0.162	18%	10%	Inactive/Little Activity	Low expression	6A
501	466	0.446	0.122	22%	7%	None	Active	6
502	467	0.608	0.324	30%	20%	None	Active	6
503	468	1.094	0.648	54%	39%	None	Active	6

EX1133 (Naismith), ¶¶ 216, 218 (citing EX2165 (US-457)), ¶ 0362; Reply, 36

Rescue Mutations Are Not Described or Predictable

4. Dr. Petsko's Rescue-Mutation Theory is Not Supported by the Common Disclosure or the Literature

210. In ¶¶ 374–383, Dr. Petsko suggests that a POSA could use the data in the common disclosure and a PH20 homology model to identify "rescue" mutations that restore activity lost from deleterious substitutions, and that examining structural interactions would allow a POSA to predictably design such activity restoring combinations. This is not described in the common disclosure. There also is no technique or passage in the common disclosure that could provide a basis for Dr. Petsko's theory that a POSA could reliably identify rescue mutations to restore activity in inactive modified PH20 mutants. Instead, it only proposes methods that discovers multiply-modified PH20 variants after performing iterative rounds of mutagenesis and experimental testing, in which libraries of PH20 variants are generated, assayed for activity, and used to guide further mutation.[234] This is not a technique that attempts to predict rescue mutations in advance.

211. Petsko's rescue mutation theory would not have yielded predictable results based on what was known as of 2011. Although the general concept of "rescue mutations" was recognized, there was no reliable way to predict in advance which additional mutations would restore activity in a given protein, let alone PH20. Available approaches—including (i) mutagenesis-and-screening methodologies, (ii) computational or in silico modeling, and (iii) rational or semi-rational design—were inherently uncertain. Dr. Petsko's focus on local structural interactions would likely miss many—if not most—potential rescue mutations for a given "inactive" mutant, as such mutations are often distributed throughout a protein and act through indirect effects on stability or dynamics, rather than through obvious local structural corrections. The rational or semi-rational approaches that Dr. Petsko proposes also were known to succeed only in a fraction of cases and still required experimental testing to confirm restoration of function.[235]

EX1133 (Naismith), ¶¶ 210-211; Reply, 39

Halozyme Sur-Reply: What A POSA "Could" Or "Would" Do

As Dr. Petsko testified, "[t]aken 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 a POSA seeking to generate a modified PH20 that retains hyaluronidase activity." EX2070, ¶322. As he testified at deposition based on the patent data:

> Q. So your opinion is that a POSA could use the data from testing single mutants in the patent to select 15 or 20 substitutions in the PH20 sequence, and you would be able to predict whether that mutated 15 or 20 residue-mutated PH20 will exhibit the activity that you are hoping to get?
>
> **A. My opinion is that you could do so**….
>
> ***

Sur-Reply, 8

Q. So your opinion is that a POSA could use the data from testing single mutants in the patent to select 15 or 20 substitutions in the PH20 sequence, and you would be able to predict whether that mutated 15 or 20 residue-mutated PH20 will exhibit the activity that you are hoping to get?

A. My opinion is that you could do so. My opinion is also that you would almost never do so. You would probably rarely, if ever, make that many substitutions. You wouldn't need to.

EX1131 (Petsko Dep.), 111:9-19; Sur-Reply, 8

Merck Correctly Represented the Disclosure

Petitioner contends that the patent mandates a random screening approach to making multiply mutated PH20s (Reply, 35), and thus that Drs. Petsko and Simpson contradict the common disclosure by using the same "prevalent" method used by Petitioner's experts. Pet., 99-106; Reply, 8-9. But Petitioner misunderstands the specification. The experiment that Petitioner identifies involves testing for stability—not activity— and that approach is called out as exemplary only. EX1026, 47:11-18, 141:7-33 150:1-26. The patent in no way mandates it.

Sur-Reply, 13

The method can be performed a plurality of times, whereby the steps of the method are repeated 1, 2, 3, 4, or 5 times. The method provided herein also is iterative. In one example, after the method is performed, any identified modified hyaluronan-degrading enzyme can be modified or further modified to increase or optimize the activity.

EX1026, (EX1001), 141:30-33; EX1003, ¶ 174; Petition, 80

In any of the methods provided herein for identifying or selecting a modified hyaluronan-degrading enzyme that exhibits stability, the method is performed *in vitro*. Also provided are any of the methods that are iterative, whereby the steps of the method are repeated a plurality of times, wherein in each repetition, further modified hyaluronan-degrading enzymes of a selected modified hyaluronan-degrading enzyme are generated and tested, whereby the modified hyaluronan-degrading enzyme is evolved to exhibit increased stability under a denaturation condition. Also provided herein is a modified hyaluronan-degrading enzyme identified by any of the methods provided herein.

EX1026 ('731 App.), 47:11-18; Sur-Reply 13

D. METHODS FOR IDENTIFYING MODIFIED HYALURONAN-DEGRADING ENZYMES WITH ALTERED PROPERTIES OR ACTIVITIES

Provided herein are methods for identifying a modified or variant hyaluronan-degrading enzyme, such as a modified hyaluronidase or modified PH20 polypeptide, that exhibits an altered activity or property compared to an unmodified hyaluronan-degrading enzyme. In particular, the methods provided herein can be used to screen for one or more

EX1026 ('731 App.), 140:15-20; Sur-Reply 13

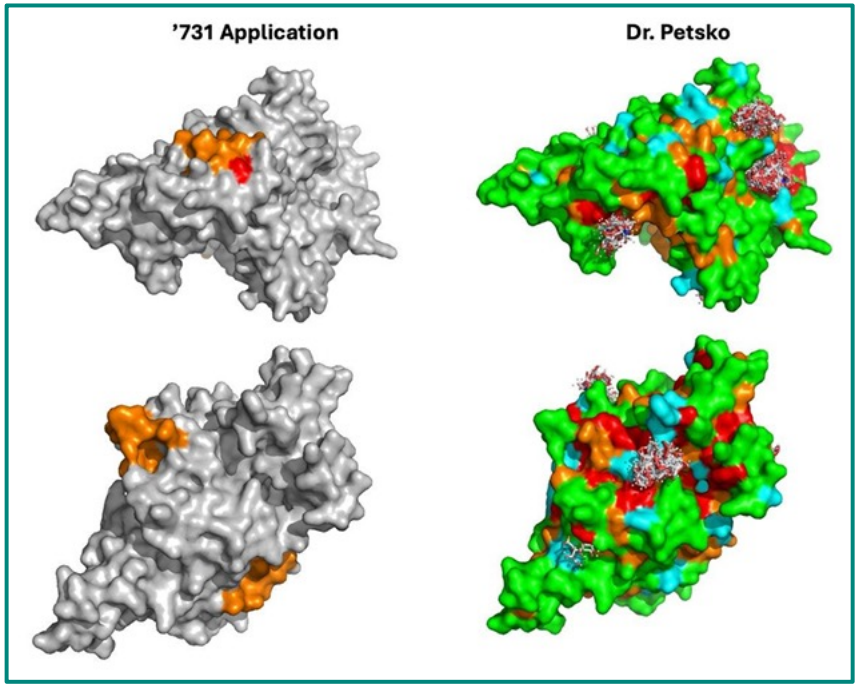
The Data for the Single Substitution Mutants Does Not Reflect Structural Features

But as Dr. Petsko explains, the intolerant positions (and the tolerant positions) are not randomly scattered throughout the protein but rather are clustered, reflecting structural features rather than random epistastic effects. EX1131, 157:5-25; EX2070, ¶¶334342.

Sur-Reply, 16

230. Dr. Petsko relies on potential HA binding sites that differ from those identified in the common disclosure and on analyses derived from the information not disclosed there. I find as of the date of this report, no experimental identification of such additional extended HA sites on PH20. The common disclosure does not reference AutoDock Vina tool or comparable computational tool for identifying HA binding sites. The hypothesis he offers is one for future experimental work, not guidance a POSA would have derived from the common disclosure.

EX1133 (Naismith), ¶ 230; Reply, 38-39



EX1133 (Naismith), ¶ 229; Reply, 39

As Dr. Petsko testified: "the POSA would not start that way at all. They would start with a much more focused program making use of the data in the patent," as set out above. EX1131, 106:21-25, 113:20-114:24. That is because POSA could "reliably predict" actives and practice the claims without "undue" experimentation.

Sur-Reply, 19

Q. And is -- is your opinion that the POSA would believe that any amino acid substitution tolerated at a particular position, for example, in the dark green ones, would be tolerated in any PH20 mutant regardless of how many changes or where those changes were in the protein?

A. Most of the time, yes. If the other changes were in adjacent residues, residues in contact with that one, then you would want to look at the structure.

Q. Okay. So that's something you'd have to kind of do on a case-by-case basis?

A. You would do that on a case-by-case basis when you were trying to make the mutants that had the purposes that you intended.

EX1131, 160:22-161:11

Q. -- is it your view that a protein that had those 15 mutations in them would fold, regardless of which amino acids were put in each position?

A. That would depend on which mutations you picked. If you picked a mutation that only had -- a residue that was only mutated to one or two others, you might be hesitant to make that prediction without first checking with the structure.

EX1131, 161:15-24

*He testified that "properly" folded meant "stably" folded, which he said meant exhibiting activity at **8 hours**. EX2301, 138:6-141:19, 180:2-184:24, 190:9-19, 200:9-16, 250:17-25. Of course, the patent's activity data was measured after 90 minutes. EX1001, Example 3.*

Sur-Reply, 4, 22

Q. Okay. So how can you tell if the folded enzyme is sufficiently stable to retain its folded state?

A. You would look at different time points. A protein which -- in the assay they talk about overnight for example. That could have been one of the things you could do to see if the protein was stable overnight or for a few hours.

EX2301, Naismith Deposition, 137:7-14

Q. Okay. I think in the Example 3 activity assay they were using an incubation time of 90 minutes?

A. Yes.

Q. Is that what you would consider?

A. Yes, but they had isolated the protein too. So there is more to it than just, you know, they purified it. Sorry, they took the extract I should say, not "purified it". They took the extract. So protein has a bit of time, not just 90 minutes.

EX2301, Naismith Deposition, 137:15-25

Moreover, Dr. Naismith admitted at deposition that epistasis simply refers to the fact that multiple mutations will not necessarily be "additive" (EX2301, 129:17-130:18)—that is, that a POSA could not necessarily predict the precise activity level when combining mutations by looking at the effect on activity from individual mutations. But an exact prediction of activity level is not at issue here—particularly because Petitioner's 40% activity construction must be rejected.

Sur-Reply, 20

Q. Okay.
A. And those are very large, and because of that a POSA would look at that and realize that the difference between a mutation, particular introduction, that makes the protein 2.5 times more active and one that makes a protein half as active, roughly, right, I'm not going to get into the precision here. That is a really interesting issue about what is happening to the protein active site or binding or whatever, that is quite interesting. So it speaks to this problem that this mutation is doing something to the protein that we don't understand. We can see their outcome. So therefore if you combine that mutation which has done something to the active site in some way, because we can see it in the assay, with another mutation, that is where we start to worry, really would worry about epistasis because we know these are affecting at a distance in some way the active site because you can see it in the assay, and that is a big difference.

EX2301, Naismith Deposition, 293:23-294:19

When he learned at deposition the data was for every position in PH20, Dr. Hecht admitted that the patent data would in fact be useful in predicting changes in PH20. EX2076, 262:7-22, 253:9-254:12.

Q So is it your testimony that the data in the patent provide no information whatsoever that is useful to a POSA that is seeking to make, for example, a PH20 with 10 mutations, compared to wild-type?

A Once again, I'll say it's the continuum. Having zero data gives you zero insight; having more data gives you more insight.

That's not the same as saying that having X amount of data allows you to predict something accurately.

EX2076, Hecht Deposition 262:15-263:3