Supplementary tables

Supplementary Table 1. Clinical characteristics and main laboratory data from symptomatic relatives of the French index thrombophilic family.

	Reference values	II-3 (proband)				III-3						III-2
		23/03/1999	04/05/2000	19/06/2000	11/01/2008	19/02/2000	22/02/2000	07/03/2000	11/01/2008	26/09/2008	03/04/2009	11/01/2008
Treatment		Heparin	None	None	Tetrazepam	Heparin	Heparin	Heparin	Fluidione	None	Enoxaparin	None
Platelet ×10^9/L	150-400		170		162				258			347
vWF antigen (IU/ml)	0.60-1.40		2.06		1.81				0.51	0.89	0.81	0.93
FVIII (IU/ml)	0.70-1.30		1.63		1.14				0.76	0.74	0.70	0.90
FII (IU/ml)	0.70-1.30			1.10	1.03			0.87	0.43	0.94	1.01	1.16
FVII+FX (IU/ml)	0.70-1.30			0.93								
FV (IU/ml)	0.70-1.30			0.68	0.84			0.63	0.61	0.63	0.69	1.04
FVII (IU/ml)	0.70-1.30									1.19		
FX (IU/ml)	0.70-1.30				0.81				0.19	0.80	0.97	0.97
Fibrinogen (g/l)	1.5-3.5		2.99	4.07	3.78	4.13		5.59	3.04	2.82	3.68	5.23
Prothrombin level (%)	>70		87	70	91	85		44	33	88	87	91
APTT (sec)	30-40		29	33	32	39		53	50	34	36	37
Thrombin time (sec)	20-35		29	26	29	30		28	29	27	26	28
D-dimer (µg/ml)	<0.40			2.41	0.26				0.56	0.30	0.55	0.38
Plasminogen (IU/ml)	0.8-1.2		1.1									
von Kaulla test	1h45-5h		4h15	4h30								

t-Pa (ng/ml)	4.0-10.0		8	5						
PAI-1 activity (IU/ml)	0-17		9	2	4			12		7
Homocysteine (umol/l)	<14.0		17.3							
Protein C (IU/ml)	0.80-1.20	1.07		1.21	1.24	0.93	0.89	0.16	0.98	1.34
Free protein S (IU/ml)	0.65-1.10	0.68		0.74	0.70	0.98	0.75	0.25	0.64	0.89
AT functional (IU/ml)	0.80-1.20	0.52	0.83	0.75	0.82	0.80	0.66	0.85	0.85	0.98
AT antigen (g/l)	0.24-0.36		0.27	0.26		0.25				
TGA ETP (nM.min)	1500±400				2759				2254	3095

Supplementary methods

Blood collection and sample preparation

Platelet poor plasma was prepared by centrifugation of the anticoagulated blood for 10 min at 2300 g at room temperature, aliquoted and stored at -80 ºC. Genomic DNA was obtained from peripheral blood using standard commercial methods and stored at -20 ºC.

Functional methods to determine antithrombin activity

Anti-FXa assays were performed using chromogenic methods with heparin, bovine FXa, and S-2765 chromogenic substrate (HaemosIL TH, Instrumentation Laboratory, Italy). Anti-FIIa assays were performed with or without unfractionated heparin, bovine thrombin, and S-2238 chromogenic substrate (Werfen, Spain).

Genetic analysis

Whole genome sequencing was done at the Centre National de Recherche en Génomique Humaine (CNRGH, Institut de Biologie François Jacob, Evry, France), using the Illumina TruSeq DNA PCR-Free Library Preparation Kit, according to the manufacturer's instructions. After normalisation and quality control, qualified libraries were sequenced on a HiSeqX5 instrument from Illumina (Illumina Inc., USA) using a paired-end 150 bp reads strategy. FastQ sequences were aligned on human genome hg37 using the BWA-mem program.[1]

Glycosylation analysis

Cell supernatants (up to 200 µg of glycoprotein in a volume of 35 µl) were denatured with 10 µl of 250 mM phosphate buffer and 2.5 µl of 2% SDS with 1 M 2-mercaptoethanol, heated at 100 ºC for 5 minutes and cooled. TRITON x-100 (2.5 µl of 15%) (v/v) was added. Then, 2.0 µl of PNGase F (≥ 5,000 units/ml) were added and incubated overnight at 37 ºC. Samples were run in SDS-PAGE and detected as described in material and methods.

References

1. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*. 2009;25(14):1754–60.

Legends of Supplementary Figures

Supplementary figure 1. Recombinant antithrombin glycoforms secreted to the conditioned medium of HEK-EBNA cells transfected with the plasmid containing the cDNA of human antithrombin. Detection of antithrombins was done by Western blotting. If the plasmid contains the wild-type sequence (Ser169), two glycoforms of antithrombin were generated due to the inefficient glycosylation of Asn167. The mutation of p.Ser169Ala fully abolished the glycosylation at Asn167 and a single glycoform, beta, was generated. Arrows point the glycoforms. The number of N-glycans of each glycoform is also indicated.

Supplementary Figure 2. Localization of Glu227 in A) the secondary and B) tertiary structure of native and latent conformations of antithrombin. Glu227 is represented as yellow balls.

Supplementary Figure 3. *In silico* predictions of the consequences of the p.Glu227Lys mutation in antithrombin in the electrostatic potential of wild-type (WT) and mutated (Mut) molecules.

Supplementary Figure 4. Plasma antithrombin in heterozygous carriers of the p.Arg79Cys, p.Glu227Lys, and p.Glu269Lys mutations and in a healthy control. Separation of proteins were done under native and denaturing conditions and antithrombins were detected by Western blotting. Red arrows point variant forms, black arrow points the wild-type antithrombin.

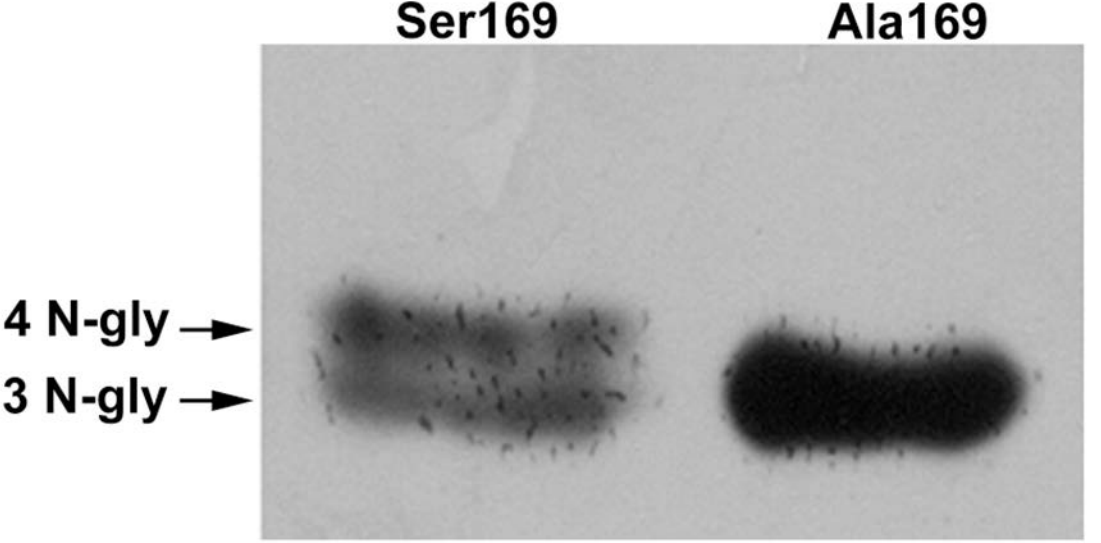
Supplementary Figure 5. Identification of the aberrant antithrombin in the Peruvian patient carrying the p.Glu227Lys mutation.

Supplementary figure 6. Effect of Lysine residues on the efficacy of N-glycosylation at different sequons of antithrombin: A) Localization of Lys residues surrounding N-glycosylation sequons of antithrombin on the wild-type sequence. Lys mutated are underlined in green, while residues mutated to Lys are underlined in blue. The glycosylated asparagines are shown in red. B) Antithrombins secreted to the conditioned medium of HEK-EBNA cells transfected with different variants in the wild-type (Ser169) or beta (Ala169) backgrounds. The position of each variant in the N-Sequon and the numbers of N-glycoforms generated are indicated. C) Effect of PNGase F treatment on the above recombinant antithrombins. Arrows point the different glycoforms and the number of N-glycans of each glycoform is indicated.

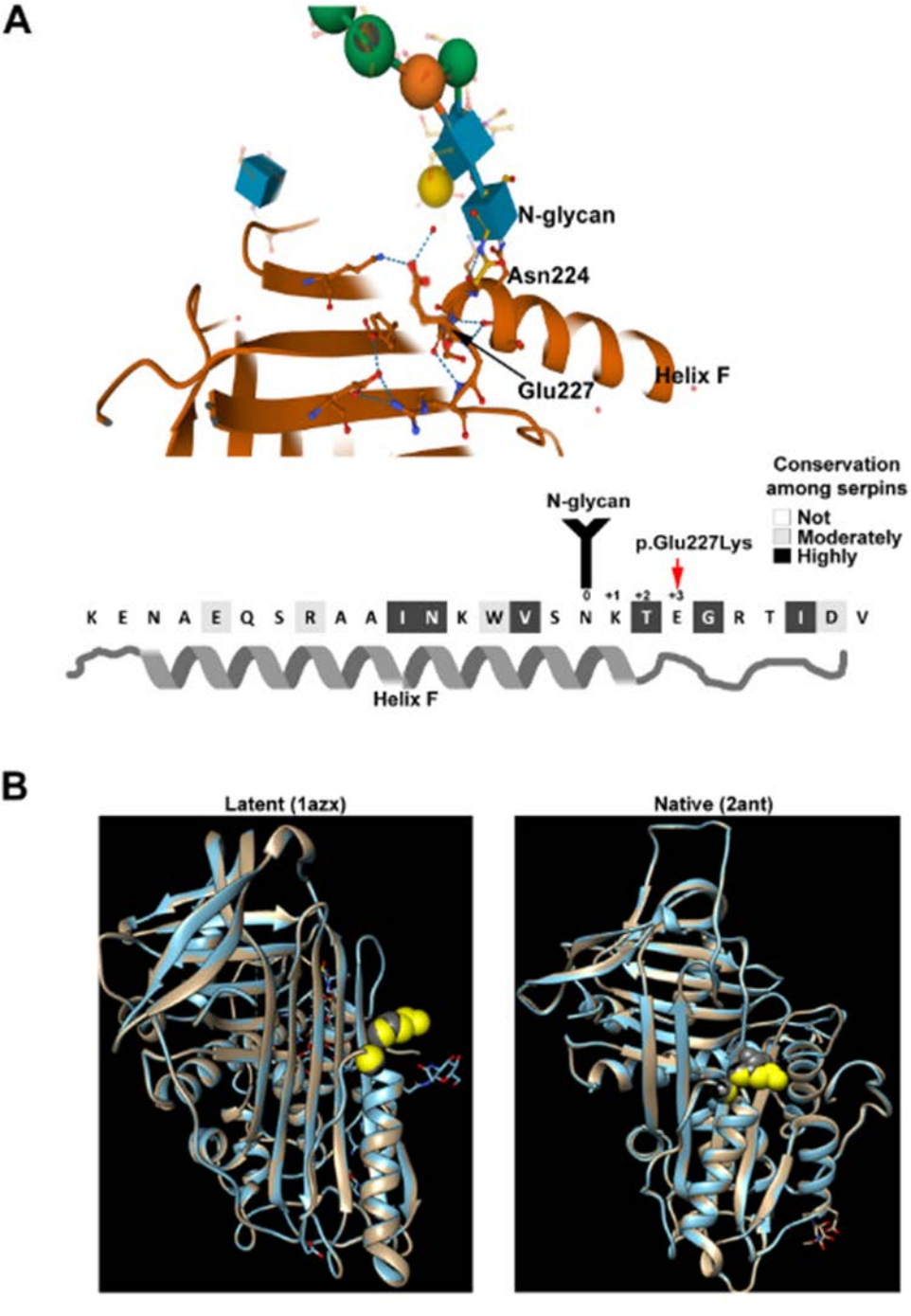
Supplementary Figure 7. Heparin affinity of beta glycoforms of wild-type and His224 recombinant antithrombins purified from conditioned medium.

Supplementary Figure 8. Representative experiment of the anti-FXa activity determined by a chromogenic assay of purified recombinant proteins of wild-type (WT) and mutant (H224). Proteins are generated in a wild-type background (Ser167) and in a beta background (Ala167) indicated by (β).

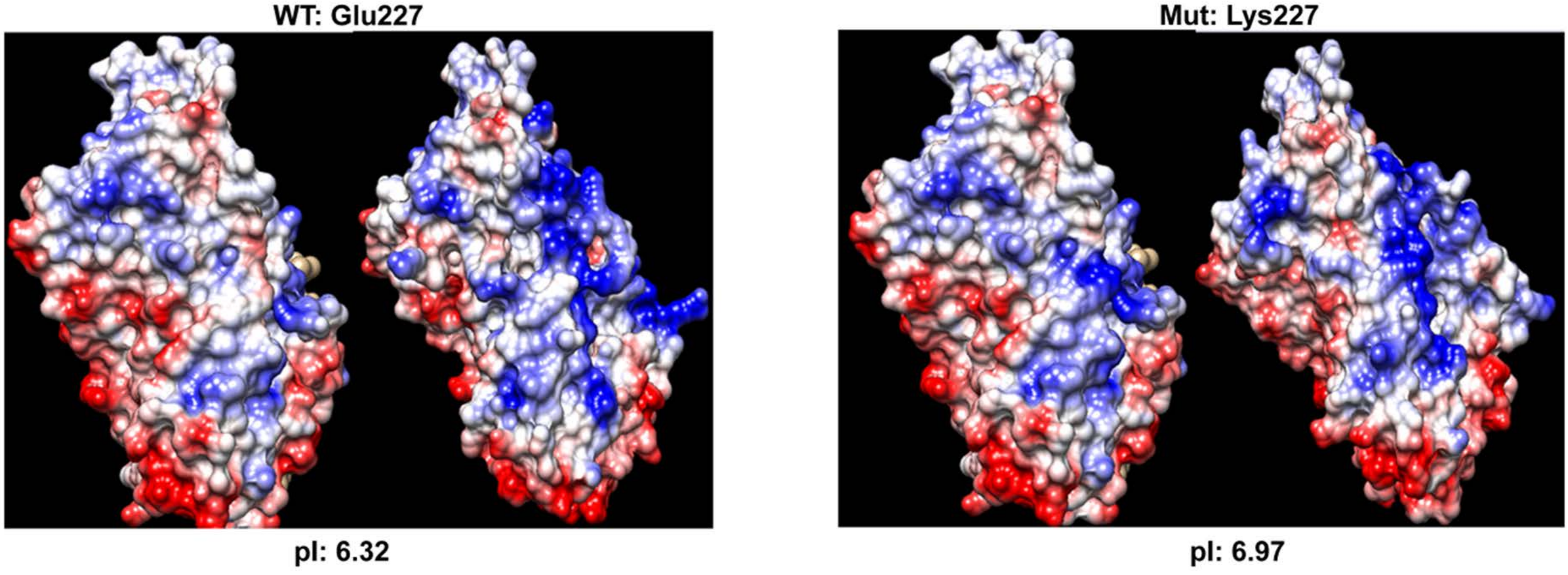
Supplementary Figure 1.



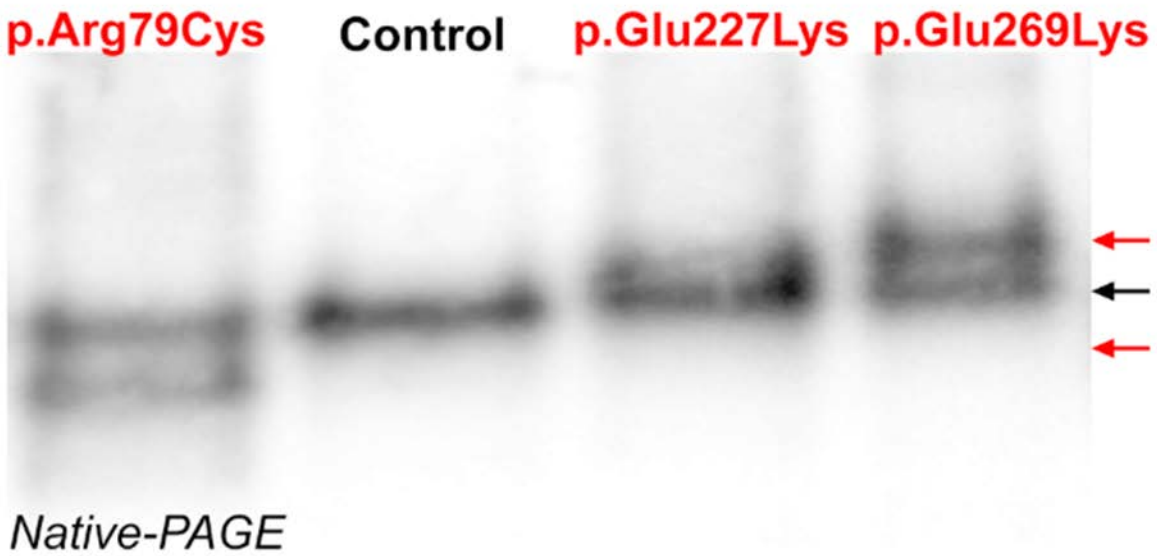
Supplementary Figure 2.

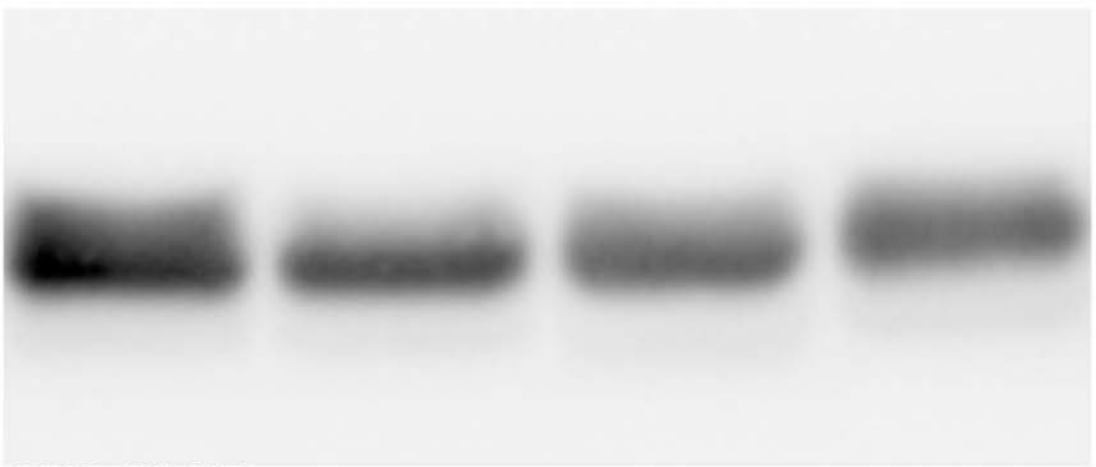


Supplementary Figure 3.

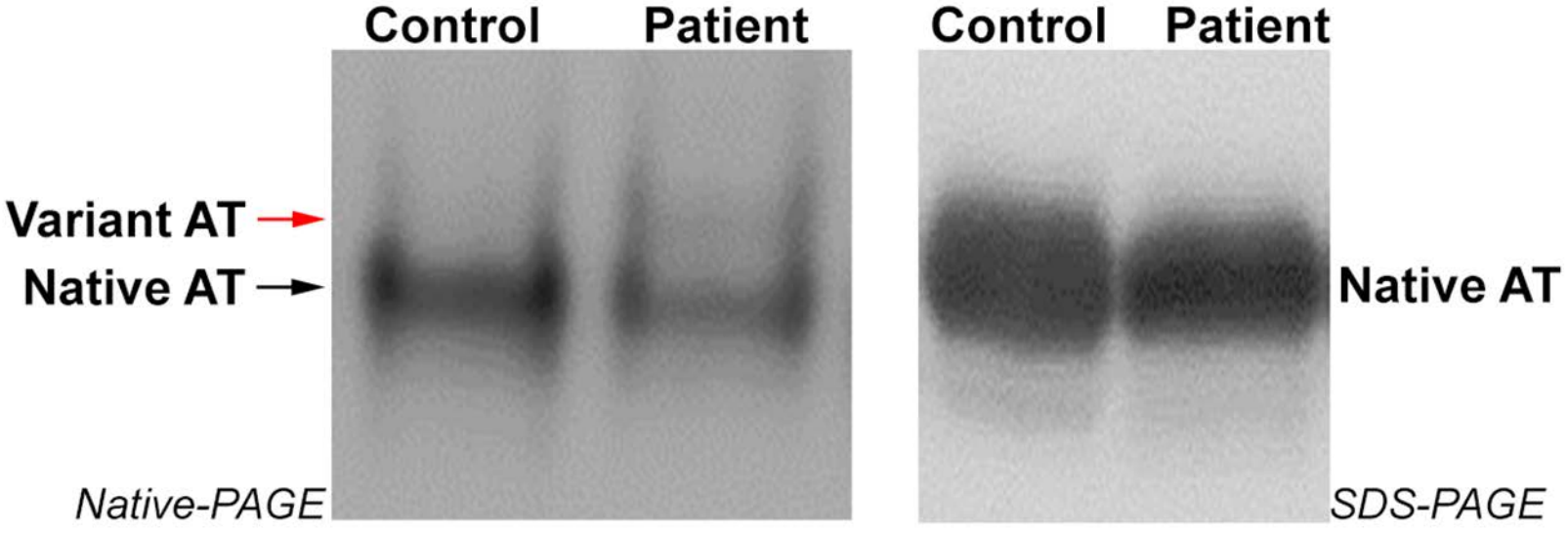


Supplementary Figure 4.

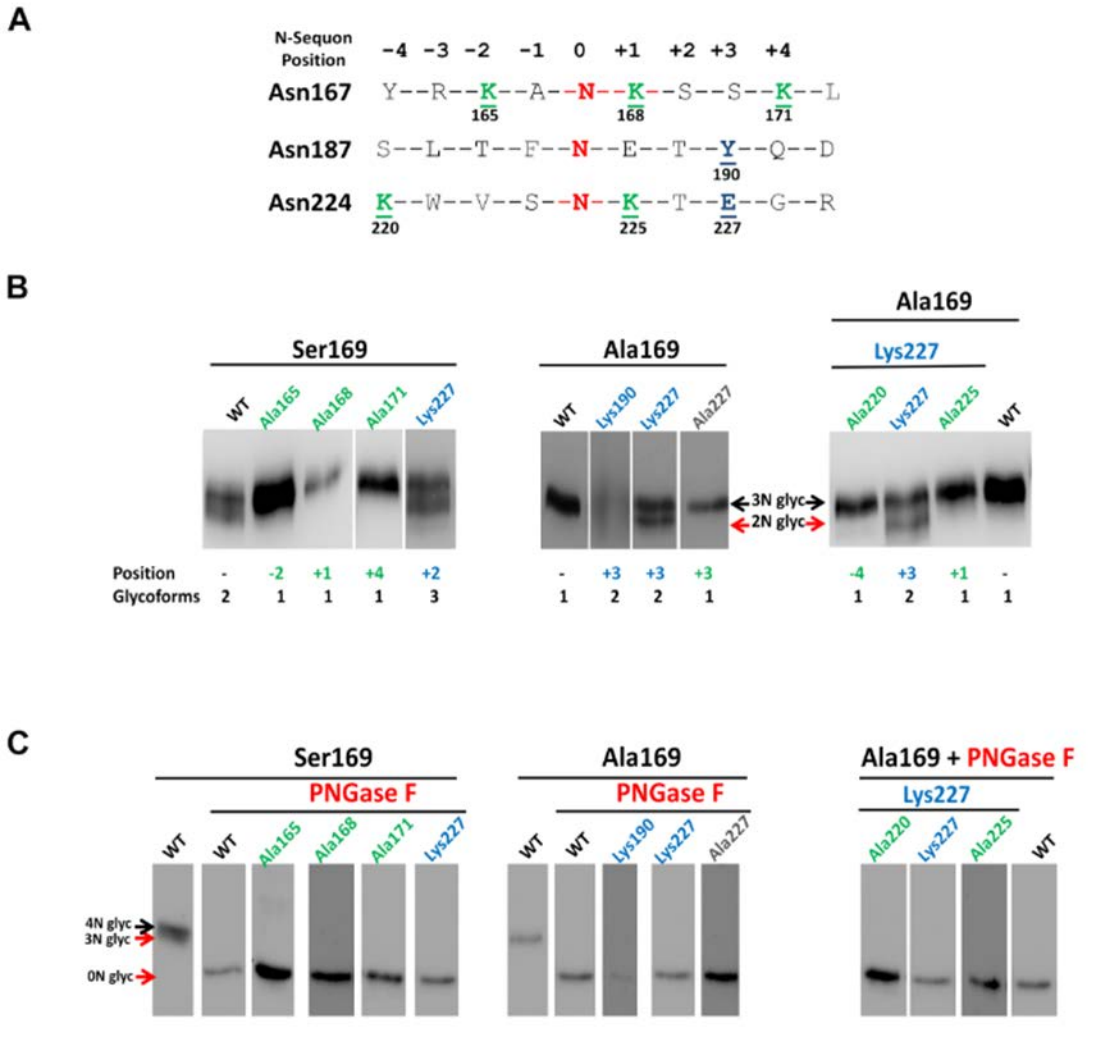




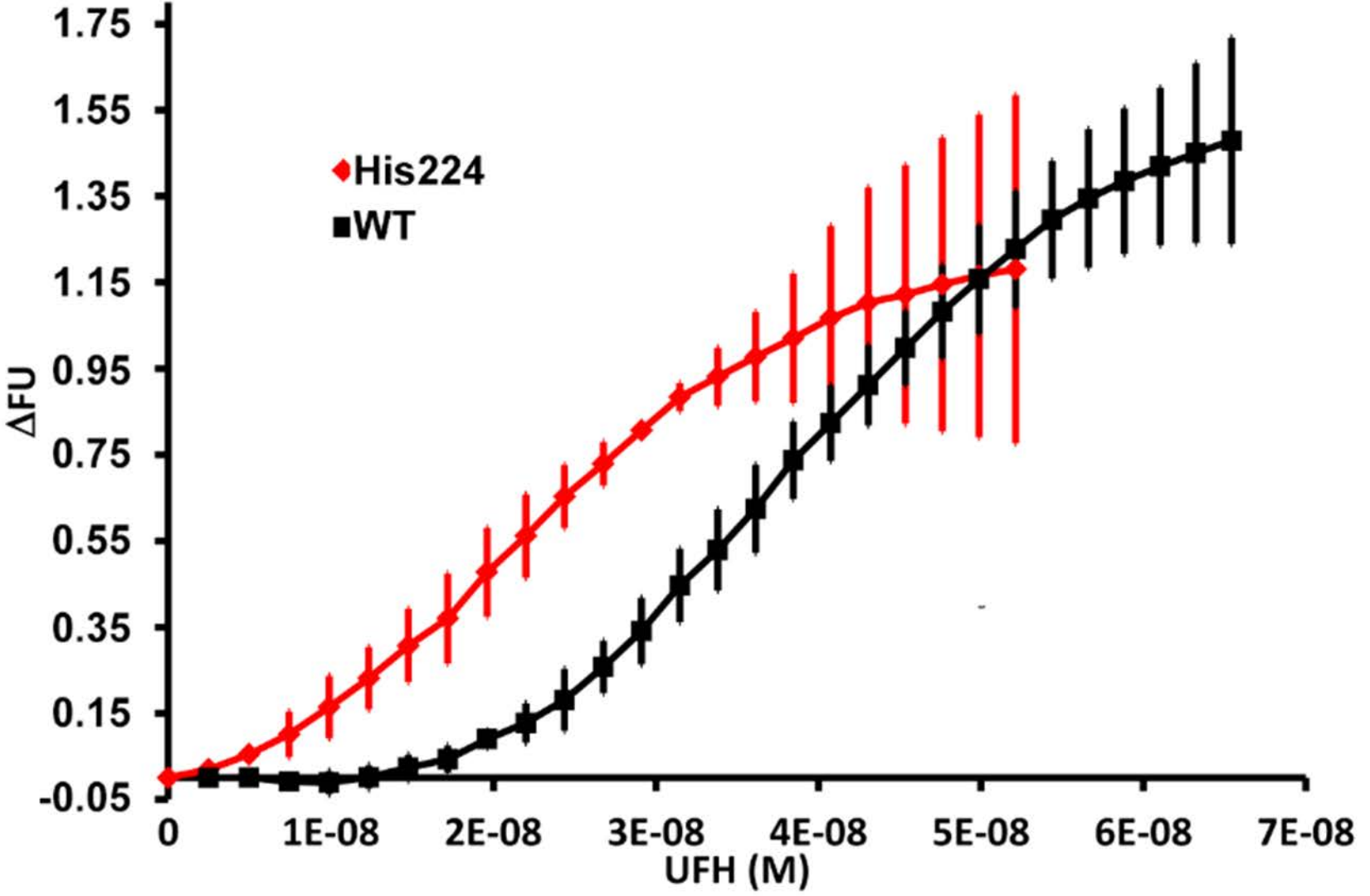
Supplementary Figure 5.

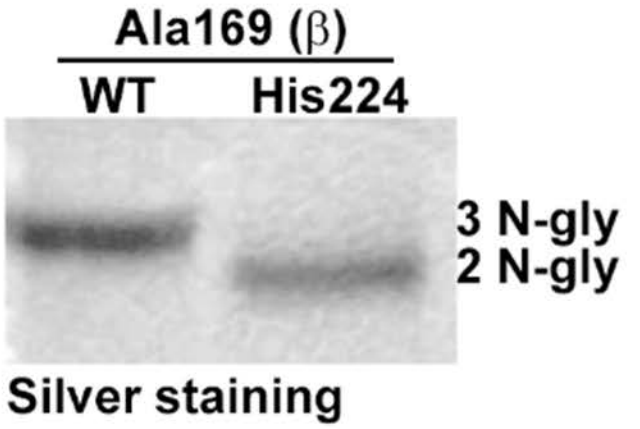


Supplementary Figure 6.

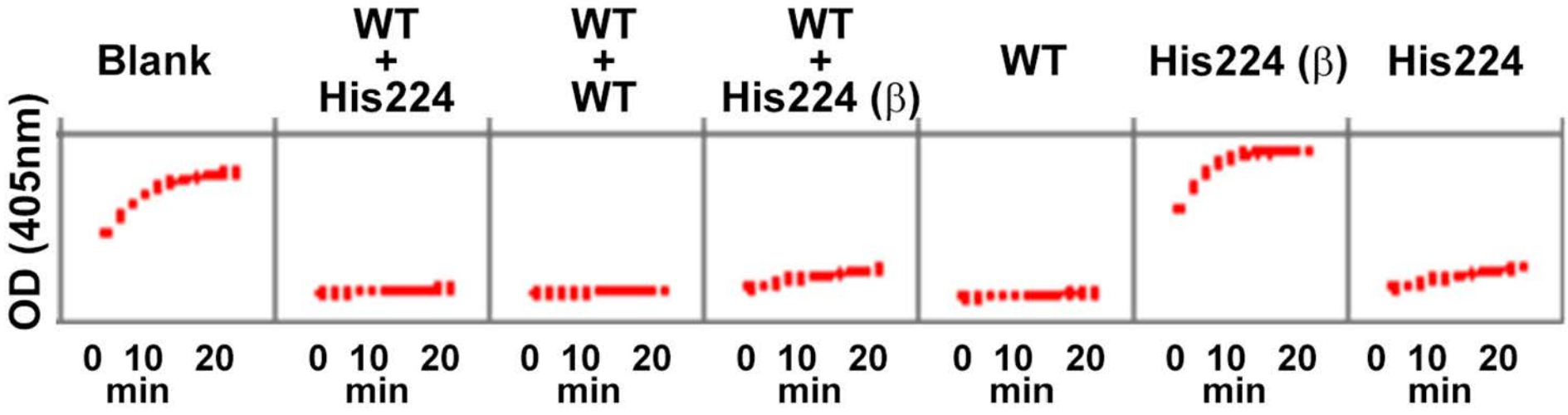


Supplementary Figure 7.





Supplementary Figure 8.



	Blank	WT + His224	WT + WT	WT + His224 (β)	WT	His224 (β)	His224
Vmax (OD/min)	14.04	0.68	0.61	4.18	0.68	12.21	5.25