The Importance of the Scaffold for *de Novo* Enzymes: A Case Study with Kemp Eliminase

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Parameterization of substrate in reactant and transition state. In order to perform simulations with 5-nitrobenzisoxazole using the AMOEBA force field, we need to obtain parameters for all atoms in the substrate molecule in the reactant and transition states. For parameterizing the transition state of this molecule, the structure of the substrate used for parameterization was reported in Ref [1], in which the C-H and N-O bonds are partially broken and the C-N bond is somewhere between a double and triple bond as shown in Figure 2 of the main text. Since the transition state structure is not at it's energy minimum, we do not minimize the structure as done in the original protocol (the reactant state structure is minimized). As can also be seen in Figure 1 of the main text, the system used for the parameterization includes not only the ligand but also a base (acetate) to better model the transition state. The overall system has a net charge of -1e.

We then use the protocol described by Ponder and Ren [2] which has 2 main components – first finding the electrostatic parameters and second, finding 'valence' parameters (bond lengths, bond angles, dihedrals). The electrostatic component is described briefly in 6 steps below.

1. Run a single point quantum mechanics-based calculation on the transition state structure using Gaussian g09 at the MP2/6-311G(1D, 1P) level of theory. This calculation returns the electron density as obtained at this relatively low level of theory.
2. Find approximate charges, dipoles and quadrupoles by running the distributed multipole analysis using GDMA on the electron density.
3. Once we have the approximate multipoles, use Tinker's POLEDIT program to break the dipole moments into permanent contributions that act between polarization groups and mutual contributions that act within and between polarization groups.
4. A second Gaussian g09 calculation is run at the MP2/6-311G(2D, 2P) level of theory to obtain a electrostatic potential.
5. In order to obtain a electrostatic potential, we create a spatial grid on which to calculate the potential using Tinker's POTENTIAL program and then compute the potential using the Gaussian CUBEGEN program.

6. Finally, using Tinker's POTENTIAL program, we fit the atomic multipoles to the MP2/6-311G(2D, 2P) electrostatic potential.

After finishing the first step, the 'valence' parameters are assigned from similar, previously parameterized organic compounds. Thus, we model the transition state of the substrate with transition state electrostatics and energy minimized state valence parameters.

The exact same protocol was used to parameterize the EL state of the 19NT inhibitor for the ketosteroid isomerase enzyme.

Calculation of dipole moment of the 3 bonds in EL and EL† states for 5-nitrobenzisoxazole. We used the monopoles and dipole moments of the parameterized 5-nitrobenzisoxazole in AMOEBA to calculate the dipole moment of the 3 bonds in each state. Table S2 lists the parameters used to calculate the dipole moment of each bond. The positive direction is as shown in Fig 2 of the main text. Since the net charge is not zero, we used Δq instead of q to calculate the dipole contribution from the monopoles.

Active site residues for KE07, KE70 and KSI. We defined active site residues to be residues that are within 5 Å from the substrate of the respective enzymes studied. The residue numbers are -

KE07: 9, 11, 48, 50, 101, 128, 169, 201, 202

KE70: 16, 18, 45, 48, 72, 103, 138, 140, 168, 202, 204

KSI: 16, 20, 40, 57, 61, 66, 86, 88, 90, 99, 101, 103, 116, 118, 120

TABLES

Table S1. *Design and laboratory directed evolution mutations for KE07 and KE70*. The computationally designed residues (red), mutated residues introduced by LDE of a given round (black) and residues after which insertions took place (green) have been listed in the table below [3 4].

Sequence	KE07 Design	KE07 Best LDE Variant	KE70 Design	KE70 Best LDE Variant
	ILE 7	Asp	**HIS 17**	
	ALA 9		**ALA 19**	
	ILE 11		**THR 20**	Ser
	VAL 12	Met	**ALA 21**	
	LYS 19		**ASP 23**	
	SER 48		**LYS 29**	Asn
	TRP 50		**THR 43**	Asn
	PHE 77	Ile	**ASP 45**	
	HIS 84		**TYR 48**	Phe
	PHE 86		**TRP 72**	Cys
	GLU 101		**SER 74**	Gly
	ILE 102	Phe	**GLY 101**	Ser
	GLN 123		**ALA 103**	
	TYR 128		**SER 138**	Ala
	ALA 130		**HIS 166**	Asn
	LYS 146	Thr	**VAL 168**	
	VAL 169		**THR 171**	
	GLY 171		**GLY 177**	
	LEU 176		**ALA 178**	Ser
	HIS 201		**LYS 197**	Asn
	GLY 202	Arg	**THR 198**	Ile
	MET 207		**ILE 202**	
	LYS 222		**ALA 204**	Val
	ASN 224	Asp	**ASP 212**	
	PHE 229	Ser	**ALA 231**	
			ALA 235	
			SER 239	Ala
			HIS 251	
k_{cat} (s^{-1})	**0.02**	**1.37**	**0.14**	**5.00**
K_M (mM)	**1.40**	**0.54**	**1.11**	**0.09**
k_{cat} / K_M ($M^{-1}s^{-1}$)	**12.2**	**2590**	**126**	**57300**

Table S2. Bond dipoles using AMOEBA force field electrostatics.

C-H bond (+ve axis from C to H)	**EL**	**EL**†
Permanent dipole (Debye)	-0.93	0.1
Charge on C (e^- units)	0.05	0.05
Charge on H (e^- units)	0.04	0.20
Charge difference (e^- units)	-0.01	0.15
Distance (Å)	1.09	1.31
Net dipole (Debye)	-1.0	1.0
C-N bond (+ve axis from N to C)	**EL**	**EL**†
Permanent dipole (Debye)	-0.1	-0.2
Charge on C (e^- units)	0.05	0.05
Charge on N (e^- units)	-0.27	-0.05
Charge difference (e^- units)	-0.32	-0.1
Distance (Å)	1.36	1.25
Net dipole (Debye)	2.0	0.4
N-O bond (+ve axis from O to N)	**EL**	**EL**†
Permanent dipole (Debye)	0.7	-1.1
Charge on O (e^- units)	0.09	-0.44
Charge on N (e^- units)	-0.27	-0.05
Charge difference (e^- units)	-0.36	0.39
Distance (Å)	1.4	1.8
Net dipole (Debye)	-1.7	2.3
N-O bond for 5-nitro (+ve axis from O to N)	**EL**	**EL**†
Permanent dipole (Debye)	0.01	0.12
Charge on O (e^- units)	-0.46188	-0.50388
Charge on N (e^- units)	0.79685	0.81965
Charge difference (e^- units)	1.25873	1.32353
Distance (Å)	1.24	1.24
Net dipole (Debye)	7.51	7.90

Table S3: Contributions of pre-organization relative to reorganization to the free energy stabilization of the transition state along the 3 bonds of the substrate 5-nitrobenzisoxazole in the EL and EL† states of KE07 and KE70 designed enzymes and best LDE variants. We evaluate the preorganization with an adiabatic step whereby the transition state dipoles change, but there is no relaxation of the structural ensemble to these changes, using the reactant state ensemble only. See Table 1 in the text for further details.

Enzyme Construct and $\Delta G^{\ddagger}$ transition state stabilization	**Fields generated for each bond**		
	C-H	**C≡N**	**O-N**
Designed KE07			
$\Delta G^{\ddagger}$ = –5.3 kcal/mole	–5.6 kcal/mole	3.1 kcal/mole	–2.8 kcal/mole
$\Delta G^{\ddagger}$ preorganization (adiabatic)	–5.8 kcal/mole	3.05 kcal/mole	–3.1 kcal/mole
$\Delta G^{\ddagger}$ reorganization	0.2 kcal/mole	0.05 kcal/mole	0.3 kcal/mole
LDE R7 Variant KE07			
$\Delta G^{\ddagger}$ = –9.9 kcal/mole	–9.1 kcal/mole	3.2 kcal/mole	–4.0 kcal/mole
$\Delta G^{\ddagger}$ preorganization (adiabatic)	–9.0 kcal/mole	3.4 kcal/mole	–4.1 kcal/mole
$\Delta G^{\ddagger}$ reorganization	–0.1 kcal/mole	–0.2 kcal/mole	0.1 kcal/mole
Designed KE70			
$\Delta G^{\ddagger}$ = –6.6 kcal/mole	–6.3 kcal/mole	3.5 kcal/mole	–3.8 kcal/mole
$\Delta G^{\ddagger}$ preorganization (adiabatic)	–6.3 kcal/mole	3.6 kcal/mole	–3.7 kcal/mole
$\Delta G^{\ddagger}$ reorganization	–0.0 kcal/mole	–0.1 kcal/mole	–0.1 kcal/mole
LDE R6 Variant KE70			
$\Delta G^{\ddagger}$ = –6.6 kcal/mole	-6.3 kcal/mole	2.1 kcal/mole	–2.4 kcal/mole
$\Delta G^{\ddagger}$ preorganization (adiabatic)	-6.4 kcal/mole	2.1 kcal/mole	-2.6 kcal/mole
$\Delta G^{\ddagger}$ reorganization	0.1 kcal/mole	~0 kcal/mole	0.2 kcal/mole

Table S4: List of top residues that contribute >10 MV/cm electric field by magnitude at the C-H, C-N, and NO bond in either the EL and EL† states for the designed enzyme KE07 enzyme and the best LDE R7 variant. Positive sign indicates field supporting bond breaking (C-H and N-O) and bond-making (C-N). Contributions to activation free energies are also provided using the dipole values reported in Table S2

KE07 Design				C-H Bond	KE07 R7 Variant			
	Electric Field					Electric Field		
Residue	**EL**	**EL†**	**ΔG‡**		**Residue**	**EL**	**EL†**	**ΔG‡**
Glu-101	86.3 (11)	103.6 (11)	-9.1(0.7)		Glu-101	142.2 (12)	144.3 (16)	-13.7 (1)
His-201	8.7 (1)	11.2 (1)	-0.9(0.1)		His-201	4.1 (1)	7.3 (1)	-0.5 (0.1)
Gly-202	1.1 (0.1)	1.5 (0.1)	-0.1(~0)		Arg-202	18.7 (1)	18.6 (2)	-1.8 (0.1)
Glu-46	6.6 (1)	5.3 (0.5)	-0.6(0.1)		Glu-46	11.2 (1)	12.1 (2)	-1.1 (0.1)
Ser-48	-7.6 (2)	-4.3 (2)	0.6(0.1)		Ser-48	-17.1 (2)	-16.1 (3)	1.6 (0.2)
Lys-222	-43.5 (6)	-46.3 (6)	4.3 (0.4)		Lys-222	-51.8 (6)	-41.5 (6)	4.5 (0.4)
Asn-224	1.2 (0.5)	1.7 (0.2)	-0.1 (~0)		Asp-224	-16.1 (2)	-10.4 (2)	1.3 (0.1)

KE07 Design				C-N Bond	KE07 R7 Variant			
	Electric Field					Electric Field		
Residue	**EL**	**EL†**	**ΔG‡**		**Residue**	**EL**	**EL†**	**ΔG‡**
Glu-101	15.5 (7)	18.7 (7)	1.1 (0.6)		Glu-101	47.4 (7)	54.4 (12)	3.5 (0.7)
His-201	3.9 (2)	5.5 (2)	0.3 (0.2)		His-201	16.3 (2)	21.2 (3)	1.1 (0.2)
Lys-222	10.6 (7)	16.8 (6)	0.7 (0.7)		Lys-222	-3.8 (3)	4.0 (7)	-0.4 (0.3)
Asn-224	2.2 (0.6)	2.9 (0.5)	0.2 (0.1)		Asp-224	-19.8 (2)	-13.6 (2)	-1.6 (0.2)

KE07 Design				N-O Bond	KE07 R7 Variant			
	Electric Field					Electric Field		
Residue	**EL**	**EL†**	**ΔG‡**		**Residue**	**EL**	**EL†**	**ΔG‡**
Glu-101	53.0 (6.0)	65.1 (2.4)	-11.5(.5)		Glu-101	58.5 (2.5)	60.0 (1.3)	-11.4 (.2)
His-201	12.7 (2.5)	15.4 (0.7)	-2.7 (.2)		His-201	7.3 (2)	7.5 (1)	-1.3 (0.2)
Gly-202	1.1 (0.1)	1.5 (0.1)	-0.2(~0)		Arg-202	21.4 (2)	21.8 (2.9)	-4.1 (0.4)
Glu-46	7.3 (1)	6.8 (0.3)	-1.3(0.1)		Glu-46	10.2 (1)	10.6 (0.3)	-2 (0.1)
Ser-48	-5.8 (1)	-3.5 (1)	0.8 (0.1)		Ser-48	-10.0 (2)	-7.7 (3)	1.7 (0.4)
Lys-222	-54.5(6)	-59.5 (3.2)	11 (0.6)		Lys-222	-45.6 (3.9)	-40.6 (2.4)	8.2 (0.4)
Asn-224	1.8 (0.7)	2.6 (0.5)	-0.4(0.1)		Asp-224	-19.2 (2.9)	-11.2 (2.3)	2.8 (0.3)

Table S5: List of top residues that contribute >10 MV/cm electric field by magnitude at the C-H, C-N, and NO bond in either the EL and EL† states for the designed enzyme KE70 enzyme and the best LDE R6 variant. Positive sign indicates field supporting bond breaking (C-H and N-O) and bond-making (C-N). Contributions to activation free energies are also provided using the dipole values reported in Table S2

KE70 Design				C-H Bond	KE70 R6 Variant			
	Electric Field					Electric Field		
Residue	**EL**	**EL†**	**$\Delta G^{\ddagger}$**		**Residue**	**EL**	**EL†**	**$\Delta G^{\ddagger}$**
His-17	30.0 (4)	47.4 (5)	-3.7 (0.3)		His-17	48.0 (4)	65.0 (4)	-5.4 (0.3)
Asp-45	16.1 (2)	17.8 (1)	-1.6 (0.1)		Asp-45	13.5 (1)	15.1 (1)	-1.4 (0.1)
Arg-70	-12.1 (2)	-13.4 (1)	1.2 (0.1)		Arg-70	-10.9 (1)	-11.5 (1)	1.1 (0.1)
Trp-72	9.0 (2)	10.8 (1)	-1 (0.1)		Cys-72	0.8 (1)	0.2 (1)	-0.1 (0.1)
Ser-138	5.3 (0.5)	8.5 (0.3)	-0.7 (~0)		Ala-138	0.7 (0.1)	0.7 (0.1)	-0.1 (~0)
Glu-142	-7.6 (0.5)	-6.9 (0.2)	0.7 (~0)		Glu-142	-6.8 (~0)	-7.4 (~0)	0.7 (~0)

KE70 Design				C-N Bond	KE70 R6 Variant			
	Electric Field					Electric Field		
Residue	**EL**	**EL†**	**$\Delta G^{\ddagger}$**		**Residue**	**EL**	**EL†**	**$\Delta G^{\ddagger}$**
His-17	22.7 (2)	28.9 (0.5)	1.6 (0.2)		His-17	20.5 (2)	25.7 (1)	1.4 (0.2)
Asp-45	6.7 (1)	6.7 (0.5)	0.5 (0.1)		Asp-45	3.0 (1)	4.4 (0.5)	0.2 (0.1)
Arg-70	-1.9 (0.8)	-1.3 (0.6)	-0.2 (0.1)		Arg-70	2.3 (1)	0.1 (0.4)	0.2 (0.1)
Trp-72	9.0 (0.9)	10.8 (0.5)	0.6 (0.1)		Cys-72	3.8 (1)	2.2 (0.4)	0.3 (0.1)
Ser-138	20.1 (2)	29.3 (1)	1.3 (0.2)		Ala-138	2.3 (0.2)	2.8 (0.3)	0.2 (~0)
Glu-142	-0.9 (1)	-1.0 (0.3)	-0.1 (0.1)		Glu-142	-0.1 (0.4)	-1.3 (0.2)	~0

KE70 Design				N-O Bond	KE70 R6 Variant			
	Electric Field					Electric Field		
Residue	**EL**	**EL†**	**$\Delta G^{\ddagger}$**		**Residue**	**EL**	**EL†**	**$\Delta G^{\ddagger}$**
His-17	4.9 (1)	7.7 (1)	-1.2 (0.1)		His-17	10.5 (1)	12.9 (1)	-2.3 (0.1)
Asp-45	10.7 (1)	12.5 (0.5)	-2.2 (0.1)		Asp-45	10.8 (0.6)	11.6 (0.3)	-2.1 (0.1)
Arg-70	-10.1 (1)	-12.0 (0.6)	2.1 (0.1)		Arg-70	-11.7 (1)	-11.2 (0.5)	2.2 (0.1)
Trp-72	7.8 (1)	11.9 (0.5)	-2 (0.1)		Cys-72	-3.6 (0.8)	-1.9 (1)	0.5 (0.1)
Ser-138	-8.4 (1)	-2.2 (0.7)	0.9 (0.1)		Ala-138	-0.5 (0.3)	-0.9 (0.3)	-0.1 (0.1)
Glu-142	-9.9 (1)	-8.7 (0.3)	1.8 (0.1)		Glu-142	-8.4 (0.6)	-9.5 (0.4)	1.7 (0.1)

Table S6: *Chemical Positioning vs. Electric Field Environment at the C-H, C-N and O-N Bonds*. The magnitude of the electric field in either the EL and EL† states for the designed KE07 enzyme and the best R7 variant. The active site is defined by residues within 5 Å from the center of the substrate, while the protein environment (scaffold) is summed over all residues outside this region. Solvent includes waters in the neck of the TIM barrel as well as the surrounding hydration and bulk water. Sum of free energy are summed over all bonds, with dominant region and bond most affected shown. Positive sign indicates field supporting bond breaking. Fields are reported in units of MV/cm

KE07 Design				C-H Bond	KE07 R7 Variant			
	Electric Field					Electric Field		
Region	**EL**	**EL†**	**ΔG‡**		**Region**	**EL**	**EL†**	**ΔG‡**
Base	86.3 (11)	103.6 (12)	-9.1 (0.7)		Base	142.2 (12)	144.3 (17)	-13.7 (1)
Active Site	1.0 (1)	11.2 (2)	-0.6 (0.1)		Active Site	2.0 (1)	8.3 (1)	-0.5 (0.1)
Scaffold	-24.1 (7)	-26.8 (7)	2.4 (0.5)		Scaffold	-40.1 (7)	-24.1 (7)	3.1 (0.5)
Solvent	-15.6 (4)	-19.2 (1)	1.7 (0.2)		Solvent	-22.6 (1)	-20.2 (2)	2.1 (0.1)

KE07 Design				C-N Bond	KE07 R7 Variant			
	Electric Field					Electric Field		
Region	**EL**	**EL†**	**ΔG‡**		**Region**	**EL**	**EL†**	**ΔG‡**
Base	15.4 (7)	18.7 (7)	1.1 (0.7)		Base	47.4 (8)	54.4 (14)	3.5 (0.8)
Active Site	18.3 (3)	26.3 (3)	1.2 (0.3)		Active Site	26.3 (3)	35.3 (4)	1.8 (0.3)
Scaffold	3.3 (7)	6.2 (6)	0.2 (0.7)		Scaffold	-26.5 (5)	-12.3 (8)	-2.3 (0.5)
Solvent	6.8 (3)	7.7 (2)	0.5 (0.3)		Solvent	2.1 (2)	0.3 (2)	0.2 (0.2)

KE07 Design				N-O Bond				
	Electric Field					Electric Field		
Region	**EL**	**EL†**	**ΔG‡**		**Region**	**EL**	**EL†**	**ΔG‡**
Base	53.0 (7)	64.1 (3)	-11.4 (0.7)		Base	58.5 (5)	60.0 (4)	-11.3 (0.6)
Active Site	4.1 (1)	14.5 (1)	-1.9 (0.1)		Active Site	11.5 (2)	17.2 (2)	-2.8 (0.3)
Scaffold	-32.0 (7)	-37.0 (4)	6.7 (0.7)		Scaffold	-35.4 (5)	-23.2 (4)	5.4 (0.6)
Solvent	-21.4 (3.8)	-19.9 (1.5)	3.9 (0.3)		Solvent	-27.4 (2.4)	-23.7 (3)	4.8 (0.4)

KE07 Design		KE07 R7 Variant	Improvement from dominant bond
Region	**∑ΔG‡ (all bonds)**	**∑ΔG‡ (all bonds)**	**ΔΔG‡ (best bond)**
Base	-19.4	-21.5	C-H bond (-4.6)
Active Site	-1.3	-2.3	N-O bond (-0.8)
Scaffold	9.3	6.2	C-N bond (-2.5)
Solvent	6.1	6.7	C-N bond (-0.3)

Table S7: *Chemical Positioning vs. Electric Field Environment at the C-H, C-N and O-N Bonds*. The magnitude of the electric field in either the EL and EL† states for the designed KE70 enzyme and the best R6 variant. The active site is defined by residues within 5 Å from the center of the substrate, while the protein environment is summed over all residues outside this region. Solvent includes waters in the neck of the TIM barrel as well as the surrounding hydration and bulk water. Sum of free energy are summed over all bonds, with dominant region and bond most affected shown. Positive sign indicates field supporting bond breaking. Fields are reported in units of MV/cm

KE70 Design				C-H Bond	KE70 R6 Variant			
	Electric Field					Electric Field		
Region	EL	EL†	$\Delta G^{\ddagger}$		Region	EL	EL†	$\Delta G^{\ddagger}$
Base	46.1 (5)	65.1 (5)	-5.3 (0.3)		Base	61.4 (4)	80.1 (4)	-6.8 (0.3)
Active Site	16.7 (2)	23.1 (2)	-1.9 (0.1)		Active Site	2.3 (1)	2.9 (1)	-0.2 (0.1)
Scaffold	-12.2 (4)	-11.3 (2)	1.1 (0.2)		Scaffold	-11.6 (2)	-9.1 (1)	1 (0.1)
Solvent	2.9 (1)	0.7 (1)	-0.2 (0.1)		Solvent	1.9 (1)	2.8 (1)	-0.2 (0.1)

KE70 Design				C-N Bond	KE70 R6 Variant			
	Electric Field					Electric Field		
Region	EL	EL†	$\Delta G^{\ddagger}$		Region	EL	EL†	$\Delta G^{\ddagger}$
Base	29.4 (3)	35.6 (2)	2.1 (0.3)		Base	23.5 (2)	30.1 (1)	1.7 (0.2)
Active Site	23.2 (4)	32.0 (4)	1.6 (0.4)		Active Site	6.1 (2)	8.9 (1)	0.4 (0.2)
Scaffold	-8.6 (4)	-11.4 (2)	-0.7 (0.4)		Scaffold	-5.8 (2)	-6.7 (2)	-0.4 (0.2)
Solvent	4.6 (1)	6.0 (1)	0.3 (0.1)		Solvent	5.9 (1)	4.7 (1)	0.5 (0.1)

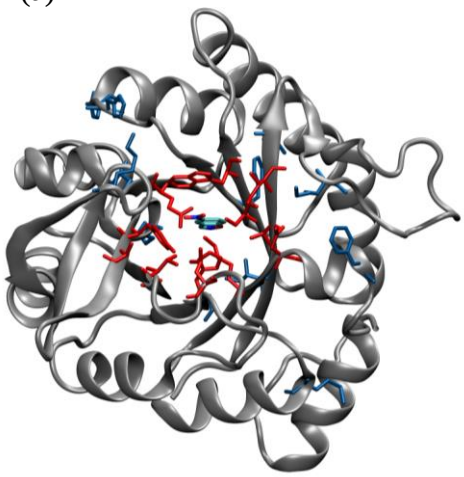
KE70 Design				N-O Bond	KE70 R6 Variant			
	Electric Field					Electric Field		
Region	EL	EL†	$\Delta G^{\ddagger}$		Region	EL	EL†	$\Delta G^{\ddagger}$
Base	15.5 (2)	20.2 (1)	-3.5 (0.2)		Base	21.3 (2)	24.4 (2)	-4.3 (0.3)
Active Site	2.0 (~0)	16.6 (1)	-2.0 (0.1)		Active Site	-2.7 (1)	1.9 (1)	~0 (0.1)
Scaffold	-9.8 (3)	-8.1 (2)	1.7 (0.3)		Scaffold	-12.6 (2)	-10.7 (1)	2.2 (0.2)
Solvent	1.1 (1)	-0.6 (1)	~0 (0.1)		Solvent	0.9 (1)	1.2 (1)	-0.2 (0.1)

KE70 Design		KE70 R6 Variant	Improvement from dominant bond
Region	$\sum \Delta G^{\ddagger}$ (all bonds)	$\sum \Delta G^{\ddagger}$ (all bonds)	$\Delta\Delta G^{\ddagger}$ (best bond)
Base	-6.7	-9.4	C-H bond (-1.5)
Active Site	-2.3	0.2	C-N bond (-1.2)
Scaffold	2.1	2.8	C-H bond (-0.1)
Solvent	0.1	-0.1	C-H bond (-0.2)

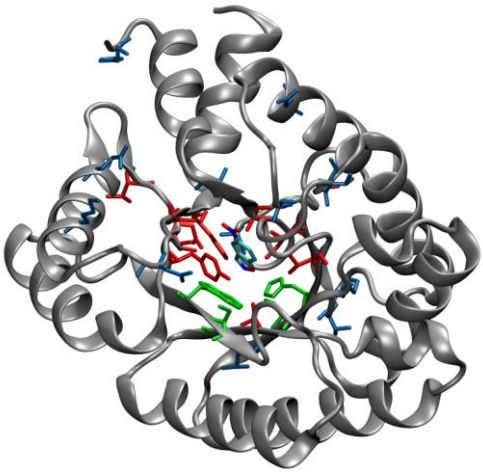
Supplementary Figures

Figure S1. *The Kemp elimination KE07 and KE70 designs*. (a) KE07 involved residues mutated from the original scaffold (red) as well as mutations introduced by LDE shown in blue. (b) KE70 involved residues mutated from the original scaffold (red) as well as mutations made during laboratory DE shown in blue. Additional design mutations via a recombination DE strategy are shown in green.

(a)



(b)



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