

The structural basis of pyridoxal-5'-phosphate-dependent β -NAD-alkylating enzymes

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Supplemental Information

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Supplementary Table 1. Data collection and refinement statistics of cryo-EM data.

	PseP-apo (EMDB-36852)(PDB 8K3I)	PseP-NAD (EMDB-36851)(PDB 8K3H)
Dataset numbers	1 dataset	1 dataset
Data collection and processing		
Microscope	Titan Krios G3i	Titan Krios G3i
Voltage (kV)	300	300
Detector	K3 with BioQuantum energy filter	K3 with BioQuantum energy filter
Magnification	105,000	105,000
Pixel size (Å) (calibrated)	0.83 (-)	0.83 (-)
Automation software	EPU	serialEM
Total exposure (e-/Å ²)	49	49
Exposure rate (e-/Å ² frame)	1.00	1.00
No. of frames	49	49
Defocus range (µm)	-1.0 to -1.8	-0.8 to -1.8
Symmetry imposed	<i>C</i> ₂	<i>C</i> ₂
No. of collected micrographs	6,587	3,006
No. of selected micrographs	6,587	3,006
No. of particles for 2D classification	2,771,336	2,478,450
No. of particles for 3D classification	643,748	1,142,873
No. of particles for 3D refinement	211,371	462,772
Map resolution (Å)	2.83	2.86
FSC threshold	0.143	0.143
Local resolution range (Å)	2.7-3.1	2.7-3.1
Refinement		
Initial model used (PDB code)	none	none
Model composition		
Non-hydrogen atoms	7,372	7,460
Protein residues	960	960
Ligand atoms	0	74
<i>B</i> factors (min/max/mean, Å ²)		
Protein	5.02/44.8/17.8	17.4/56.7/29.9
Ligand		32.2/32.5/32.4
R.m.s. deviations from ideal values		
Bond lengths (Å)		
Bond angles (°)	0.004	0.002
	0.602	0.466
Validation		
MolProbity score	1.48	1.21
Clashscore	6.04	4.01
Poor rotamers (%)	0	0
Ramachandran plot		
Favored (%)	97.2	97.9
Allowed (%)	2.9	2.1
Disallowed (%)	0	0

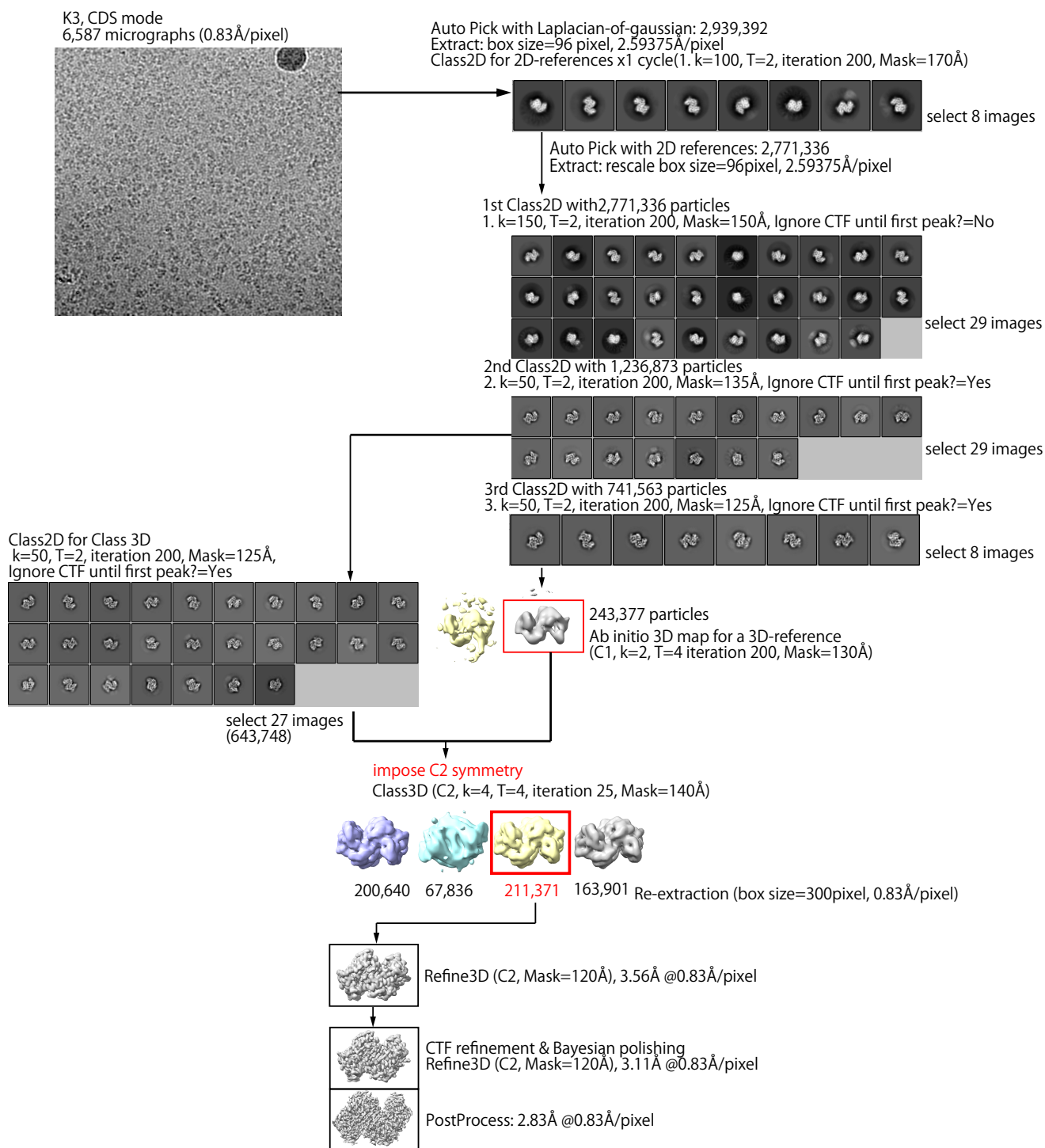
Supplementary Table 2. The tested ligand and protein for cryo-EM data in this study.

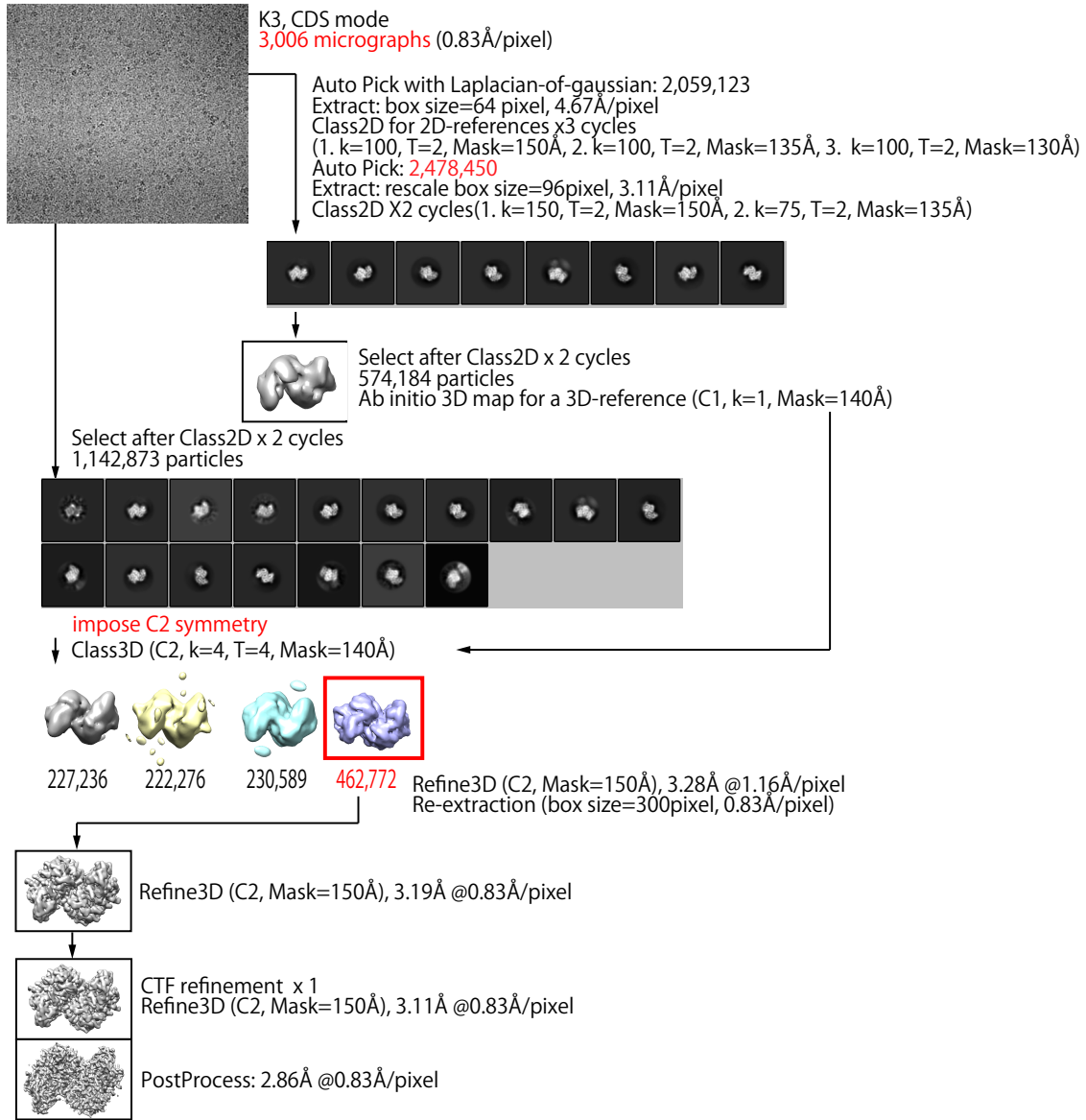
Sample	Enzyme	Ligand 1	Ligand 2	Results
1	WT	1 mM β -NAD	1 mM SAH	trace density of substrate
2	WT	20 mM β -NAD		Structure with β -NAD
3	WT	20 mM AVG		No ligand (apo)
4	WT	20 mM SAH		Low monodispersity
5	WT	20 mM SAM		Low monodispersity
6	WT	20 mM SAM	20 mM AVG ^f	Low monodispersity
7	WT	20 mM SAM	20 mM β -NAD	Low monodispersity
8	WT w/o PLP	20 mM SAM		Low monodispersity
9	Y525F	20 mM SAM		Low monodispersity
10	WT	13.6 mM SAH ^a		Low monodispersity
11	WT	5 mM SAH ^b		Low monodispersity
12	WT	4 mM SAH ^b		Low monodispersity
13	WT	2 mM SAH ^b		Low monodispersity
14	WT	3 mM D ₈ -SAM ^c	3 mM β -NAD	Low monodispersity
15	WT	20 mM SF ^d		Low monodispersity
16	WT	20 mM SF ^d	1 mM β -NAD	Low monodispersity
17	WT	20 mM SF ^d	1 mM AVG ^f	Low monodispersity
18	WT	1 mM SAM	1 mM NADH	Low monodispersity
19	WT	5 mM VG ^e	20 mM β -NAD	Low monodispersity
20	WT	10 mM VG ^e	20 mM β -NAD	Low monodispersity
21	WT	20 mM VG ^e	20 mM β -NAD	Low monodispersity

^aThe DMSO solution of SAH (stock conc 200 mM) was mixed into the enzyme. ^bThe 0.1 N HCl solution of SAH (stock conc 50 mM) was mixed into the enzyme. ^cD₈-SAM was prepared from D8 L-methionine and ATP with SAM synthetase¹. ^dsinefungin. ^evinylglycine. ^faminovinylglycine.

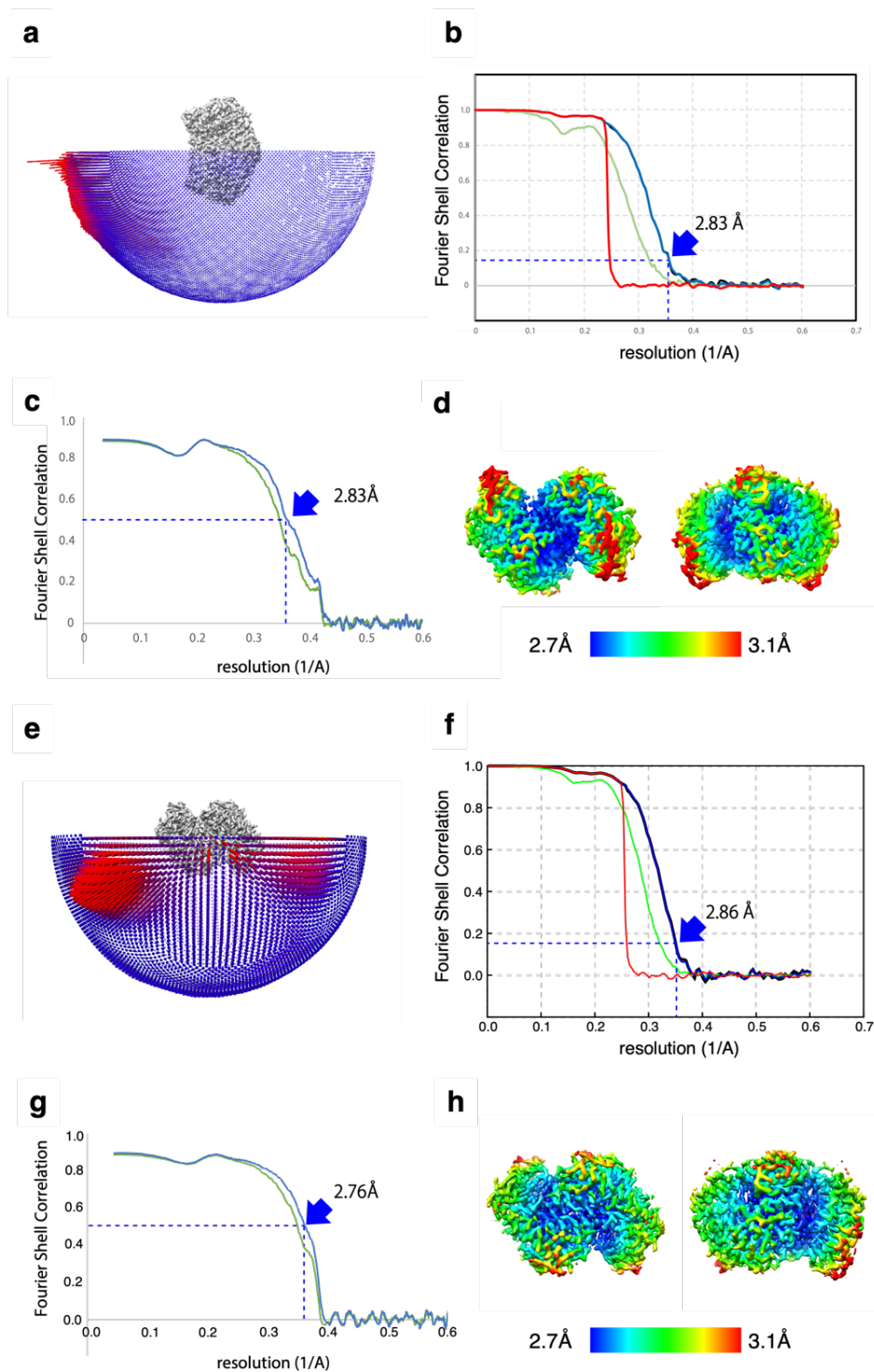
For samples 1-4, the data was obtained, and the 3D structure was obtained after the data analysis. For sample 4, no 3D structure was obtained because 2D averaging did not work. For samples 5-21, the shape of the enzyme and the ice made during sample preparation were not suitable for measurement, and the data was not obtained. The PsePQ without PLP was prepared by using hydroxylamine according to reference 2.

a

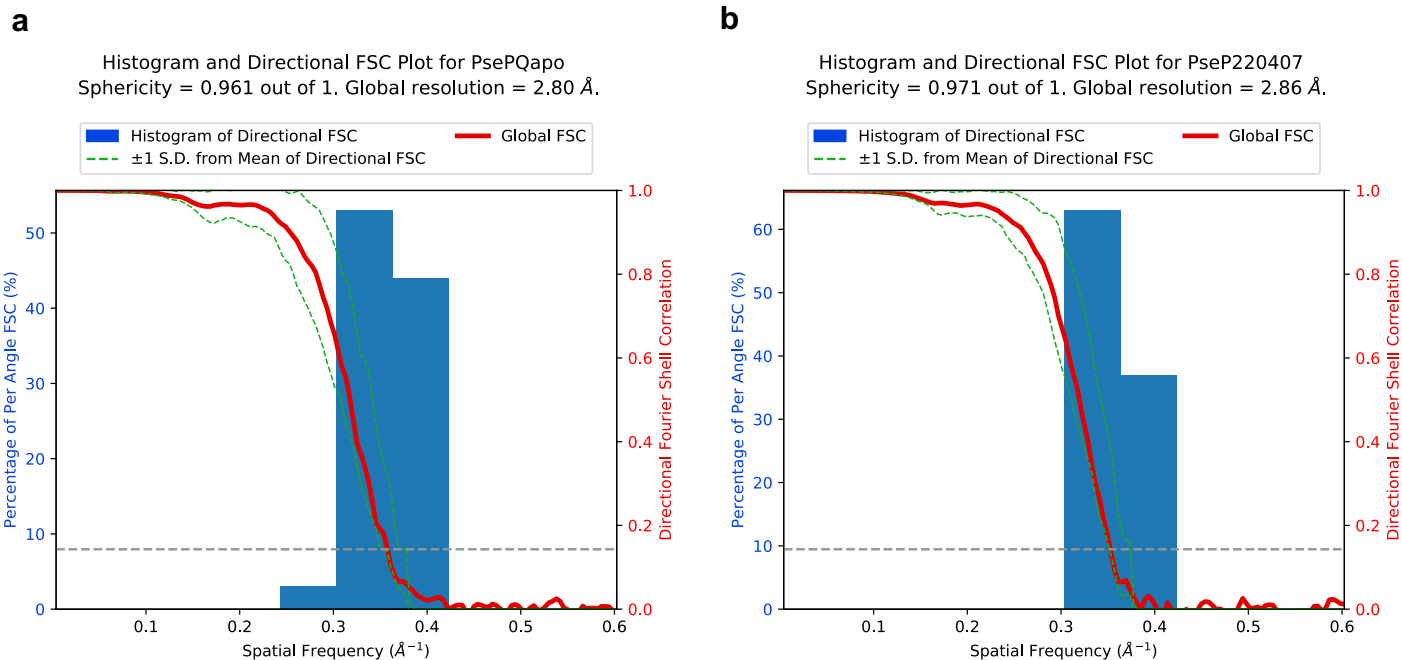


b

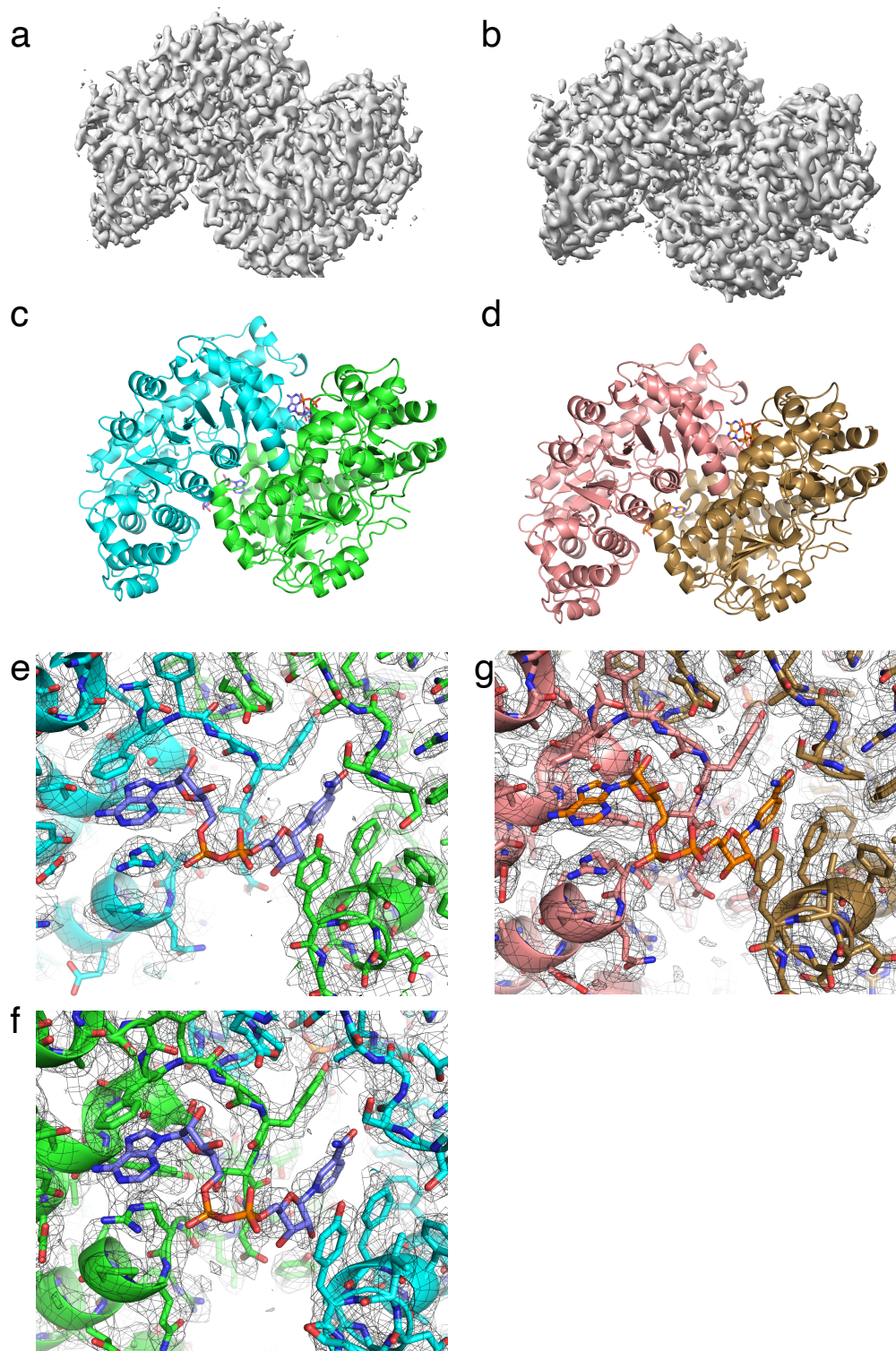
Supplementary Figure 1. Workflow for cryo-EM data-processing of the PseP structure complexed with β -NAD. Data processing workflow used to obtain the final cryo-EM map of PseP-apo (a) and PseP-NAD (b).



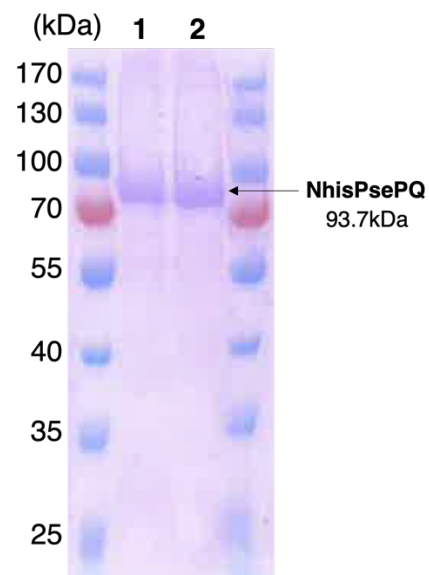
Supplementary Figure 2. Cryo-EM data-processing of PseP. Orientation distribution (a. PseP-apo, e. PseP-NAD), half sets FSC curves (b. PseP-apo, f. PseP-NAD), map-to-model FSC curve (c. PseP-apo, g. PseP-NAD), and local resolutions of the cryo-EM maps PseP colored from highest resolution (dark blue) to lowest resolution (red) (d. PseP-apo, h. PseP-NAD). In the half-sets FSC curves, FSC corrected (black), FSC unmasked (green), FSC masked (blue), and corrected FSC phase randomized (red) maps are shown.



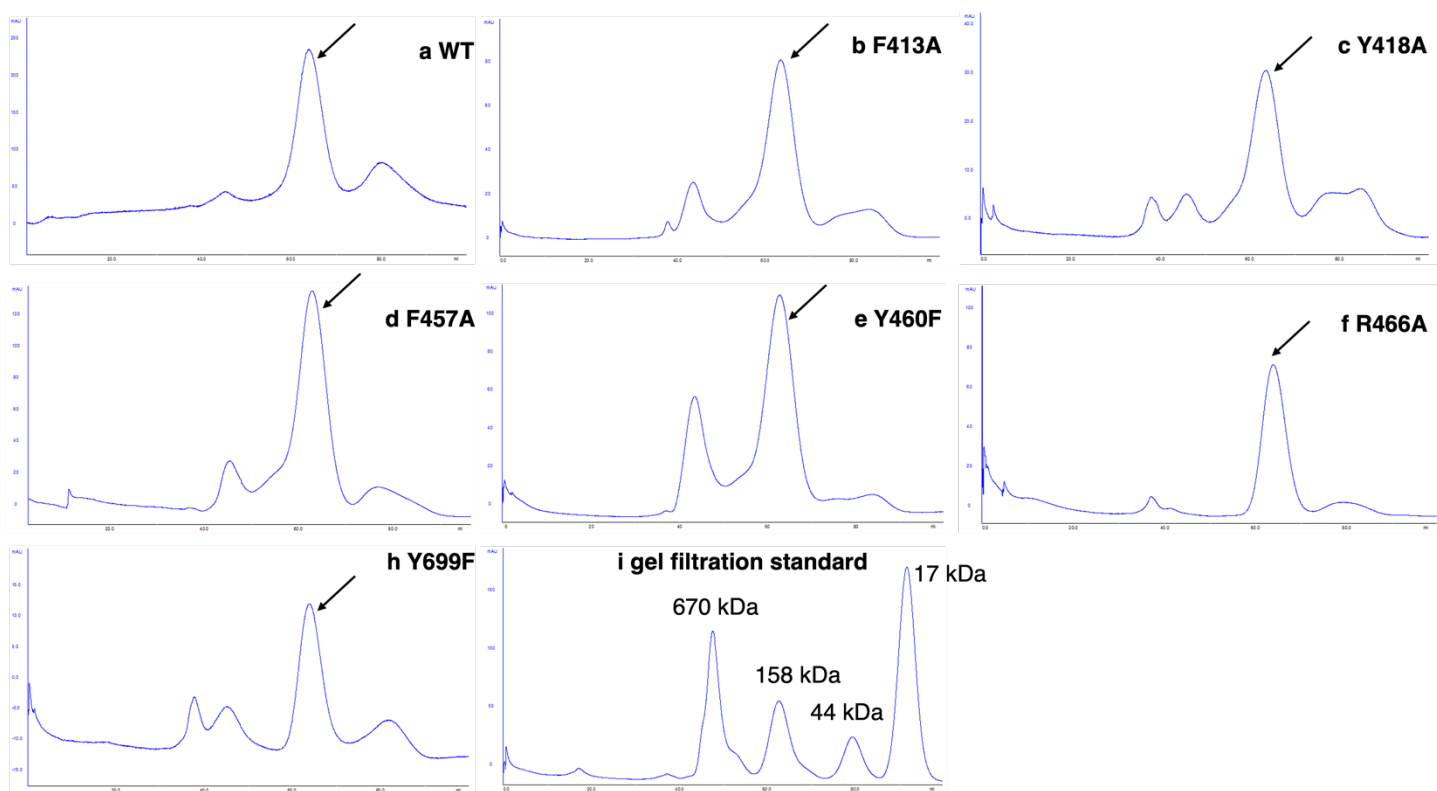
Supplementary Figure 3. 3D-FSC analysis of PseP. Histogram and directional FSC plots for **a.** Pse-apo and **b.** Pse-NAD. Plot of the directional FSC that represents a measure of directional resolution anisotropy. Shown are the global FSC (red line), the spread of directional resolution values defined by ± 1 standard deviation from the mean of the directional resolutions (area encompassed by the green dotted lines) and a histogram of 100 directional resolutions evenly sampled over the 3D FSC (blue bars). A sphericity of 0.961 and 0.971 for Pse-apo and Pse-NAD, respectively, were determined at an FSC threshold of 0.5, which indicates very isotropic angular distribution (a value of 1 stands for completely isotropic angular distribution). Directional FSC determination was performed with the 3DFSC software.



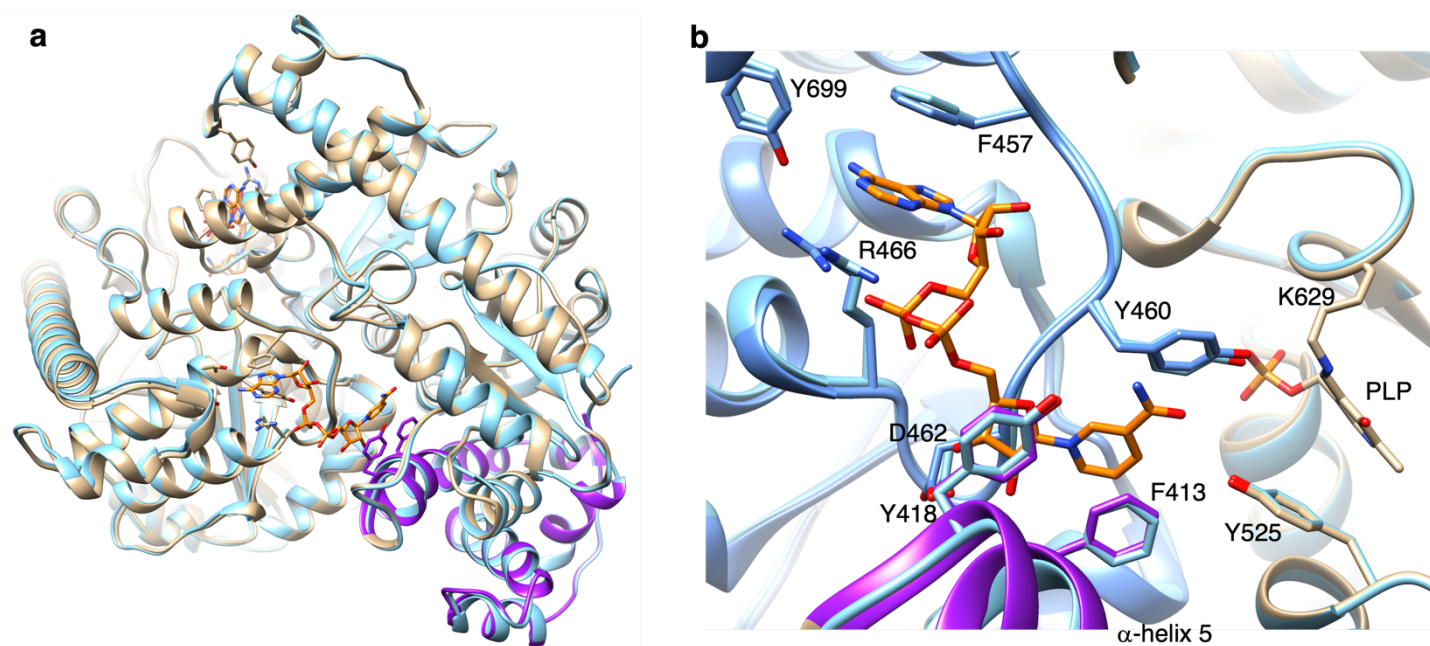
Supplementary Figure 4. Comparison of the structures with C1 and C2 symmetry. Density maps (a. C1 structure, b. C2 structure) and overall structure (c. C1 structure, d. C2 structure). (e-g) Cryo-EM density map around β -NAD⁺ binding site (e. Monomer A and f. monomer B in C1 structure and g. monomer A in C2 structure) The density is shown as black map contoured at +6.0s.



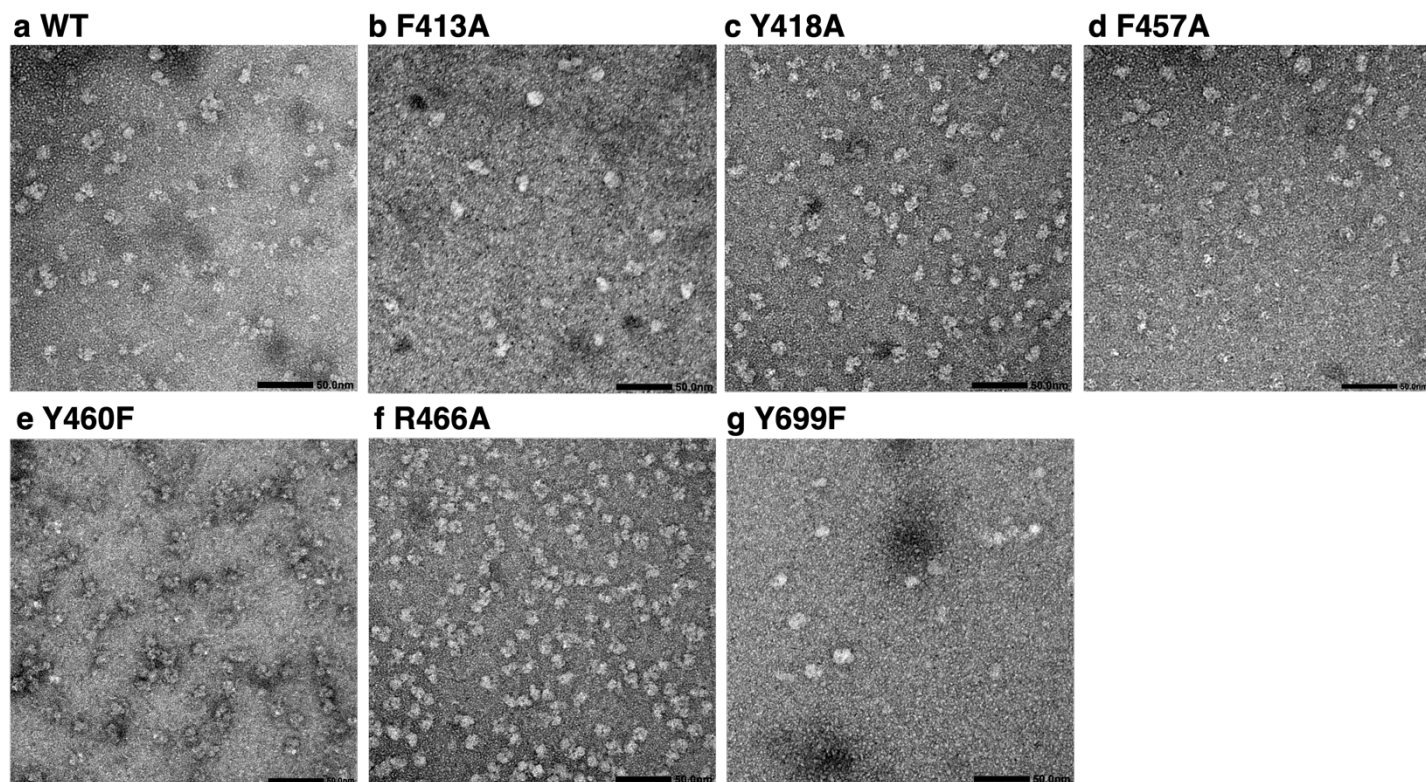
Supplementary Figure 5. SDS-PAGE analysis of WT PsePQ. 1. PsePQ after Ni-affinity and gel filtration purification, 2. After liquid ethane treatment. Experiment was repeated two times independently with similar results.



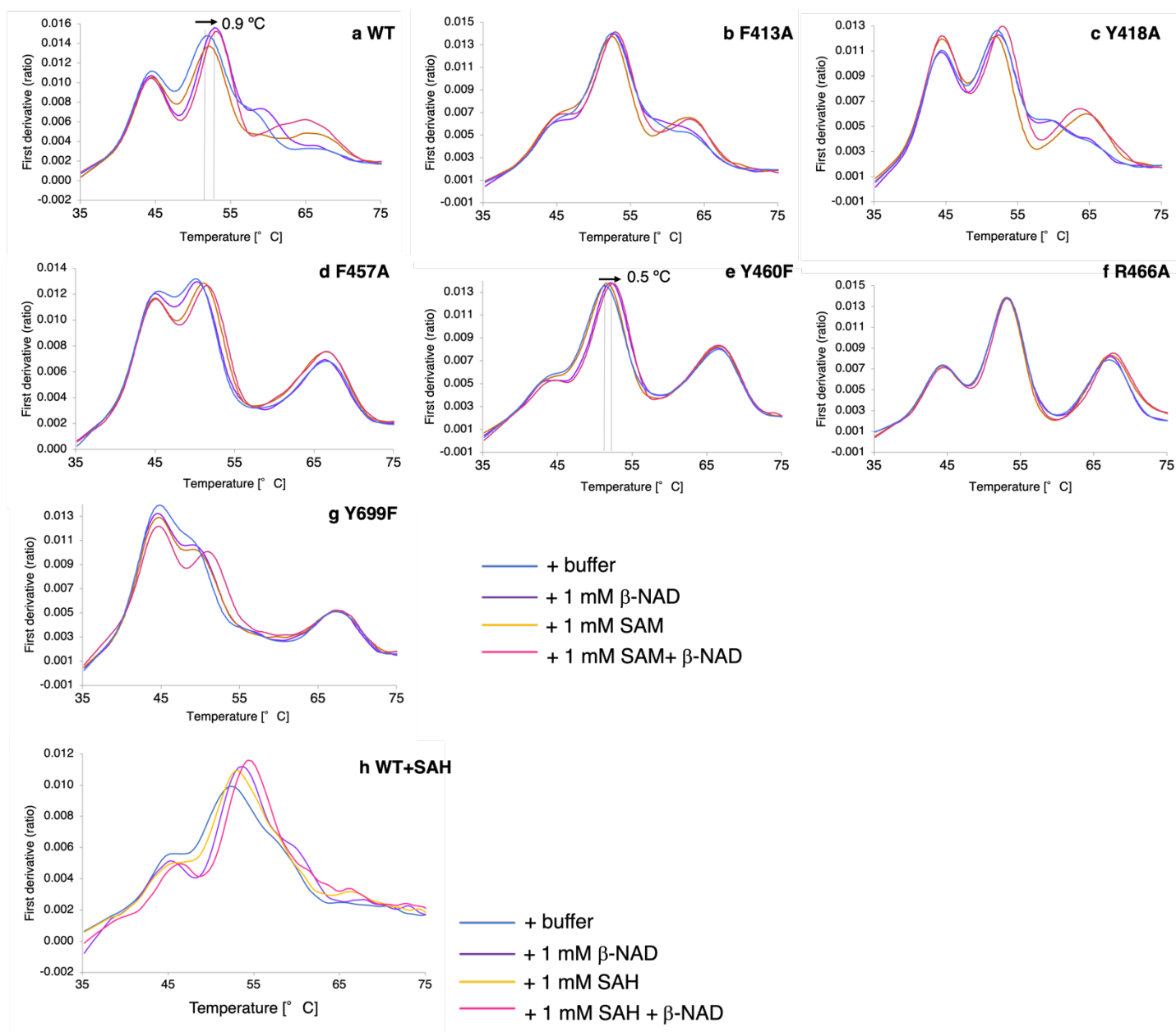
Supplementary Figure 6. Gel filtration analysis of WT and variants of PsePQ. The arrow indicates a peak of the dimer which corresponds to MW 187 kDa. Gel filtration standard is a product from Bio-rad (Catalog number 1511901).



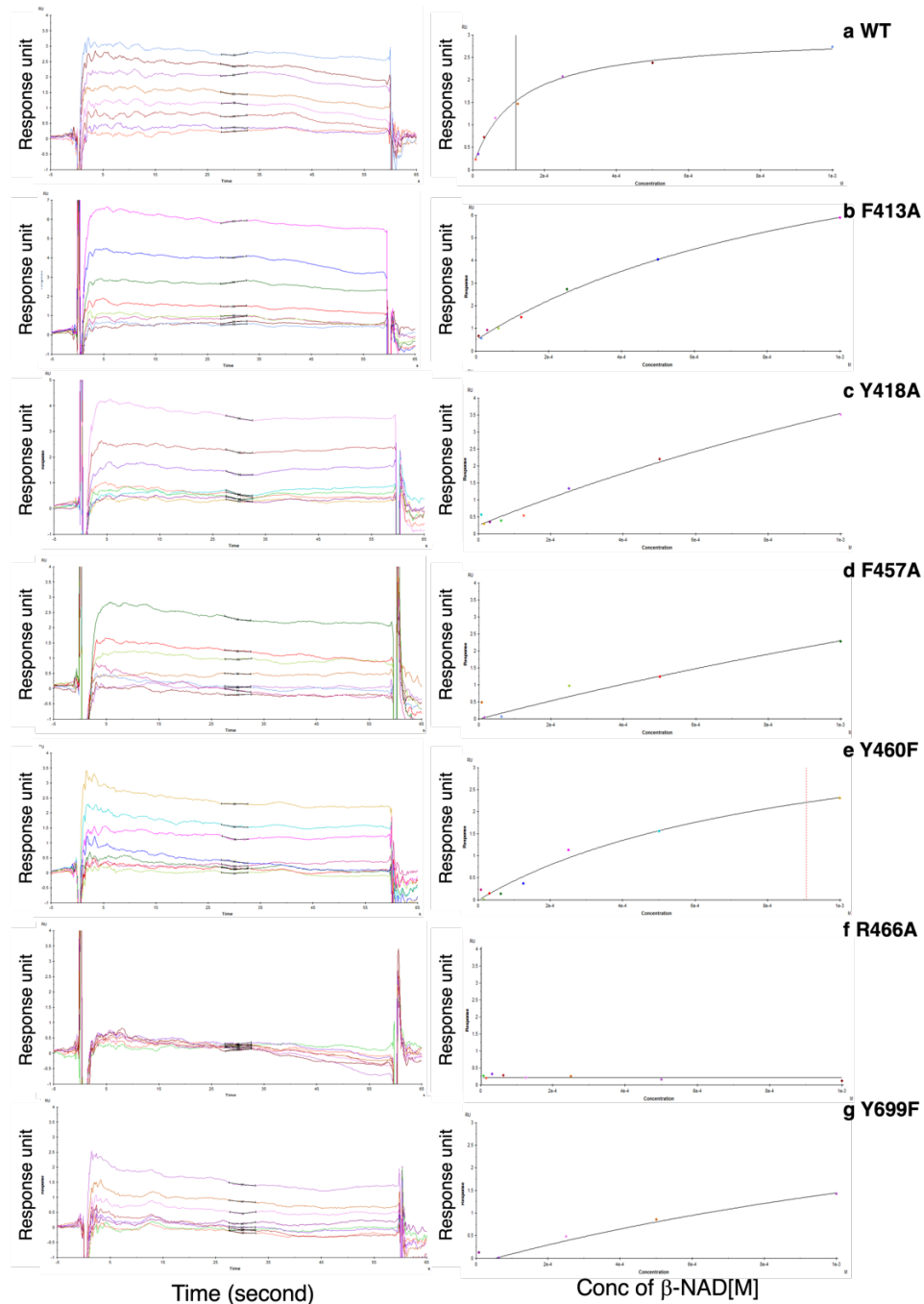
Supplementary Figure 7. Structure comparison between apo (cyan) and NAD-complex structure of PseP. The overall structure and close up view are shown in **a** and **b**, respectively. The N-terminal 85 amino acid region of SbzP is highlighted in violet.



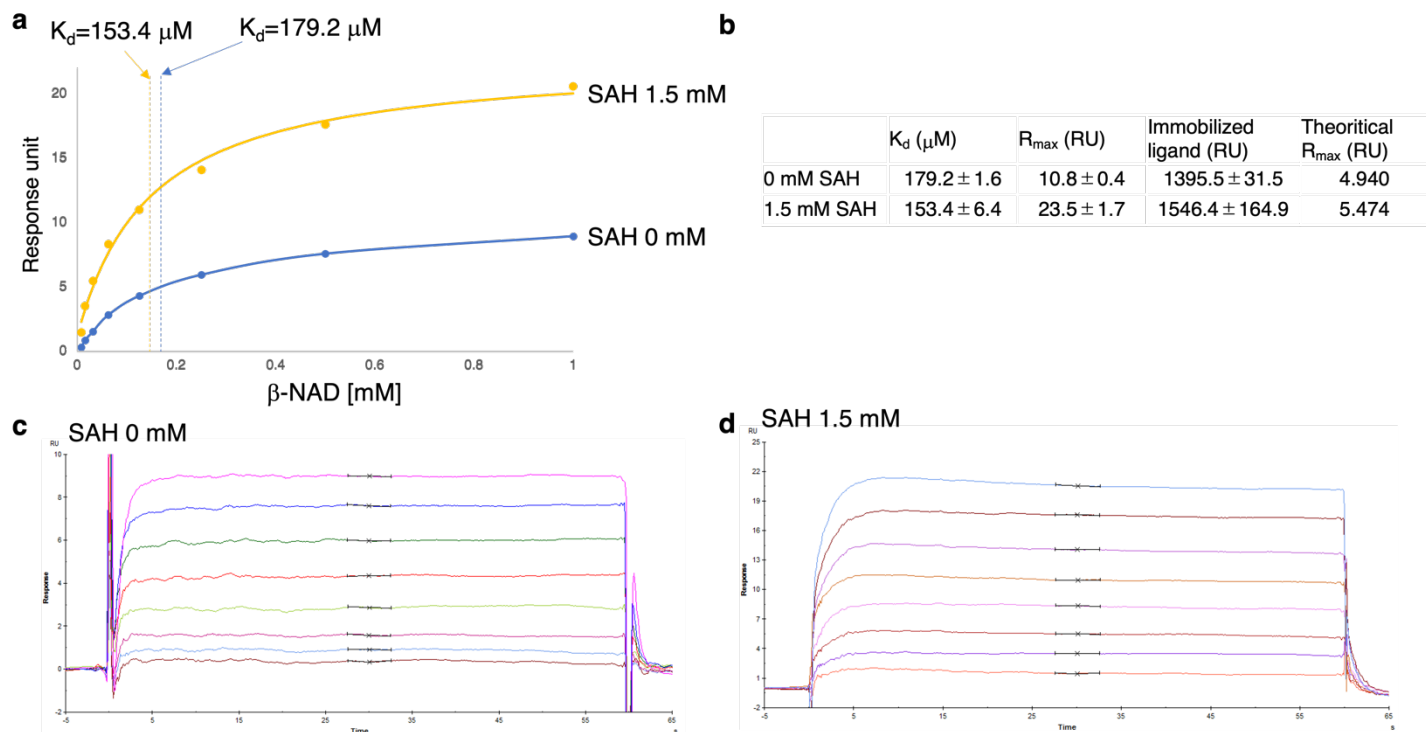
Supplementary Figure 8. Negative stain electron microscopy of WT (a) and variants (b-g) of PsePQ. Experiment was repeated two times independently with similar results.



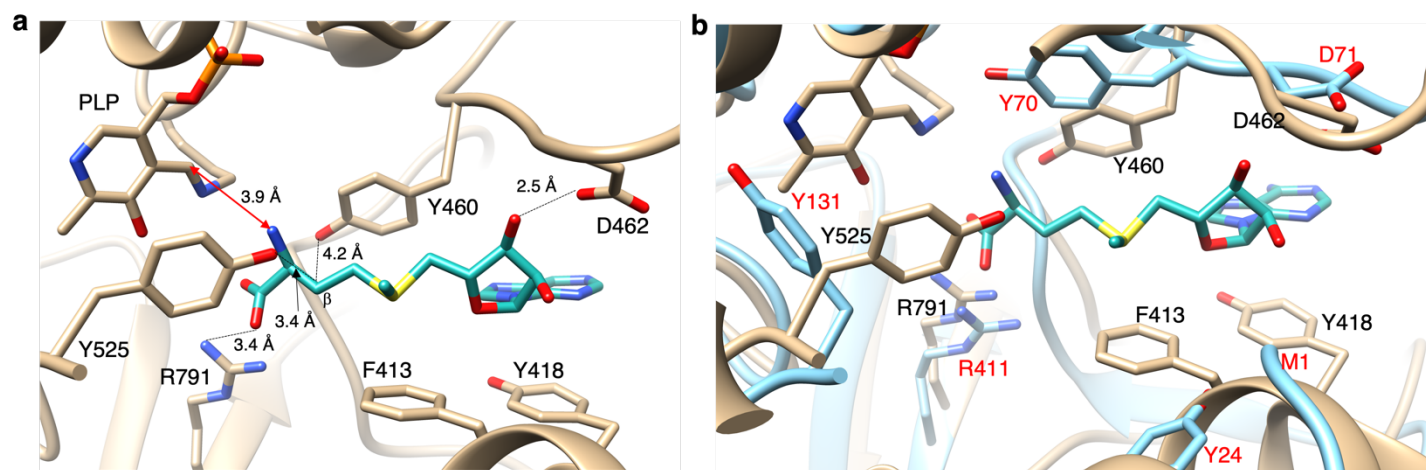
Supplementary Figure 9. Thermal shift assay of PsePQ WT and mutants. Blue line shows enzyme without ligand, purple line shows enzyme with 1.0 mM β -NAD, yellow line shows enzyme with 1.0 mM SAM, and pink line shows enzyme with 1.0 mM SAM and β -NAD (a-g). For the experiment with SAH (h), Blue line shows enzyme without ligand, purple line shows enzyme with 1.0 mM β -NAD, yellow line shows enzyme with 1.0 mM SAH, and pink line shows enzyme with 1.0 mM SAH and β -NAD.



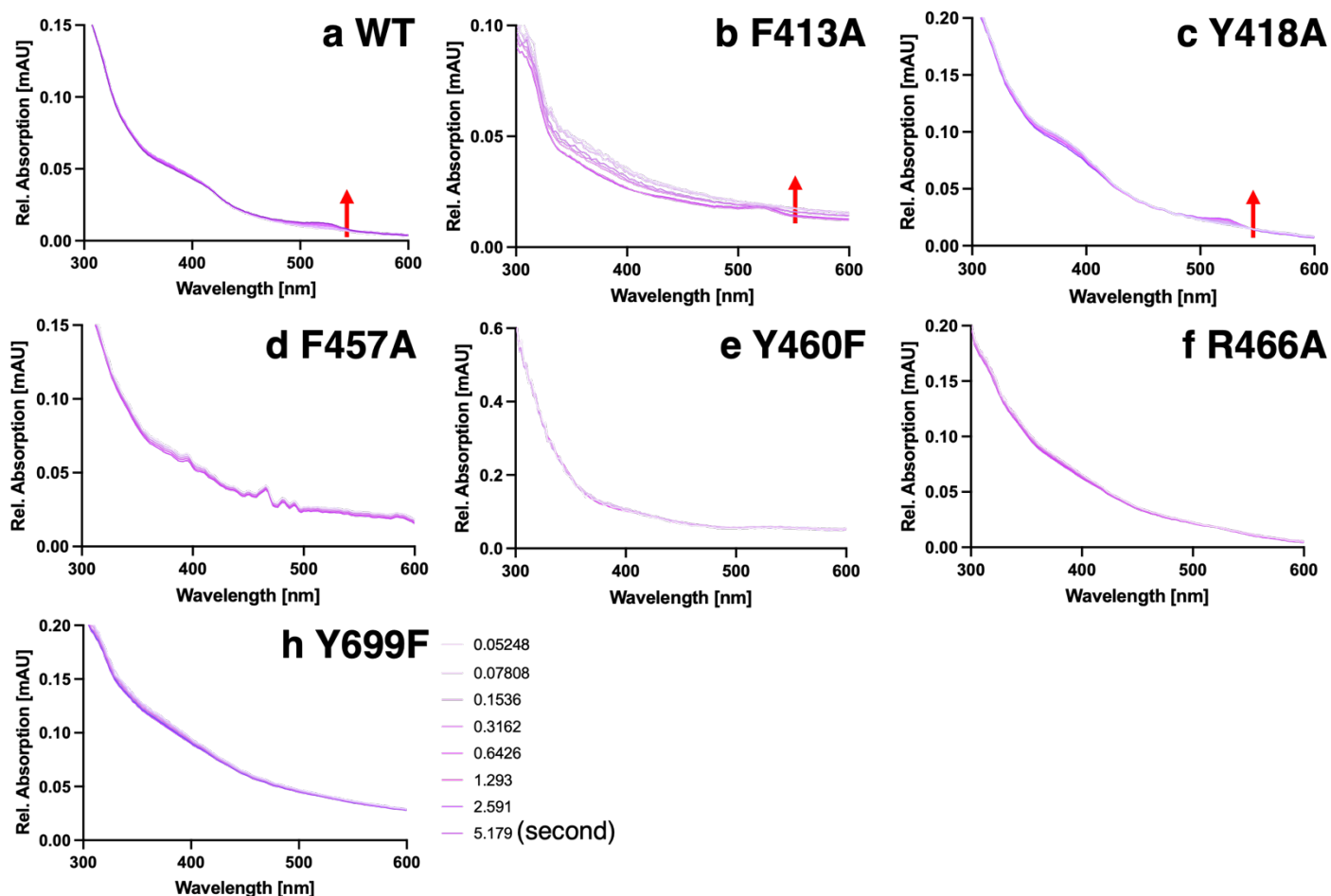
Supplementary Figure 10. SPR analysis of PsePQ WT and mutants. Red line shows enzyme without ligand and blue line shows enzyme with 1.0 mM β -NAD. All analysis was done by using the same concentration of β -NAD (7.8 μ M - 1000 μ M) as an analyte. The parameters of each figure are summarized in the table (h).



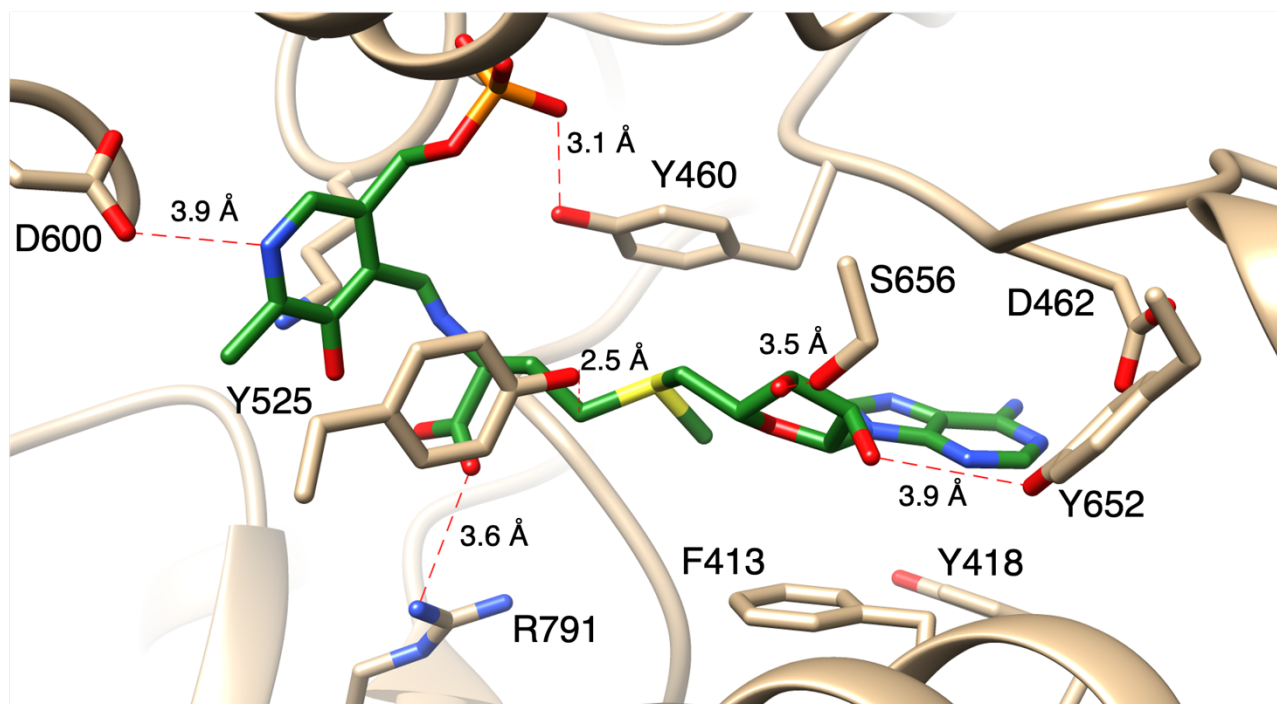
Supplementary Figure 11. SPR analysis of PsePQ WT in presence of 1.5 mM SAH. (a) The curve fitting of response unit. (b) The summary of the parameters. (c) The raw data of the protein in absence of SAH. (d) The raw data of the protein in presence of 1.5 mM SAH.



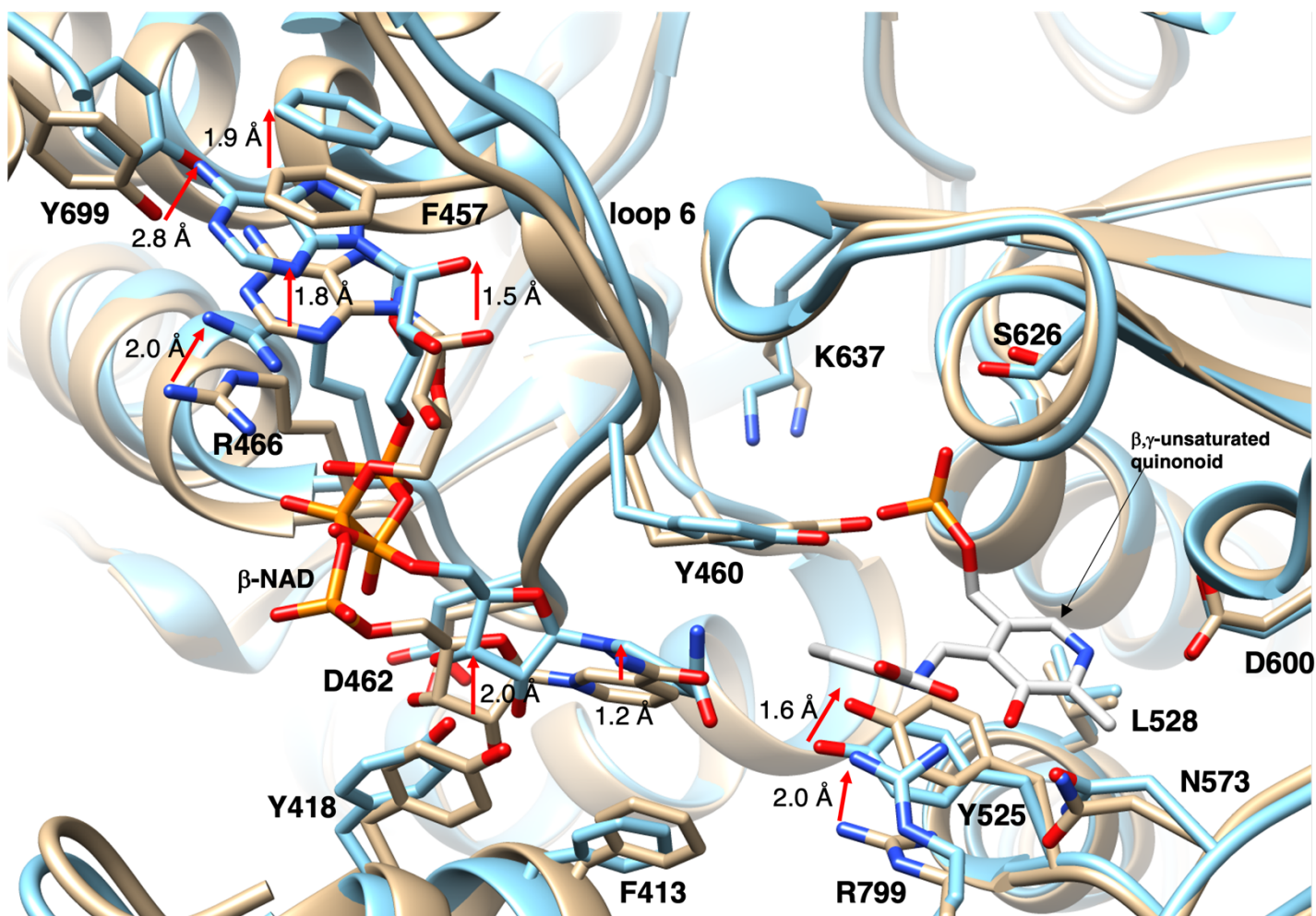
Supplementary Figure 12. The docking of SAM to the active site of PseP-apo and the comparison of the structure with Mur24. The SAM was docked within 5.0 Å distance from the PLP bound to the catalytic lysine in apo-PseP (a). The comparison of the structure shown in a and the α -fold 2 model of Mur24. The amino acid residues from Mur24 were shown in red (b). The SAM was colored in light green.



Supplementary Figure 13. Stopped flow analysis of PsePQ WT and mutants. Time-resolved photospectroscopic change of PsePQ after addition of 5.0 mM SAM solution.



Supplementary Figure 14. The docked model of PseP with PLP-SAM external aldimine

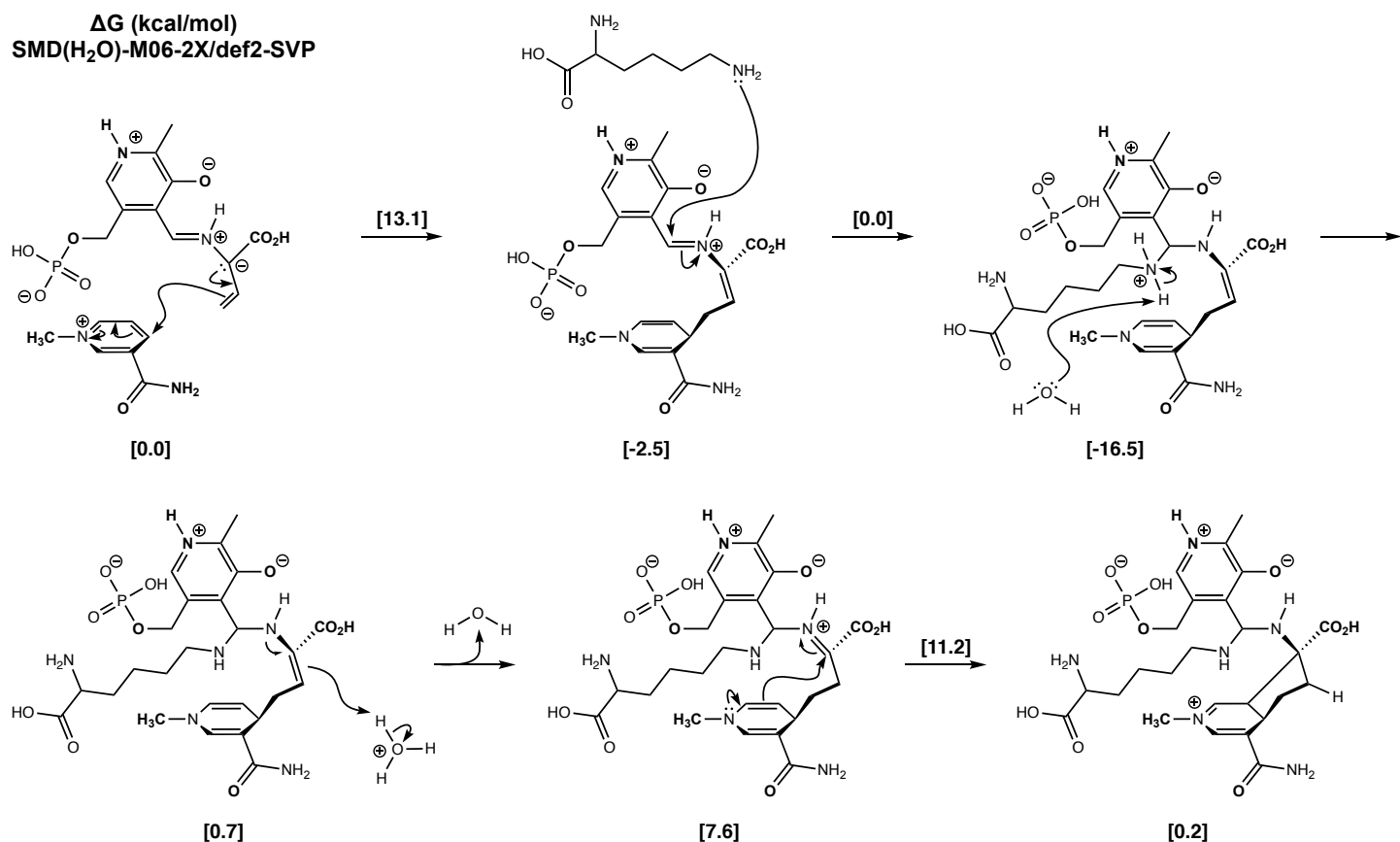


Supplementary Figure 15. The comparison of the PseP structure in cryo-EM analysis and MD simulation. The cryo structure of PseP-NAD is shown in brown, and the 400 ns snapshot of apo-PseP complexed with β -NAD in MD simulation (chain B) is shown in cyan. The structure of β,γ -unsaturated quinonoid from the MD simulation at 400 ns snapshot is shown in white.

Supplementary Note 1: Results of DFT calculations of SbzP reactions with a catalytic lysine.

Various (3+2) cycloaddition pathways were investigated computationally using the Gaussian 16 program, revision C.01³. Geometry optimizations were performed using the M06-2X functional^{4,5} along with the def2-SVP basis set^{6,7}. Calculations were performed with the solvation model based on density (SMD)¹⁰ continuum method with water ($\epsilon=78.36$). Intrinsic reaction coordinate (IRC) calculations were performed for all transition structures⁹⁻¹¹.

Results from these DFT calculations support a stepwise (3+2) cycloaddition that involves intermolecular protonation of an alkene after formation of the first C-C bond, yielding a conjugated carbocation intermediate (Supplementary Figure 16). This is followed by nucleophilic attack by catalytic lysine and subsequent protonation of the resulting enamine. A second C-C bond-forming event completes the cycloaddition. A Zundel cation was utilized as the hydrated excess proton in our calculations to provide a reasonable model of complexed hydronium as an acid¹².



Supplementary Figure 16. The DFT computed energies for the SbzP reaction with a catalytic lysine.

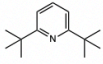
Supplementary Note 2: Results of DFT calculations of SbzP reactions without a catalytic lysine.

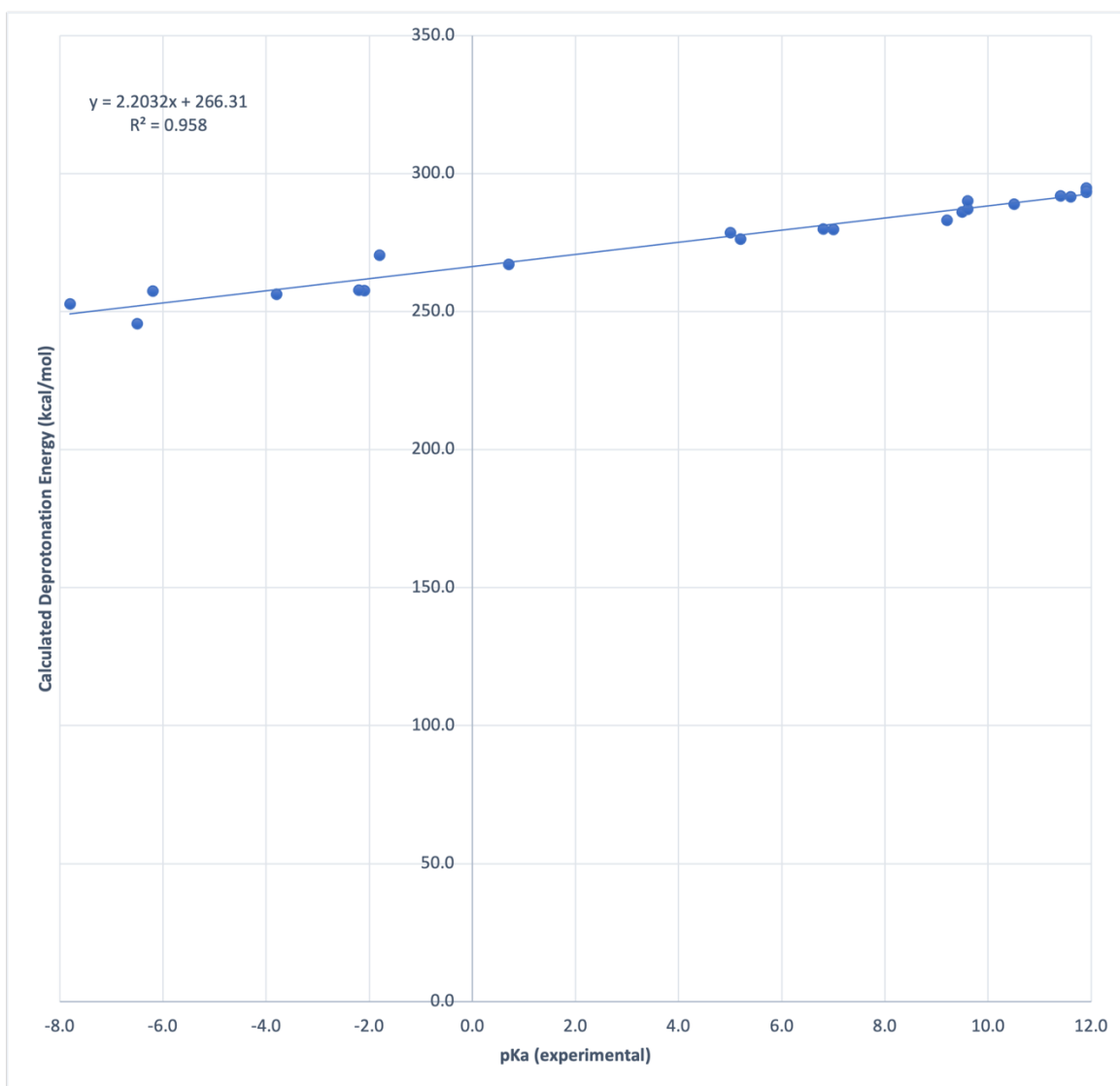
We investigated the feasibility of alkene (enamine) protonation by calculating the pK_a of the conjugate acid (Supplementary Table 3 and Supplementary Figure 17)¹³⁻¹⁵. Using the equation $y = 2.2032x + 266.31$ (y = calculated deprotonation energy, $x = pK_a$), we calculated the protonated alkene (iminium ion) to have a pK_a of -1.1 using the calculated deprotonation free energy of 264.0 kcal/mol. This is similar to the pK_a of protonated water (-1.7). It is reasonable that this alkene acts as a base in the proposed intermolecular protonation step, as water is utilized as an adequate base in numerous mechanisms. Since the identity of the proton source has not been definitively confirmed and our DFT calculations were aimed at assessing feasibility, not exact energetics, we used H_3O^+ as a simple but reasonable model.

It should be noted that a thermodynamic sink is introduced in the proposed mechanism due to the structure generated after nucleophilic attack (-16.5 kcal/mol) and the following water-mediated proton transfer events. Although water is used in this study as a model base, it is likely that enzymatic assistance of proton transfer events after nucleophilic attack will minimize or eliminate this effect.

A possible mechanistic pathway that does not involve nucleophilic attack by lysine was also calculated (Supplementary Figure 18-19). Both C-C bond-forming events possess small energy barriers that are of similar magnitude to those in the pathway which includes lysine. Additionally, we calculated the pK_a of the protonated alkene to be -2.5 with a deprotonation free energy of 261.0 kcal/mol, similar to the pK_a value of -1.1 in the mechanism with lysine.

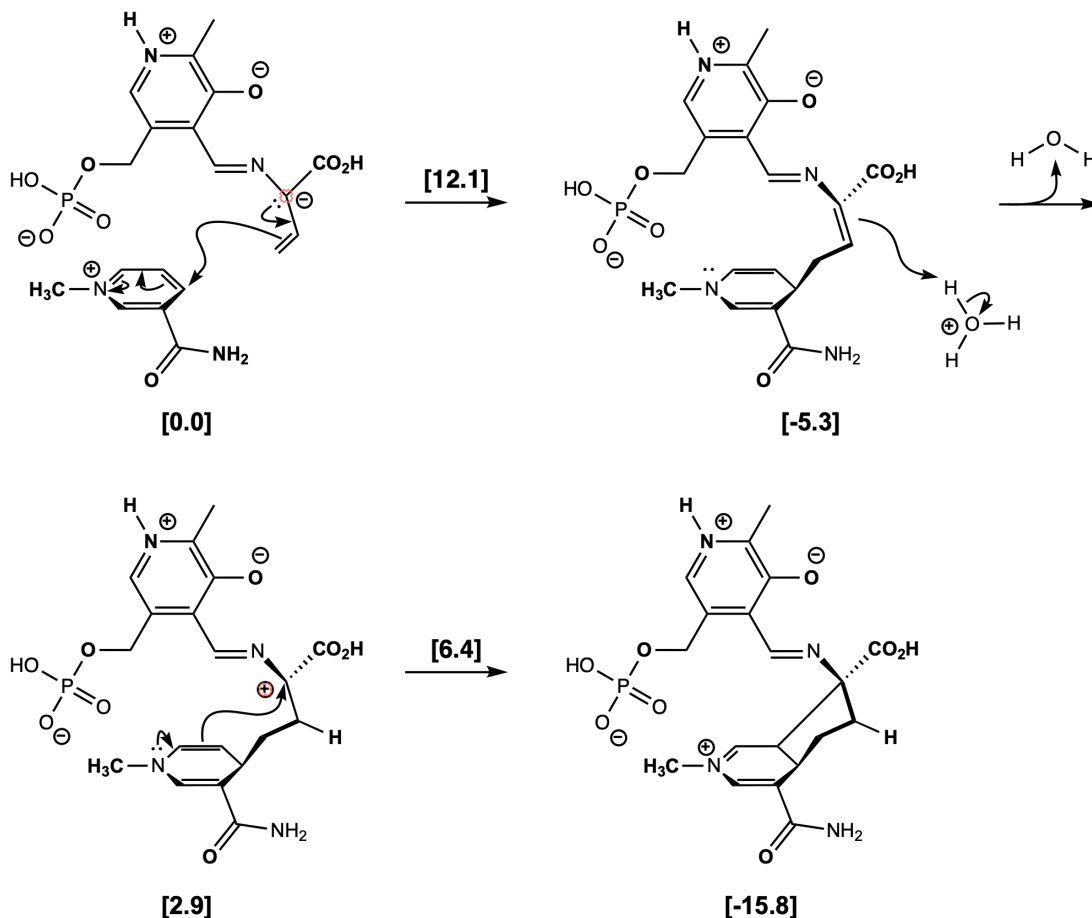
Supplementary Table 3. Calculated deprotonation energies of the conjugate acids of various bases.

Acid	pK _a (experimental)	Calculated Deprotonation Energy (kcal/mol)
	-7.8	252.9
	-6.5	245.7
	-6.2	257.6
	-3.8	256.4
	-2.2	257.8
	-2.1	257.8
	-1.8	270.5
	0.7	267.2
	5.0	278.7
	5.2	276.4
	6.8	280.0
	7.0	279.8
	9.2	283.2
	9.5	286.2
	9.6	290.2
	9.6	287.3
	10.5	289.1
	11.4	292.0
	11.6	291.6
	11.9	294.8
	11.9	293.7
	11.9	293.4



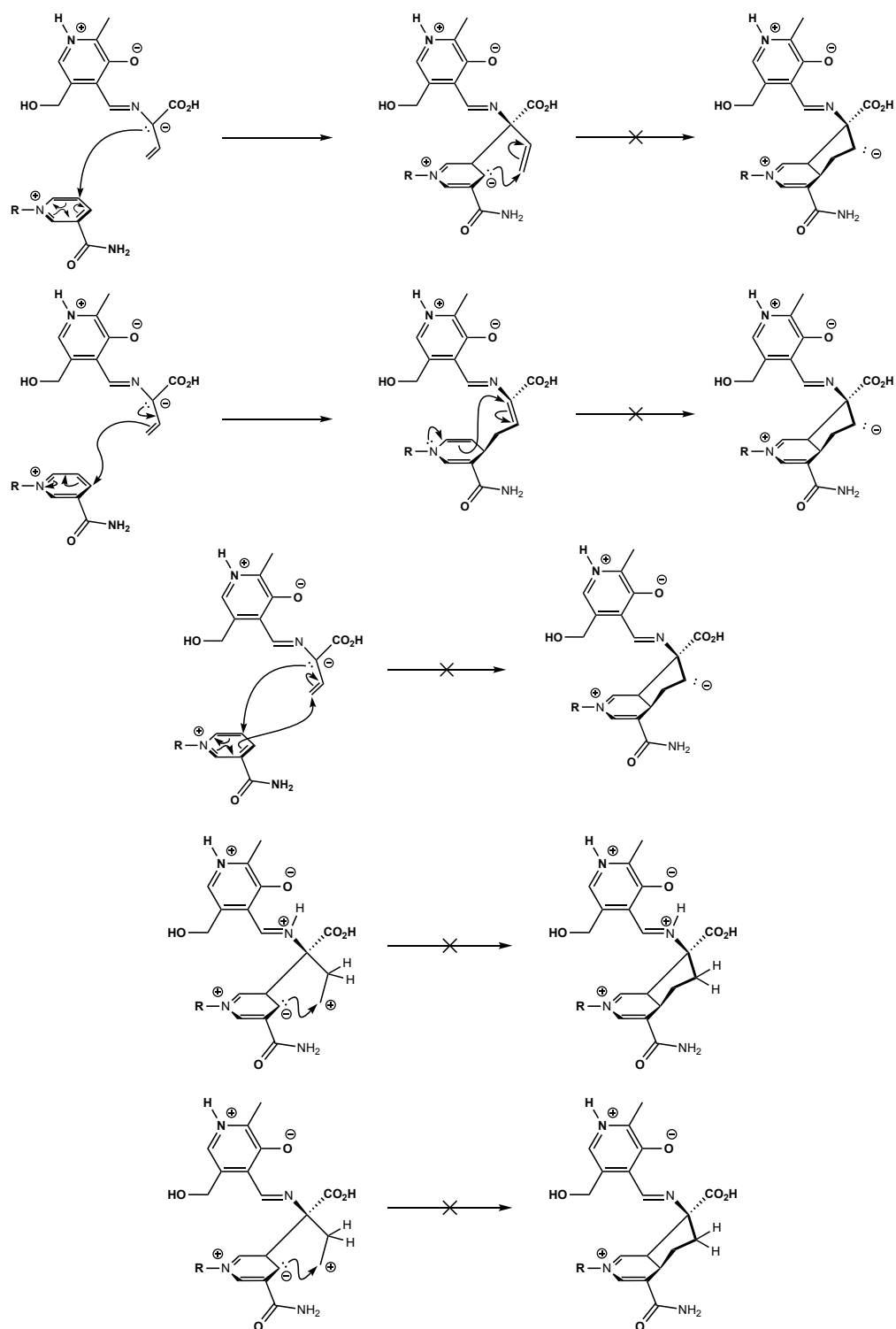
Supplementary Figure 17. Graph of experimental pKa values vs calculated deprotonation energies.

ΔG (kcal/mol)
SMD(H₂O)-M06-2X/def2-SVP



Supplementary Figure 18. DFT computed energies for the Sb₂P reaction without a catalytic lysine.

Attempts to locate stepwise and concerted pathways that do not involve protonation of the alkene were also investigated. We located transition structures corresponding to only one initial C-C bond formation in a stepwise (3+2) cycloaddition, but never the second C-C bond formation needed to complete the cyclization. Attempts at locating transition structures for concerted mechanisms that do not involve alkene protonation were also unsuccessful. These mechanisms are unlikely due to generation of an alkyl carbanion that is not a minimum on the potential energy surface.



Supplementary Figure 19. Investigated pathways that were not supported by DFT calculations.

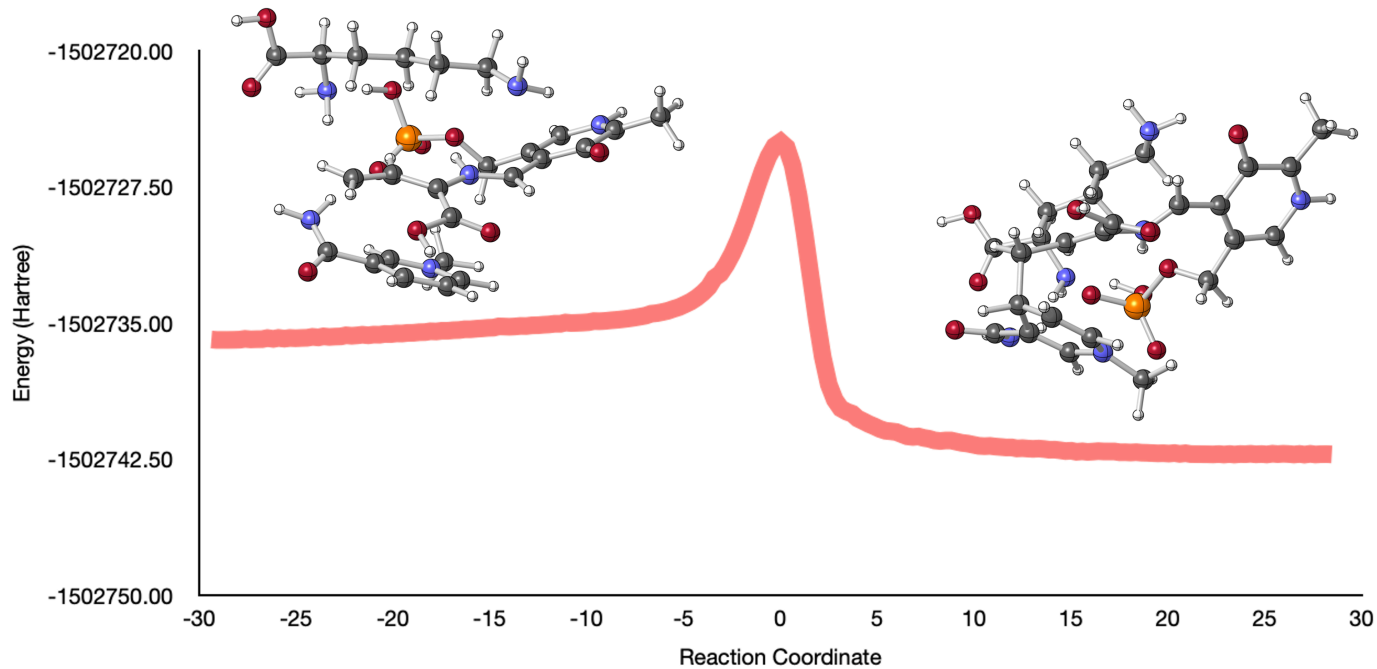
Energies and frequencies for each reported structure can be found in Supplementary Tables 4 and 5. IRC plots can be found in Supplementary Figures 20-24. Computed structures can be accessed at the ioChem-BD repository: <https://iochem-bd.bsc.es/browse/handle/100/305422>

Supplementary Table 4. Energies and lowest frequencies of the structures in the DFT mechanisms.

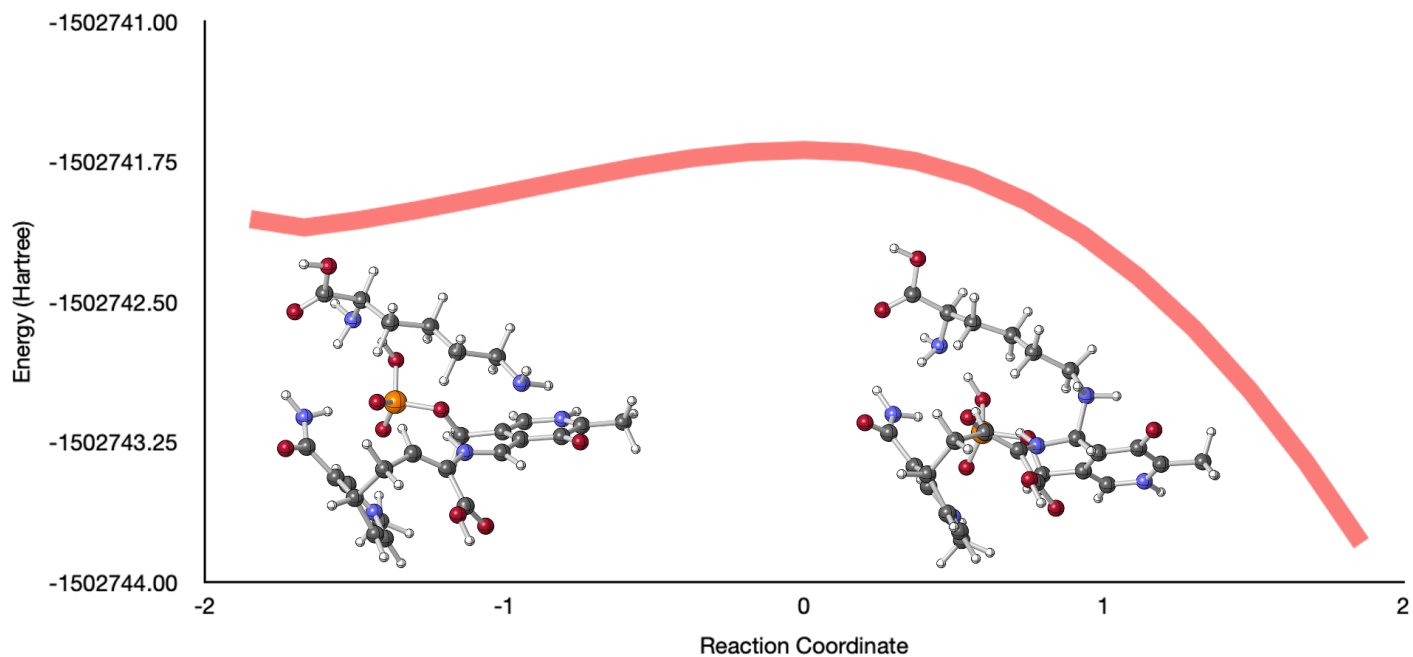
Filename	G (Hartree)	E (Hartree)	Lowest frequency (cm ⁻¹)
mechanism1_INT1	-1897.4547850	-1897.3630100	25.24
mechanism1_TS1	-1897.4354450	-1897.3448390	-408.92
mechanism1_INT2	-1897.4633030	-1897.3727830	22.96
mechanism1_INT3	-1897.881738	-1897.787606	15.56
mechanism1_TS2	-1897.8761700	-1897.7857700	-218.04
mechanism1_INT4	-1897.9115970	-1897.8196440	12.71
mechanism2_INT1	-2394.1990920	-2394.0759950	17.12
mechanism2_TS1	-2394.1782540	-2394.0574490	-474.88
mechanism2_INT2	-2394.2030440	-2394.0822990	11.11
mechanism2_TS2	-2394.1991230	-2394.0818630	-175.58
mechanism2_INT3	-2394.2253440	-2394.1091460	13.66
mechanism2_INT4	-2393.7663940	-2393.6511570	16.37
mechanism2_INT5	-2394.1870490	-2394.0679980	10.24
mechanism2_TS3	-2394.1813110	-2394.0640850	-293.10
mechanism2_INT6	-2394.1987480	-2394.0798430	9.99
two_water	-152.6603890	-152.6290240	133.13
zundel_cation	-153.0919720	-153.0627760	172.45

Supplementary Table 5. Energies and lowest frequencies of the structures used in the pK_a calculations.

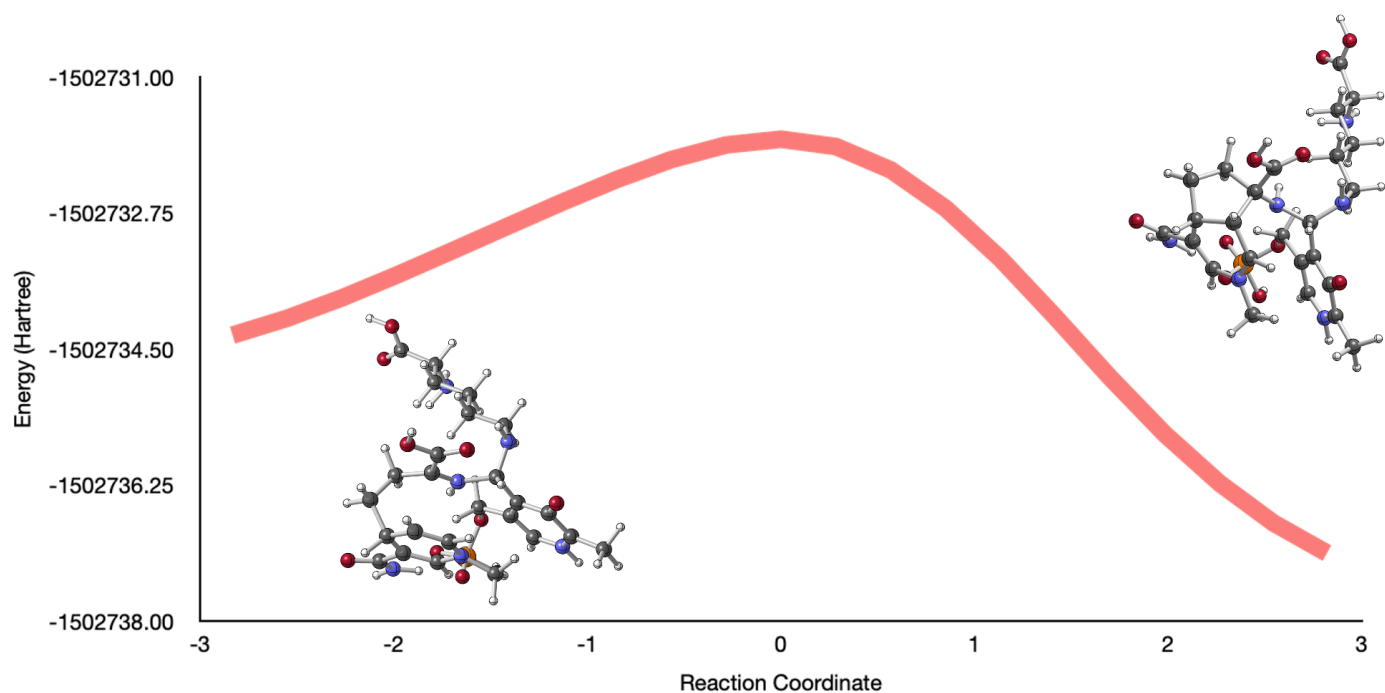
Filename	G (Hartree)	E (Hartree)	Lowest frequency (cm ⁻¹)
1_neutral	-367.9766680	-367.9334350	59.75
1_protonated	-368.4373420	-368.3942010	88.95
2_neutral	-446.4514600	-446.4011220	67.03
2_protonated	-446.9076150	-446.8566950	58.60
3_neutral	-328.7365350	-328.6948780	109.62
3_protonated	-329.1943120	-329.1523060	91.54
4_neutral	-289.5009230	-289.4630980	155.89
4_protonated	-289.9684120	-289.9283790	63.84
5_neutral	-407.2068070	-407.1591310	53.78
5_protonated	-407.6766510	-407.6266330	45.54
6_neutral	-328.7389930	-328.6980760	108.48
6_protonated	-329.2070240	-329.1639740	67.79
8_neutral	-328.7409330	-328.7004190	84.75
8_protonated	-329.2062910	-329.1651300	101.14
9_neutral	-367.9786120	-367.9344900	48.27
9_protonated	-368.4433150	-368.3993820	81.69
10_neutral	-289.5148770	-289.4771650	144.45
10_protonated	-289.9772870	-289.9395270	116.63
11_neutral	-420.2686840	-420.2294790	57.97
11_protonated	-420.6717200	-420.6321330	81.06
12_neutral	-384.3408240	-384.3002970	52.05
12_protonated	-384.7513440	-384.7103920	67.62
13_neutral	-346.2602420	-346.2226140	95.74
13_protonated	-346.6517500	-346.6129660	76.98
14_neutral	-154.7706180	-154.7409340	200.55
14_protonated	-155.1791520	-155.1487240	156.15
15_neutral	-232.0758070	-232.0413520	13.21
15_protonated	-232.4865730	-232.4538840	155.15
16_neutral	-115.5484680	-115.5224400	330.28
16_protonated	-115.9592620	-115.9327230	287.66
17_neutral	-552.8029230	-552.7691540	186.37
17_protonated	-553.2340480	-553.1992780	181.28
19_neutral	-381.6827130	-381.6389600	28.54
19_protonated	-382.1340420	-382.0920240	76.01
20_neutral	-225.9240880	-225.8942530	613.48
20_protonated	-226.3699790	-226.3399480	638.77
21_neutral	-247.9325600	-247.9009270	391.47
21_protonated	-248.3730830	-248.3412450	398.53
22_neutral	-561.8409410	-561.7844290	27.23
22_protonated	-562.2851180	-562.2294640	40.60
23_neutral	-326.4213100	-326.3811900	75.51
23_protonated	-326.8674780	-326.8273240	79.29
24_neutral	-707.3953130	-707.3603430	182.44
24_protonated	-707.8211580	-707.7859460	178.84



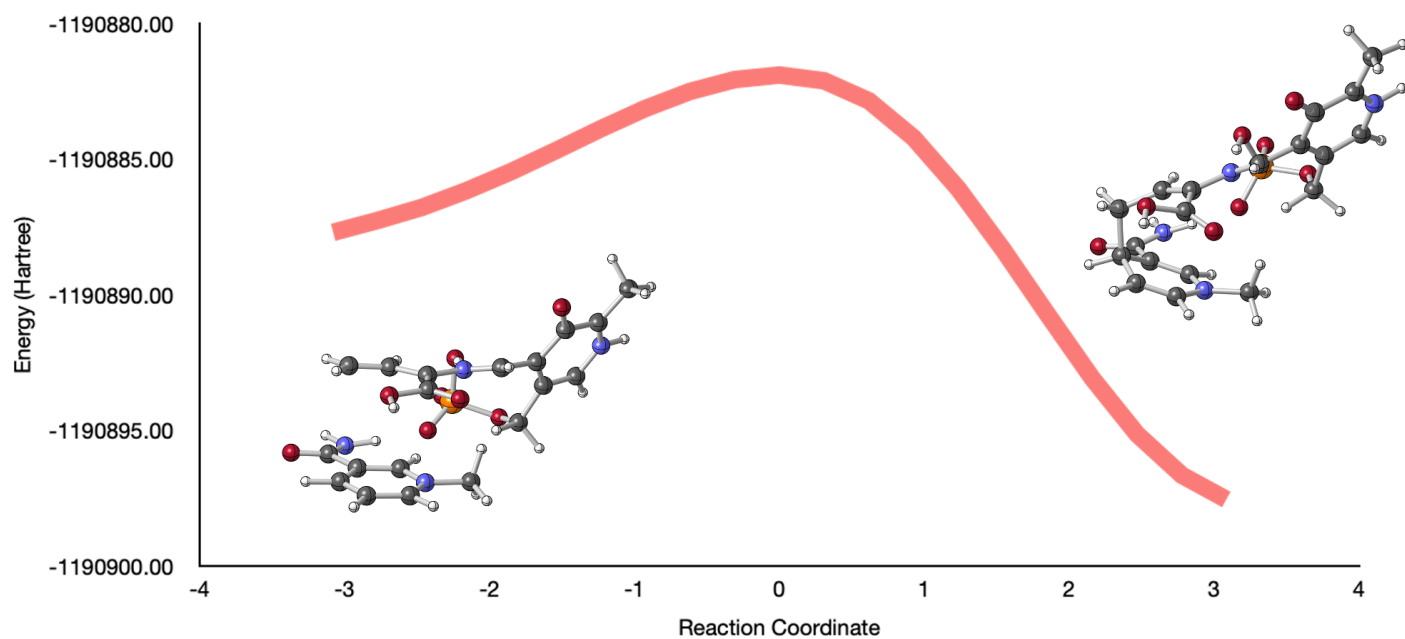
Supplementary Figure 20. IRC plot of lysine_mechanism_TS1. Color code: C grey, H white, O red, N blue.



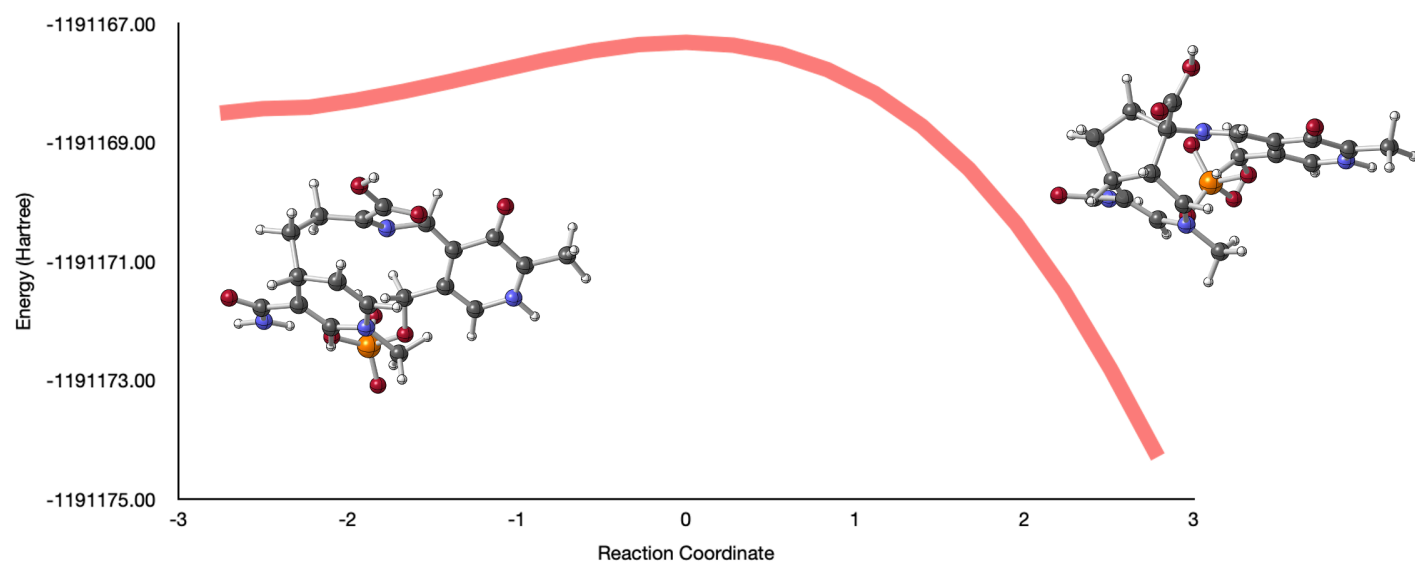
Supplementary Figure 21. IRC plot of lysine_mechanism_TS2. Color code: C grey, H white, O red, N blue.



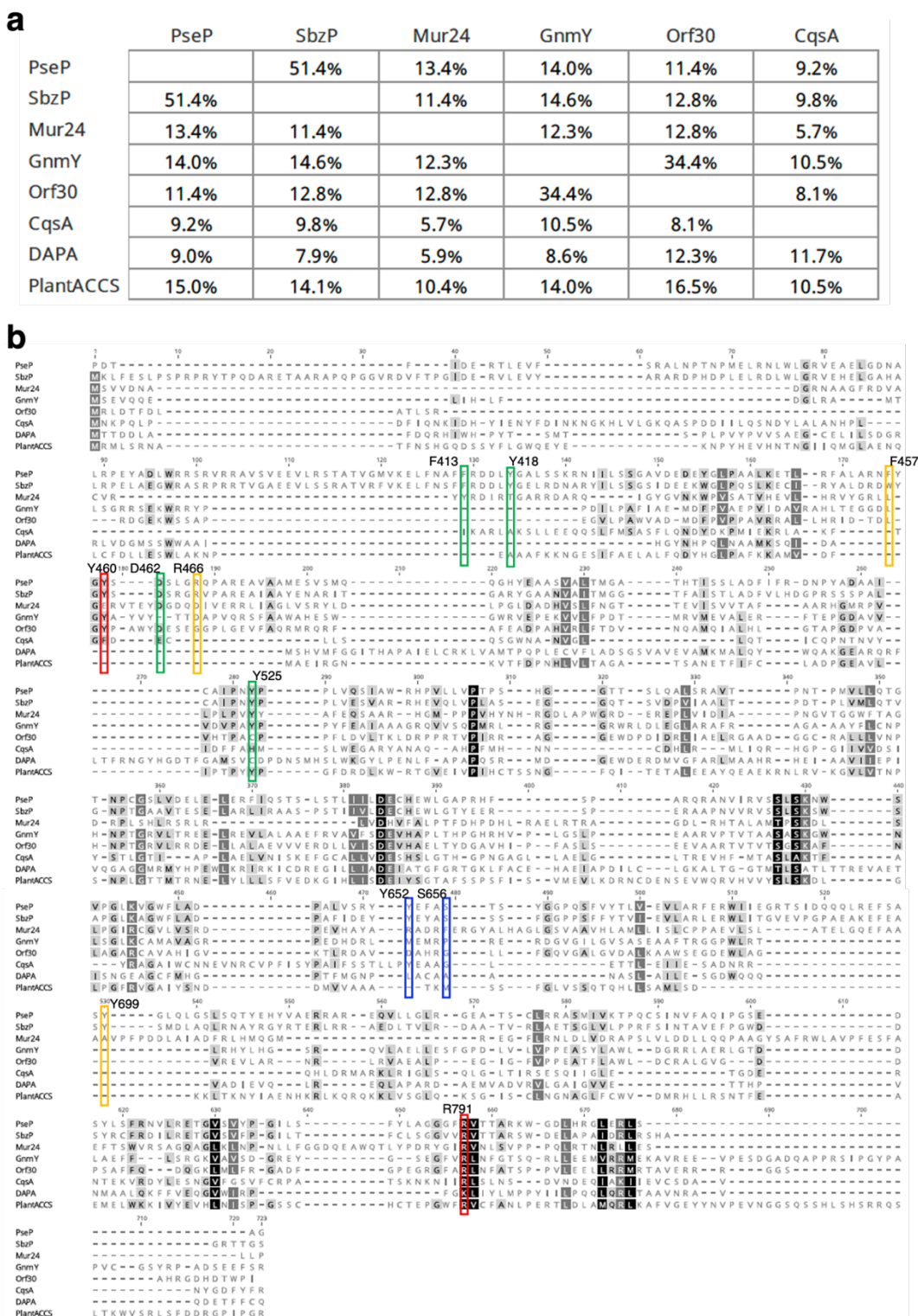
Supplementary Figure 22. IRC plot of lysine_mechanism_TS3. Color code: C grey, H white, O red, N blue.



Supplementary Figure 23. IRC plot of no_lysine_mechanism_TS1. Color code: C grey, H white, O red, N blue.



Supplementary Figure 24. IRC plot of no_lysine_mechanism_TS2. Color code: C grey, H white, O red, N blue.



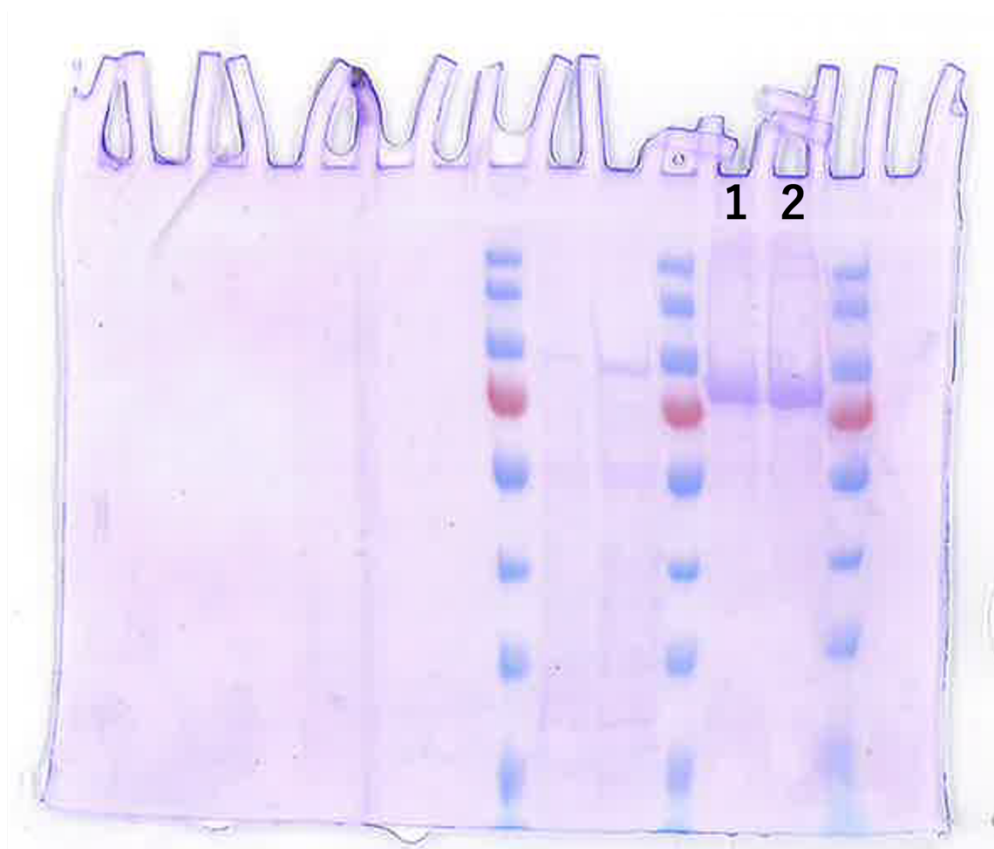
Supplementary Figure 25. The amino acid identity (a) and alignment of the PLP enzymes which accept SAM as a substrate (b). Red frames show the residues conserved for the binding of β , γ -unsaturated quinonoid, green frames show the residues for binding SAM in PseP, yellow frames show the residues for binding β -NAD and SAM, and blue frames show the residues for binding for PLP-SAM external aldimine in PseP.

Supplementary Table 6 Bacterial strains, vectors and primers used in this study.

Strains	Description	Source
<i>Streptomyces lividans</i> TK21	Heterologous expression host	John Innes Centre
Vectors	Description	Source
pQTEV	Vector for protein expression in <i>E. coli</i>	Addgene
pHSA81	Vector for protein expression in <i>S. lividans</i>	Dr. M. Kobayashi
pHSA81-PsePQ	Vector for protein expression in <i>S. lividans</i> .	Reference 2
Primers	Sequence (5' → 3')	Description
PsePQF413A_f	gctgttcaacgcttttGCccgcatgacctgtacggc	Primers for F413A mutation on <i>psePQ</i>
PsePQF413A_r	gccgtacaggtcatcgcgGCaaaagcgttgaacagc	
PsePQY418A_f	ttcccgcatgacctgGCcggcgccctgagttctaagc	Primers for Y418A mutation on <i>psePQ</i>
PsePQY418A_r	gcttagaactcagggcgccgGCcaggtcatcgcgaaa	
PsePQF457A_f	ttgccctggcgagaaatGCctacggctattcgactctc	Primers for F457A mutation on <i>psePQ</i>
PsePQF457A_r	gagagtccgaatagccgtagGCatttctcgccagggcaa	
PsePQY460F_f	cgagaaatttctacggcttttcggactctctggggcgctc	Primers for Y460F mutation on <i>psePQ</i>
PsePQY460F_r	gacgcccagagagtcgaaaagccgtagaaatttctcg	
PsePQR466A_f	gctattcggactctctggggGCtcagccggcgcgagagg	Primers for R466A mutation on <i>psePQ</i>
PsePQR466A_r	cctctcgccgggctgaGCcccagagagtcggaatagc	
PsePQY418F_f	TTTCCGCGATGACCTGTTCCGGCGCCCTGAGTTCTAAGC	Primers for Y418F mutation on <i>psePQ</i>
PsePQY418F_r	GCTTAGAACTCAGGGCGCCGAACAGGTCATCGCGGAAA	
PsePQY525A_f	GCGATCTGCGCAATTCCCAACTTTCCACCGCTGGTTCAG	Primers for Y525A mutation on <i>psePQ</i>
PsePQY525A_r	CTGAACCAGCGGTGGAAAGTTGGGAATTGCGCAGATCGC	
PsePQY699F_f	cgcgaaatttccgccagctTtgactgcagttgggcagcc	Primers for Y699F mutation on <i>psePQ</i>
PsePQY699F_r	ggctgccaactgcagccaAagctggcggaattcgcg	
pQTEV-pHSA81NdeI	AAAGGAATGAGCATatgaaacatcaccatcaccatcacc	Primers for cloning from pQTEV to pHSA81
pQTEV-pHSA81HindIII	TAGTGCTAGCAAGCTTctatcaacaggagtcgaagctcagc	

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Source Data 1. Unprocessed SDS-PAGE analysis of WT PsePQ. Lane 1. PsePQ after Ni-affinity and gel filtration purification, Lane 2. After liquid ethane treatment.