Table 4-1. Summary of design calculations for Kemp elimination enzymes. The residues allowed for each required catalytic contact are indicated along with the actual catalytic residue chosen from the active site search in parentheses. The positions allowed to change identity during the active site search and repacking calculations are indicated as design residues. Float residues are allowed to change conformation but not identity.

		required contacts, allowed residues (actual catalytic contact)				repacking residues		
design	scaffold	base	π-stacking	H-bond	active site search design residues	design	float	mutations
HG-2	1GOR	D (D126)	F/W (T44W)	S/T/Y (S265T)	17, 42, 44, 81, 83, 126, 129, 170, 172, 209, 234, 236, 237, 265, 267	42, 21, 81, 83, 84, 125, 130, 172, 234, 236, 267	16, 17, 46, 47, 50, 79, 87, 90, 170, 207, 209, 239, 275 276	Q22M, T44W, R81G, H83G, T84M, N130G, N172M, A234S, T236L, E237M, T265S, W267F
1THF-1	1THF	E (S101E)	F/W (L50W)	K (L222K)	7, 9, 11, 48, 50, 101, 126, 128, 130, 169, 171, 176, 199, 201, 222, 224	9, 48, 78, 101, 103, 126, 128, 130, 169, 171, 199, 201	7, 11, 176	C9G, V48G, L50W, T78S, S101E, N103M, Q128W, D130A, L169A, T171V, I199G, S201M, L222K
1THF-2	1THF	E (S101E)	F/W (L50W/A128W)	S/T/Y (S201)	"	9, 48, 78, 126, 128, 130, 169, 171, 222	7, 11, 176, 199, 224	C9G, V48G, L50W, T78S, S101E, A128W, D130A, L169A, T171L, L222V
1A53-1	1A53	E (L231E)	F/W (K110W)	S/T/Y (L131S)	51, 81, 83, 89, 108, 110, 112, 131, 133, 157, 159, 178, 180, 182, 184, 210, 211, 231	51, 81, 108, 157, 159, 180, 210	83, 89, 112, 133, 178, 182, 184, 211	E51I, S81G, K110W, L131S, L157G, E159W, N180G, E210S, L231E
1A53-2	1A53	E (G178E)	F/W (K110W/E210W)	none	"	51, 81, 83, 108, 131, 157, 159, 180, 211, 231	8, 53, 89, 108, 112, 133, 182, 184	E51A, S81A, L83A, K110W, L131A, K157A, E159V, G178E, N180A, E210E, S211Q, L231G
1A53-3	1A53	E (L157E)	F/W (K110W/E210W)	none	"	81, 83, 108, 131, 178, 180, 211, 231	8, 53, 89, 112, 129, 133, 182, 184	L51E, S81A, L83A, K110W, L131A, L157E, E159I, N180M, E210W, S211Q, L231G

Table 4-2. Physical characteristics of protein variants.

	molecular weight (g/mol)	ε ($M^{-1}cm^{-1}$)	formal charge	# amino acids
1THF	29185	10360	-8	266
KE07	29350	17120	-5	266
1THF-1	29245	21480	-7	266
1THF-2	29225	21480	-8	266
1A53	30070	16360	0	261
KE59	29969	21920	+1	261
1A53-1	29974	27480	+1	261
1A53-2	29969	27480	+1	261
1A53-3	30071	27480	+1	261
1GOR	34564	55280	-4	318
HG-2	34440	55280	-4	318

Table 4-3. Experimental characterization of designed Kemp elimination enzymes.

parent	mutation	scaffold	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})	k_{cat}/k_{uncat} [a]	T_m (°C)
HG-2	-	1GOR	NA	NA	130	NA	46.6
HG-2	D127N	1GOR	NA	NA	2	NA	51.0
HG-2	S265A	1GOR	NA	NA	54	NA	45.8
HG-2	K50A	1GOR	NA	NA	37	NA	49.3
HG-2	G81A	1GOR	NA	NA	17	NA	46.7
HG-2	S265T	1GOR	0.68 ± 0.4	1.6 ± 0.1	430	5.9E+05	47.9
1A53-2	-	1A53	0.012 ± 0.002	0.74 ± 0.2	16	1.0E+04	ND
1A53-3	-	1A53	0.015 ± 0.003	0.90 ± 0.03	17	1.3E+04	ND
1THF-2	-	1THF	NA	NA	5	NA	73.7
KE07 [b, c]	-	1THF	0.018 ± 0.001	1.4 ± 0.1	13	1.6E+04	NA
KE07 [b, d]	-	1THF	0.0089 ± 0.006	0.79 ± 0.09	11	7.7E+04	ND
KE70 [b, c]	-	1JCL	0.16 ± 0.05	2.1 ± 2	78	1.4E+05	NA
KE70 [b, d]	-	1JCL	0.070 ± 0.003	0.50 ± 0.06	146	6.0E+04	ND

ND = not determined
NA = not applicable
[a] k_{uncat} under the assay conditions was determined to be 1.16×10^{-6} s^{-1} by Rothlisberger *et al*.
[b] These enzymes were designed by Rothlisberger *et al*.
[c] The kinetic constants were determined by Rothlisberger *et al*.
[d] The kinetic constants were determined as a part of this thesis work.

Table 4-4. Design summary of Kemp elimination enzymes. MD prediction and experimental detection of activity are indicated.

					# mutations		activity	
name	**scaffold**	**base**	**π-stack**	**H-bond**	**from wt**	**from active design**	**MD**	**experimental**
KE59[a]	1A53	L231E	K110W	L131S	10			
1A53-1	1A53	L231E	K110W/E159W	L131S	9	8 (KE59)	no	no
KE10[a]	1A53	G178E	E210W	-	11			
1A53-2	1A53	G178E	K110W/E210W	-	12	9 (KE10)	yes	yes
1A53-3	1A53	L157E	K110W/E210W	-	11	-	yes	yes
KE07[a]	1THF	S101E	L50W	L222K	13			
1THF-1	1THF	S101E	K50W/A128W	L222K	13	9 (KE07)	yes	no
1THF-2	1THF	S101E	L50W/A128W	S201	10	10 (KE07)	yes	yes
HG-2	1GOR	D126	T44W	T265S	12	-	yes	yes
HG2-S265T	1GOR	D126	T44W	T265	11	1 (2.2.0)	increase	higher k_{cat}/K_m
HG2-G81A	1GOR	D126	T44W	T265S	13	1 (2.2.0)	decrease	decrease
HG2-K50A	1GOR	D126	T44W	T265S	13	1 (2.2.0)	decrease	decrease

[a] These KE enzymes were designed by Rothlisberger *et al*.

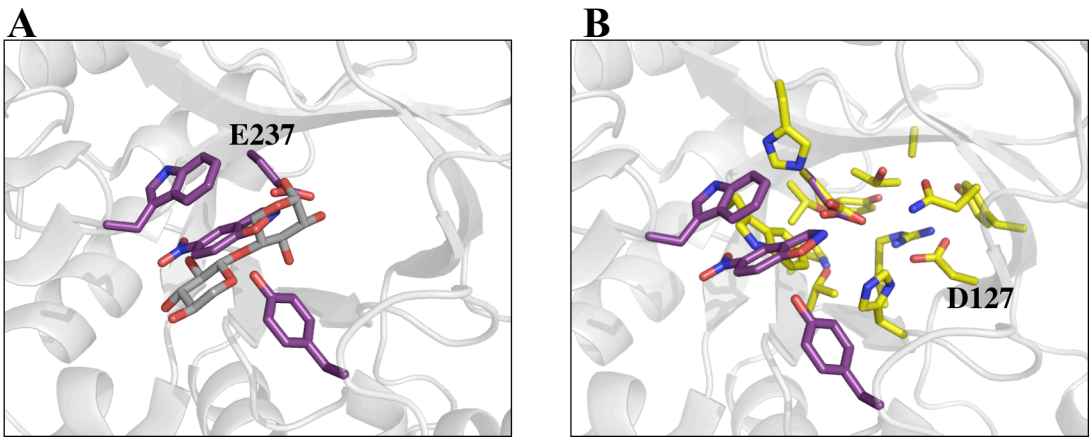


Figure 4-1. Active site locations of first- and second-generation designs in TAX. **(A)** The active site of first generation design 1.2.1 (purple) is in the native binding pocket for xylobiose (grey). **(B)** The active site of second-generation design HG-2 (yellow) is located deeper into the barrel of the scaffold.

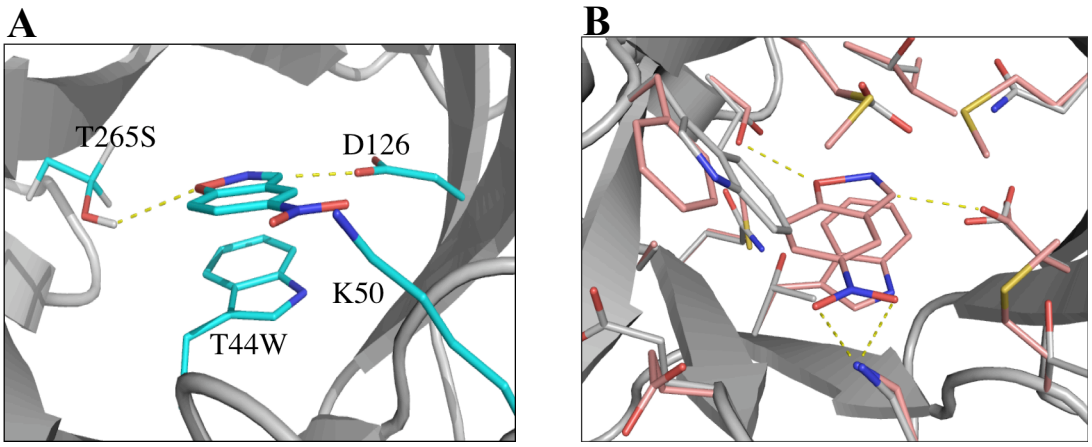


Figure 4-2. Active site of HG-2. (A) Catalytic residues identified through the active site search. **(B)** Repacked active site (pink) overlaid with the wild-type residues (grey).

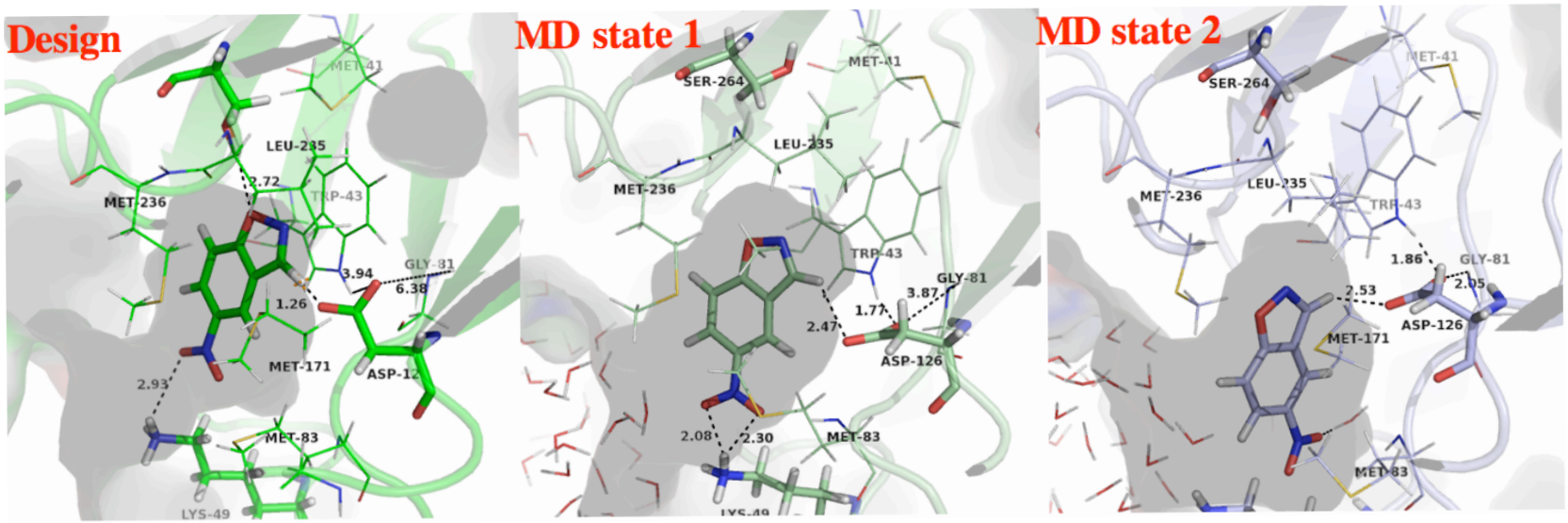


Figure 4-3. Active site structures of HG-2 during the MD simulation. Three active site configurations are shown: the initial configuration, a representative active site from state 1, and a representative active site from state 2.

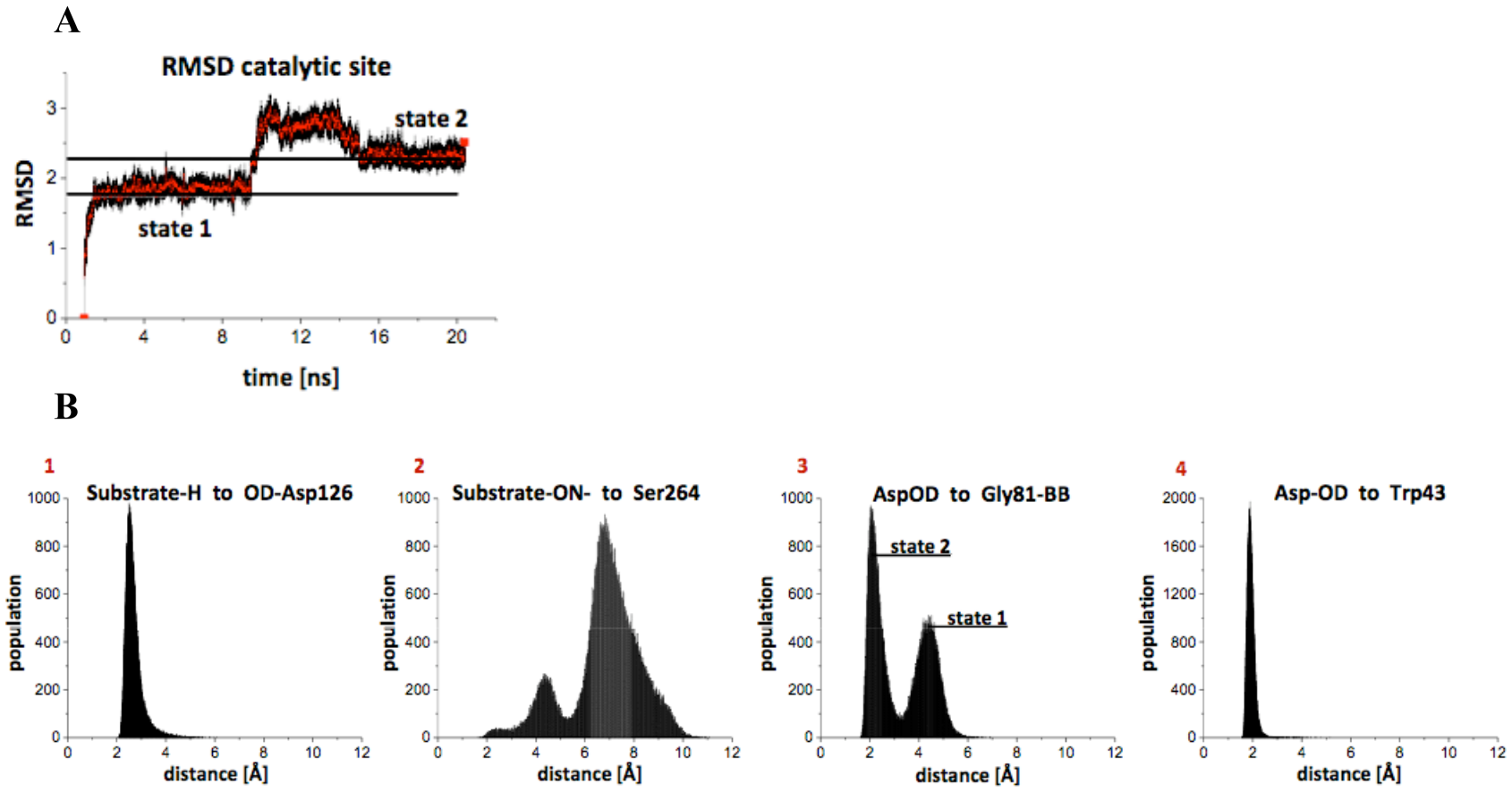
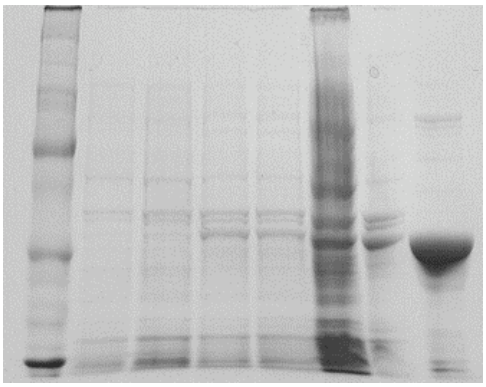
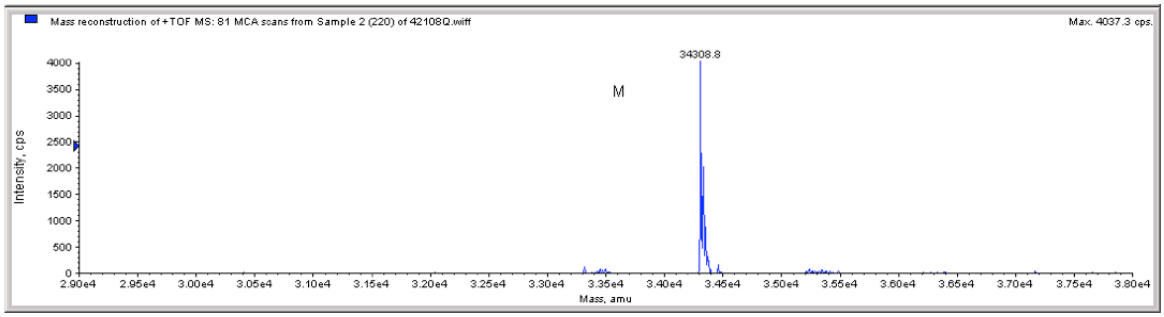


Figure 4-4. Distance distributions of HG-2. (A) Catalytic site RMSD with respect to the initial configuration as the simulation progresses. **(B)** Distance distributions for important catalytic contacts.

A



B



C

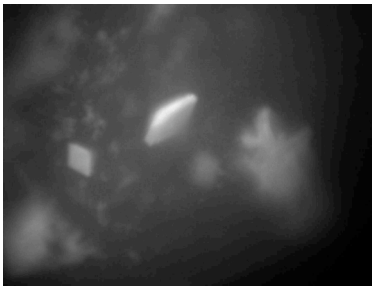


Figure 4-5. Expression and purification of HG-2. **(A)** SDS-PAGE gel of HG-2 expression and purification. Lane 1: MW marker. Lanes 2 and 3: uninduced cells. Lanes 4 and 5: cells 18 hours after IPTG induction. Lane 6: lysate supernatant. Lane 7: lysate pellet. Lane 8: Ni-NTA purification elution. **(B)** Electrospray mass spec. Expected mass: 34308.7, actual mass: 34308.8. **(C)** UV image of HG-2 crystals grown in 0.1 M MES, pH 6.5, 1.6 M Magnesium Sulfate with 9.5 mg/mL HG-2.

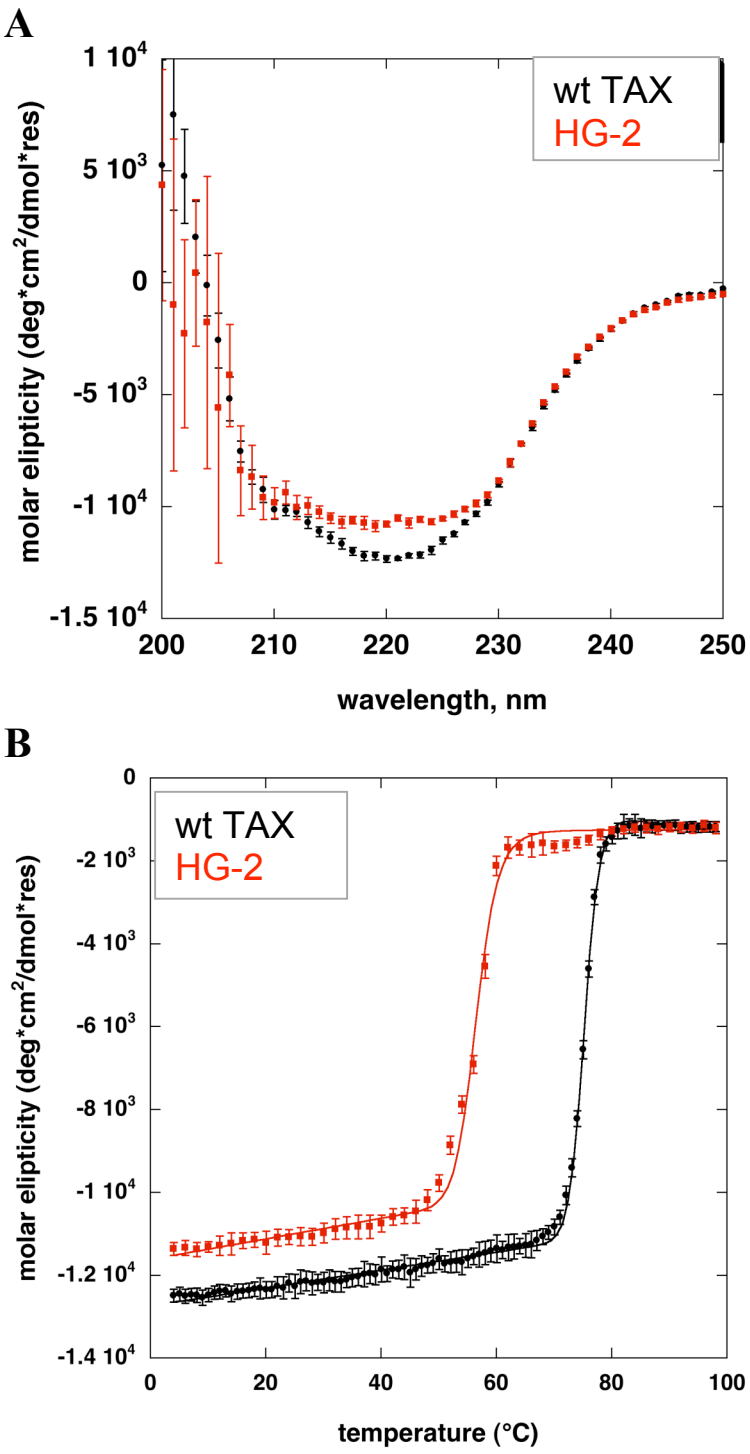


Figure 4-6. CD analysis of wild-type TAX and HG-2. (A) Far-UV wavelength scan. **(B)** Thermal denaturation. All experiments were carried out with 10 μM protein in 25 mM HEPES pH 7.25, 100 mM NaCl.

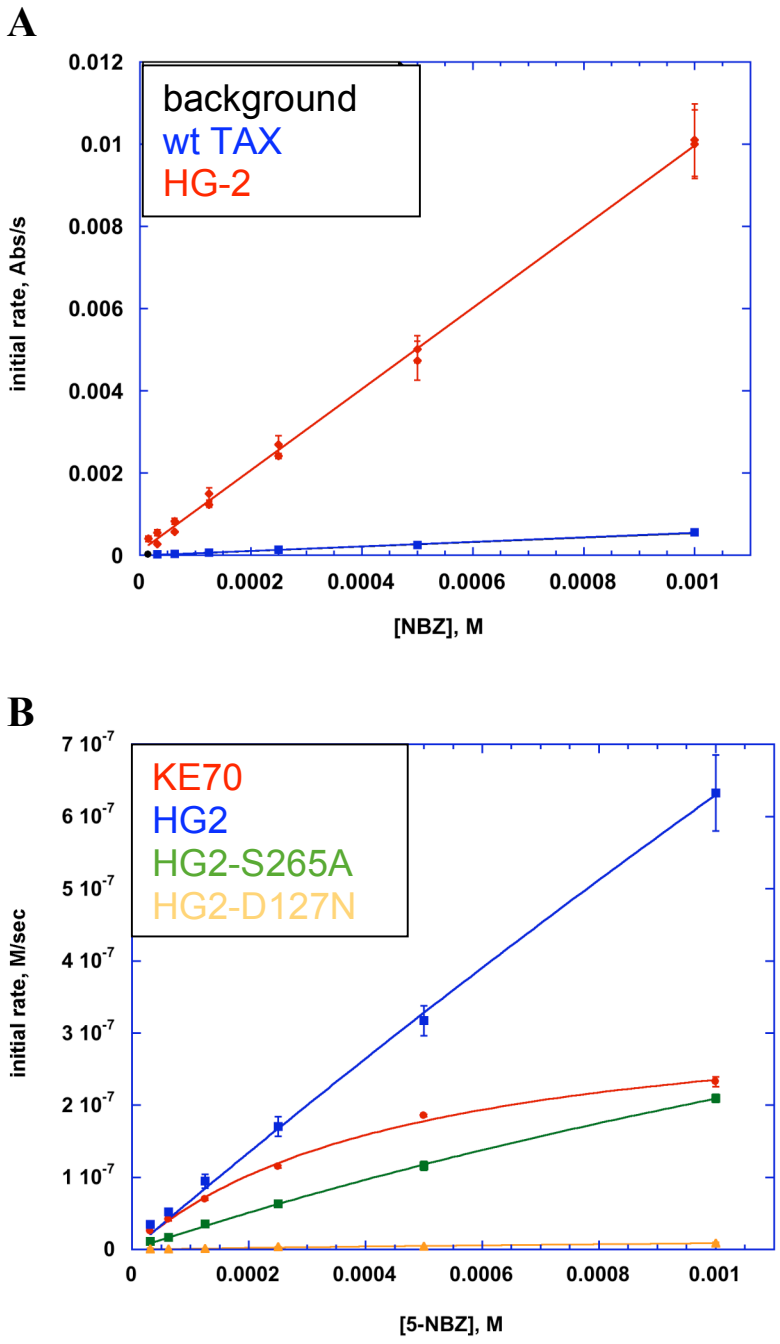


Figure 4-7. Kinetic characterization of second-generation enzymes. 5 μM protein is used in 25 mM HEPES pH 7.25, 100 mM NaCl, 27°C. **(A)** Michaelis-Menten plots of design HG-2 (red), wild-type TAX (blue) and background reaction (black). **(B)** Michaelis-Menten plots of KE70 (red), HG-2 (blue), and HG-2 knock out mutants HG-2-S265A (green) and HG-2-D127N (yellow).

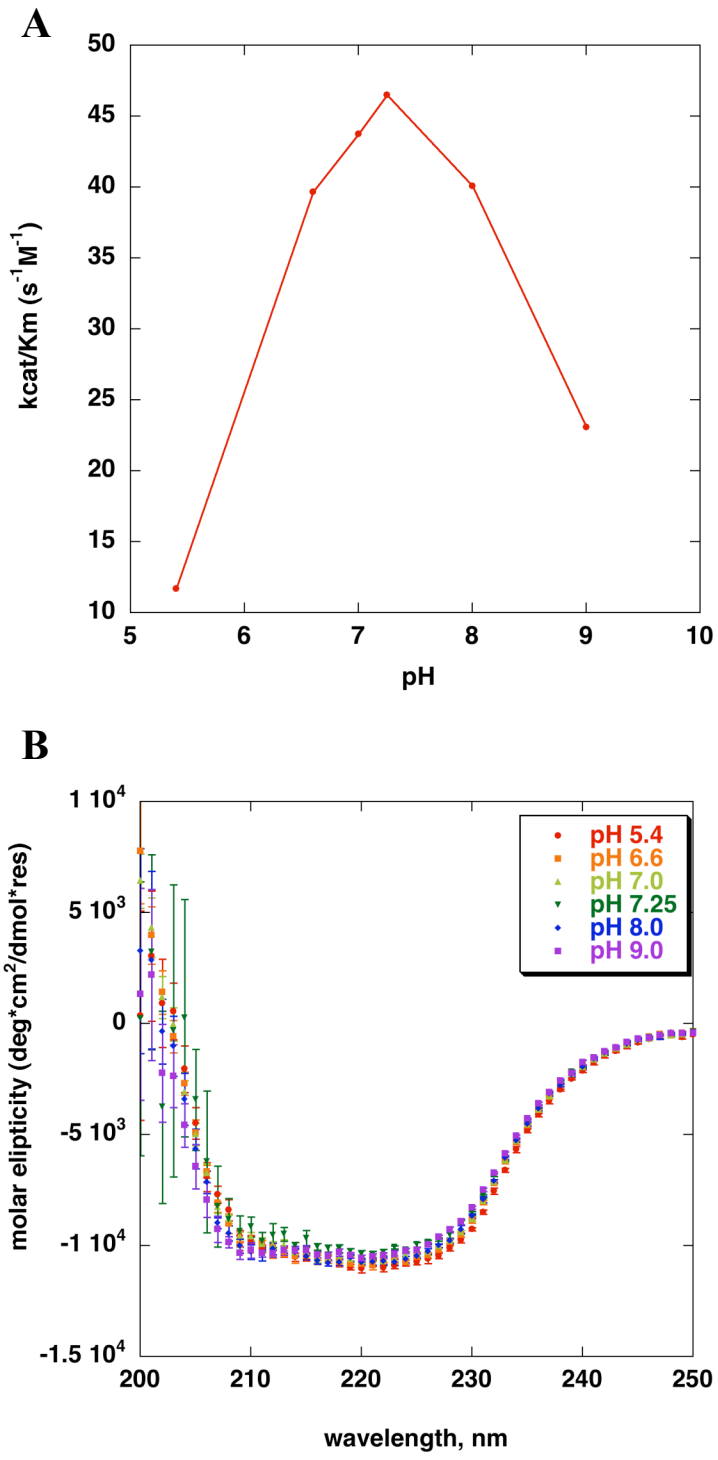


Figure 4-8. Effect of pH on the activity and structure of HG-2. (A) pH-rate profile of HG-2. **(B)** Far-UV CD wavelength scan of HG-2 at various pHs.

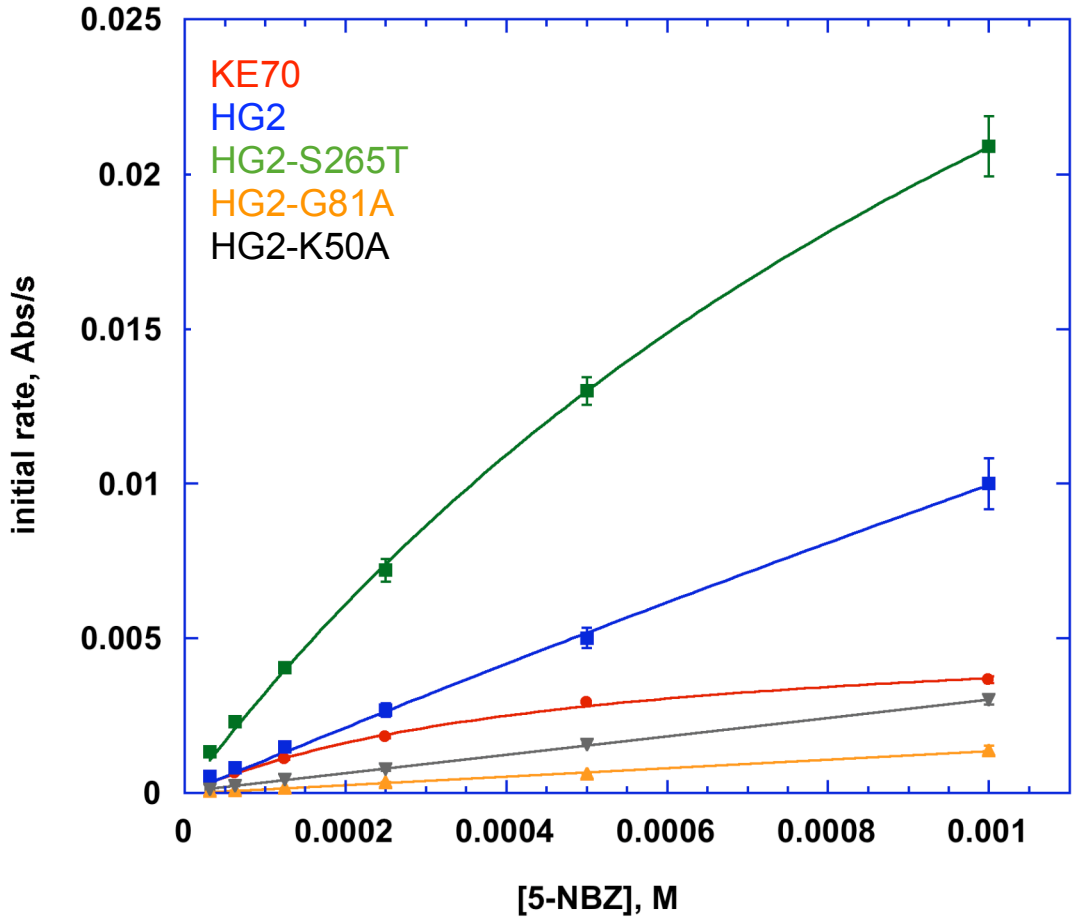


Figure 4-9. Kinetic characterization of third-generation enzymes. Michaelis-Menten plots of KE70 (red), design HG-2 (blue), 220-S265T (green), G81A (yellow), and 220-K50A (grey). 5 μM protein was used and the reactions were carried out in 25 mM HEPES pH 7.25, 100 mM NaCl, 27°C.

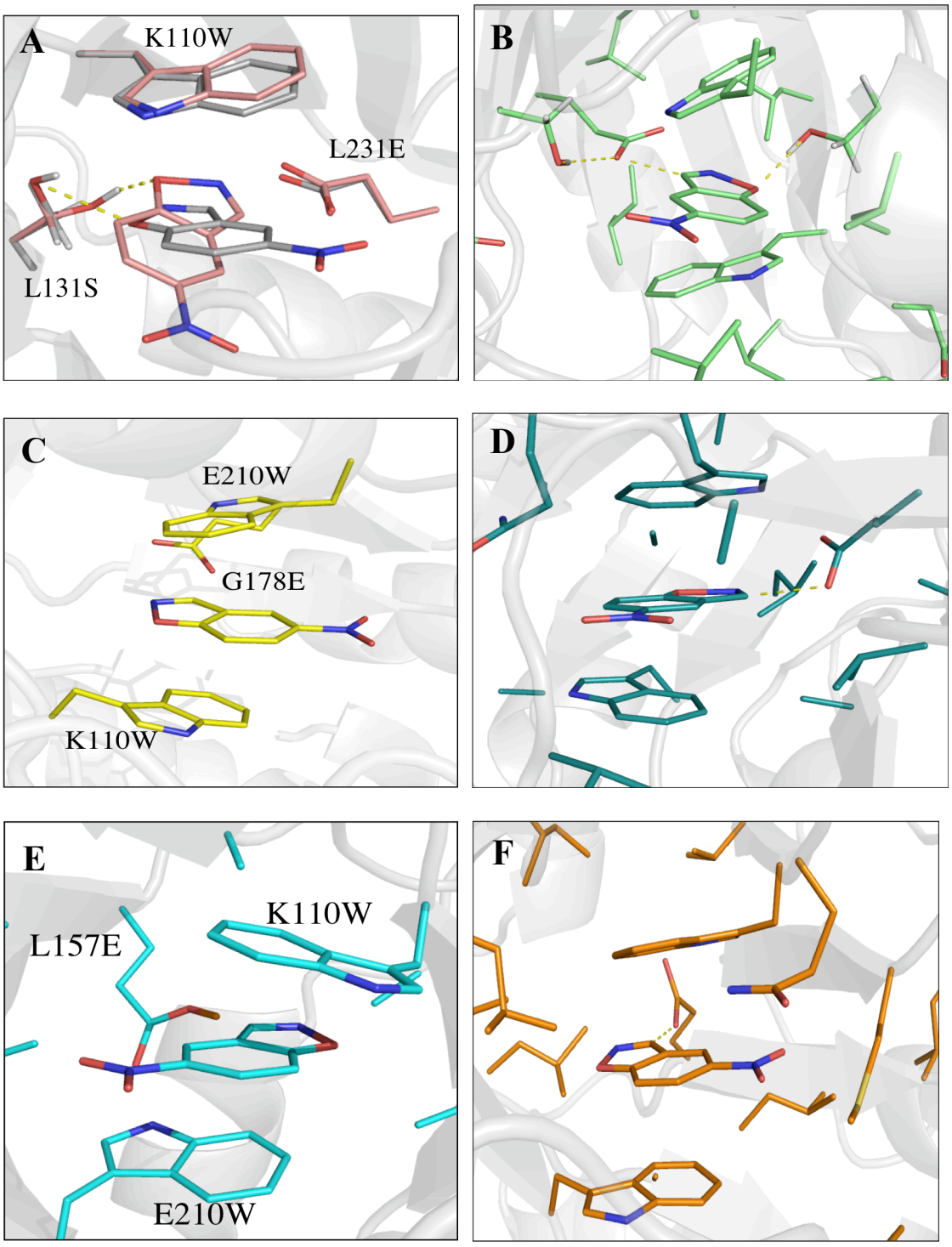


Figure 4-10. Active sites of designs in scaffold 1A53. **(A)** Catalytic residues of 1A53-1 (pink) overlaid with those from KE59 (grey). **(B)** Repacked active site of 1A53-1. **(C)** Catalytic residues of 1A53-2. **(D)** Repacked active site of 1A53-2. **(E)** Catalytic residues of 1A53-3. **(F)** Repacked active site of 1A53-3.

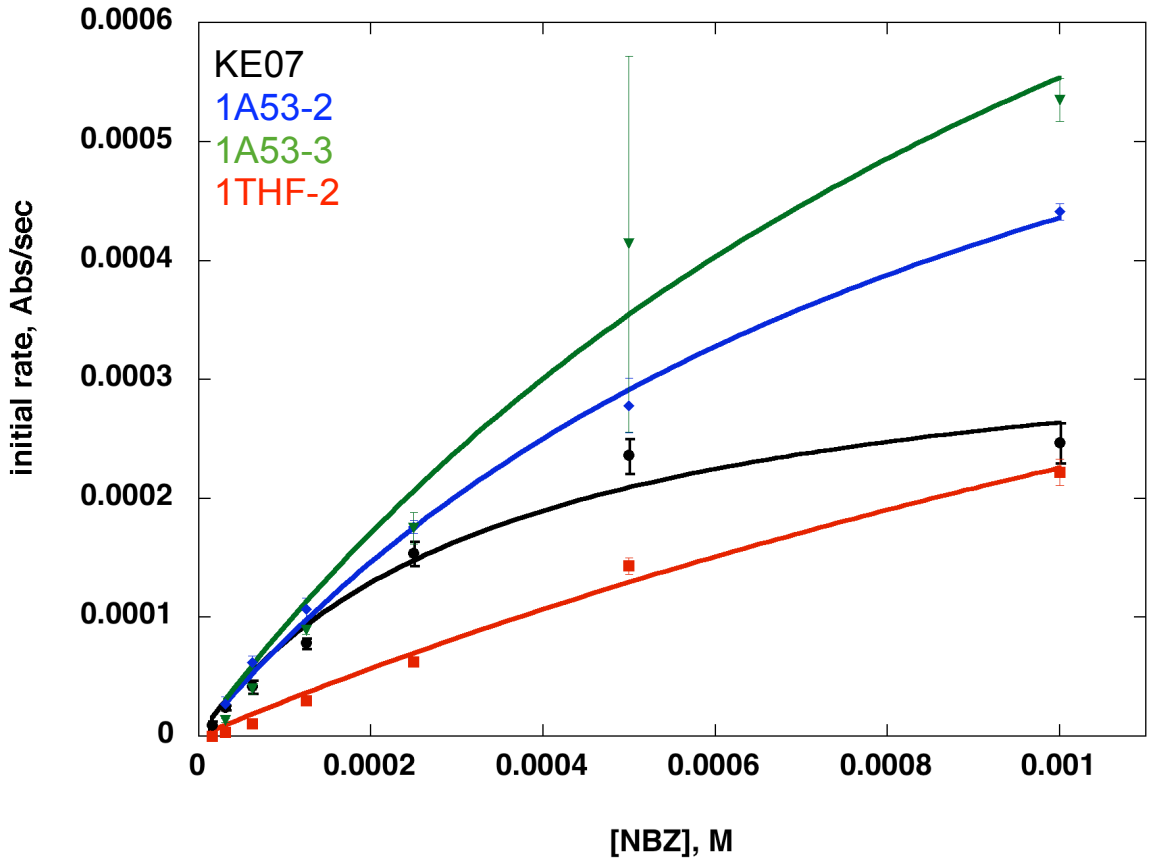


Figure 4-11. Kinetic characterization of designs in scaffolds 1A53 and 1THF. Standard errors are calculated from three measurements.

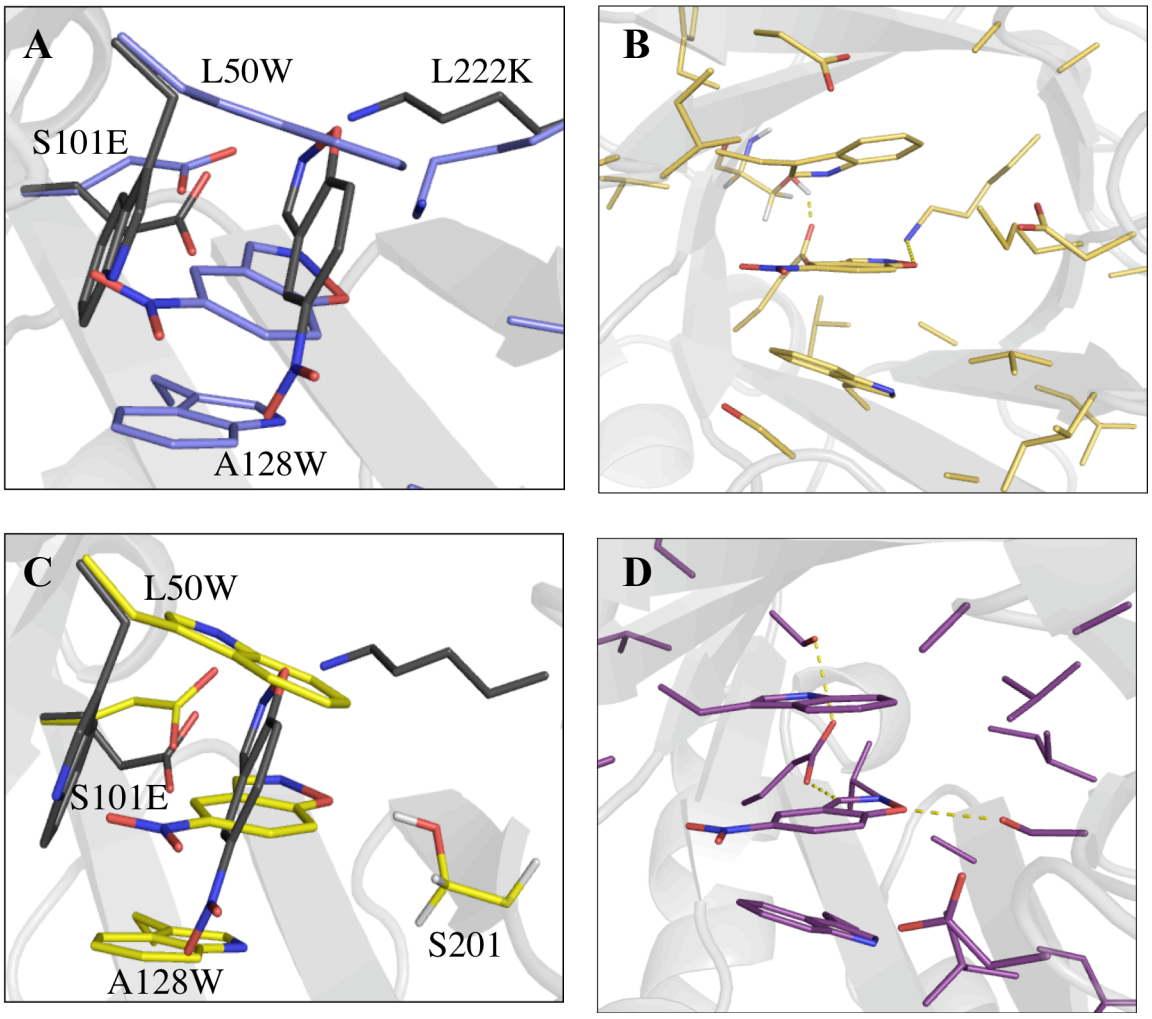


Figure 4-12. Active sites of designs in scaffold 1THF. (A) Catalytic residues of 1THF-1 (blue) overlaid with those from KE07 (grey). **(B)** Repacked active site 1THF-1. **(C)** Catalytic residues of 1THF-2 (yellow) overlaid with those from KE07 (grey). **(D)** Repacked active site of 1THF-2.

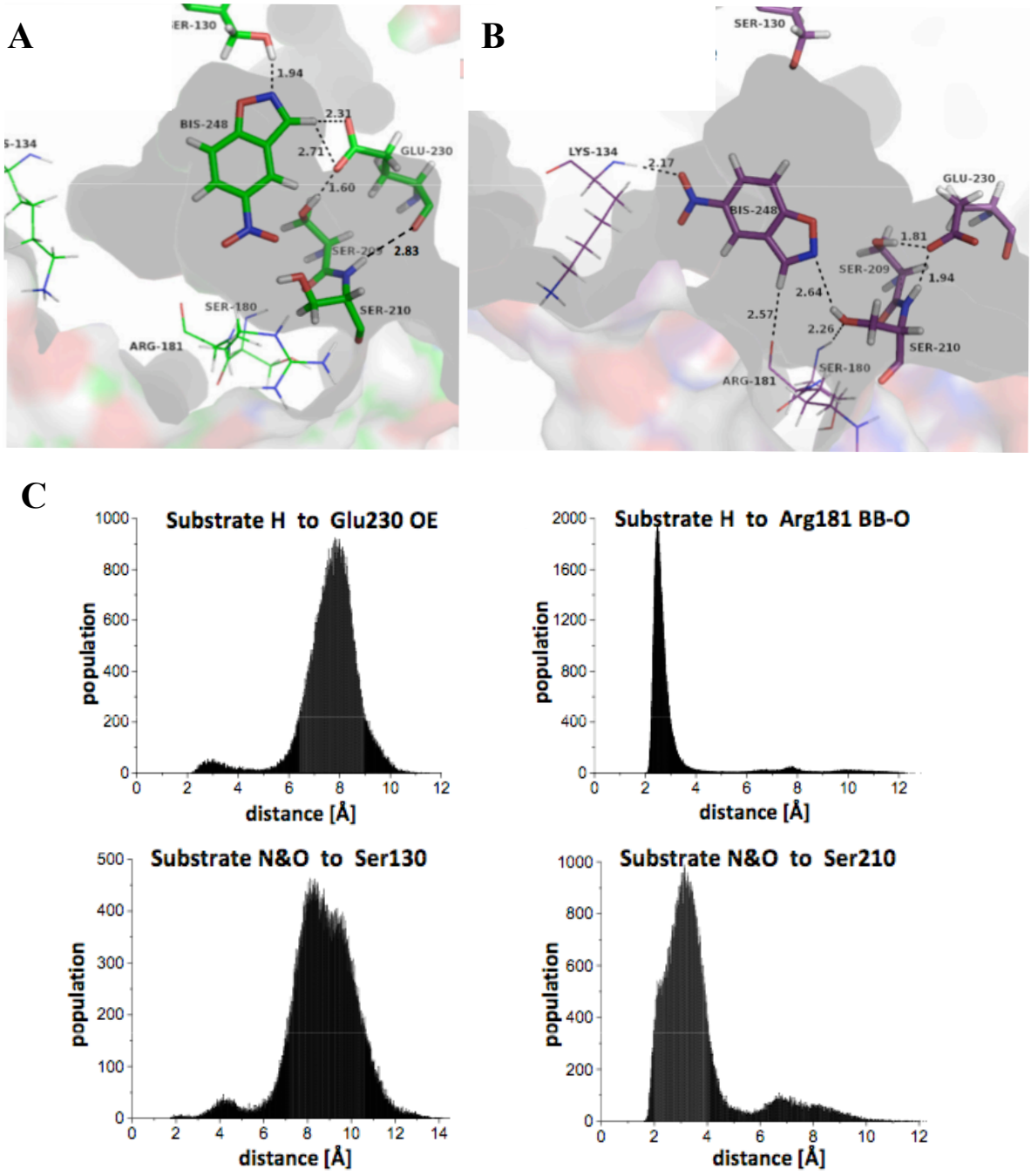


Figure 4-13. MD analysis of 1A53-1. **(A)** Initial active site configuration of 1A53-1. **(B)** Representative configuration after MD simulation. **(C)** Distance distributions of contacts between the substrate and catalytic residues.

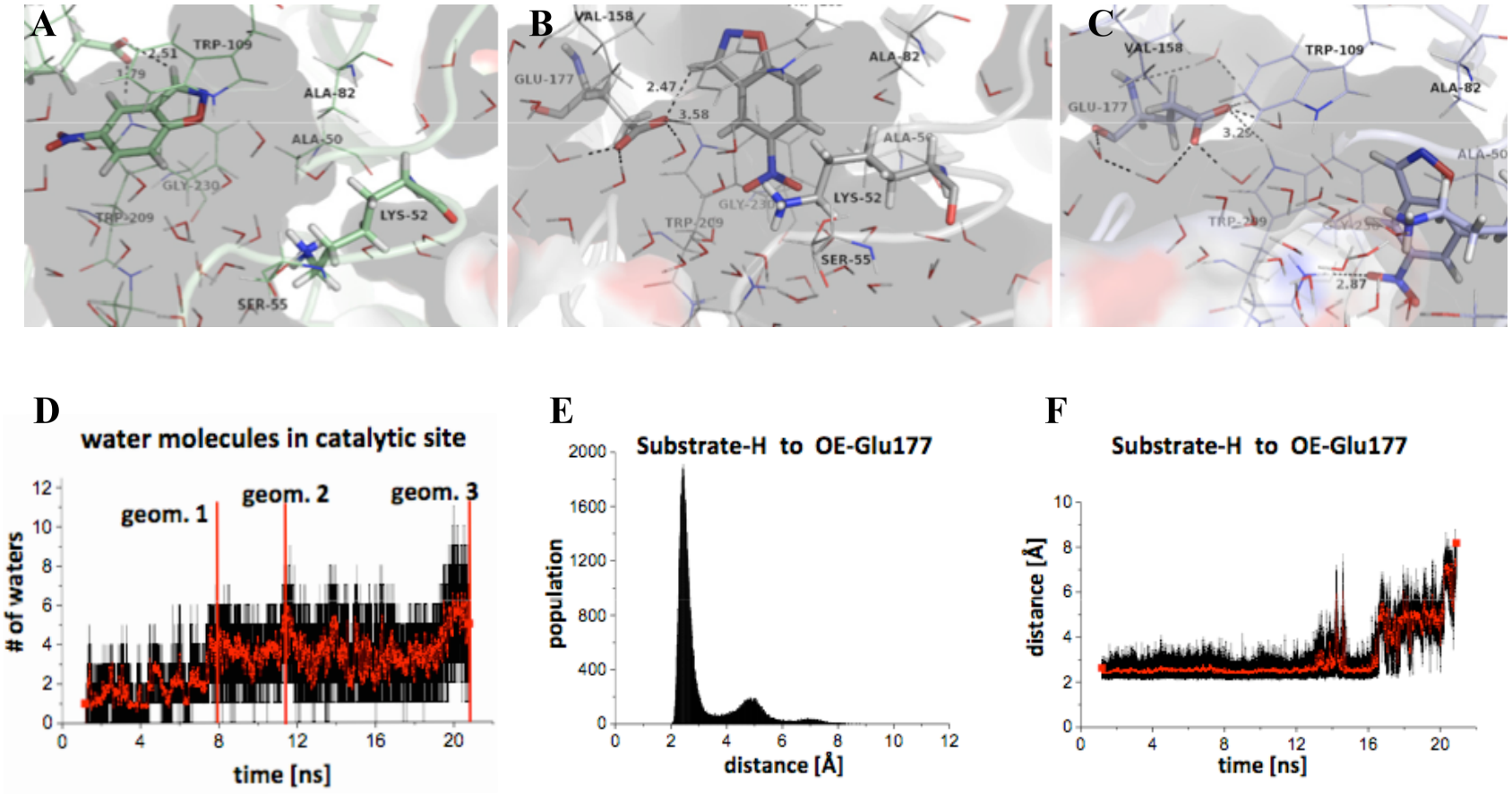


Figure 4-14. MD analysis of 1A53-2. **(A)** Initial active site configuration of 1THF-1. **(B)** Representative configuration midway through MD simulation (12 ns). **(C)** Representative geometry after MD simulation. **(D)** Number of water molecules that enter the active site over the course of the simulation. **(E)** Distance distribution of substrate-base contact. **(F)** Distance of the substrate-base contact over the course of the simulation.

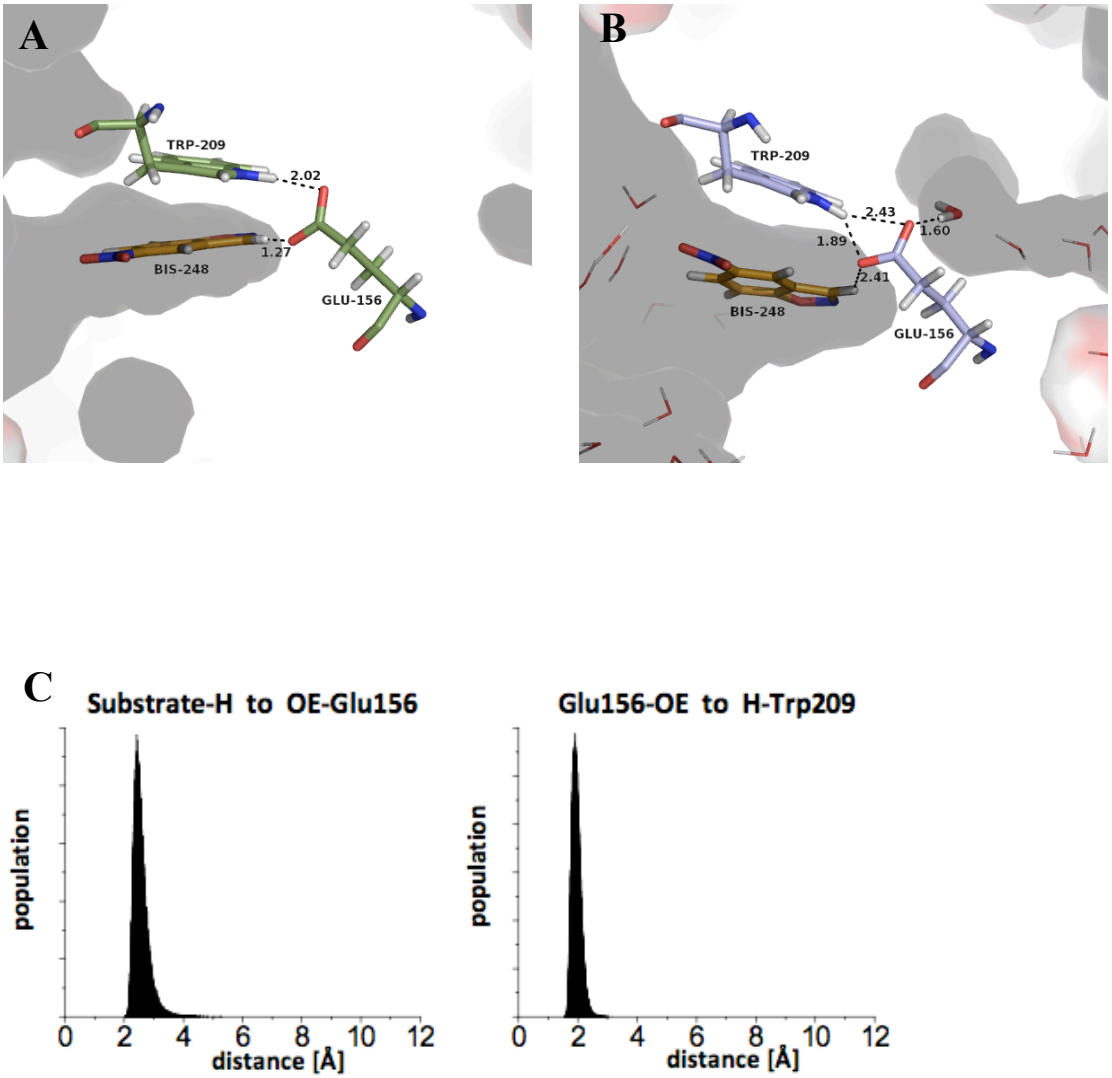


Figure 4-15. MD analysis of 1A53-3 **(A)** Initial active site configuration of 1A53-3. **(B)** Representative configuration after MD simulation. **(C)** Distance distributions of contacts between the substrate and catalytic residues.

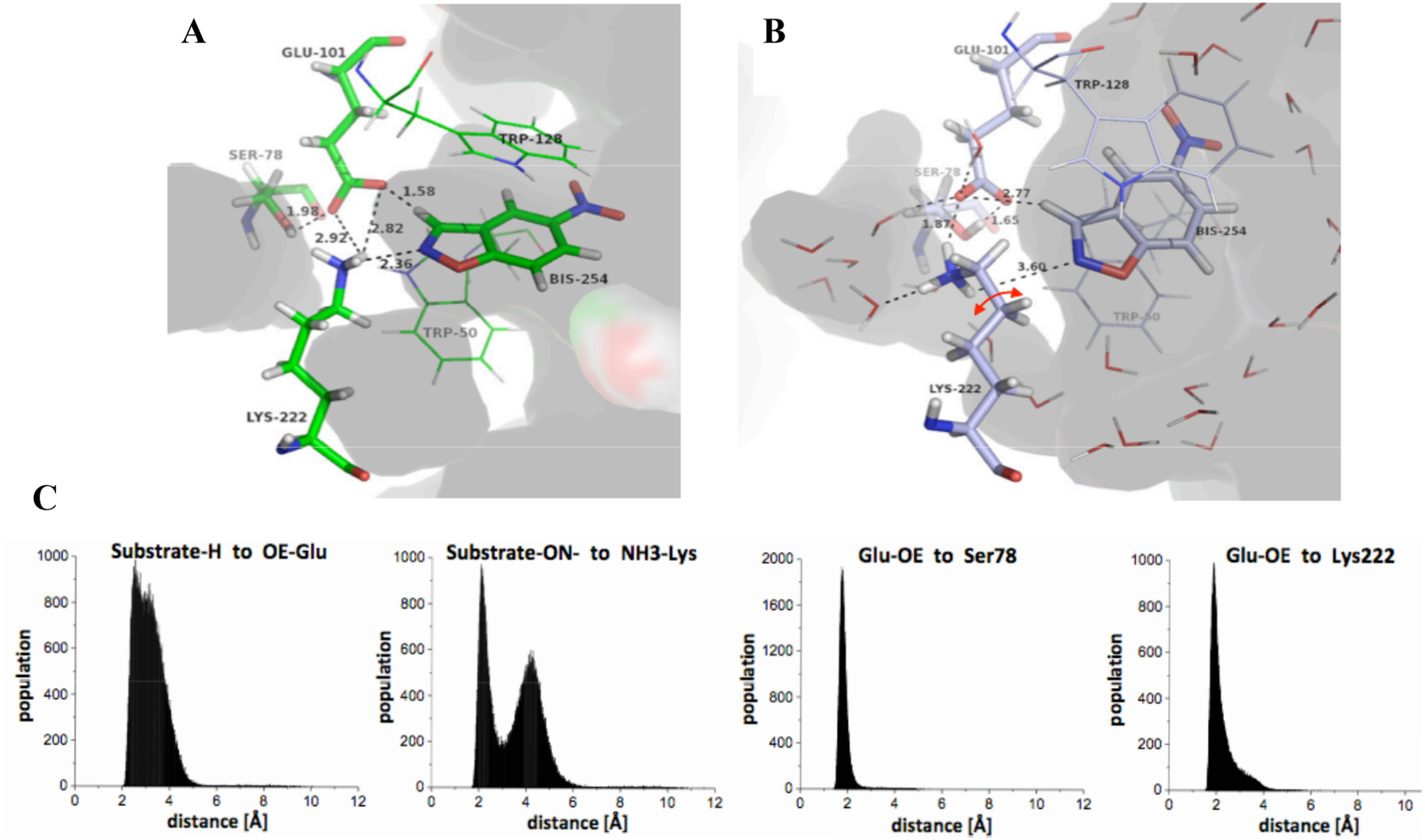


Figure 4-16. MD analysis of 1THF-1. **(A)** Initial active site configuration of 1THF-1. **(B)** Representative configuration after MD simulation. **(C)** Distance distributions of contacts between the substrate and catalytic residues.

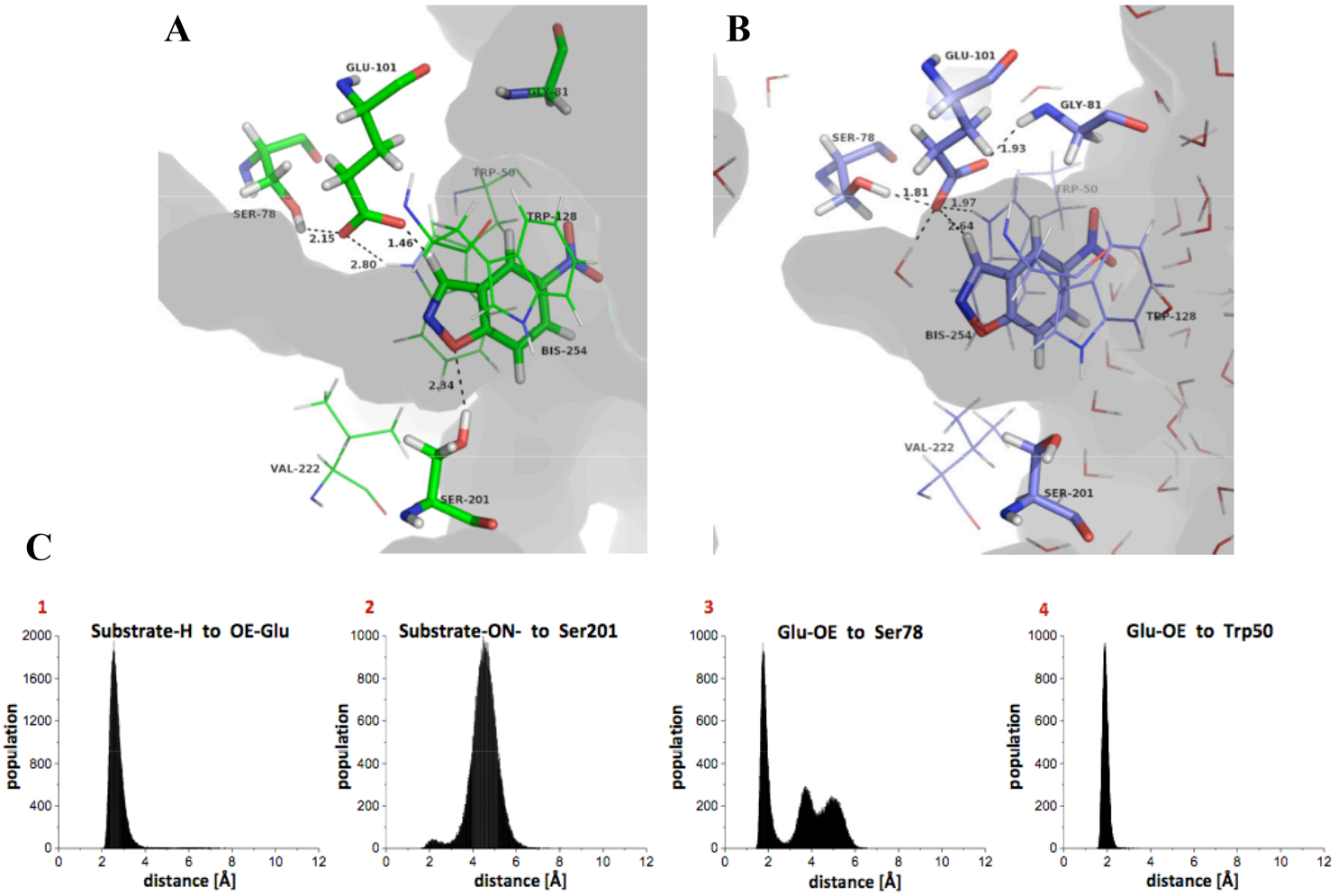


Figure 4-17. MD analysis of 1THF-2. **(A)** Initial active site configuration of 1THF-2. **(B)** Representative configuration after MD simulation**. (C)** Distance distributions of contacts between the substrate and catalytic residues.