Table S1 The predicted value and apparent melting temperature of single point mutants

Enzyme Variant	I-Mutant 3.0 (kcal.mol^{-1})	FoldX (kcal.mol^{-1})	Rosetta (kcal.mol^{-1})	T_m^{app} (℃)	ΔT_m^{app} (℃)
WT	/	/	/	58.7±0.7	0
N186W	0.47	-1.23	-1.27	55.6±0.7	-3.1
N264W	0.35	-1.05	-1.07	57.2±0.8	-1.5
T18W	0.51	-1.39	-1.2	57.8±0.6	-0.9
D238L	0.86	-1.57	-2.79	57.8±0.0	-0.9
D238R	0.4	-1.55	-1.24	58.0±0.5	-0.7
T18I	0.67	-2.08	-1.20	58.1±0.4	-0.6
D238F	0.72	-1.48	-1.18	58.2±0.6	-0.5
E220I	0.45	-2.13	-1.17	58.7±0.2	0
D226L	0.33	-0.73	-1.43	58.7±0.1	0
D238P	0.15	-1.40	-1.82	58.7±0.3	0
D238W	0.63	-0.66	-5.78	58.7±0.1	0
D238Y	0.53	-1.43	-1.81	58.7±0.4	0
E220V	0.12	-1.85	-1.32	58.8±0.4	0.1
S114Y	0.38	-1.75	-2.66	58.9±0.5	0.2
S114V	0.66	-1.06	-1.86	59.1±0.3	0.4
S114F	0.5	-2.04	-2.37	59.2±0.2	0.5
N120L	0.48	-0.70	-1.26	59.2±0.6	0.5
A8L	0.09	-1.05	-1.93	59.3±0.5	0.6
N186L	0.69	-1.23	-1.92	59.3±0.3	0.6
N227L	0.08	-1.62	-1.45	59.3±0.5	0.6
N120R	0.25	-1.38	-1.27	59.4±0.8	0.7
S114W	0.31	-1.76	-3.05	59.5±0.6	0.8
Q233I	0.08	-0.73	-2.08	59.5±0.2	0.8
E230R	0.2	-0.72	-1.50	59.9±0.4	1.2
E230M	0.6	-3.15	-3.27	60.0±0.3	1.3
E230F	0.58	-1.41	-3.59	60.3±0.2	1.6
E230Y	0.28	-0.84	-3.49	60.8±0.6	2.1
T22I	0.27	-1.46	-1.80	61.1±0.9	2.4
E230P	0.00	-1.02	-3.91	61.2±0.4	2.5
E230W	0.42	-1.09	-1.42	61.4±0.2	2.7
E230C	0.01	-1.05	-2.17	62.0±0.3	3.3
E230T	0.15	-0.51	-3.62	62.5±0.5	3.8
T18K	0.14	-1.84	-1.06	63.2±0.0	4.5
E230L	0.62	-2.44	-3.07	64.1±0.2	5.4
E230V	0.51	-0.99	-3.93	64.1±0.3	5.4
E230I	0.60	-2.13	-4.72	64.4±0.2	5.7

Table S2 Primers used for construction of mutants

Enzyme Variant	Forward Primer (5’-3’)	Reverse Primer (5’-3’)
N186W	gcgaccctgcctttgcctggtacgttgttagcaccgg	ccggtgctaacaacgtaccaggcaaaggcagggtcgc
N264W	tactttggtatctggacaggcctct	tgtccagataccaaagtacgagaga
T18W	gagttggcagatagtgtagtgtaatacaacaattcattgatttcttgcgaggtag	ctacctcgcaagaaatcaatgaattgttgtattacactacactatctgccaactc
D238L	ctgttcaggtctgcacaagcctcctggaaacctctgattgctc	gagcaatcagaggtttccaggaggcttgtgcagacctgaacag
D238R	gcaatcagaggtttccagacggcttgtgcagacctgaac	gttcaggtctgcacaagccgtctggaaacctctgattgc
T18I	ctcgcaagaaatcaatgaattgatttattacactacactatctgcca	tggcagatagtgtagtgtaataaatcaattcattgatttcttgcgag
D238F	tgttcaggtctgcacaagctttctggaaacctctgattgc	gcaatcagaggtttccagaaagcttgtgcagacctgaaca
E220I	gctattgtcagtaatccaatactcaatgccagcgtggagaaaaccaaaag	cttttggttttctccacgctggcattgagtattggattactgacaatagc
D226L	gctggcgaggagtattggattactctgaatagcccagagactg	cagtctctgggctattcagagtaatccaatactcctcgccagc
D238P	ctgttcaggtctgcacaagcccactggaaacctctgattgctc	gagcaatcagaggtttccagtgggcttgtgcagacctgaacag
D238W	ctgttcaggtctgcacaagctggctggaaacctctgattgctc	gagcaatcagaggtttccagccagcttgtgcagacctgaacag
D238Y	gtctgcacaagctatctggaaacctc	gaggtttccagatagcttgtgcagac
E220V	gtcagtaatccaatactcaacgccagcgtggagaaaacc	ggttttctccacgctggcgttgagtattggattactgac
S114Y	cgttttgaacttccccgtaatagtccaggaatcccttgtgt	acacaagggattcctggactattacggggaagttcaaaacg
S114V	gtacacaagggattcctggacgtgtacggggaagttcaaaacgag	ctcgttttgaacttccccgtacacgtccaggaatcccttgtgtac
S114F	acacaagggattcctggacttttacggggaagttcaaaac	gttttgaacttccccgtaaaagtccaggaatcccttgtgt
N120L	gaacagtagcaacaagctccagttgaacttccccgtaactgtccag	ctggacagttacggggaagttcaactggagcttgttgctactgttc
A8L	ttcttgcgaggtagccagacggataccaccattaatgctcattgct	agcaatgagcattaatggtggtatccgtctggctacctcgcaagaa
N186L	gcgaccctgcctttgccctgtacgttgttagcaccgg	ccggtgctaacaacgtacagggcaaaggcagggtcgc
N227L	gaggagtattggattactgacctgagcccagagactgttcaggtc	gacctgaacagtctctgggctcaggtcagtaatccaatactcctc
N120R	gaacagtagcaacaagctcacgttgaacttccccgtaactgtccag	ctggacagttacggggaagttcaacgtgagcttgttgctactgttc
S114W	gactggtacggggaagttcaaaacg	tgaacttccccgtaccagtccagga
Q233I	atcgcttgtgcagacaataacagtctctgggctattgtcagtaatc	gattactgacaatagcccagagactgttattgtctgcacaagcgat
E230R	ttggattactgacaatagcccacgtactgttcaggtctgcacaag	cttgtgcagacctgaacagtacgtgggctattgtcagtaatccaa
E230M	tgtgcagacctgaacagtcattgggctattgtcagtaatcc	ggattactgacaatagcccaatgactgttcaggtctgcaca
E230F	attggattactgacaatagcccatttactgttcaggtctgcacaagc	gcttgtgcagacctgaacagtaaatgggctattgtcagtaatccaat
E230Y	ggattactgacaatagcccatatactgttcaggtctgcaca	tgtgcagacctgaacagtatatgggctattgtcagtaatcc
T22I	tcaatgaattgacttattacactattctatctgccaactcgtactgccg	cggcagtacgagttggcagatagaatagtgtaataagtcaattcattga
E230P	gattactgacaatagcccaccgactgttcaggtctgcaca	tgtgcagacctgaacagtcggtgggctattgtcagtaatc
E230W	gattactgacaatagcccatggactgttcaggtctgcaca	tgtgcagacctgaacagtccatgggctattgtcagtaatc
E230C	ttggattactgacaatagcccatgcactgttcaggtctgcacaag	cttgtgcagacctgaacagtgcatgggctattgtcagtaatccaa
E230T	ttggattactgacaatagcccaaccactgttcaggtctgcacaag	cttgtgcagacctgaacagtggttgggctattgtcagtaatccaa
T18K	cctcgcaagaaatcaatgaattgaaatattacactacactatctgccaac	gttggcagatagtgtagtgtaatatttcaattcattgatttcttgcgagg
E230L	gattactgacaatagcccactgactgttcaggtctgcaca	tgtgcagacctgaacagtcagtgggctattgtcagtaatc
E230V	actgacaatagcccagtgactgttcaggtctgc	gcagacctgaacagtcactgggctattgtcagt
E230I	tagcccaattactgttcaggtctgc	agtaattgggctattgtcagtaatc
S56C	gcatttgtatcatagatgagcgtacaccaagtcttgataatcttgagat	atctcaagattatcaagacttggtgtacgctcatctatgatacaaatgc
N63C	gtgcaaccattgcacatgtatcatagatgagcgtgctccaa	ttggagcacgctcatctatgatacatgtgcaatggttgcac
V189C	accctgcctttgccaactacgtttgtagcaccggcatt	aatgccggtgctacaaacgtagttggcaaaggcagggt
D238C	gcaatcagaggtttccagacagcttgtgcagacctgaac	gttcaggtctgcacaagctgtctggaaacctctgattgc

Table S3 Prediction and stability effect of potential disulfide bonds

SS-bond	Predicted by[a]	FoldX (kcal.mol^{-1})	Visual inspection	SS-bond	Predicted by[a]	FoldX (kcal.mol^{-1})	Visual inspection
5-236	1	-0.01	Failed	72-74	2/3	0.51	/
10-12	3	2.14	/	73-136	3	4.34	/
10-13	1/3/4	2.46	/	74-139	4	-0.90	Failed
20-172	1/2/3/4	6.82	/	77-140	1/3/4	6.22	/
21-263	4	1.21	/	95-109	1/3/4	0.98	/
23-78	4	5.59	/	96-106	1/2/3	3.57	/
25-263	4	2.71	/	97-109	1/2/3/4	1.05	/
31-35	1/4	5.30	/	101-102	3	3.19	/
31-39	1/2/3	0.43	/	102-187	4	3.95	/
33-54	3/4	2.54	/	115-149	2/3/4	5.05	/
33-63	3	2.54	/	115-152	1/3	8.18	/
34-56	1/4	1.23	/	118-122	4	5.87	/
36-51	4	2.11	/	133-136	1/3/4	4.31	/
36-54	4	-0.37	Failed	137-168	3	1.78	/
37-39	2/3	1.15	/	139-170	3/4	0.35	/
49-69	4	3.98	/	140-171	1/4	4.22	/
50-68	1	2.21	/	160-162	3	-0.69	Failed
52-53	3	2.94	/	166-192	1/2/4	2.93	/
52-66	1/2/3	4.77	/	171-195	3/4	6.22	/
54-65	1/3/4	2.39	/	182-216	3/4	4.03	/
56-63	4	-1.00	Passed	189-218	4	0.96	/
57-117	1/2/3	0.34	/	189-238	1/3/4	-2.42	Passed
57-118	4	2.12	/	210-212	3	2.80	/
60-114	4	1.12	/	223-233	3	4.04	/
60-117	1/4	0.92	/	226-231	1/2/4	-0.73	Failed
65-78	4	5.89	/	241-243	3	2.59	/
69-72	4	0.93	/	264-269	1/4	-0.39	Failed
69-74	1	1.16	/	267-269	3	0.02	/

[a] 1, SSBOND; 2, DbD2; 3, MODIP; 4, BridgeD.

Table S4 Quantifying disulfide bonds

RML variant	Free thiol/protein (mol/mol)		Total number of free cysteines	Deduced number of SS-bonds
	-DTT	+DTT		
T18K/T22I/E230I	0.91±0.08	6.68±0.21	1	3
S56C/N63C	0.93±0.10	8.74±0.35	1	4
V189C/D238C	0.92±0.06	8.87±0.58	1	4
S56C/N63C/V89C/D238C	0.95±0.12	10.22±0.49	1	5

Table S5 Details of various non-bonded interactions of wild-type residues followed by mutant residues

Wild: Thr18

Hydrogen bonding interaction

Donor				Acceptor				Type	Distance_DA	Distance_HA	DHA_Angle	HAAA_Angle	DAAA_Angle
Chain	Res.No	Res.ID	Atom	Chain	Res.No	Res.ID	Atom						
A	18	THR	N	A	14	ILE	O	MM	2.99	2.13	143.5	146.1	155.3
A	18	THR	OG1	A	14	ILE	O	SM	2.83	1.85	164.6	135.5	138.4
A	22	THR	N	A	18	THR	O	MM	2.93	2	152.5	147.7	152.9
A	22	THR	OG1	A	18	THR	O	SM	2.87	1.87	179.2	145.3	145.4

Mut: Lys18

Hydrogen bonding interaction

Donor				Acceptor				Type	Distance_DA	Distance_HA	DHA_Angle	HAAA_Angle	DAAA_Angle
Chain	Res.No	Res.ID	Atom	Chain	Res.No	Res.ID	Atom						
A	18	LYS	N	A	14	ILE	O	MM	2.88	1.99	146.3	144.7	154.4
A	22	ILE	N	A	18	LYS	O	MM	2.92	2.06	142.8	140.5	150.6

Cation-pi interactions

Cationic residue			Aromatic residue			ASA		SS	
Chain	Res.No	Res.ID	Chain	Res.No	Res.ID	Cation	Aromatic	Cation	Aromatic
A	18	LYS	A	261	PHE	39.9	5.1	H	T

Wild: Thr22

Hydrogen bonding interaction

Donor				Acceptor				Type	Distance_DA	Distance_HA	DHA_Angle	HAAA_Angle	DAAA_Angle
Chain	Res.No	Res.ID	Atom	Chain	Res.No	Res.ID	Atom						
A	22	THR	N	A	18	THR	O	MM	2.93	2	152.5	147.7	152.9

A	22	THR	OG1	A	18	THR	O	SM	2.87	1.87	179.2	145.3	145.4
A	26	ASN	N	A	22	THR	O	MM	2.76	1.79	162.3	149.6	154.6

Mut: Ile22

Hydrogen bonding interaction

Donor				Acceptor				Type	Distance_DA	Distance_HA	DHA_Angle	HAAA_Angle	DAAA_Angle
Chain	Res.No	Res.ID	Atom	Chain	Res.No	Res.ID	Atom						
A	22	ILE	N	A	18	LYS	O	MM	2.92	2.06	142.8	140.5	150.6
A	22	ILE	N	A	19	TYR	O	MM	3.07	2.45	119.7	96.1	111
A	26	ASN	N	A	22	ILE	O	MM	2.86	1.9	158.5	147.3	153.8

Hydrophobic interactions

Residue 1			Residue 2			SS		ASA	
Chain	Res.No	Res.ID	Chain	Res.No	Res.ID	Res1	Res2	Res1	Res2
A	22	ILE	A	45	ALA	H	T	11.3	40.2
A	22	ILE	A	263	ILE	H	E	11.3	11.2

Wild: Glu230

Hydrogen bonding interaction

Donor				Acceptor				Type	Distance_DA	Distance_HA	DHA_Angle	HAAA_Angle	DAAA_Angle
Chain	Res.No	Res.ID	Atom	Chain	Res.No	Res.ID	Atom						
A	227	ASN	ND2	A	230	GLU	OE2	SS	2.41	1.46	155.7	162.7	171.5

Mut: Ile230

Hydrophobic interactions

Residue 1			Residue 2			SS		ASA	
Chain	Res.No	Res.ID	Chain	Res.No	Res.ID	Res1	Res2	Res1	Res2
A	14	ILE	A	230	ILE	H	E	14.2	36.2

A	224	ILE	A	230	ILE	E	E	0	36.2
A	230	ILE	A	232	VAL	E	E	36.2	2
A	230	ILE	A	261	PHE	E	T	36.2	5.1

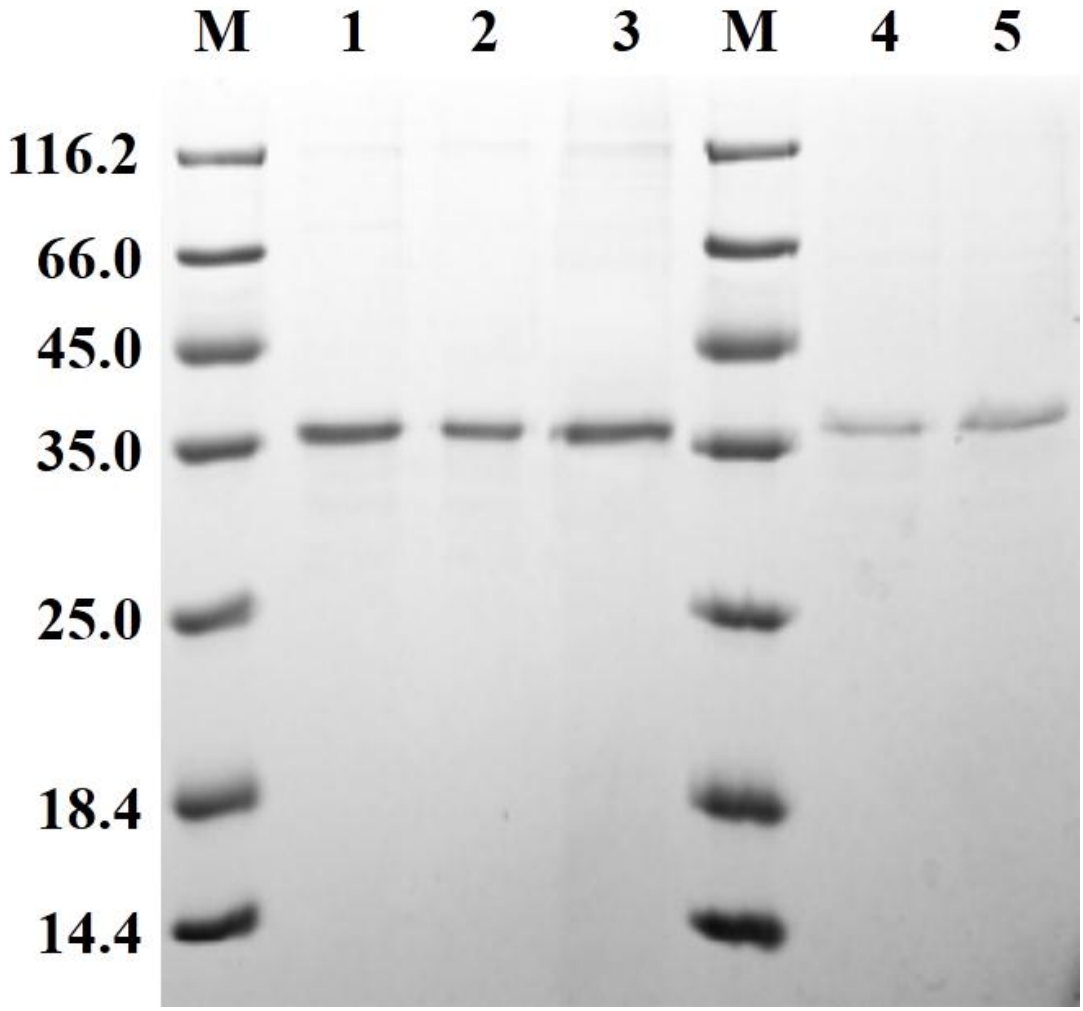

Fig. S1 SDS-PAGE of T18K/T22I/E230I and disulfide bond mutants (M: Marker, 1: wild-type RML; 2: T18K/T22I/E230I; 3: T18K/T22I/E230I/S56C/N63C; 4: T18K/T22I/E230I/V189C/D238C, 5: M7.)

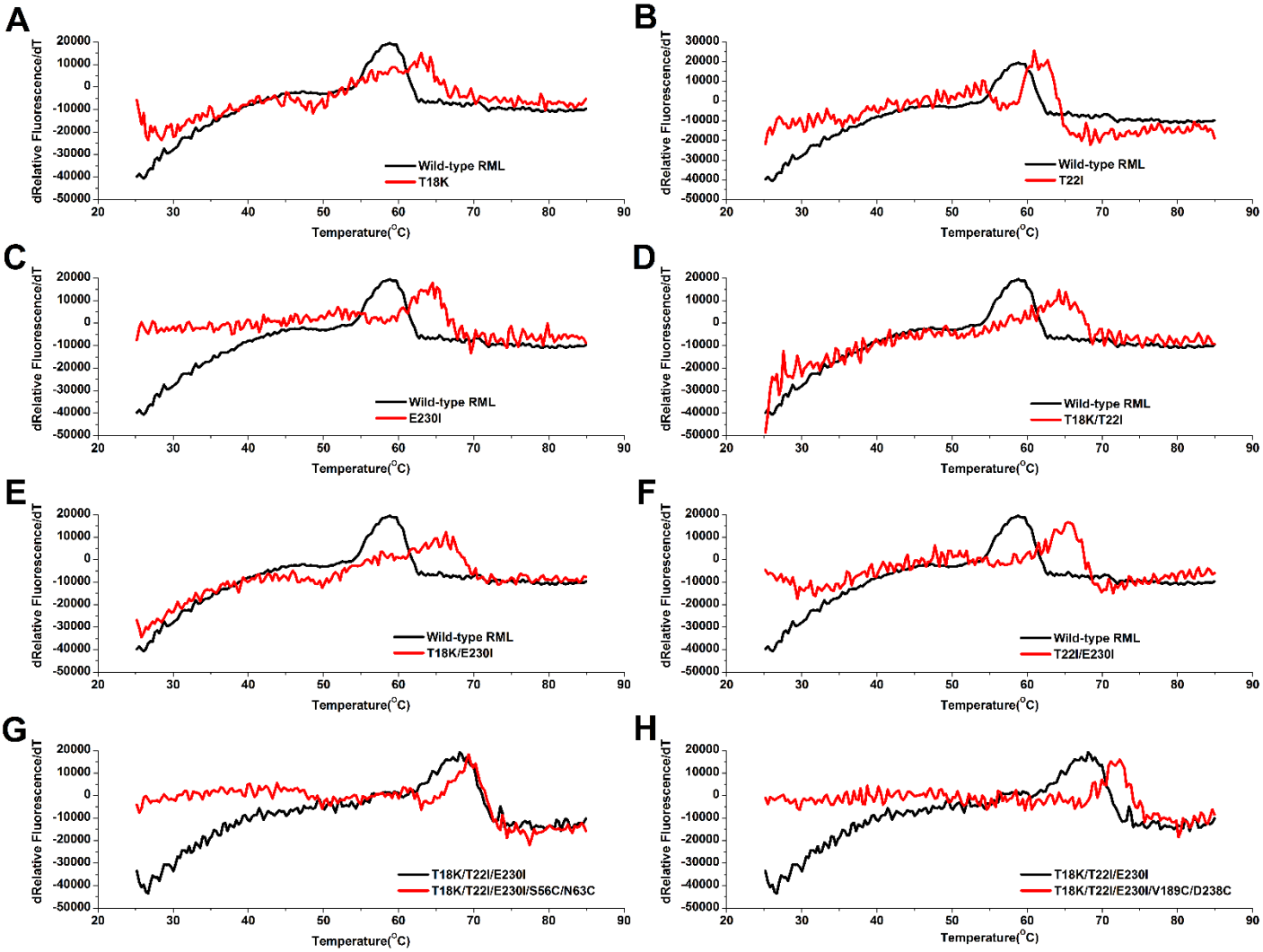

Fig. S2 The apparent melting temperatures of WT and variants measured by thermofluor method. (A: Wild-type RML and T18K mutant; B: Wild-type RML and T22I mutant; C: Wild-type RML and E230I mutant; D: Wild-type RML and T18K/T22I mutant; E: Wild-type RML and T18K/E230I mutant; F: Wild-type RML and T22I/E230I mutant; G: T18K/T22I/E230I mutant and T18K/T22I/E230I/S56C/N63C mutant; H: T18K/T22I/E230I mutant and T18K/T22I/E230I/V189C/D238C mutant.)