Supplementary Information for:

Mechanistic conformational and substrate selectivity profiles emerging in the evolution of enzymes via parallel trajectories.

Christos S. Karamitros[1], Kyle Murray[2], Yoichi Kumada[3], Kenneth A. Johnson[4], Sheena D'Arcy[2], George Georgiou[1,4,5,6]

[1]Department of Chemical Engineering, University of Texas at Austin (UT Austin), Austin, Texas, USA

[2]Department of Chemistry and Biochemistry, The University of Texas at Dallas, Richardson, Texas, USA

[3]Department of Molecular Chemistry and Engineering, Kyoto Institute of Technology, Kyoto, Japan

[4]Department of Molecular Biosciences, University of Texas at Austin (UT Austin), Austin, Texas, USA

[5]Department of Biomedical Engineering, University of Texas at Austin (UT Austin), Austin, TX, USA.

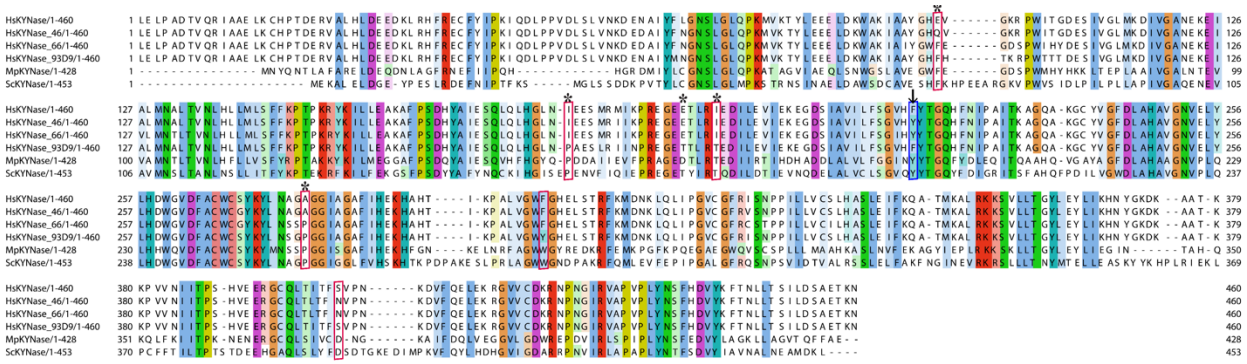
[6]Institute for Cellular and Molecular Biology, The University of Texas at Austin (UT Austin), Austin, Texas, USA

Correspondence should be sent to: George Georgiou: gg@che.utexas.edu

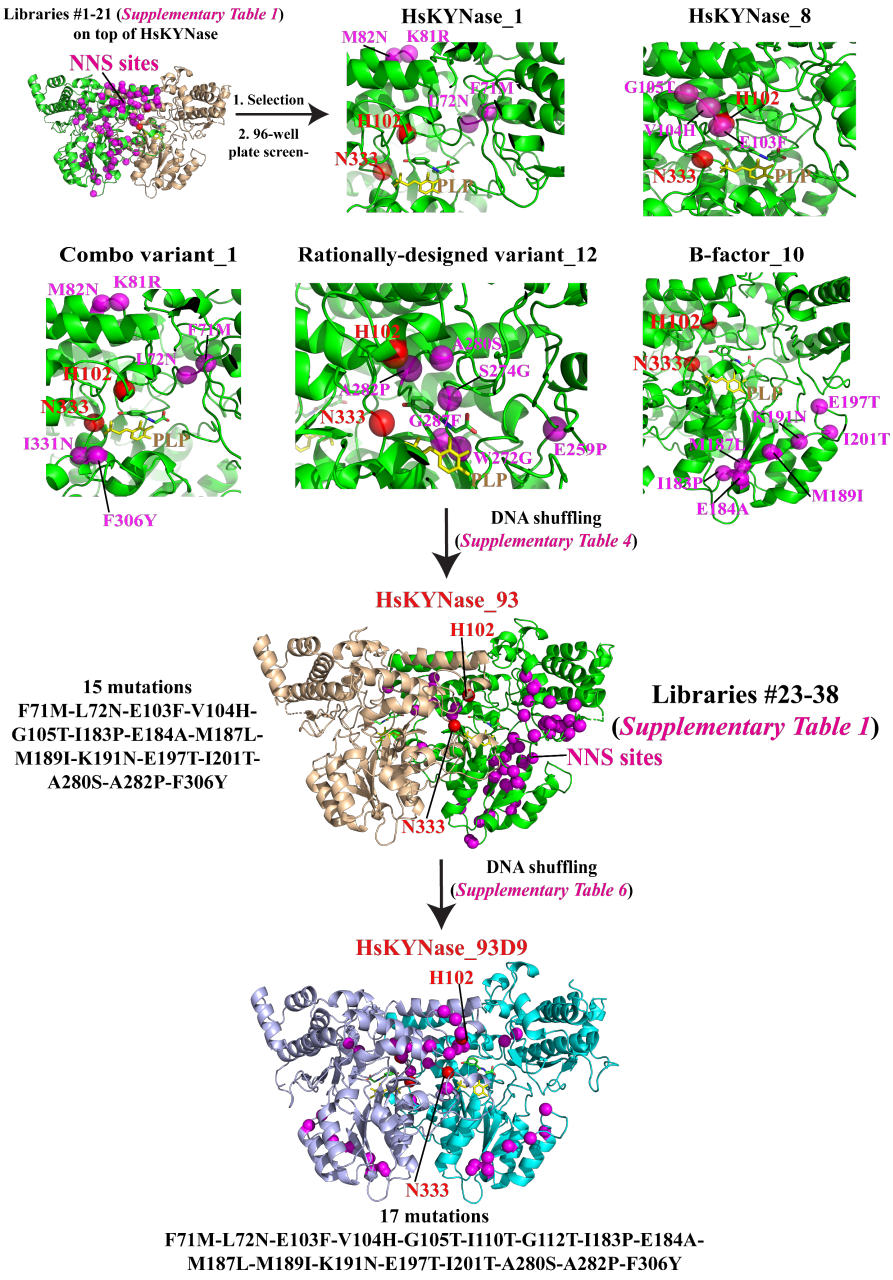
Supplementary Information includes:

Supplementary Figures 1-10

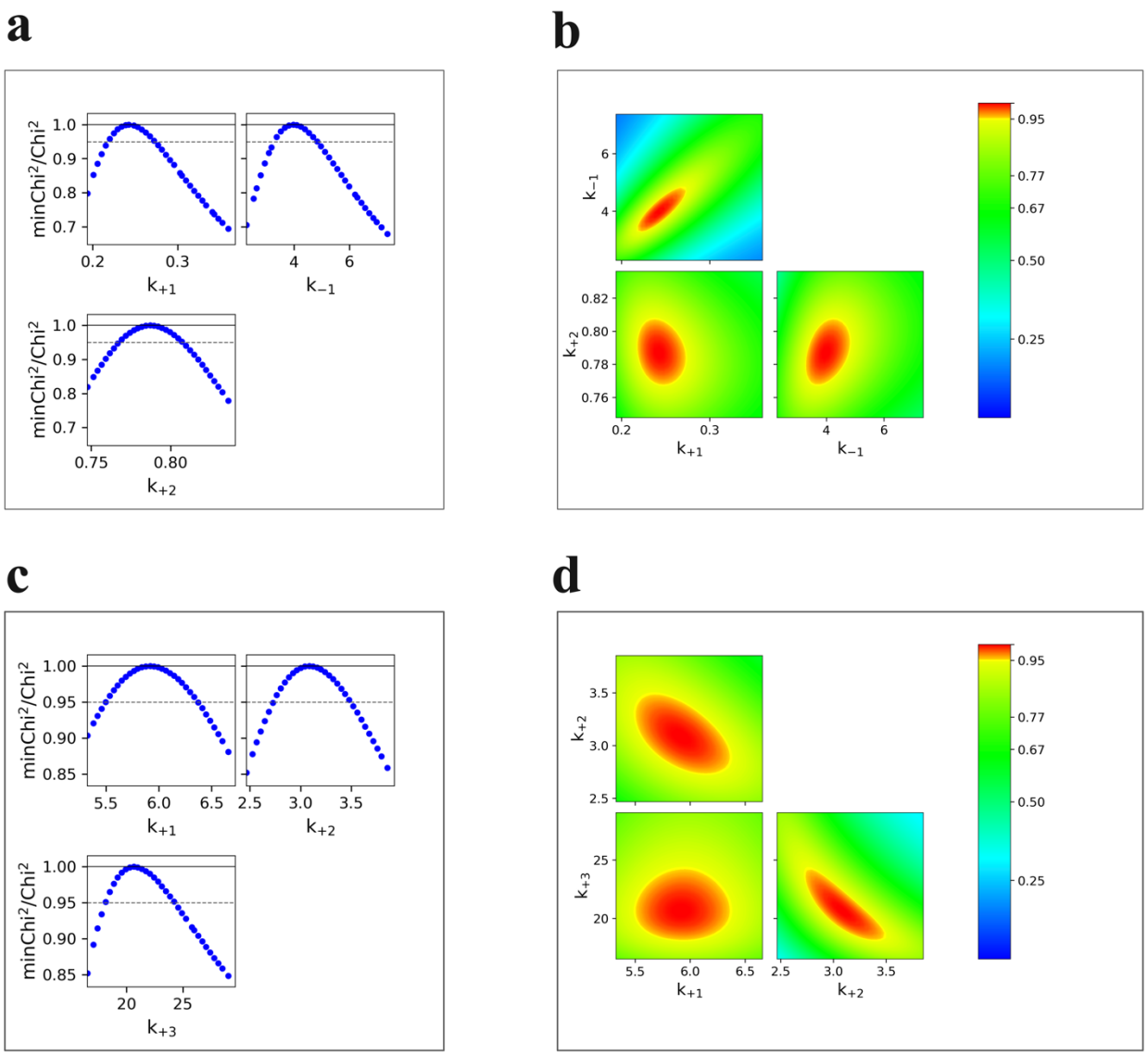
Supplementary Tables 1-11



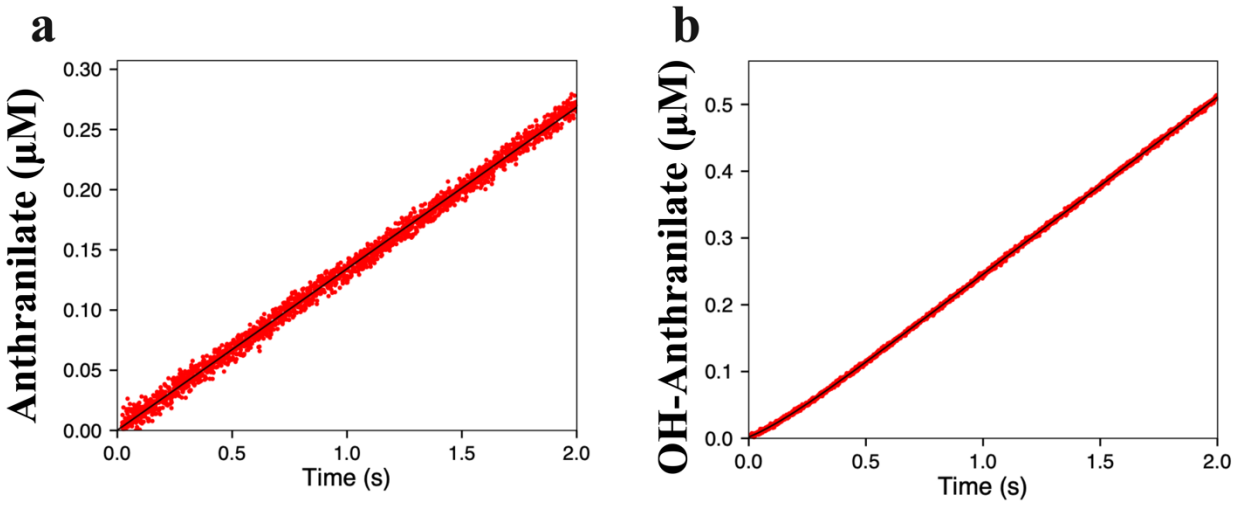
Supplementary Figure 1. **Amino acid sequence alignment of HsKYNase, HsKYNase_46, HsKYNase_66, HsKYNase_93D9, MpKYNase and ScKYNase.** The blue rectangular frame indicated by the arrow shows the amino acid substitution shared by HsKYNase_46 and ScKYNase. The red rectangular frames indicate residues that are shared by the eukaryotic OH-KYN-preferred ScKYNase and the prokaryotic KYN-preferred *Mucilaginibacter paludis* KYNase (MpKYNase). Asterisks denote the five mutations of HsKYNase_93D9 that are found in ScKYNase and MpKYNase. The alignment was performed using the CLUSTALW algorithm of the Snapgene program (Version 6.05) and the coloring of the residues is based on their properties as per CLUSTALW (conservation cutoff for the coloring was set at >80%). The alignment file was exported as FASTA and the final figure shown here was prepared by JalView.



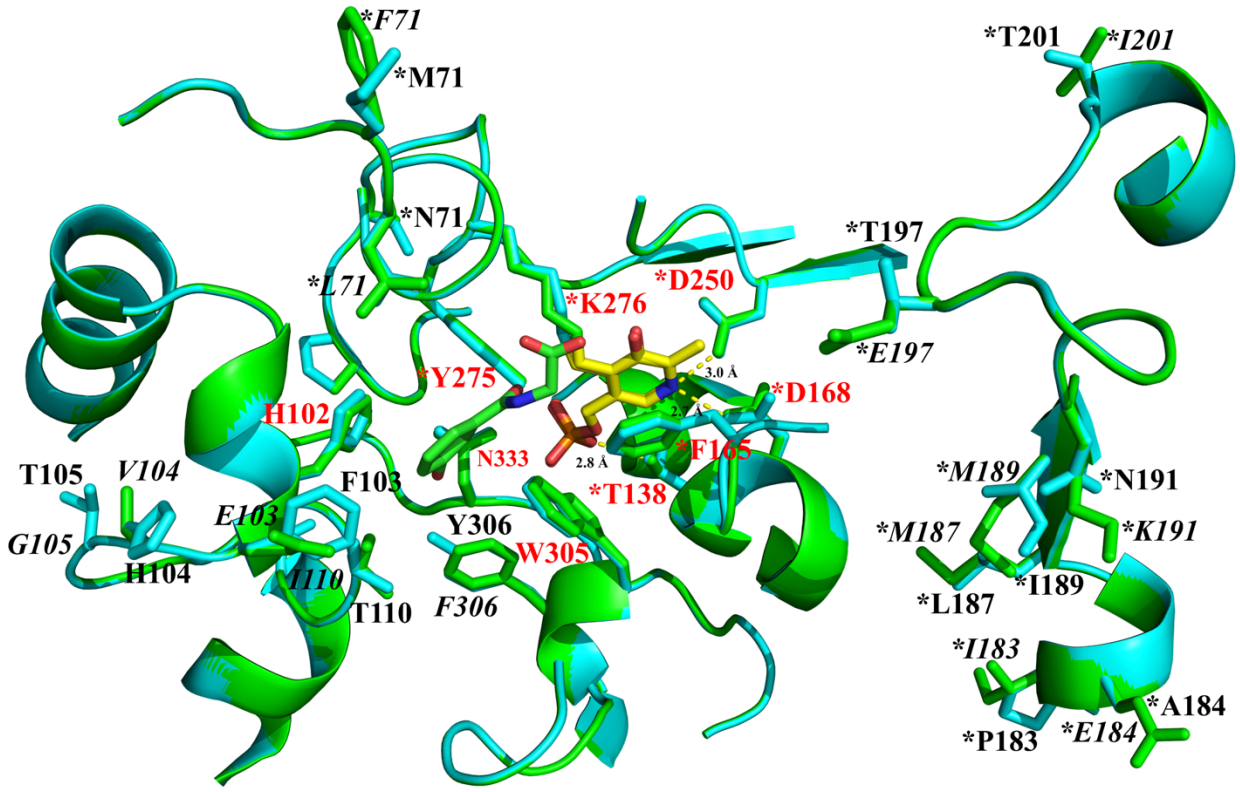
Supplementary Figure 2. Detailed workflow of the evolutionary trajectory leading to the identification of HsKYNase_93D9. Fully randomized (NNS) amino acid residues of HsKYNase and HsKYNase_93 are shown as magenta spheres. For simplicity, the targeted residues of only one of the monomers are shown. Mutations in evolutionary intermediates, namely HsKYNase_1, HsKYNase_8, Combo variant_1, rationally designed variant_12 and B-factor_10 found in HsKYNase_93 (Supplementary Table 4) are shown in green cartoon representations. Further NNS randomization on HsKYNase_93 sites and DNA shuffling of variants (Supplementary Table 6) led to the identification of HsKYNase_93D9 which harbors seventeen amino acid substitutions. The two monomers in HsKYNase_93D9 harboring 17 mutations are shown in cyan light and in dark blue respectively and the mutations (on both monomers) are depicted as magenta spheres. The two monomers of HsKYNase_93 are shown in wheat and green color respectively.



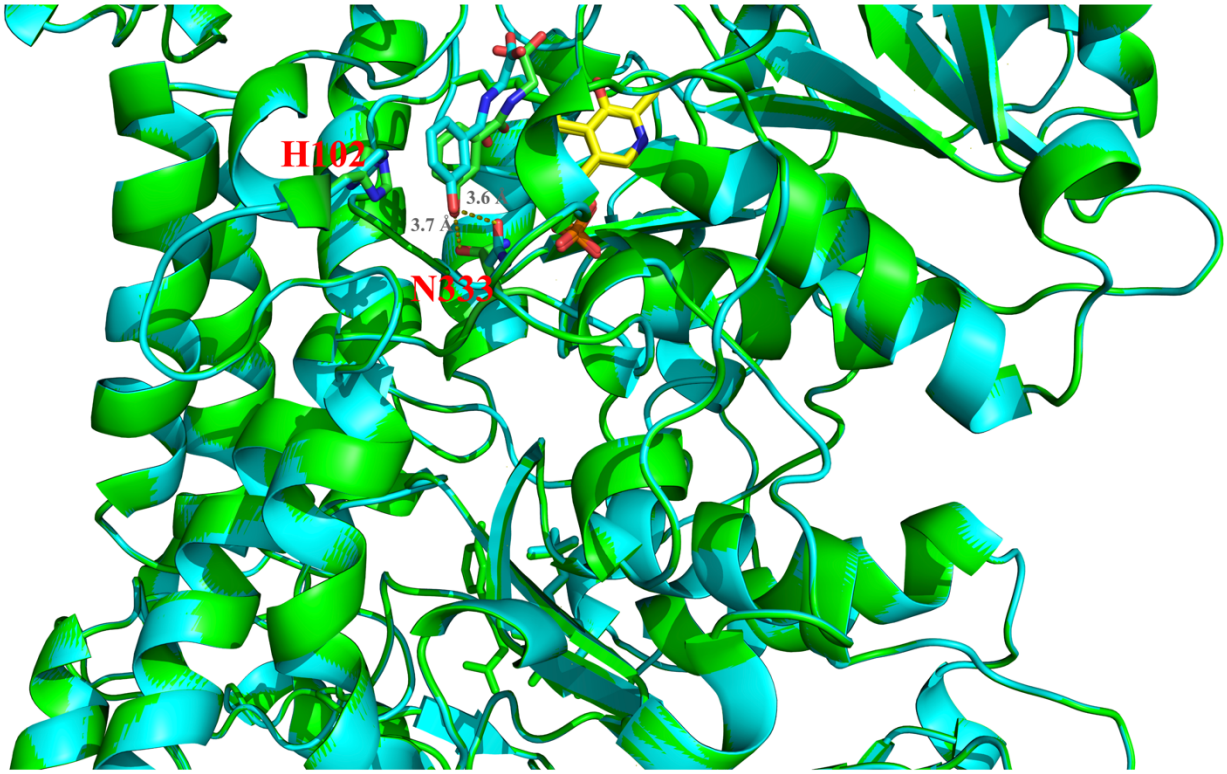
Supplementary Figure 3. Confidence contour analysis of the global fitting of the pre-steady-state kinetic data shown in main Figure 2. a-b. 1D and 2D FitSpace confidence contour analysis respectively, from the global fit of the stopped-flow kinetic data for the reaction of HsKYNase_93D9 with KYN. The simulation data and the respective models are shown in Fig. 2a, b. **c-d**. 1D and 2D FitSpace confidence contour analysis respectively from the global fit of the data for the reaction of HsKYNase_66 with KYN (Fig. 2d, e). All rate constants are expressed as $\mu M^{-1}s^{-1}$ (k_1) and s^{-1} (k_{-1}, k_2, k_3).



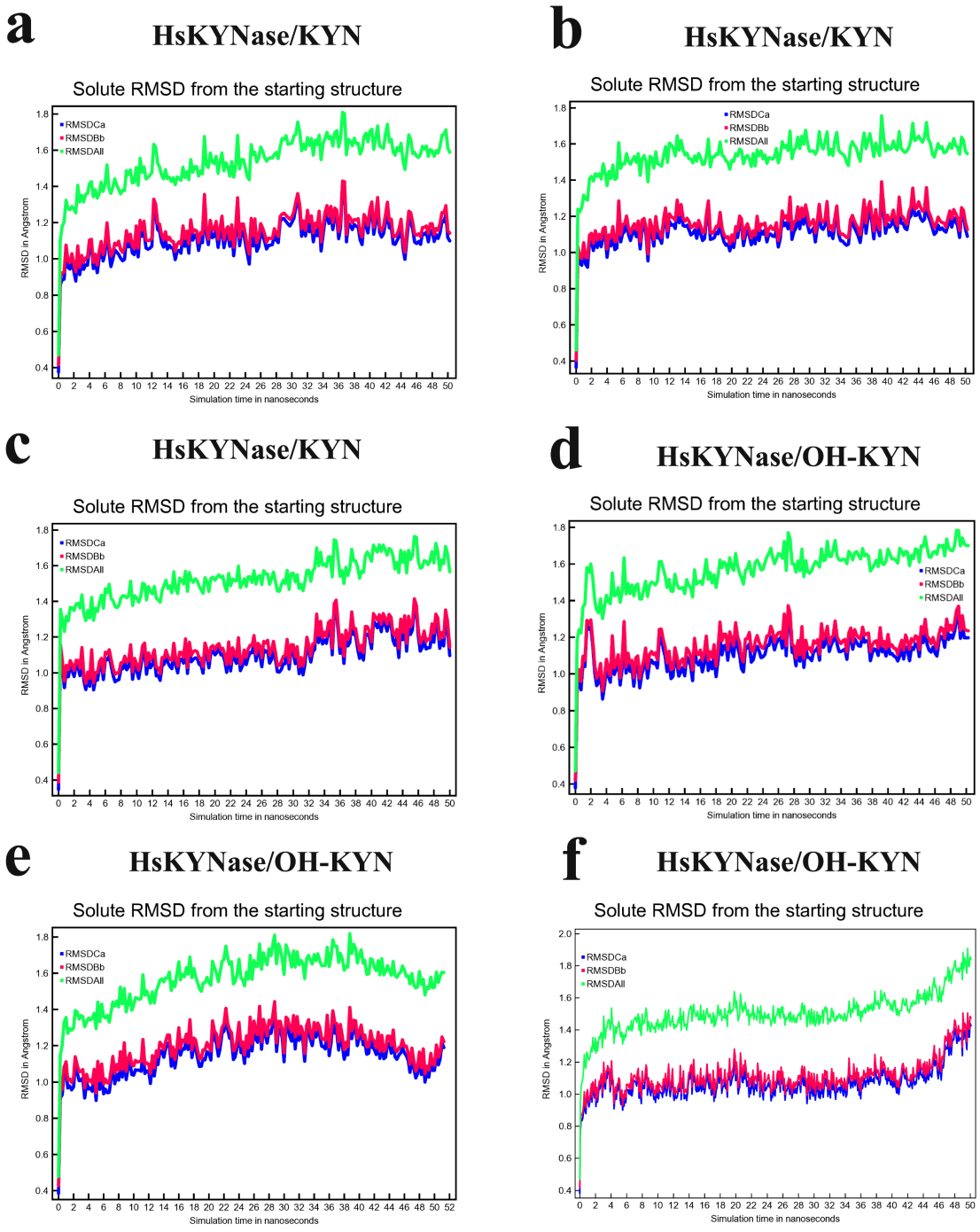
Supplementary Figure 4. **Pre-steady-state kinetic traces of HsKYNase_93D9-H102W/N333T variant during reaction with KYN or OH-KYN**. **a**. Rapid formation of AA after mixing 9 μM enzyme with 500 μM KYN. **b**. Pre-steady-state OH-AA formation upon mixing 9 μM HsKYNase_93D9-H102W/N333T with 500 μM OH-KYN. Experimental red traces represent the average of five measurements and the black line is the analytical fit to the single-exponential function as described in Methods. All reactions were performed in PBS, pH 7.4 at 37 °C.



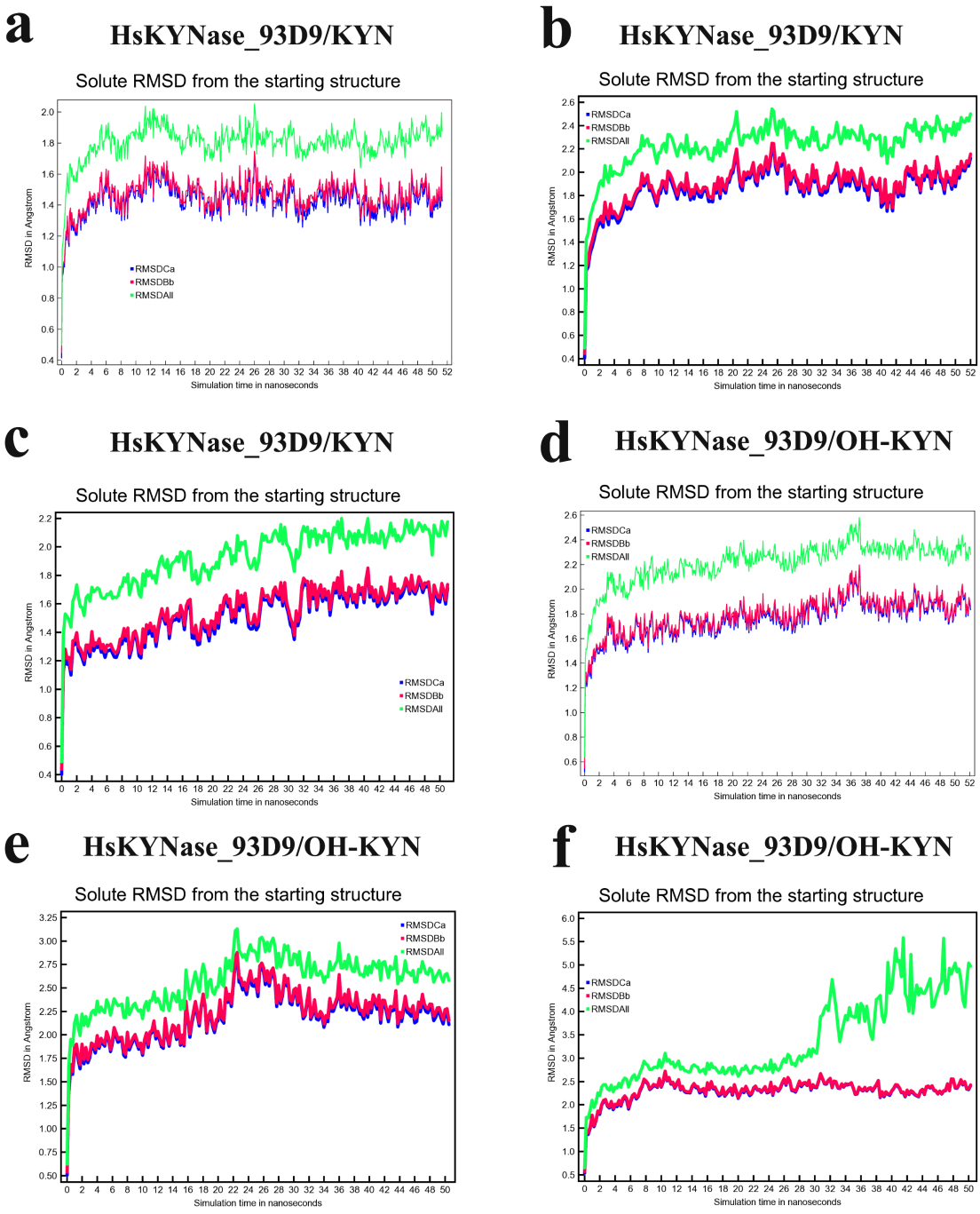
Supplementary Figure 5. HsKYNase_93D9 homology model. Overlaid structures of HsKYNase (PBD entry: 3E9K) and HsKYNase_93D9 homology model focusing on the active site and mutated regions. HsKYNase and HsKYNase_93D9 are shown in green and cyan, respectively. Side chains of mutated residues are shown in bold black font whereas in red font are key active site residues shared by both enzyme species. The asterisk denotes residues which are contributed from the "second" monomer and HsKYNase's wild-type residues are shown in italics font.



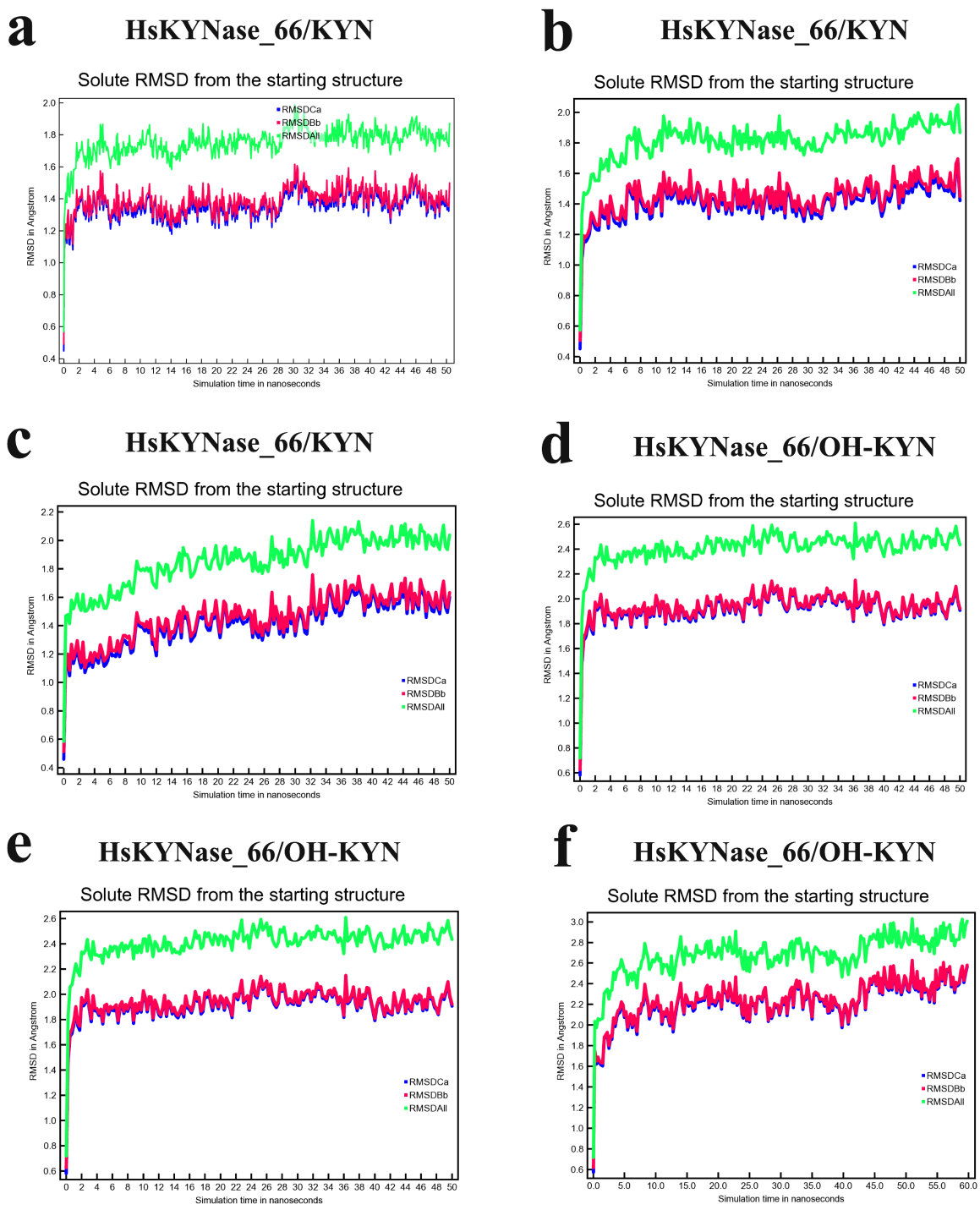
Supplementary Figure 6. Docking of 3-hydroxy-hippuric acid inhibitor into the HsKYNase_93D9 active site. Overlaid active sites of HsKYNase (PDB: 3E9K, shown in green) in complex with 3-hydroxy-hippuric acid inhibitor and HsKYNase_93D9 (homology model shown in cyan). The docked inhibitor into the active site of HsKYNase_93D9 is shown as cyan sticks colored by atom sticks (nitrogen: blue, phosphate: orange, oxygen: red). The distances between the inhibitor and the N333 residue are shown as yellow dashed lines (3.7 Å and 3.6 Å for HsKYNase and HsKYNase_93D9 respectively). The side chains of the motif H102-N333 residues are shown as sticks. The PLP is also shown as yellow sticks.



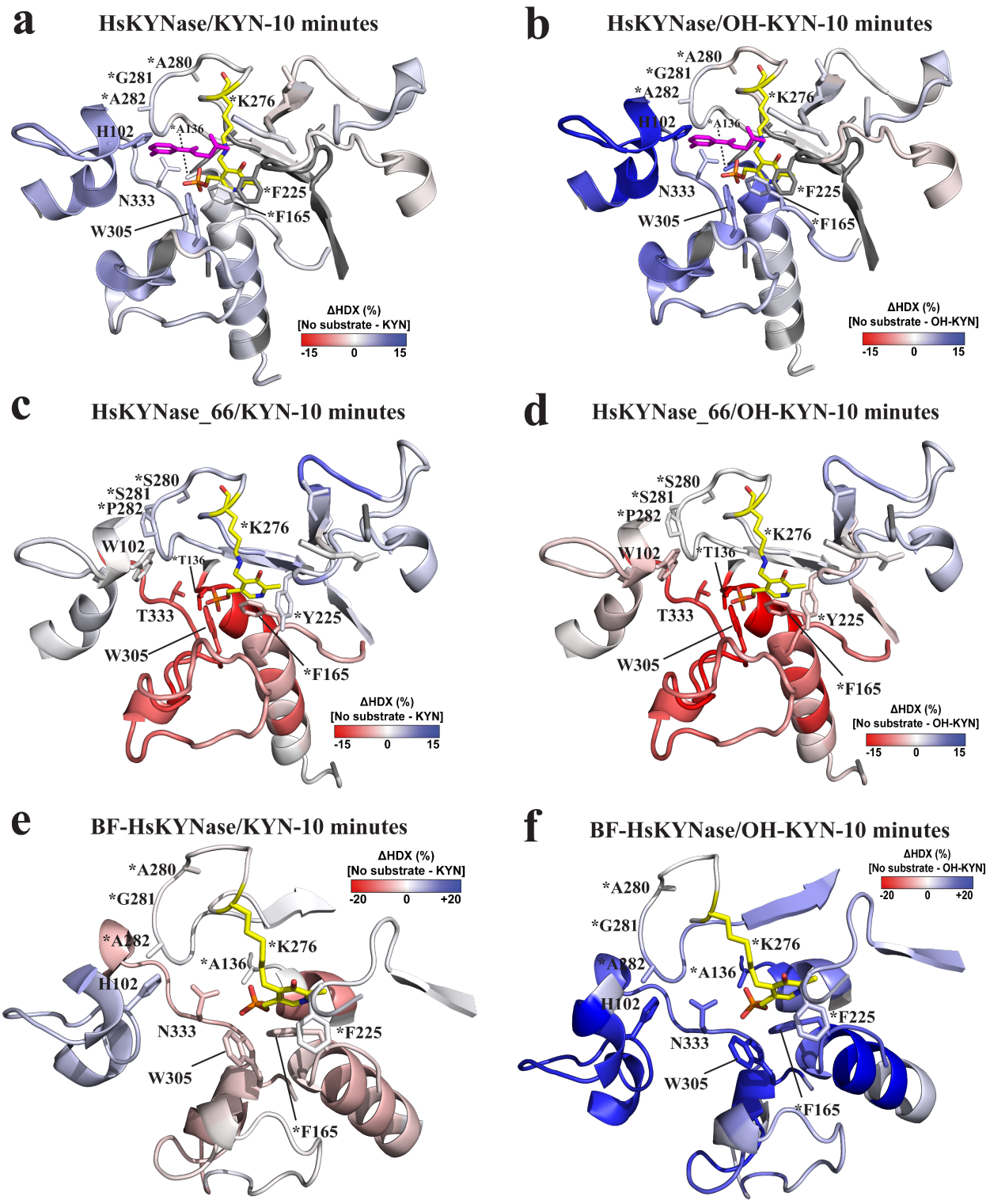
Supplementary Figure 7. RMSD graphs showing the solute RMSD from the starting structure as a function of simulation time for HsKYNase. **a-c**. Replicate runs for HsKYNase/KYN complex and **d-f**. MD simulation runs for the HsKYNase/OH-KYN complex. Each plot displays the RMSDC*α*, backbone (RMSDBb) and all-heavy atom including that from the ligand as well (RMSDAll) in blue, red and green traces respectively. The RMSD plots indicate that the simulations reach equilibrium and are stable over 50 nanoseconds. The trajectories shown in panels a and d were chosen for further analysis of the enzyme-ligand interactions and are shown in the main text.



Supplementary Figure 8. RMSD graphs showing the solute RMSD from the starting structure as a function of simulation time for HsKYNase_93D9. **a-c**. Replicate runs for HsKYNase_93D9/KYN complex and **d-f**. replicate runs for the HsKYNase_93D9/OH-KYN complex. Traces are colored as in Supplementary Fig. 7 above. The trajectories shown in panels a and d were chosen for further analysis of the enzyme-ligand interactions and are shown in the main text.



Supplementary Figure 9. RMSD graphs showing the solute RMSD from the starting structure as a function of simulation time for HsKYNase_66. **a-c**. Replicate runs for HsKYNase_66/KYN complex and **d-f**. replicate runs for the HsKYNase_66/OH-KYN complex. Traces are colored as in Supplementary Fig. 7 and 8 above. The trajectories shown in panels a and d were chosen for further analysis of the enzyme-ligand interactions and are shown in the main text.



Supplementary Figure 10. **Summary of HDX-MS protein dynamics for wild-type HsKYNase, HsKYNase_66 and BF-HsKYNase**. **a-b**. Zoom-in on the active site of HsKYNase (PDB: 3E9K) colored by the difference in fractional D-uptake (-15 to +15%) between no substrate and with KYN or OH-KYN respectively after 10 minutes exposure to deuterium[1] . **c-d**. Zoom-in on the active site of HsKYNase_66

(PDB: 7S3V) during reaction for 10 minutes with KYN and OH-KYN respectively[2]. **e-f**. Zoom-in on the active site of BF-HsKYNase during reaction for 10 minutes with KYN and OH-KYN respectively[3]. Key active site- and important PLP-interacting residues at regions with significant exchange are labeled in all panels and atom sticks are color-coded (nitrogen: blue, phosphate: orange, oxygen: red). Asterisk indicates that residue is contributed by the "second" KYNase chain. HDX-MS experiments for each protein were conducted in the same conditions but independently. The data were reproduced upon permission of the respective journals cited here. HsKYNase data were reprinted with permission from Karamitros, C. S. *et al.* Conformational Dynamics Contribute to Substrate Selectivity and Catalysis in Human Kynureninase. *ACS Chem. Biol.* **15**, 3159–3166 (2020). Copyright 2020, American Chemical Society.

Supplementary Tables

Supplementary Table 1. Summary of all the mutant libraries that were generated and subjected to genetic selection and 96-well plate screening in the present study.

#	Type of library	Sites targeted and fixed/allowed mutations	HsKYNase template	Screening method
1	Saturated mutagenesis (NNS)	T297-A301-V303	Wild-type HsKYNase	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
2	Saturated mutagenesis (NNS)	A295-H296-I298-T297	Wild-type HsKYNase	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
3	Saturated mutagenesis (NNS)	N127-E128-K129	Wild-type HsKYNase	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
4	Saturated mutagenesis (NNS)	F71-L72-K81-M82	Wild-type HsKYNase	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
5	Saturated mutagenesis (NNS)	F149-K150-K154-Y156	Wild-type HsKYNase	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
6	Saturated mutagenesis (NNS)	K84-T85-Y86-E88	Wild-type HsKYNase	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
7	Saturated mutagenesis (NNS)	E90-K93-V104-K106-R107	Wild-type HsKYNase	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
8	Saturated mutagenesis (NNS)	V339-C340-S341-H343-E347-T353	Wild-type HsKYNase	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
9	Saturated mutagenesis (NNS)	I97-A98-A99-Y100	Wild-type HsKYNase	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
10	Saturated mutagenesis (NNS)	E103-V104-G105	Wild-type HsKYNase	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
11	Saturated mutagenesis (NNS)	G105-K106-R107-P108	Wild-type HsKYNase	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
12	Saturated mutagenesis (NNS)	P108-I110-T111-S115	Wild-type HsKYNase	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
13	Saturated mutagenesis (NNS)	Y100-E103-P108-I110-T111	Wild-type HsKYNase	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
14	Saturated mutagenesis (NNS)	V326-I331-P334	Wild-type HsKYNase	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
15	Saturated mutagenesis (NNS)	F329-R330-I331-P334	Wild-type HsKYNase	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
16	Saturated mutagenesis (NNS)	M187-M189-K191	Wild-type HsKYNase	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
17	Saturated mutagenesis (NNS)	E259	Wild-type HsKYNase	96-well plate spectrophotometric assay
18	Fixed mutations and partial saturation mutagenesis	T138S-V139NDT-H142F–L143K	Wild-type HsKYNase	96-well plate spectrophotometric assay
19	Fixed mutations and partial saturation mutagenesis	Y156NNS-K157V-L160A/T-A162R/T-K163S/T-A164N	Wild-type HsKYNase	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
20	Fixed mutations and pairwise mutagenesis	F249W-A254S/T-V255A-N257A-E259P	Wild-type HsKYNase	96-well plate spectrophotometric assay
21	Fixed mutations and pairwise mutagenesis	W272G-S274G/S/T-A280G/S-A282P-G287F	Wild-type HsKYNase	96-well plate spectrophotometric assay
22	DNA shuffling-1	Recombination of genes	*see separate Table 4*	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
23	Saturated mutagenesis (NNS)	F149-K150-K154-Y156	HsKYNase_93	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay

24	Saturated mutagenesis (NNS)	Y156-K157-L160-A162-K163	HsKYNase_93	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
25	Saturated mutagenesis (NNS)	L219-F220-S221-G222	HsKYNase_93	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
26	Saturated mutagenesis (NNS)	H230-F231-N232D-P234	HsKYNase_93	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
27	Saturated mutagenesis (NNS)	P234-K238-Q241	HsKYNase_93	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
28	Saturated mutagenesis (NNS)	A242-K243-Y246-F249	HsKYNase_93	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
29	Saturated mutagenesis (NNS)	G284-I285	HsKYNase_93	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
30	Saturated mutagenesis (NNS)	H224-F225	HsKYNase_93	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
31	Saturated mutagenesis (NNS)	I331	HsKYNase_93	96-well plate spectrophotometric assay
32	Saturated mutagenesis (NNS)	S394-H395-V396-E397	HsKYNase_93	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
33	Saturated mutagenesis (NNS)	T404-I405-T406-F407-S408	HsKYNase_93	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
34	Saturated mutagenesis (NNS)	N411-D413-Q416-E419	HsKYNase_93	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
35	Saturated mutagenesis (NNS)	K427-N429-N431-G432-A436	HsKYNase_93	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
36	Saturated mutagenesis (NNS)	A99-I110-G112	HsKYNase_93	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
37	Saturated mutagenesis (NNS)	K106-R107-I110-G112	HsKYNase_93	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
38	Error prone PCR (epPCR)	Random incorporation of mutations	HsKYNase_93	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
39	DNA shuffling-2	Recombination of genes	*see separate Table 6*	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
40	Saturated mutagenesis (NNS)	E90-K93-I97	HsKYNase_93D9	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
41	Saturated mutagenesis (NNS)	T111-D113-E114	HsKYNase_93D9	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
42	Saturated mutagenesis (NNS)	V339-C340-E347-K350-T353	HsKYNase_93D9	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
43	Saturated mutagenesis (NNS) & pairwise	Y306-H308H/Y-E309-L310-S311	HsKYNase_93D9	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
44	Saturated mutagenesis (NNS & NDT)	C327-F329-R330-I331(NDT)	HsKYNase_93D9	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
45	Saturated mutagenesis (NNS)	I405-S408-N411	HsKYNase_93D9	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
46	Pairwise based on primary sequence of bacterial MpKYNase	K315-R330	HsKYNase_93D9	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay
47	Error prone PCR (epPCR)	Random incorporation of mutations	HsKYNase_93D9	*E. coli* Δ*TrpE* high throughput selection & 96-well plate spectrophotometric assay

Supplementary Table 2. HsKYNase variants_1-27 that were isolated from NNS combinatorial mutagenesis using HsKYNase as the parental species. Mutations from the most highly active variants were rationally combined yielding Combo variants_1-6. Amino acid substitutions F306Y, I331C and I3331N were identified from a previous study[2]. KYN steady-state kinetic parameters were obtained from purified enzyme preparations in PBS, pH 7.4 at 37 ºC. Key variants are shown in green bold fonts in all Supplementary Tables.

Variant name	Amino acid substitutions	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($M^{-1}s^{-1}$)	Fold-HsKYNase
wild-type HsKYNase	no mutations	0.12±0.02	1200±120	100±26	1
HsKYNase_1	**F71M-L72N-K81R-M82N (consensus sequence)**	**1.12±0.07**	**650±100**	**1720±285**	**17.2**
HsKYNase_2	F149Y-K150R-K154R	0.45±0.04	1400±230	320±60	3.2
HsKYNase_3	F149Y-K150N-K154T	0.70±0.07	2000±320	350±66	3.5
HsKYNase_4	K84R-T85N-Y86S-E88L	0.57±0.05	1500±300	380±83	3.8
HsKYNase_5	V339F-C340P-H343R-E347L-T353G (consensus sequence)	0.25±0.01	1000±120	250±32	2.5
HsKYNase_6	I97L-A98G-Y100A	0.36±0.09	1300±450	280±120	2.8
HsKYNase_7	I97V-A98G-A99V-Y100H	0.30±0.03	1000±120	300±70	3
HsKYNase_8	**E103F-V104H-G105T**	**0.85±0.02**	**500±33**	**1700±120**	**17**
HsKYNase_9	F329Y-I331T	0.38±0.05	1800±350	210±50	2.1
HsKYNase_10	M187L-M189I-K191S	0.25±0.01	500±35[a]	500±40	5
HsKYNase_11	M187L-M189V-K191N	0.23±0.01	440±40[b]	520±52	5.2
HsKYNase_12	T297G-A301G	0.27±0.03	930±300	290±100	2.9
HsKYNase_13	T297L-A301P	0.33±0.06	1300±250	250±66	2.5
HsKYNase_14	A295H-H296L-T297V-I298V	0.25±0.015	1200±140	210±27	2.1
HsKYNase_15	A295H-H296R-T297S-I298L	0.10±0.025	1600±350	63±21	0.6
HsKYNase_16	N127R-E128P-K129S	0.20±0.02	930±230	215±57	2.1
HsKYNase_17	F149Y-K150R-K154T	0.67±0.06	2300±330	290±50	2.9
HsKYNase_18	A98G-Y100A	0.36±0.07	1300±550	280±130	2.8
HsKYNase_19	E103F-V104E-G105S	0.63±0.02	620±55	1000±94	10
HsKYNase_20	E259P (consensus sequence)	0.40±0.05	800±100	500±88	5
HsKYNase_21	E103F-V104S-G105T	*only sequenced (high activity on plates but lower than HsKYNase_8 from same library)*			
HsKYNase_22	A295L-H296P-T297A-I298K	*only sequenced (marginally higher activity than wild type HsKYNase on plates; similar to HsKYNase_14)*			
HsKYNase_23	A295H-H296V-T297D-I298F	*only sequenced (marginally higher activity than wild type HsKYNase on plates; similar to HsKYNase_14)*			
HsKYNase_24	A295H-H296H-T297H-I298L	*only sequenced (marginally higher activity than wild type HsKYNase on plates; similar to HsKYNase_14)*			
HsKYNase_25	K84A-T85M-Y86G-E88G	*only sequenced (high activity on plates but lower than HsKYNase_4 from same library)*			
HsKYNase_26	K84A-T85D-Y86R-E88G	*only sequenced (high activity on plates but lower than HsKYNase_4 from same library)*			
HsKYNase_27	K84S-T85V-Y86A-E88G	*only sequenced (high activity on plates but lower than HsKYNase_4 from same library)*			

Combo variant_1	**F71M-L72N-K81R-M82N-I331N-F306Y**	**0.69±0.05**	**430±45**	**1600±200**	**16**
Combo variant_2	F71M-L72N-K81R-M82N-E103F-V104H-G105T	1±0.065	250±18	4000±390	40
Combo variant_3	F71M-L72N-K81R-M82N-I331C	0.24±0.04	400±30	600±110	6
Combo variant_4	F71M-L72N-K81R-M82N-I331N	0.63±0.05	400±30	1575±170	16
Combo variant_5	E103F-V104H-G105T-I97V-A98G-A99V-Y100H	0.70±0.04	550±45	1270±126	13
Combo variant_6	F71M-L72N-K81R-M82N-K84R-T85N-Y86S-E88L	0.65±0.06	500±30	1300±143	13

[a]This value represents KS as the variant displayed sigmoidal kinetics with a Hill coefficient of $n_H \sim 1.95$

[b]This value represents KS as the variant displayed sigmoidal kinetics with a Hill coefficient of $n_H \sim 2.6$

Supplementary Table 3. Rationally designed variants_1-15 were constructed based on phylogenetic analysis and mutational data from previous studies[2]. Steady-state kinetic parameters were calculated from assays performed in PBS, pH 7.4 at 37 ºC. Key variants are shown in green bold fonts.

#	Strategy of mutagenesis	Amino acid substitutions	HsKYNase template	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($M^{-1}s^{-1}$)	Fold-HsKYNase
1	Rationally designed variant_1	W272G-S274G-A280S-A282P-G287F	Wild-type HsKYNase	0.50±0.06	1000 ±180	500± 110	5
2	Rationally designed variant_2	W272G-S274S-A280S-A282P-G287F	Wild-type HsKYNase	0.53± 0.05	1300 ±250	400±85	4
3	Rationally designed variant_3	W272G-S274A-A280G-A282P-G287F	Wild-type HsKYNase	0.38± 0.032	750± 170	500± 120	5
4	Rationally designed variant_4	W272G-S274A-A280S-A282P-G287F	Wild-type HsKYNase	0.25± 0.016	600± 120	420±88	4.2
5	Rationally designed variant_5	W272G-S274T-A280S-A282P-G287F	Wild-type HsKYNase	0.17± 0.014	850± 130	200±35	2
6	Rationally designed variant_6	W272G-S274G-A280G-A282P-G287F	Wild-type HsKYNase	0.16± 0.008	480± 50	330±38	3.3
7	Rationally designed variant_7	N232D-Q241H	Wild-type HsKYNase	0.35± 0.03	1000 ±200	350±76	3.5
8	Rationally designed variant_8	H224N-F225Y	Wild-type HsKYNase	Was not kinetically characterized-only 96-well plate assessment using cell lysate (~2.5X active than wild-type)			
9	Rationally designed variant_9	F225Y	Wild-type HsKYNase	Was not kinetically characterized-only 96-well plate assessment using cell lysate (~2.5X active than wild-type)			
10	Rationally designed variant_10	E259P-W272G-S274A-A280G-A282P-G287F	Wild-type HsKYNase	Was not kinetically characterized-only 96-well plate assessment using cell lysate (~3X active than wild-type)			
11	Rationally designed variant_11	E259P-W272G-S274A-A280S-A282P-G287F	Wild-type HsKYNase	Was not kinetically characterized-only 96-well plate assessment using cell lysate (~3X active than wild-type)			
12	**Rationally designed variant_12**	**E259P-W272G-S274G-A280S-A282P-G287F**	Wild-type HsKYNase	Was not kinetically characterized-only 96-well plate assessment using cell lysate (~3X active than wild-type)			
13	Rationally designed variant_13	E259P-W272G-A280G-A282P-G287F	Wild-type HsKYNase	Was not kinetically characterized-only 96-well plate assessment using cell lysate (~3X active than wild-type)			
14	Rationally designed variant_14	N232D-Q241H-E259P	Wild-type HsKYNase	Was not kinetically characterized-only 96-well plate assessment using cell lysate (~2.5X active than wild-type)			
15	Rationally designed variant_15	G432I-V435F-A436G-P437F	Wild-type HsKYNase	Insoluble protein			

Supplementary Table 4. Genes of HsKYNase variants that were shuffled, and screening of the resulting library led to the isolation of HsKYNase_93. Group 1 includes variants that were isolated from NNS combinatorial mutagenesis whereas group 2 includes variants whose mutations were rationally combined. B-factor variants are from[3]. All genes were mixed at 1:1 molar ratio prior to DNase-I treatment. Key variants are shown in green bold fonts.

GROUP 1-Variants isolated from combinatorial saturation mutagenesis

Variant name	Amino acid substitutions
HsKYNase_1	**F71M-L72N-K81R-M82N**
HsKYNase_2	F149Y-K150R-K154R
HsKYNase_3	F149Y-K150N-K154T
HsKYNase_4	K84R-T85N-Y86S-E88L
HsKYNase_5	V339F-C340P-S-H343R-E347L-T353G
HsKYNase_6	I97L-A98G-Y100A
HsKYNase_7	I97V-A98G-A99V-Y100H
HsKYNase_8	**E103F-V104H-G105T**
HsKYNase_9	F329Y-I331T
HsKYNase_10	M187L-M189I-K191S
HsKYNase_11	M187L-M189V-K191N
B-factor_1	I183P-E184A
B-factor_2	K191R-E197S-I201T
B-factor_3	K191S-E197T-I201T
B-factor_4	N375A-Y376C-K378G
B-factor_5	K380G-A382G-K384R-P386S
B-factor_6	K380G-A382G-K384R
B-factor_7	D413S-Q416T-E419L
B-factor_8	D413C-Q416T-E419L
B-factor_9	K427M-N429E-G432A-A436T
GROUP 2-Variants with rationally combined mutations	
Combo variant_1	**F71M-L72N-K81R-M82N-I331N-F306Y**
Combo variant_2	F71M-L72N-K81R-M82N-E103F-V104H-G105T
Combo variant_3	F71M-L72N-K81R-M82N-I331C
Combo variant_4	F71M-L72N-K81R-M82N-I331N
Combo variant_5	E103F-V104H-G105T-I97V-A98G-A99V-Y100H
Combo variant_6	F71M-L72N-K81R-M82N-K84R-T85N-Y86S-E88L
Rationally designed variant_8	H224N-F225Y
Rationally designed variant_9	F225Y
Rationally designed variant_10	E259P-W272G-S274A-A280G-A282P-G287F

Rationally designed variant_11	E259P-W272G-S274A-A280S-A282P-G287F
Rationally designed variant_12	**E259P-W272G-S274G-A280S-A282P-G287F**
Rationally designed variant_13	E259P-W272G-A280G-A282P-G287F
Rationally designed variant_14	E259P-N232D-Q241H
B-factor_10	**I183P-E184A-M187L-M189I-K191N-E197T-I201T**
B-factor_11	I183P-E184A-M187L-M189I-K191S-E197T-I201T
B-factor_12	N375A-Y376C-K378G-K380G-A382G-K384R-P386S
B-factor_13	N375A-Y376C-K378G-K380G-A382G-K384G-P386G
B-factor_14	N411R-D413F-N375A-Y376C-K378G
B-factor_15	N411R-D413F-K427M-N429E-G432A-A436T
B-factor_16	N411R-D413F-Q416T-E419L
B-factor_17	N411R-D413C-Q416T-E419L
B-factor_18	N411R-D413S-Q416T-E419L
B-factor_19	N375A-Y376C-K378G-K380G-A382G-K384G-P386S-N411R-D413S-Q416T-E419L-K427M-N429E-G432A-A436T
B-factor_20	N375A-Y376C-K378G-K380G-A382G-K384R-P386S-N411R-D413S-Q416T-E419L-K427M-N429E-G432A-A436T
B-factor_21	N375A-Y376C-K378G-K380G-A382G-K384G-P386S-N411R-D413L-Q416T-E419L-K427M-N429E-G432A-A436T
B-factor_22	N375A-Y376C-K378G-K380G-A382G-K384G-P386S-N411R-D413F-Q416T-E419L-K427M-N429E-G432A-A436T
B-factor_23	N375A-Y376C-K378G-K380G-A382G-K384G-P386T-N411R-D413I-Q416T-E419L-K427M-N429E-G432A-A436T
B-factor_24	N375A-Y376C-K378G-K380G-A382G-K384R-P386G-N411R-D413S-Q416T-E419L-K427M-N429E-G432A-A436T
B-factor_25	N375A-Y376C-K378G-K380G-A382G-K384G-P386G-N411R-D413F-Q416T-E419L-K427M-N429E-G432A-A436T
B-factor_26	N375A-Y376C-K378G-K380G-A382G-K384G-P386G-N411R-D413I-Q416T-E419L-K427M-N429E-G432A-A436T

Supplementary Table 5. KYN steady-state-kinetic parameters of HsKYNase_94-102 variants that were isolated from NNS combinatorial mutagenesis using HsKYNase_93 as the parental species. HsKYNase_93 parental protein and key variants are shown in red and green bold fonts respectively.

Variant name	Amino acid substitutions	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($M^{-1}s^{-1}$)	Fold-HsKYNase
HsKYNase_93 (Parental)	**F71M-L72N-E103F-V104H-G105T-I183P-E184A-M187L-M189I-K191N-E197T-I201T-A280S-A282P-F306Y**	**1.5**±0.1	**110±15**	**13600**± 2000	**136**
HsKYNase_94	F71M-L72N-E103F-V104H-G105T-F149Y-K150R-K154R-I183P-E184A-M187L-M189I-K191N-E197T-I201T-A280S-A282P-F306Y	1.3±0.025	210±14	6190±430	62
HsKYNase_95	F71M-L72N-E103F-V104H-G105T-F149Y-K150R-K154M-Y156H-I183P-E184A-M187L-M189I-K191N-E197T-I201T-A280S-A282P-F306Y	1.25±0.12	190±60	6580± 2100	66
HsKYNase_96	F71M-L72N-E103F-V104H-G105T-F149Y-K150H-K154M-I183P-E184A-M187L-M189I-K191N-E197T-I201T-A280S-A282P-F306Y	1.15±0.01	170±10	6760±245	68
HsKYNase_97	F71M-L72N-E103F-V104H-G105T-I183P-E184A-M187L-M189I-K191N-E197T-I201T-H230Y-N232D-P234S-A280S-A282P-F306Y	1±0.07	130±40	7700± 2400	77
HsKYNase_98	F71M-L72N-E103F-V104H-G105T-I183P-E184A-M187L-M189I-K191N-E197T-I201T-A280S-A282P-F306Y-N411S-D413F-Q416S	1.42±0.02	300±13	4730±220	47
HsKYNase_99	**F71M-L72N-A99G-E103F-V104H-G105T-I110T-G112T-I183P-E184A-M187L-M189I-K191N-E197T-I201T-A280S-A282P-F306Y**	**0.73±0.02**	**65±14**	**11230± 2450**	**112**
HsKYNase_100	F71M-L72N-A99V-E103F-V104H-G105T-I110V-G112S-I183P-E184A-M187L-M189I-K191N-E197T-I201T-A280S-A282P-F306Y	1.5±0.033	200±17	7520±660	75
HsKYNase_101	F71M-L72N-E103F-V104H-G105T-K106P-R107E-I110T-G112L-I183P-E184A-M187L-M189I-K191N-E197T-I201T-A280S-A282P-F306Y	1.35±0.04	165±16	8200±620	82
HsKYNase_102	F71M-L72N-E103F-V104H-G105T-K106R-R107S-I110V-G112Q-I183P-E184A-M187L-M189I-K191N-E197T-I201T-A280S-A282P-F306Y	1.64±0.04	220±17	7450±600	74

Supplementary Table 6. Genes of HsKYNase variants which were shuffled, and screening of the library led to the identification of HsKYNase_93D9. Variant HsKYNase_99 harbors the two mutations I110T-G112T which were found in HsKYNase_93D9. Rationally designed variants were constructed by incorporating previously identified beneficial mutations (underlined in bold)[2] on HsKYNase_93.

Gene #	Variant name	Amino acid substitutions
1	**HsKYNase_93**	**F71M-L72N-E103F-V104H-G105T-I183P-E184A-M187L-M189I-K191N-E197T-I201T-A280S-A282P-F306Y**
2	HsKYNase_94	F71M-L72N-E103F-V104H-G105T-F149Y-K150R-K154R-I183P-E184A-M187L-M189I-K191N-E197T-I201T-A280S-A282P-F306Y
3	HsKYNase_95	F71M-L72N-E103F-V104H-G105T-F149Y-K150R-K154M-Y156H-I183P-E184A-M187L-M189I-K191N-E197T-I201T-A280S-A282P-F306Y
4	HsKYNase_96	F71M-L72N-E103F-V104H-G105T-F149Y-K150H-K154M-I183P-E184A-M187L-M189I-K191N-E197T-I201T-A280S-A282P-F306Y
5	HsKYNase_97	F71M-L72N-E103F-V104H-G105T-I183P-E184A-M187L-M189I-K191N-E197T-I201T-H230Y-N232D-P234S-A280S-A282P-F306Y
6	HsKYNase_98	F71M-L72N-E103F-V104H-G105T-I183P-E184A-M187L-M189I-K191N-E197T-I201T-A280S-A282P-F306Y-N411S-D413F-Q416S
7	**HsKYNase_99**	**F71M-L72N-A99G-E103F-V104H-G105T-I110T-G112T-I183P-E184A-M187L-M189I-K191N-E197T-I201T-A280S-A282P-F306Y**
8	HsKYNase_100	F71M-L72N-A99V-E103F-V104H-G105T-I110V-G112S-I183P-E184A-M187L-M189I-K191N-E197T-I201T-A280S-A282P-F306Y
9	HsKYNase_101	F71M-L72N-E103F-V104H-G105T-K106P-R107E-I110T-G112L-I183P-E184A-M187L-M189I-K191N-E197T-I201T-A280S-A282P-F306Y
10	HsKYNase_102	F71M-L72N-E103F-V104H-G105T-K106R-R107S-I110V-G112Q-I183P-E184A-M187L-M189I-K191N-E197T-I201T-A280S-A282P-F306Y
11	Rationally designed variant on HsKYNase_93	F71M-L72N-E103F-V104H-G105T-I183P-E184A-M187L-M189I-K191N-E197T-I201T-**E259P**-A280S-A282P-F306Y
12	Rationally designed variant on HsKYNase_93	F71M-L72N-E103F-V104H-G105T-I183P-E184A-M187L-M189I-K191N-E197T-I201T-**N232D-Q241H**-A280S-A282P-F306Y
13	Rationally designed variant on HsKYNase_93	F71M-L72N-E103F-V104H-G105T-I183P-E184A-M187L-M189I-K191N-E197T-I201T-A280S-A282P-**F306W**
14	Rationally designed variant on HsKYNase_93	F71M-L72N-E103F-V104H-G105T-I183P-E184A-M187L-M189I-K191N-E197T-I201T-**F225Y**-A280S-A282P-F306Y
15	Rationally designed variant on HsKYNase_93	F71M-L72N-E103F-V104H-G105T-I183P-E184A-M187L-M189I-K191N-E197T-I201T-**N232D-Q241H-E259P**-A280S-A282P-F306Y
16	Rationally designed variant on HsKYNase_93	F71M-L72N-E103F-V104H-G105T-I183P-E184A-M187L-M189I-K191N-E197T-I201T-**F225Y-N232D-Q241H**-A280S-A282P-F306Y
17	Rationally designed variant on HsKYNase_93	F71M-L72N-**A99G**-E103F-V104H-G105T-**I110T-G112T**-I183P-E184A-M187L-M189I-K191N-E197T-I201T-A280S-A282P-**F306W**
18	Rationally designed variant on HsKYNase_93	F71M-L72N-E103F-V104H-G105T-**K106R-R107S-I110V-G112Q**-I183P-E184A-M187L-M189I-K191N-E197T-I201T-A280S-A282P-**F306W**

Supplementary Table 7. The most highly active HsKYNase variants including HsKYNase_93D9 (shown in bold red) that were isolated from the DNA shuffling library described in Supplementary Table 6.

Variant name	Amino acid substitutions	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($M^{-1}s^{-1}$)	Fold-HsKYNase
HsKYNase_93D9	**F71M-L72N-E103F-V104H-G105T-I110T-G112T-I183P-E184A-M187L-M189I-K191N-E197T-I201T-A280S-A282P-F306Y**	**1.45±0.07**	**85±0.01**	**17000± 2200**	**170**
HsKYNase_93C3	F71M-L72N-A99G-E103F-V104H-G105T-K106R-R107S-I110V-G112L-I183P-E184A-M187L-M189I-K191N-E197T-I201T-Q241H-E259PA280S-A282P-F306Y	1.4±0.04	150±15	9330±970	93
HsKYNase_93F8	F71M-L72N-A99G-E103F-V104H-G105T-I110V-G112S-I183P-E184A-M187L-M189I-K191N-E197T-I201T-A280S-A282P-F306Y	1.6±0.05	220±20	7300±700	73

Supplementary Table 8. Rate constants calculated from the global fit analysis of the reaction of HsKYNase_93D9 with KYN (data are shown in Fig. 2a and b in the main text).

Rate constant	Lower limit[α]	Upper limit[b]	%Range[c]	Best fit
k_1 ($\mu M^{-1}s^{-1}$)	0.22	0.27	10	0.24
k_{-1} (s^{-1})	3.31	4.83	19	4
k_2 (s^{-1})	0.77	0.81	2.5	0.79

[α,b]Lower and upper limits represent a threshold of 5% deviation from the minimal Sum Square Error (SSE) in the confidence contours. [c]The percentage range was calculated by dividing the mean of the range by the best fit values as follows: (upper-lower)/(2×best fit). This represents the allowable variation of each best fit value as a percentage.

Supplementary Table 9. Rate constants calculated from the global fit analysis of the reaction of HsKYNase_66 with KYN (data are shown in Fig. 2d and e in the main text).

Rate constant	Lower limit[a]	Upper limit[b]	%Range[c]	Best fit
k_1 ($\mu M^{-1}s^{-1}$)	5.5	6.4	7.6	5.9
k_2 (s^{-1})	2.78	3.28	8	3.1
k_3 (s^{-1})	17.5	24.8	17.3	21

[a,b]Lower and upper limits represent a threshold of 5% deviation from the minimal Sum Square Error (SSE) in the confidence contours. [c]The percentage range was calculated by dividing the mean of the range by the best fit values as follows: (upper-lower)/(2×best fit). This represents the allowable variation of each best fit value as a percentage.

Supplementary Table 10. Steady-state kinetic properties of HsKYNase_93D9 against KYN and OH-KYN in D_2O, pD 7.4 at 37 ºC.

k_{cat} (s^{-1})	K_M (µM)	k_{cat}/K_M ($M^{-1}s^{-1}$)
KYN		
0.16±0.012	250±49	640±134
OH-KYN		
0.135±0.007	50±8	(2.7±0.45) × 10^3

Supplementary Table 11. Summary of HDX-MS experimental parameters

Data Set	HsKYNase_93D9
HDX reaction details	PBS ± substrate[a]
	pH_{read} = 7.0
HDX time course (min)	1, 10 , 100 at 37°C
HDX control samples	Unlabeled HsKYNase_93D9
Back-exchange (mean)	~30%
# of peptides	132
Sequence coverage	94%
Average peptide length / Redundancy	12.5 / 3.73
Replicates (biological or technical)	3 (technical)
Repeatability	0.07 (average standard deviation)
Significant difference	ΔHDX greater than 0.3 Da
	p-value≤0.01

[a]KYN and OH-KYN were 3 mM

Supplementary References

1. Karamitros, C. S. *et al.* Conformational Dynamics Contribute to Substrate Selectivity and Catalysis in Human Kynureninase. *ACS Chem. Biol.* **15**, 3159–3166 (2020).
2. Blazeck, J. *et al.* Bypassing evolutionary dead ends and switching the rate-limiting step of a human immunotherapeutic enzyme. *Nat Catal* **5**, 952–967 (2022).
3. Karamitros, C. S. *et al.* Leveraging intrinsic flexibility to engineer enhanced enzyme catalytic activity. *Proc Natl Acad Sci U S A* **119**, e2118979119 (2022).