Table S1. Crystallization conditions of lactoglobulin mutants.

Mutant	Crystallization conditions
L39K	drop: 2 µl of protein 26 mg/ml, 1 µl 3.0 M ammonium sulfate in 0.5 M Tris-HCl pH 8.5, 0.5 µl 10 mM TET well: 500 µl of 3.0 M ammonium sulfate in 0.5 M Tris-HCl pH 8.5
L39Y	drop: 2 µl of protein 24 mg/ml, 1 µl 2.2 M ammonium sulfate in 0.5 M Tris-HCl pH 7.1, 0.5 µl 10 mM TET well: 500 µl 2.2 M ammonium sulfate in 0.5 M Tris-HCl pH 7.1
I56F	drop: 2 µl of protein 12 mg/ml, 1 µl 2.4 M ammonium sulfate in 0.5 M Tris-HCl pH 8.0, 0.5 µl 10 mM TET well: 500 µl 2.4 M ammonium sulfate in 0.5 M Tris-HCl pH 8.0
L58F	drop: 2 µl of protein 10 mg/ml, 1 µl 2.8 M ammonium sulfate in 0.5 M Tris-HCl pH 8.0, 0.5 µl 10 mM TET well: 500 µl 2.8 M ammonium sulfate in 0.5 M Tris-HCl pH 8.0
F105A	drop: 2 µl of protein 22 mg/ml, 1 µl 2.6 M ammonium sulfate in 0.5 M Tris-HCl pH 8.5, 0.5 µl 10 mM TET well: 500 µl 2.6 M ammonium sulfate in 0.5 M Tris-HCl pH 8.5
F105L	drop: 2 µl of protein 23 mg/ml, 1 µl 2.4 M ammonium sulfate in 0.5 M Tris-HCl pH 8.0, 0.5 µl 10 mM TET well: 500 µl of 3.0 M ammonium sulfate in 0.5 M Tris-HCl pH 8.0
M107L	drop: 3 µl protein 27 mg/ml, 1 µl 1.34 M tri-sodium citrate in 0.5 Tris-HCl pH 8.0, 0.5 µl 10 mM TET well: 300 µl of 1.34 M tri-sodium citrate in 0.5 M Tris pH 8.0

Table S2. Statistics of data collection and structure refinement.

Mutant	**L39K**	**L39Y**	**I56F**	**L58F**	**F105L**	**F105A**	**M107L**
PDB ID	**7BGZ**	**7BH0**	**7BF8**	**7BF7**	**7BGX**	**7BGA**	**7BF9**
TET present in the β-barrel?	**NO/(fatty acid^{endo})***	**NO/(fatty acid^{endo})***	**NO/(surface site)****	**YES**	**NO**	**NO/(fatty acid^{endo})***	**YES**
Data processing							
Space group	***P*3$_2$21**	***P*3$_2$21**	***P*2$_1$2$_1$2$_1$**	***P*3$_2$21**	***P*3$_2$21**	***P*3$_2$21**	***P*3$_2$21**
Unit cell parameters a, b, c [Å]	53.56, 53.56, 110.88	53.27, 53.27, 109.89	53.83, 69.66, 79.24	53.34, 53.34, 111.87	52.79, 52.79, 108.82	53.34, 53.34, 112.31	53.02, 53.02, 111.56
Resolution limits [Å] (last shell)	14.26 – 2.40 (2.49 – 2.40)	13.47 – 2.10 (2.16 – 2.10)	14.62 – 1.80 (1.84 – 1.80)	13.77 – 2.10 (2.17 – 2.10)	19.65 – 2.00 (2.05 – 2.00)	15.40 – 1.90 (1.95 – 1.90)	14.73 - 1.80 (1.84 - 1.80)
No. of reflections	26 085 (1 866)	29 075 (1 819)	152 301 (6 789)	29 028 (1 691)	40 044 (2 350)	40 802 (2 162)	44 886 (1 989)
No. of uniques	7 446 (754)	11 001 (862)	28 251 (1 650)	11 130 (982)	12 244 (910)	15 168 (1 025)	17 194 (1 019)
Multiplicity	3.5 (2.5)	2.7 (2.1)	5.4 (4.1)	2.6 (2.0)	3.3 (2.6)	2.7 (2.1)	2.6 (2.0)
I/σI	12.9 (1.4)	7.7 (2.0)	12.3 (1.5)	7.9 (1.6)	11.0 (1.7)	12.0 (1.4)	13.8 (2.2)
R merge [%]	0.057 (0.653)	0.058 (0.223)	0.108 (0.812)	0.061 (0.285)	0.055 (0.637)	0.067 (0.492)	0.035 (0.428)
Completeness [%]	98.1 (97.6)	99.4 (98.5)	99.8 (100)	98.8 (96.4)	98.8 (99.3)	99.7 (99.8)	99.0 (99.9)
CC(1/2)	0.998 (0.714)	0.998 (0.884)	0.996 (0.701)	0.997 (0.805)	0.999 (0.465)	0.997 (0.677)	0.999 (0.351)
Mosaicity [°]	1.07	1.30	0.94	1.25	0.93	1.02	0.99
Structure refinement							
No. reflections (test)	6 362 (1 075)	9 915 (1 066)	27 180 (1 032)	10 058 (1 049)	11 123 (1 088)	14 091 (1 036)	16 136 (1 054)
R/R_{free} [%]	19.4/26.6	20.0/27.5	19.2/22.1	20.3/24.9	20.0/24.6	18.5/24.3	18.4/22.3
Rmsd bonds [Å]/angles [°]	0.011/1.779	0.010/1.686	0.011/1.606	0.010/1.722	0.012/1.836	0.011/1.638	0.011/1.602
Ramachandran (%) favored/allowed/outliers	94/6/0	94/6/0	98/2/0	95/5/0	97/3/0	95/4/1	97/3/0

* **fatty acid^{endo}** – endogenous fatty acid trapped in the β-barrel

** TET found on protein surface, not in the β-barrel

Table S3. Classification of new BLG variants according to shape of the binding pocket and ability to interact with fatty acids and tetracaine.

shape of the binding pocket	elongated (WT-like)					reduced	
Mutant:	**L39K**	**L39Y**	**F105A***	**L58F**	**M107F**	**I56F**	**F105L**
β-barrel available for external ligand?	no	no	no	**yes**	**yes**	**yes**	**yes**
binds fatty acid in the β-barrel?	*endo***	*endo***	*endo***	**yes**	**yes**	no	**yes**
binds tetracaine in the β-barrel?	-	-	-	**yes**	**yes**	no	no
binds tetracaine on the surface?	no	no	no	no	no	**yes**	no

**shape can be classified as elongated; however, it is slightly enlarged in the region of F105A substitution*

***endo – β-barrel blocked by endogenous fatty acid, not available for tested ligands*

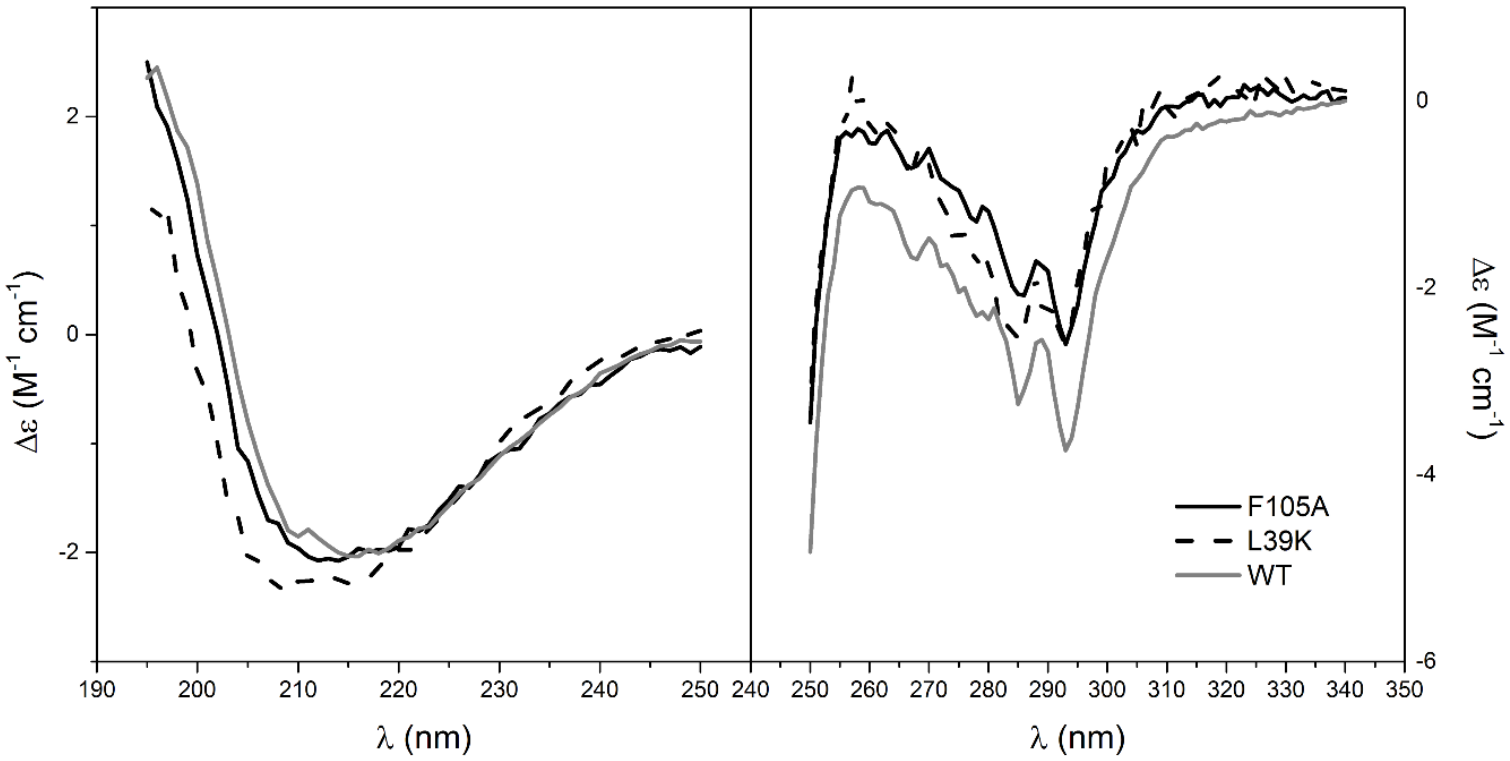

Fig. S1. Far-UV (left panel) and near-UV (right panel) CD spectra of F105A and L39K variants in 50 mM phosphate buffer pH 6.5 at room temperature. Spectrum of WT comes from (Loch et al., 2016).

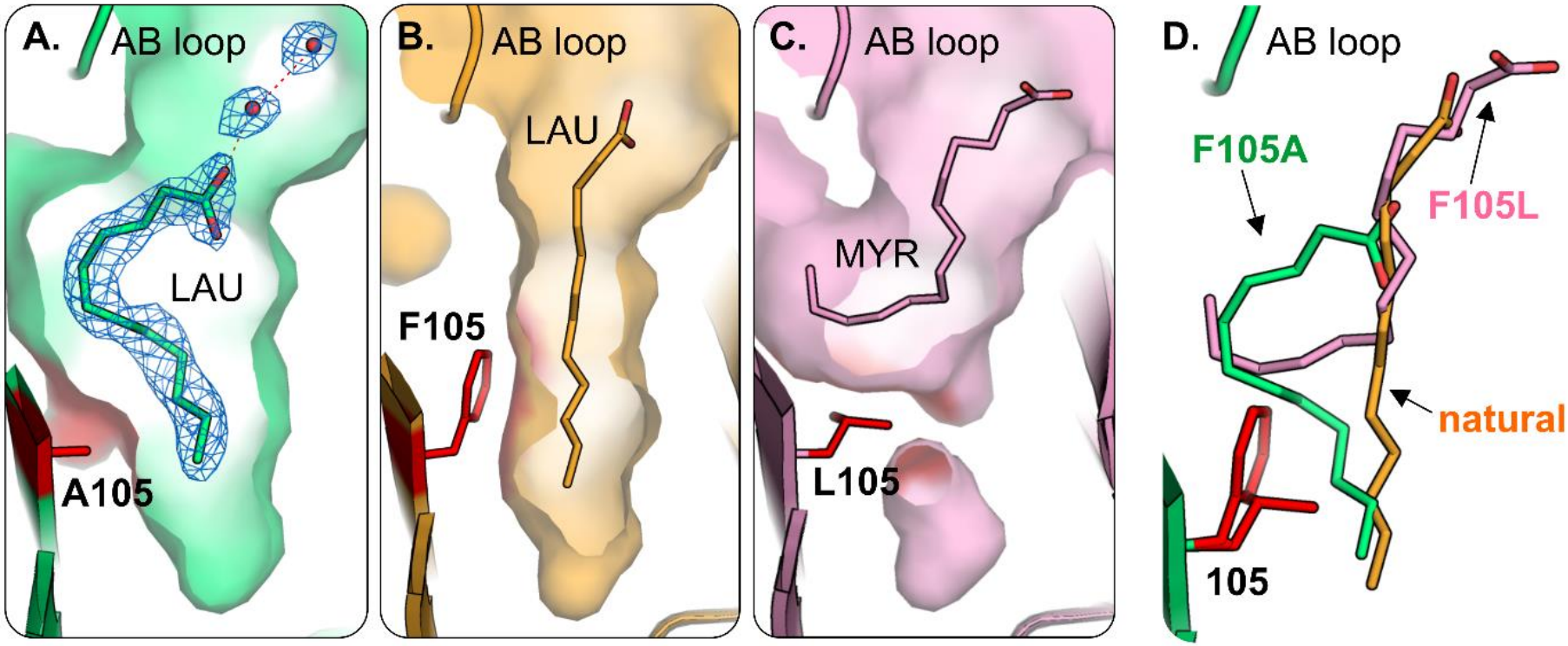

Fig. S2. The binding pocket shape and conformation of fatty acid (LAU – lauric acid, MYR – myristic acid) bound in the β-barrel of variant (A) F105A, (B) natural protein (PDB ID: 3UEU) and (C) F105L (PDB ID: 6RWQ). (D) Superposition of fatty acid molecules bound to F105A (green), F105L (pink) and natural (orange). 2FoFc map is contoured at 1.00σ level.

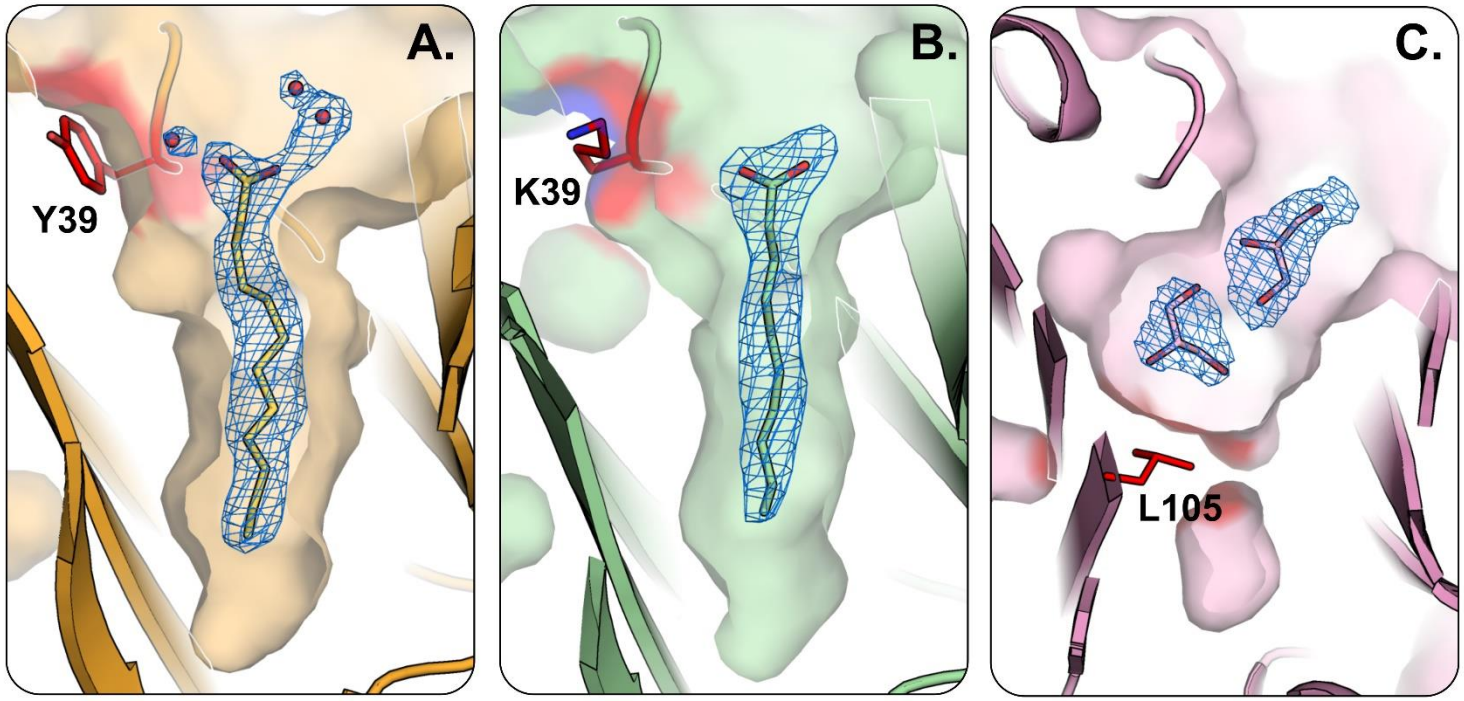

Fig.S3. Crystal structures of BLG mutants co-crystallized with tetracaine but not containing tetracaine. (A) Mutant L39Y with endogenous lauric acid bound in the β-barrel. (B) Mutant L39K with endogenous decanoic acid bound in the β-barrel. (C) Mutant F105L with cryoprotectant molecules (glycerol) bound in the modified β-barrel. 2FoFc map are contoured at 1.00σ level.

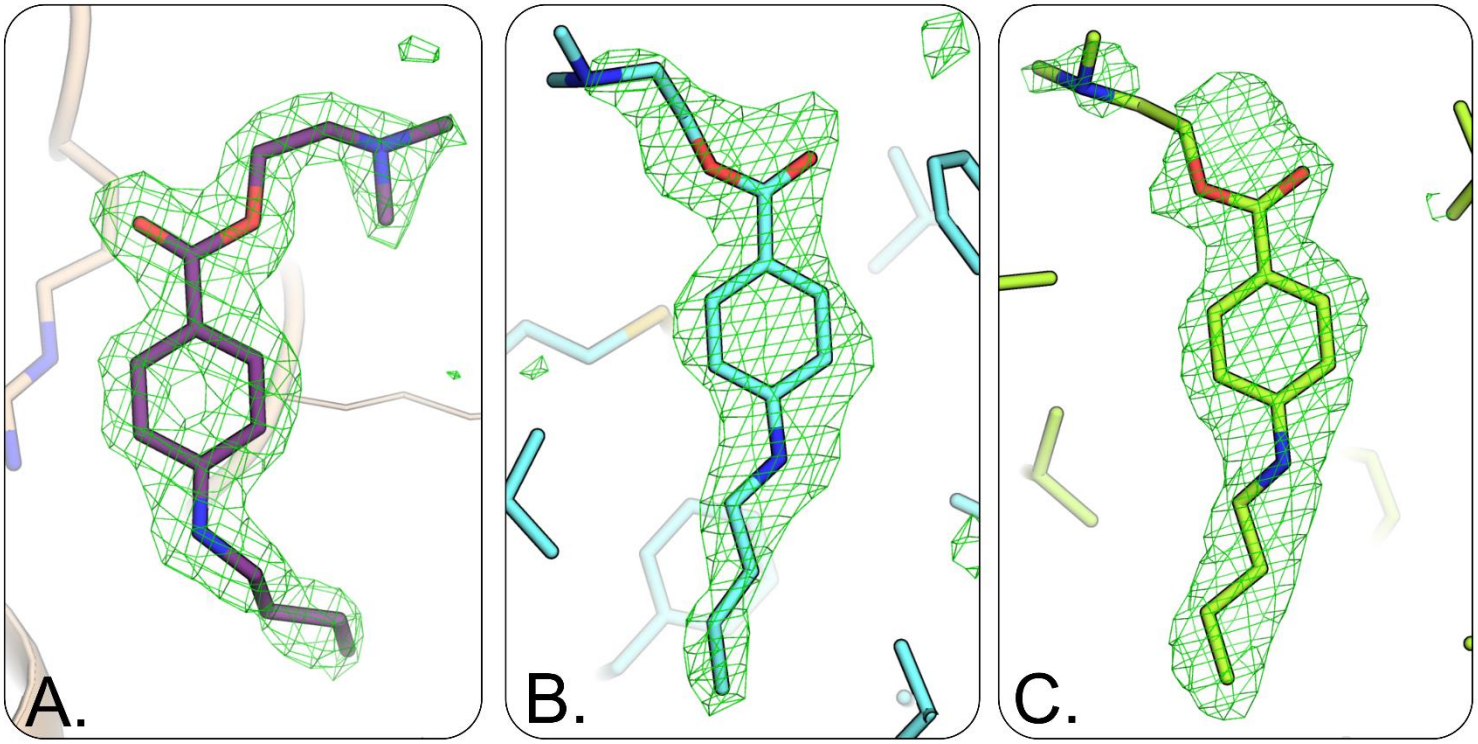

Fig. S4. Omit maps at 3.00 σ level confirming presence of tetracaine molecules in the structures of mutants (A) I56F, (B) L58F and (C) M107L.

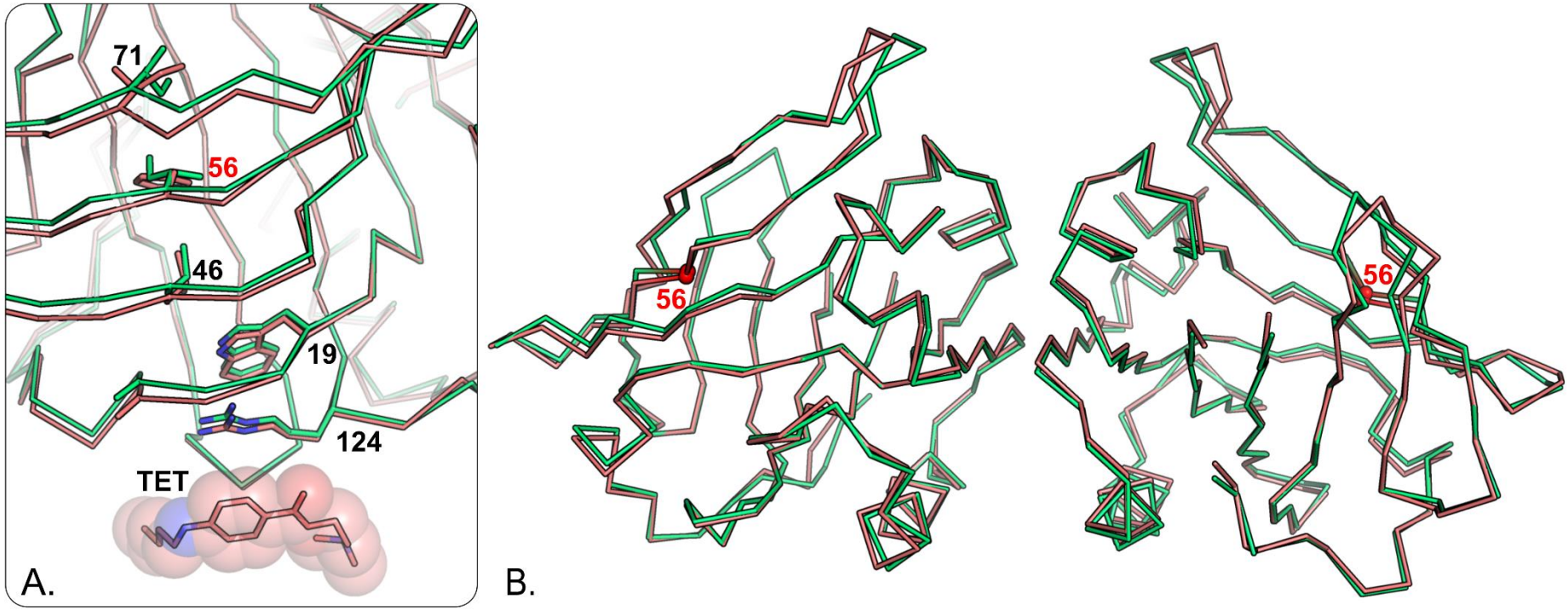

Fig. S5. Superposition of WT dimer (green, PDB ID: 6QI6) and I56F dimer (pink). Atomic shifts are visible especially in the region of the ligand binding (A) but also for the entire dimer molecule (B). Cα superposition was performed using program *LSQKAB* form the *CCP4* package.