Electronic Supplementary Material (ESI) for Physical Chemistry Chemical Physics.

Determination of nitroxide spin label conformations via PELDOR and X-ray crystallography

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Supporting Information

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Table S1. Experimental parameters of the PELDOR experiments on the azurin mutants.

Mutant	$\Delta\nu$ (MHz)	ν_{det} (GHz)	ν_{pump} (GHz)	B_0 (T)	$t_{\pi/2}$ (ns)	t_{π} (ns)	t_{pump} (ns)
T21R1	160	9.820069	9.660195	0.3442	16	32	18
	220	9.820435	9.600171	0.3419	16	32	20
	440	9.730215	9.290105	0.3309	16	32	50
	560	9.730316	9.170579	0.3267	16	32	60
T30R1	160	9.720280	9.560145	0.3405	10	20	24
	220	9.720340	9.500095	0.3384	10	20	26
	440	9.720201	9.280175	0.3306	10	20	48
	560	9.720984	9.160650	0.3263	10	20	60
T61R1	160	9.820359	9.660465	0.3441	16	32	18
	220	9.823008	9.602100	0.3419	16	32	20
	440	9.725438	9.285308	0.3308	16	32	46
	560	9.817735	9.257722	0.3297	16	32	48
D69R1	160	9.729975	9.570155	0.3408	16	32	24
	220	9.730461	9.510207	0.3387	16	32	26
	440	9.730301	9.290196	0.3309	16	32	46
	560	9.730095	9.170255	0.3268	16	32	60
T96R1	160	9.720336	9.560065	0.3404	16	32	26
	220	9.720634	9.500550	0.3384	16	32	30
	440	9.720378	9.290358	0.3310	16	32	48
	560	9.720773	9.160600	0.3263	16	32	60
S100R1	160	9.820003	9.660270	0.3442	16	32	18
	220	9.820569	9.600676	0.3419	16	32	22
	440	9.79212	9.371925	0.3338	16	32	44
	560	9.819427	9.258965	0.3298	16	32	46

Table S2. Data collection and refinement statistics for azurin T30R1.

Parameter	Crystal form I	Crystal form II
PDB ID	5I26	5I28
Wavelength (Å)	0.8943	0.8943
Resolution range (Å)	37.36 - 1.89	18.22 - 1.95
Space group	C 1 2 1	C 1 2 1
Unit cell	86.6 68.8 93.4 90 96.8 90	242.5 105.6 100.1 90 114.4 90
Total reflections	364655 (33359)	705691 (68273)
Unique reflections	39652 (3975)	166299 (16352)
Multiplicity	9.1 (8.4)	4.2 (4.2)
Completeness (%)	90.41 (89.72)	99.63 (98.30)
Mean I/sigma(I)	61.22 (5.83)	9.00 (0.69)
Wilson B-factor	19.61	39.70
R-merge	0. 18 (2.78)	0.163 (2.609)
CC1/2	0.99 (0.16)	0.99 (0.21)
R-work	0.194	0.212
R-free	0.240	0.271
Number of non-hydrogen atoms	4342	17050
macromolecules	4031	16180
ligands	4	22
water	307	848
Protein residues	509	2048
RMS (bonds)	0.008	0.008
RMS (angles)	1.15	1.19
Ramachandran favored (%)	98	97
Ramachandran outliers (%)	0	0.1
Clashscore	8.26	6.56
Average B-factor	28.00	48.40
macromolecules	27.80	48.40
ligands	18.80	52.10
solvent	31.10	48.30

* Statistics for the highest-resolution shell are shown in parentheses.

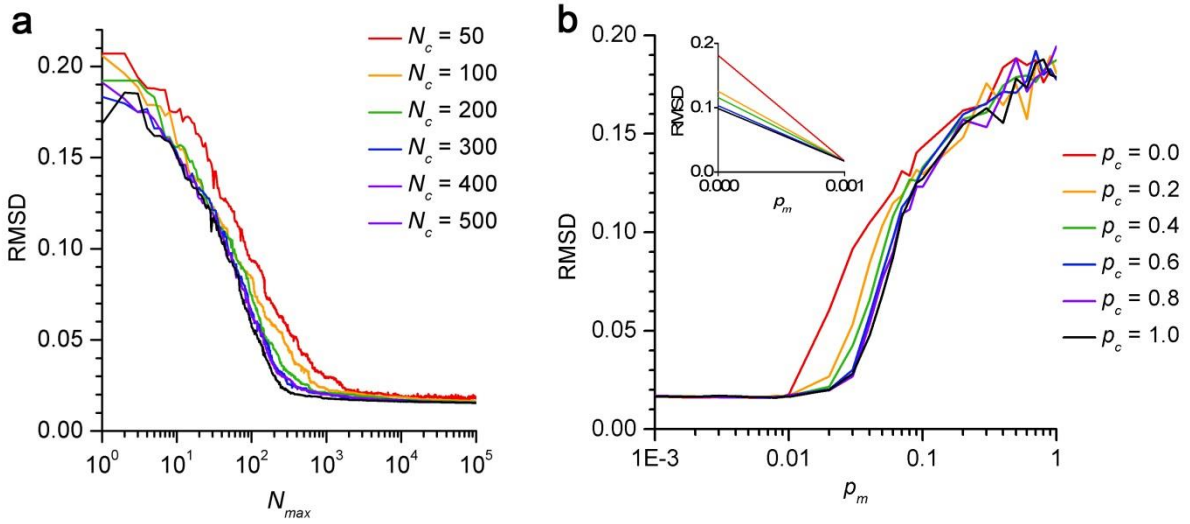


Fig. S1. Tuning the genetic algorithm for the azurin mutant T21R1. RMSD between the experimental and simulated PELDOR signals was calculated as a function of **(a)** N_c and N_{max} setting $N_g = 20$, $p_m = 0.01$, $p_c = 0.5$, **(b)** p_m and p_c setting $N_g = 20$, $N_c = 500$, $N_{max} = 10^4$.

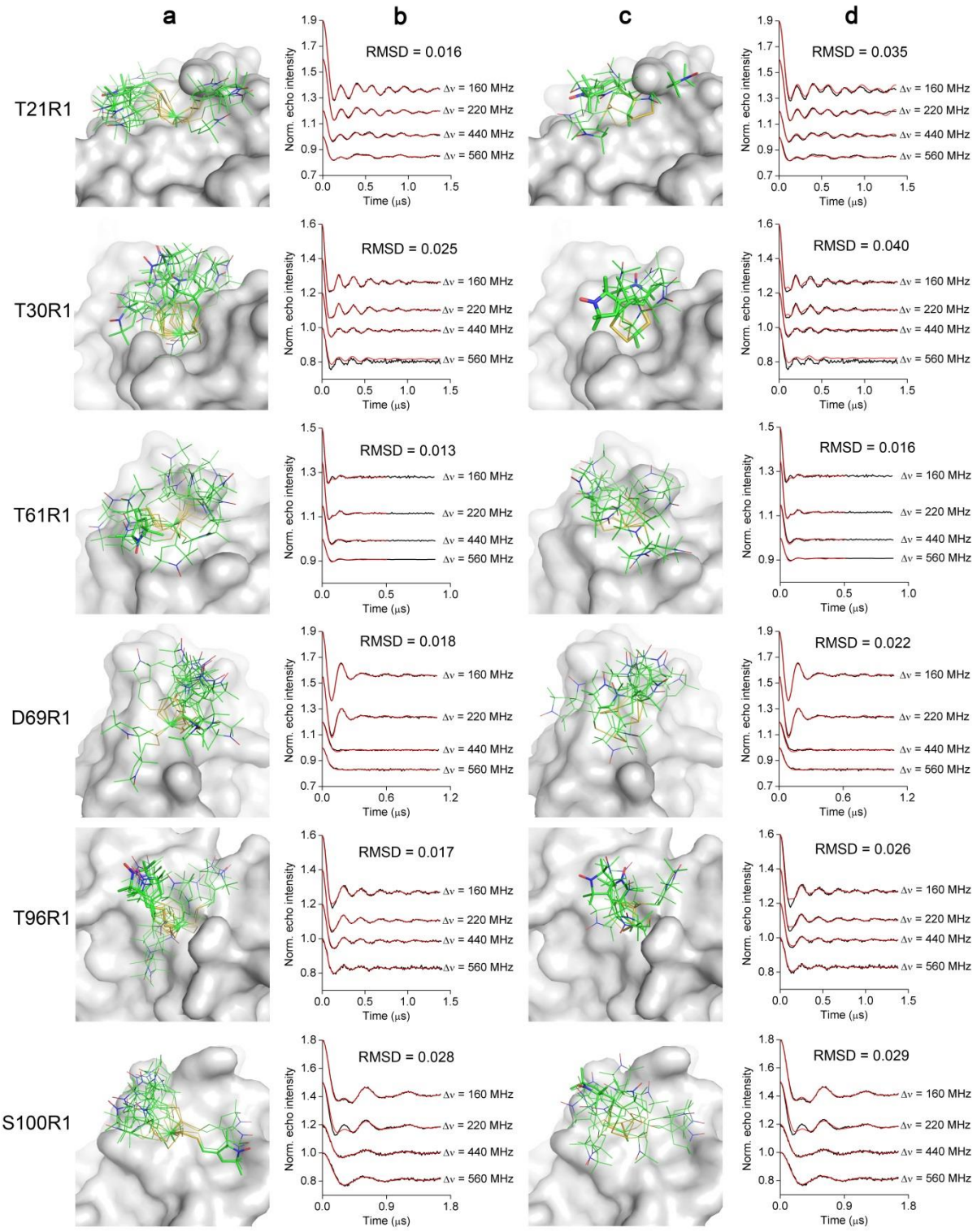


Fig. S2 Results of optimization of the R1 conformers for the six azurin mutants. **(a)** Optimized mtsslWizard-generated R1 conformers and **(b)** simulation (red) of the experimental PELDOR time traces (black) for them. **(c)** Optimized MMM-generated R1 conformers and **(d)** simulation (red) of the experimental PELDOR time traces (black) for them. RMSDs of the simulated PELDOR time traces from the experimental PELDOR time traces are shown on top of the PELDOR time traces.

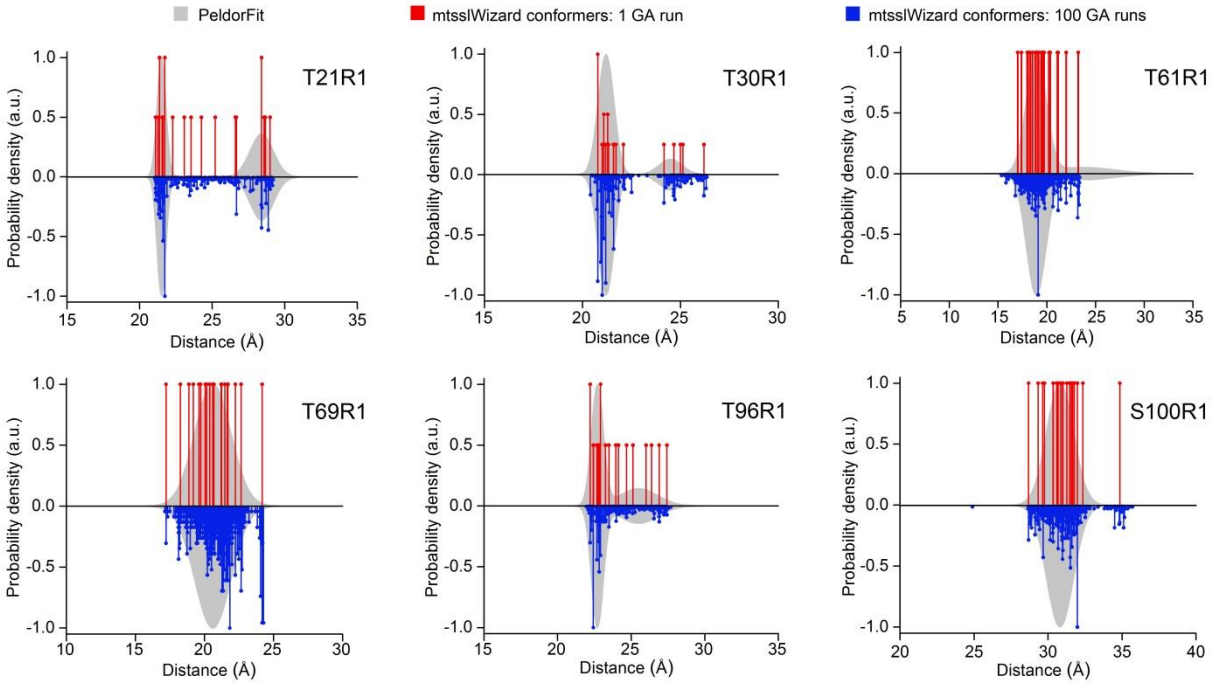


Fig. S3 The Cu^{2+}-nitroxide distance distributions, which are calculated for optimized mtsslWizard-generated R1 conformers after a single run of the genetic algorithm (GA) (red lines) and after 100 consecutive runs (blue lines). The distance distributions from the previous PeldorFit analysis are shown as grey shades.

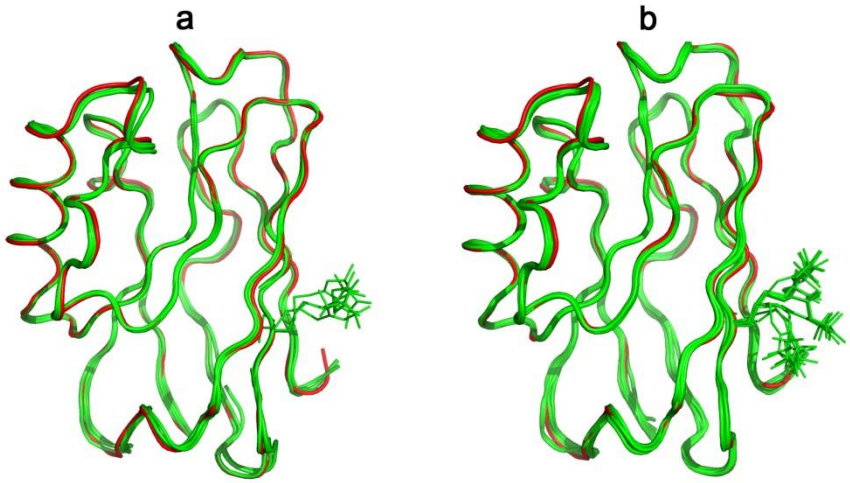
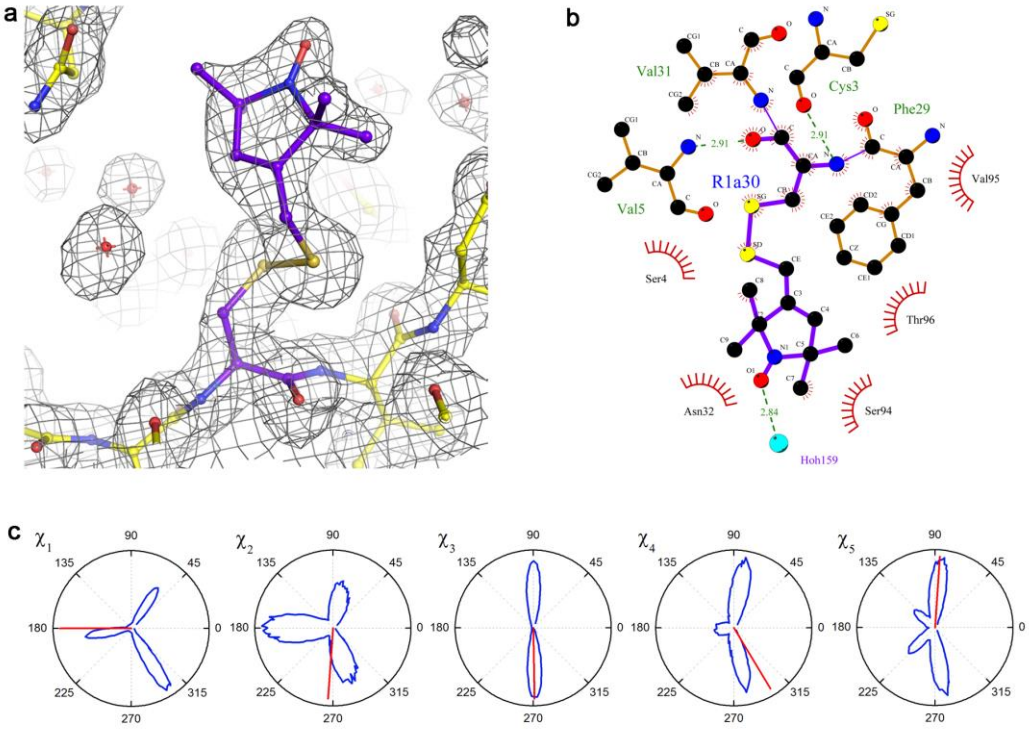


Fig. S4 A superposition of the X-ray crystal structures of the native azurin (PDB 1E67) and azurin mutant T30R1 **(a)** of form A and **(b)** of form B.

T30R1, crystal I, chain A (B-factor = 22.9 Å^2)



T30R1, crystal I, chain B (B-factor = 31.9 Å^2)

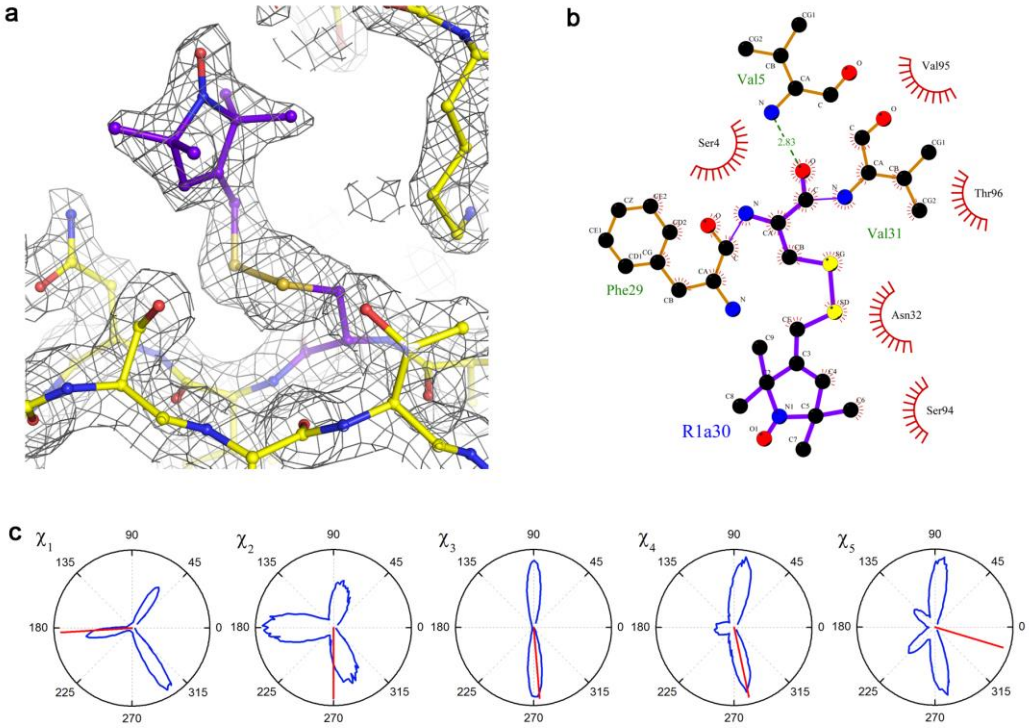
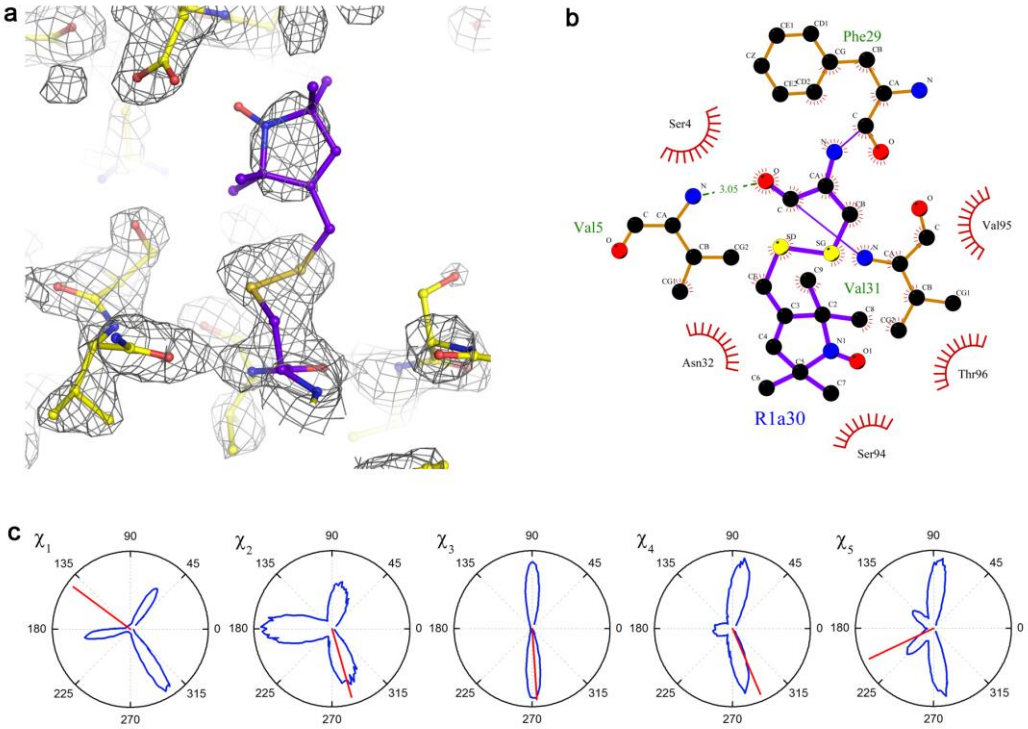


Fig. S5: (caption see below)

T30R1, crystal I, chain C (B-factor = 62.4 Å^2)



T30R1, crystal I, chain D (B-factor = 50.5 Å^2)

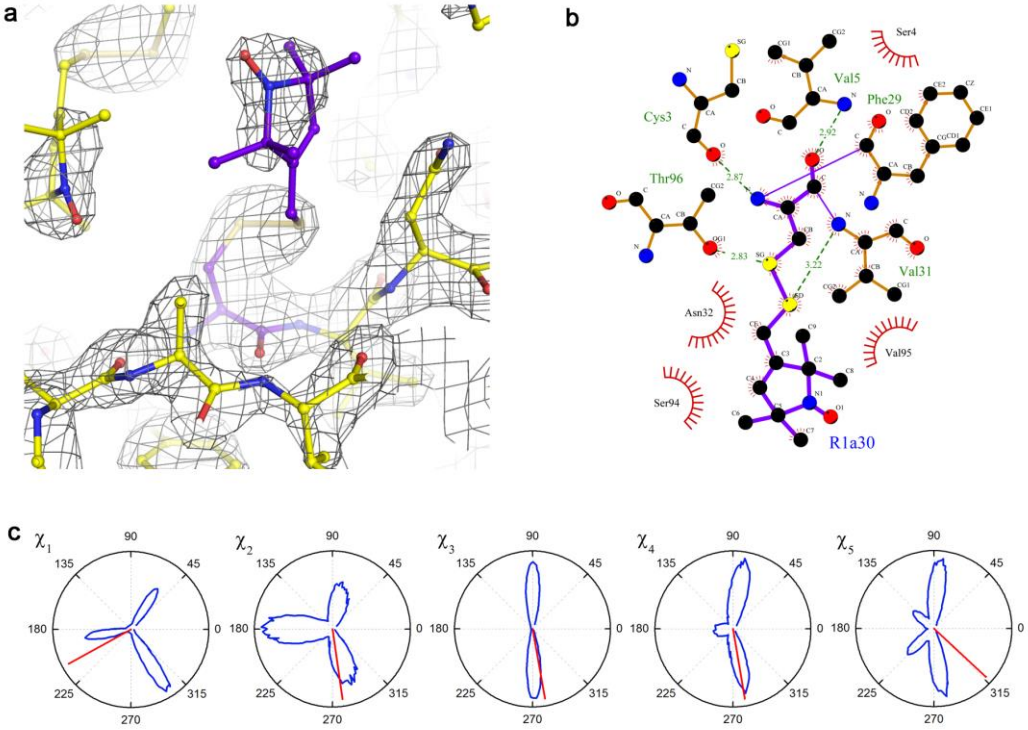
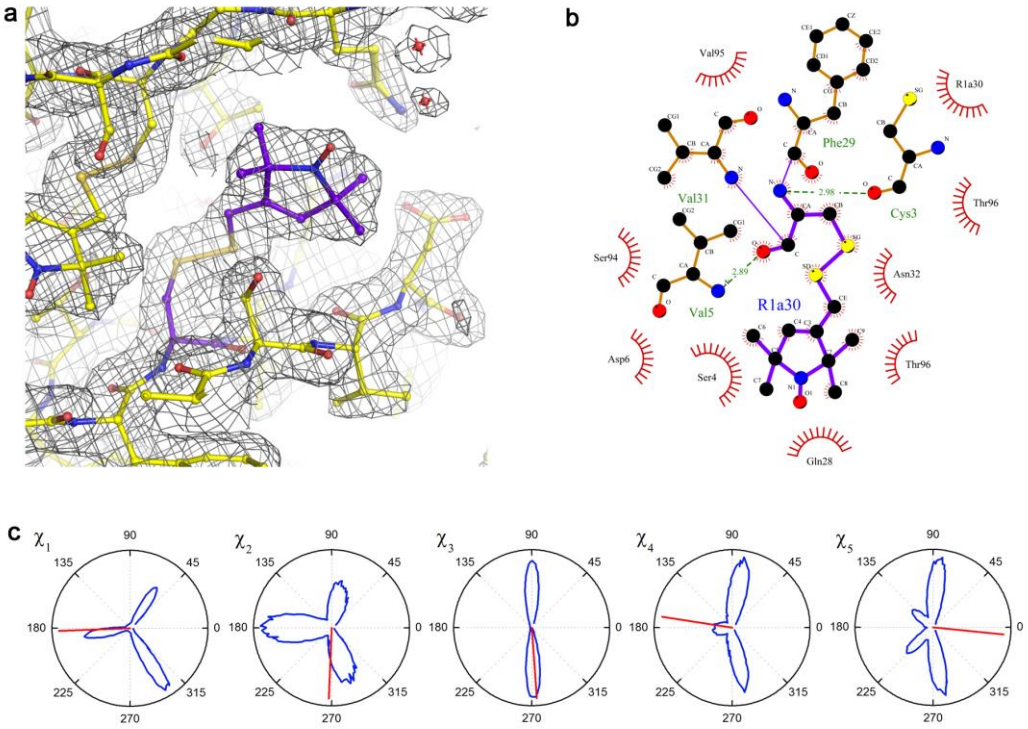


Fig. S5 continued: (caption see below)

T30R1, crystal II, chain A (B-factor = 39.5 Å^2)



T30R1, crystal II, chain B (B-factor = 35.1 Å^2)

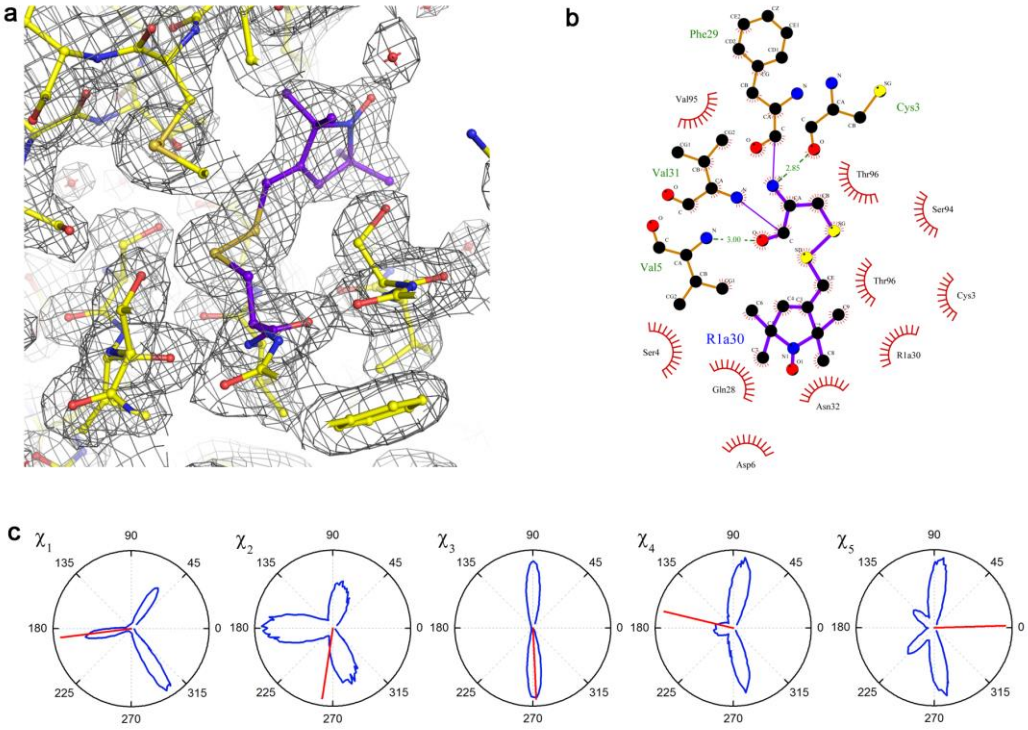
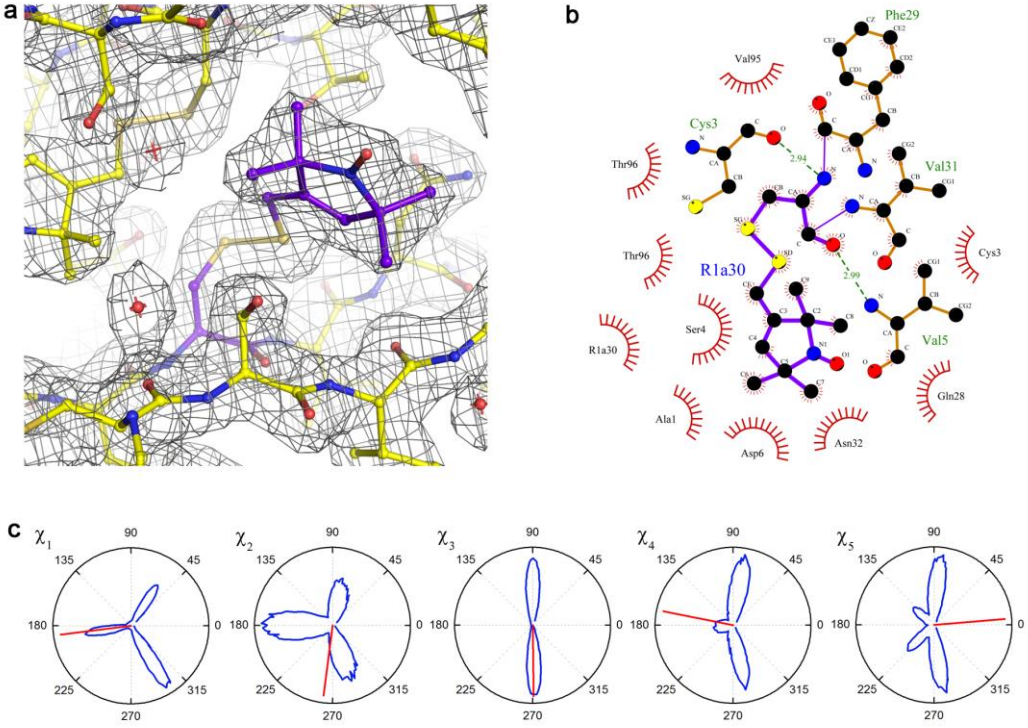


Fig. S5 continued: (caption see below)

T30R1, crystal II, chain C (B-factor = 35.6 Å^2)



T30R1, crystal II, chain D (B-factor = 71.1 Å^2)

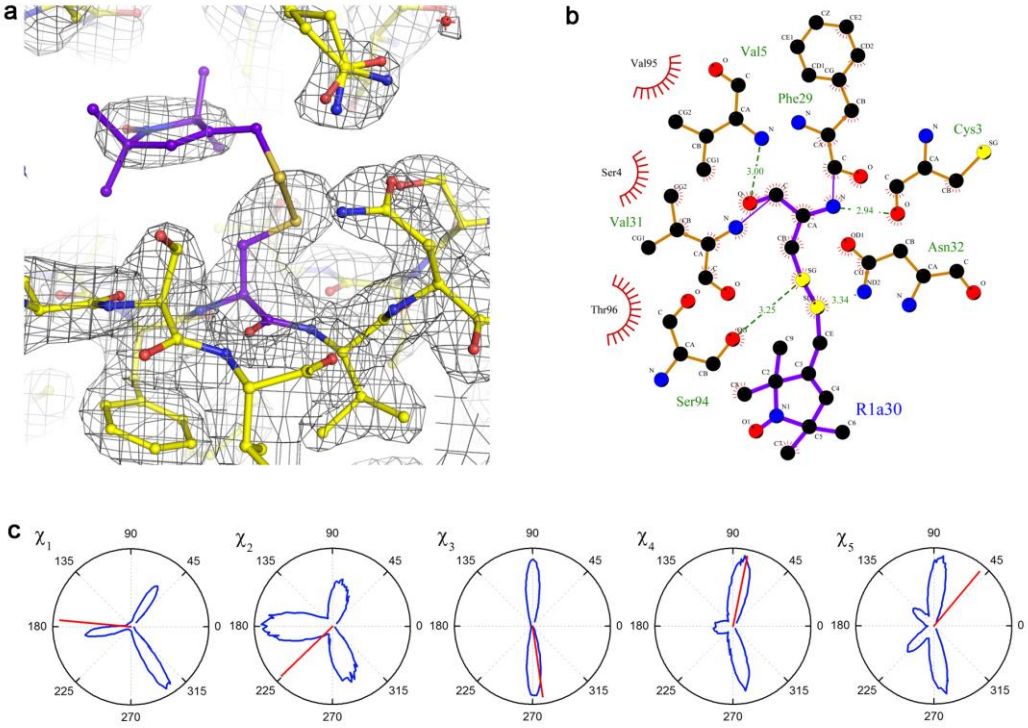
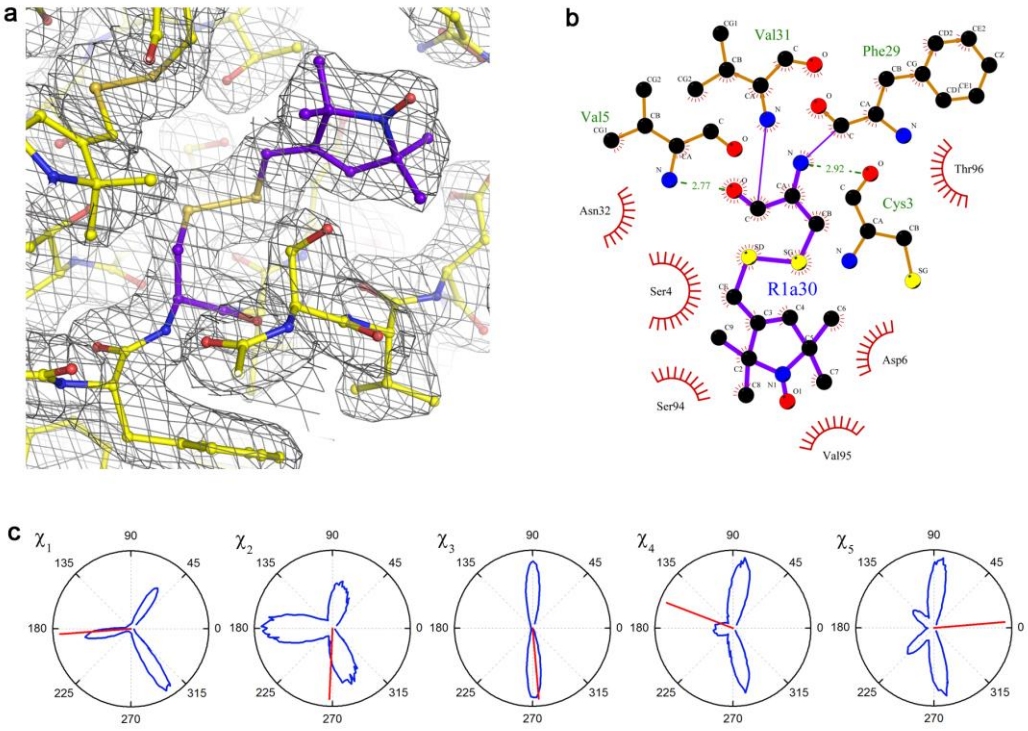


Fig. S5 continued: (caption see below)

T30R1, crystal II, chain E (B-factor = 39.7 Å^2)



T30R1, crystal II, chain F (B-factor = 51.0 Å^2)

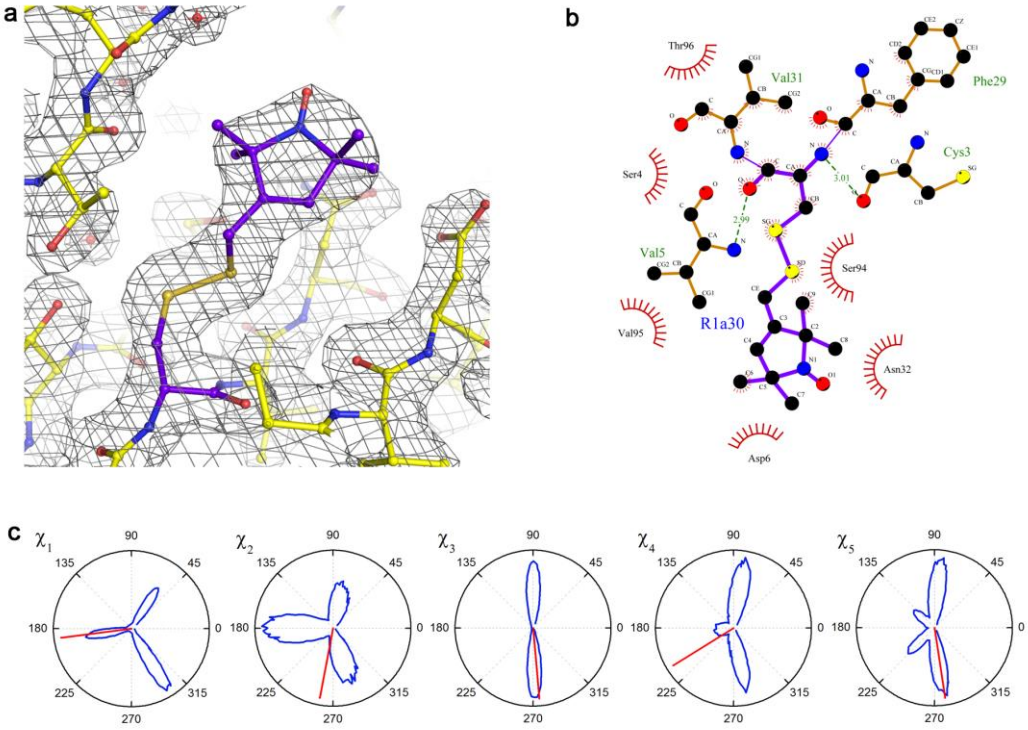
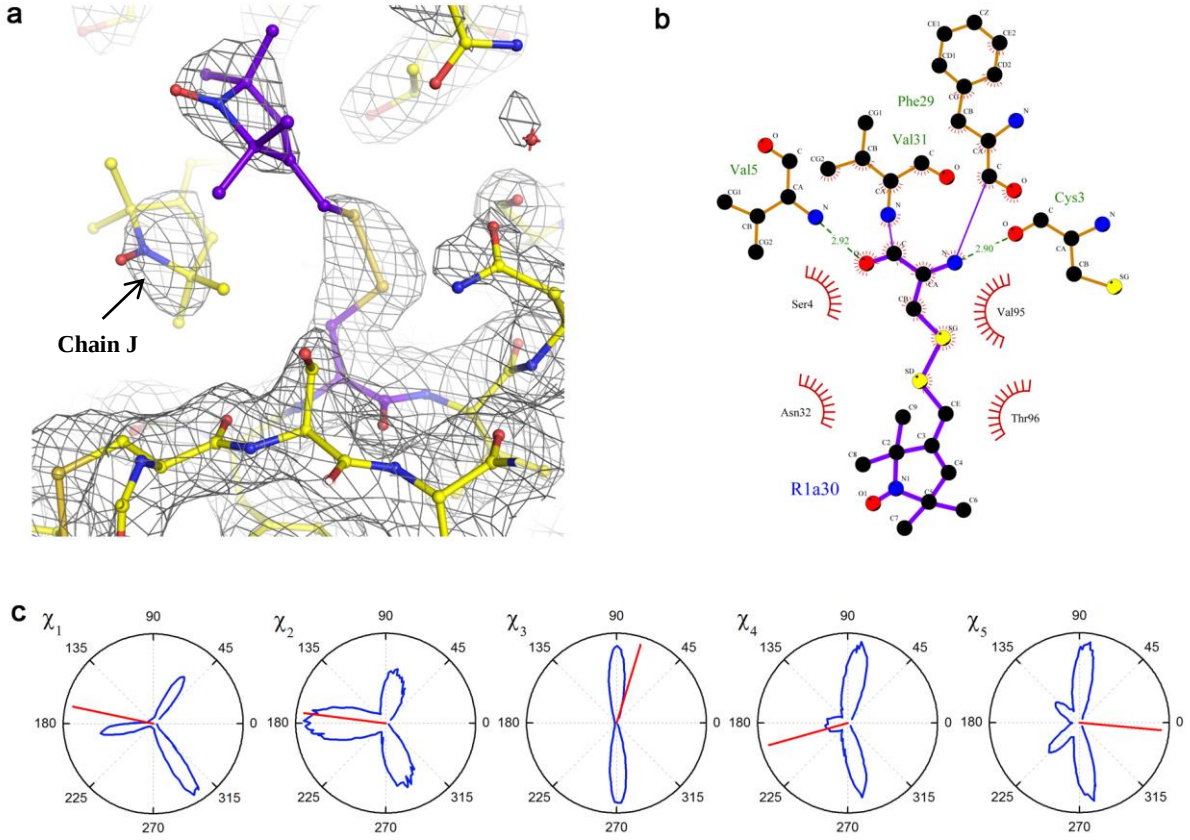


Fig. S5 continued: (caption see below)

T30R1, crystal II, chain G (B-factor = 83.6 Å^2)



T30R1, crystal II, chain H (B-factor = 69.5 Å^2)

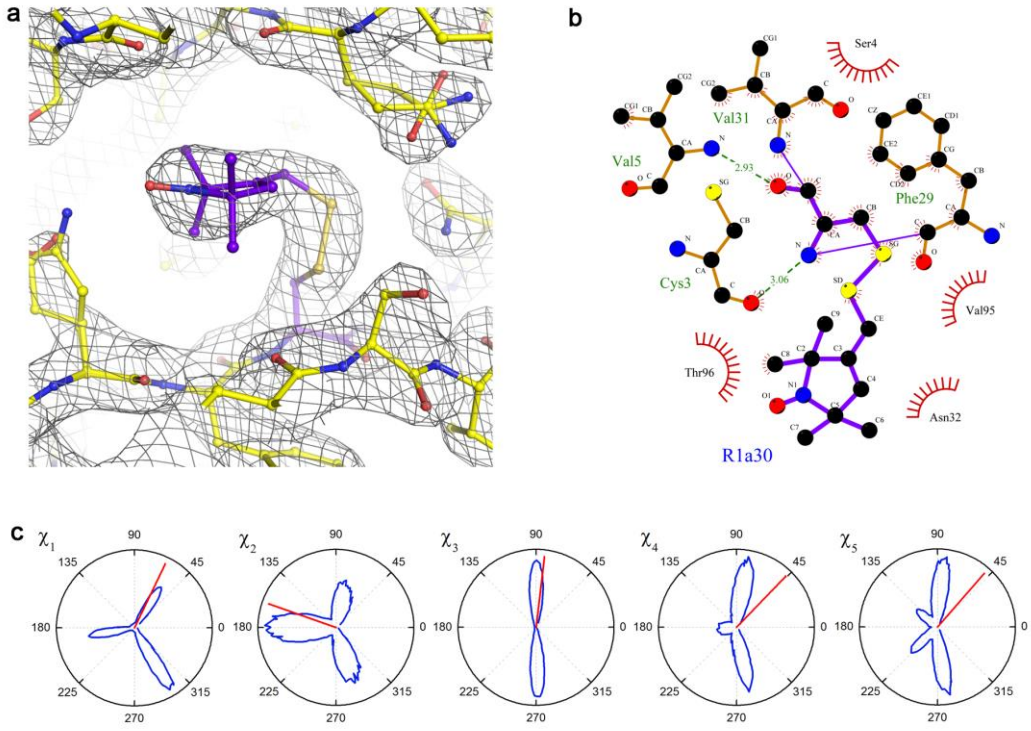
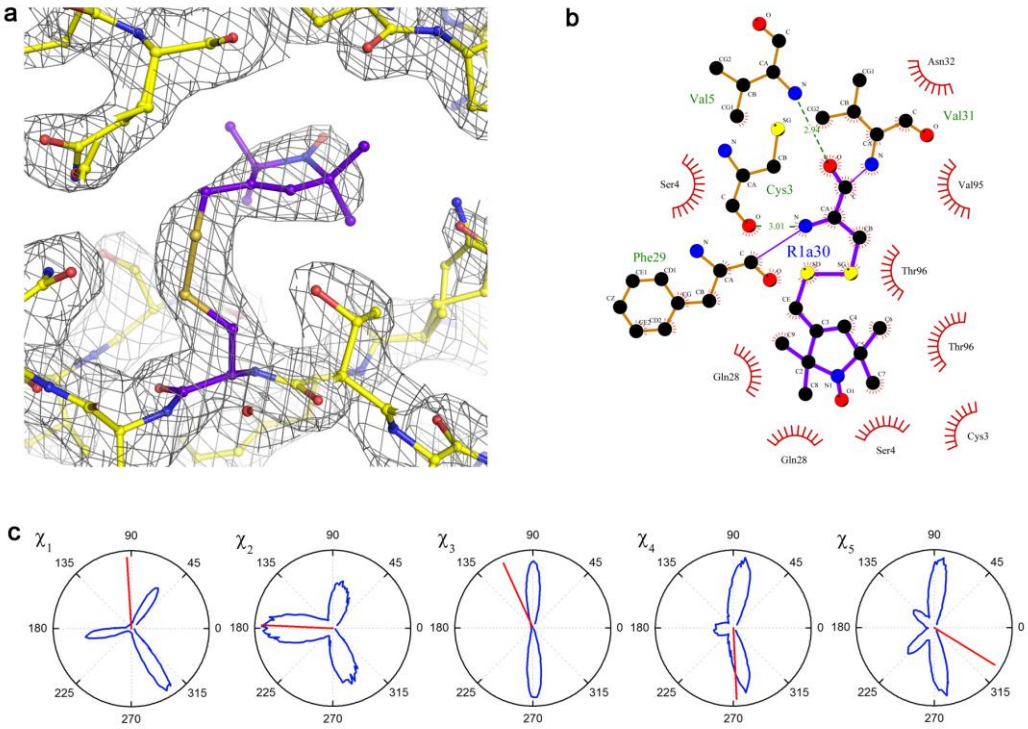


Fig. S5 continued: (caption see below)

T30R1, crystal II, chain I (B-factor = 70.3 Å^2)



T30R1, crystal II, chain J (B-factor = 81.9 Å^2)

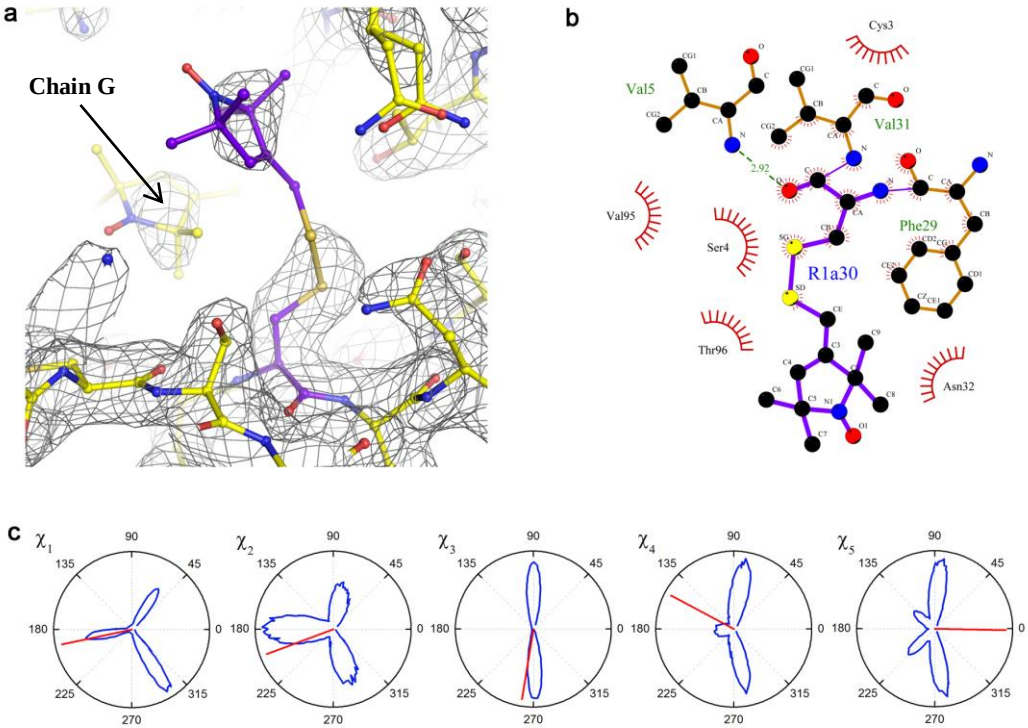
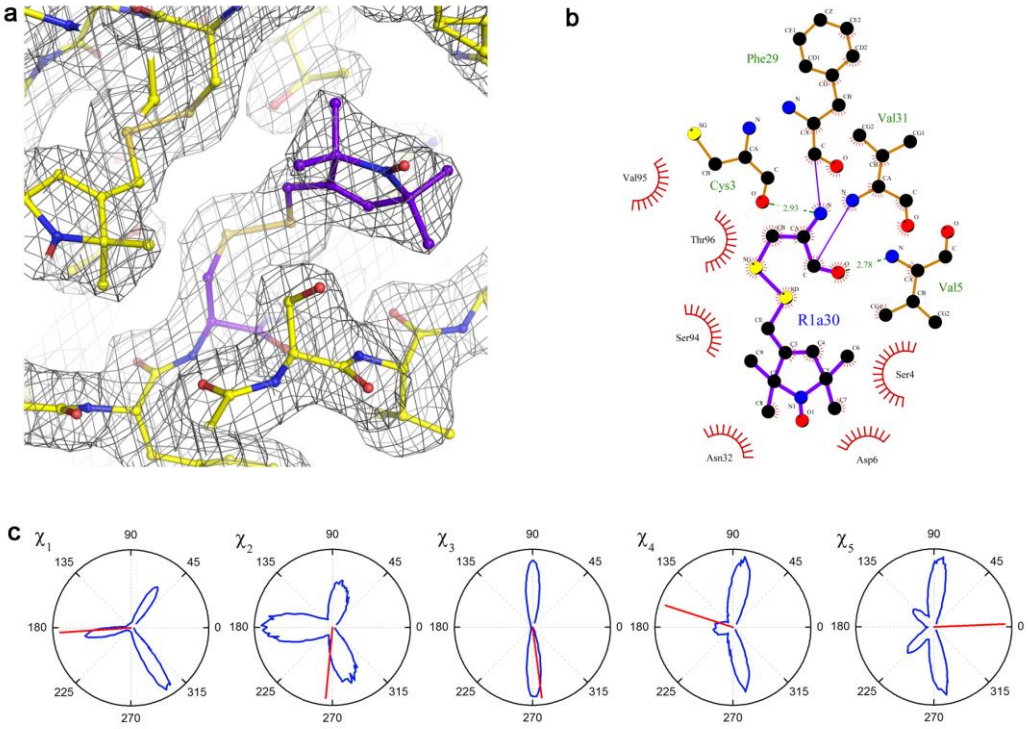


Fig. S5 continued: (caption see below)

T30R1, crystal II, chain K (B-factor = 42.2 Å^2)



T30R1, crystal II, chain L (B-factor = 53.8 Å^2)

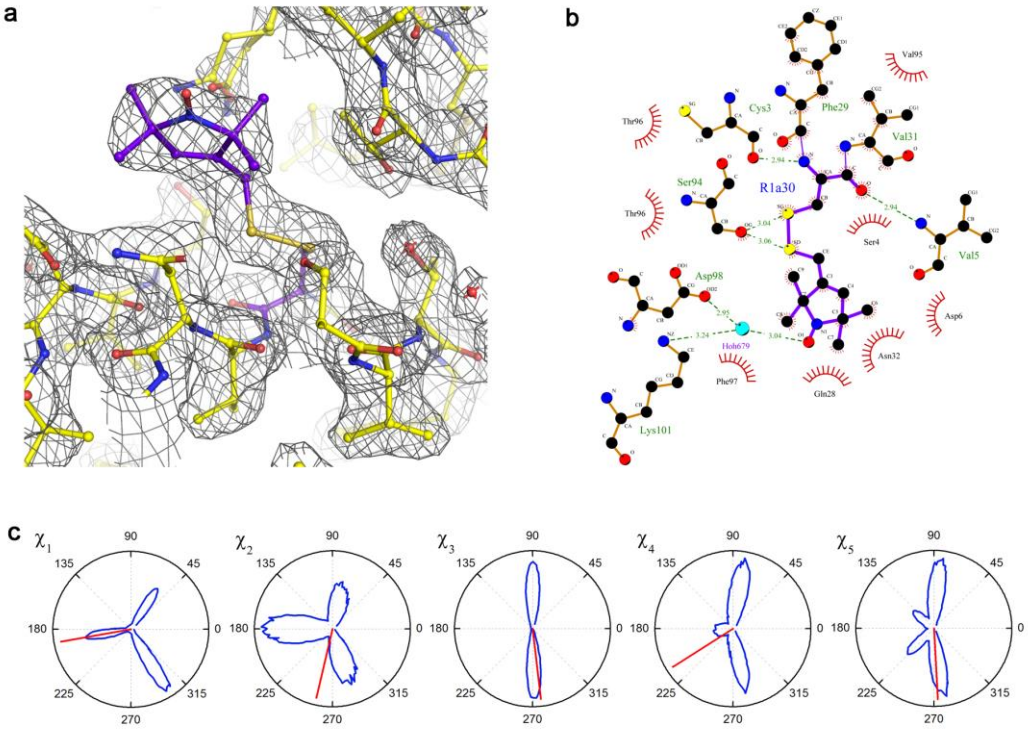
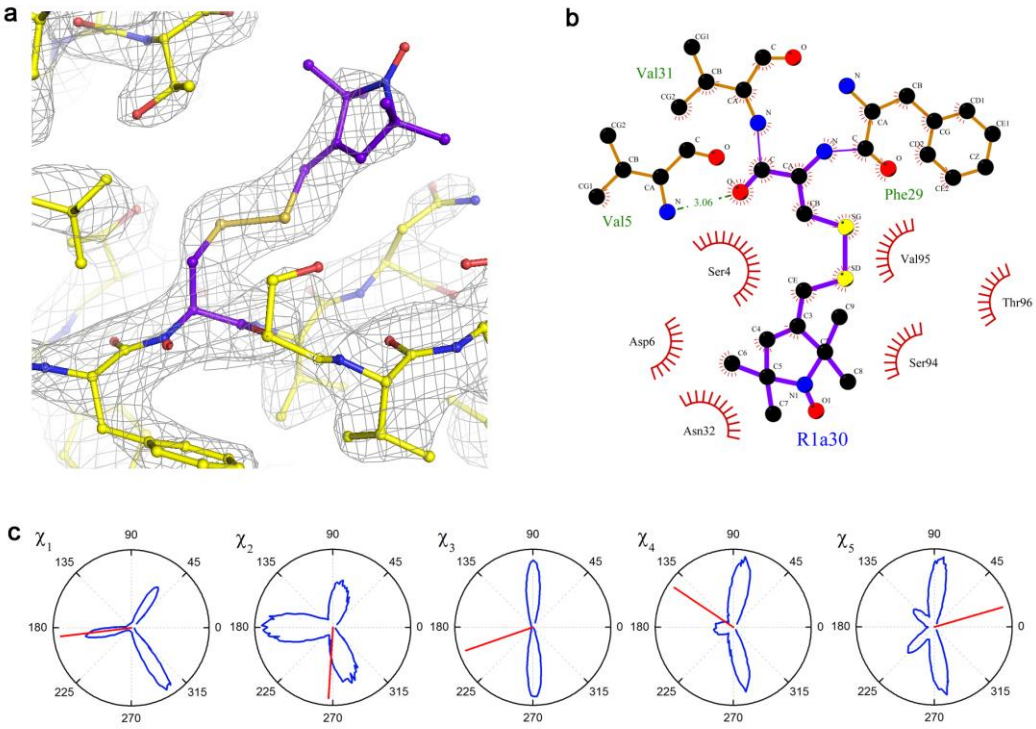
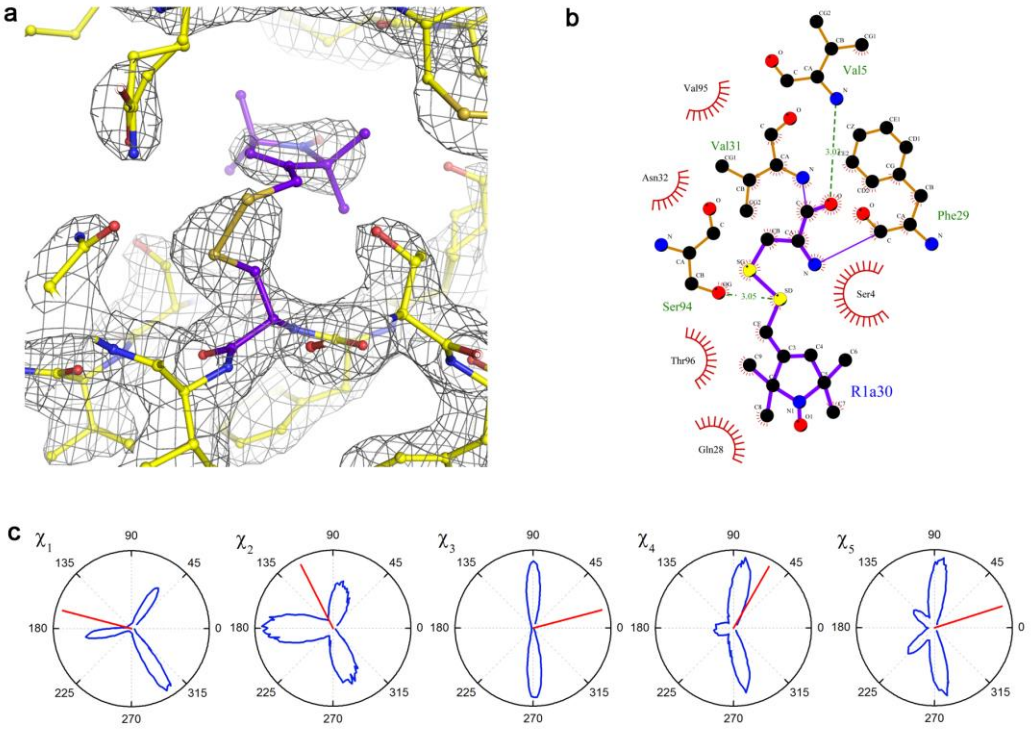


Fig. S5 continued: (caption see below)

T30R1, crystal II, chain M (B-factor = 79.3 Å^2)



T30R1, crystal II, chain N (B-factor = 71.4 Å^2)



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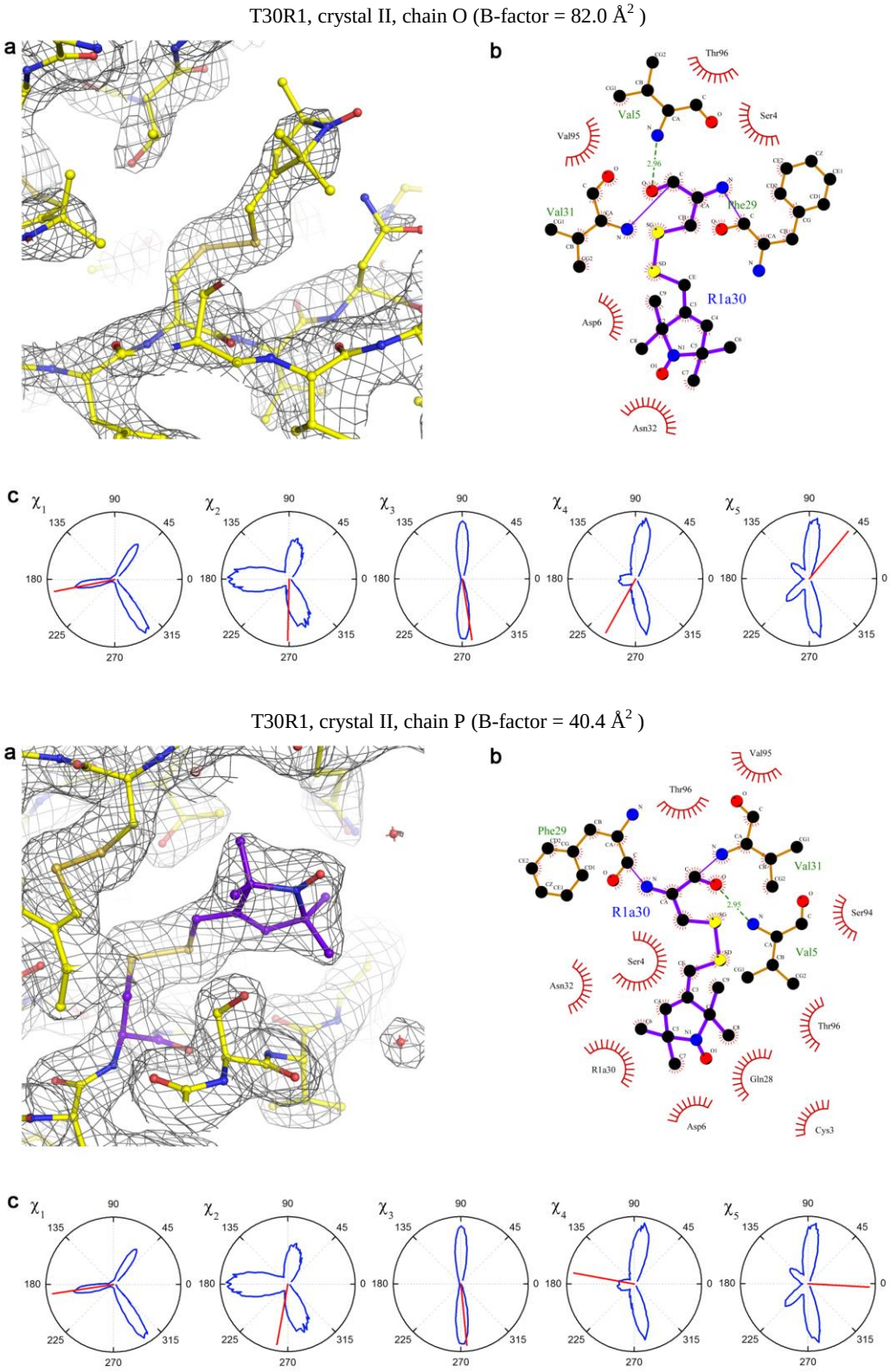

Fig. S5 Twenty X-ray conformers of the R1 side chain in T30R1. **(a)** Electron density of the R1 side chain. The protein is shown as a ball and stick model. The R1 side chain is highlighted in purple. The refined 2Fo-Fc electron density is shown as a grey mesh contoured at 1.0σ. The average B-factor of the side chain is given. Because only a single side chain was modelled for each individual monomer, the occupancy was set to 1.0 in each case. **(b)** Interaction topology diagram. Hydrophobic interactions are indicated as red arcs, polar interactions as dashed lines. Covalent bonds are shown as thick lines. **(c)** The dihedral angles χ_1- χ_5 that are determined for each X-ray conformer compared (red line) to the corresponding distributions from the MMM rotamer library (blue shapes) [Y. Polyhach, E. Bordignon and G. Jeschke, *Phys. Chem. Chem. Phys.*, 2011, **13**, 2356].

Table S3. Dihedral angles of the R1 side chain in crystal structures of the azurin mutant T30R1.

Mutant, crystal form, chain	Quality of electron density[a]	χ_1 [°] [b]	χ_2 [°] [b]	χ_3 [°] [b]	χ_4 [°] [b]	χ_5 [°] [b]	Rotamer[c]
T21R1, I, A	+	-176	78	95	-60	88	*t(p)pmp*
T21R1, I, B	+	-176	73	95	-53	88	*t(p)pmp*
T21R1, I, C	+	-174	-143	93	169	101	*t(t)ptp*
T21R1, I, D	+	180	-140	100	175	96	*t(t)ptp*
T30R1, I, A	+	180	-94	-89	-59	86	*t(m)mmp*
T30R1, I, B	+	-176	-90	-85	-78	-17	*tmmm(m)*
T30R1, I, C	-	(143)	(-74)	(-86)	(-67)	(-155)	
T30R1, I, D	0	-151	-82	-80	(-80)	(-43)	*tmm*
T30R1, II, A	+	-178	-92	-86	171	-6	*t(m)mt(m)*
T30R1, II, B	+	-173	-99	-87	167	2	*t(m)mt(p)*
T30R1, II, C	+	-173	-97	-89	169	5	*t(m)mt(p)*
T30R1, II, D	-	175	(-136)	(-82)	(79)	(50)	*t*
T30R1, II, E	+	-176	-92	-85	159	5	*t(m)mt(p)*
T30R1, II, F	+	-173	-101	-85	-148	-81	*t(m)m(t)m*
T30R1, II, G	0	168	173	(73)	(-164)	(-5)	*tt*
T30R1, II, H	0	64	161	83	46	49	*ptpp(p)*
T30R1, II, I	0	93	178	114	-87	-32	*(p)tpm(m)*
T30R1, II, J	-	-168	-160	(-99)	(152)	(-1)	*tt*
T30R1, II, K	+	-176	-95	-82	162	2	*t(m)mt(p)*
T30R1, II, L	+	-170	-103	-83	-148	-87	*t(m)m(t)m*
T30R1, II, M	0	-173	-93	(-161)	(146)	(16)	*t(m)*
T30R1, II, N	0	165	117	(15)	(60)	(18)	*t(p)*
T30R1, II, O	0	-169	-91	-81	-119	51	*t(m)m(m)p*
T30R1, II, P	+	-171	-100	-84	170	-3	*t(m)mt(m)*

[a] The quality of electron density is classified as: "+" meaning all atoms are clearly visible; "0" meaning slight disorder but overall conformation is clear; "-" meaning the side chain is disordered.

[b] The values in brackets denote the angles that cannot be precisely determined from the electron density.

[c] The *t,p,m* notation of the rotamer states: for the angles χ_1, χ_2, χ_4 – p = 60°±30°, m = 300±30°, t = 180°±30°; for the angles χ_3, χ_5 – p = 90°±30°, m = -90±30° [Z. Guo, D. Cascio, K. Hideg K, T. Kálái and W. L. Hubbell, *Protein Sci.*, 2008, **17**, 228; G. Jeschke, *Prog. Nucl. Magn. Reson. Spectrosc.*, 2013, **72**, 42]. The values in brackets denote the angles falling outside the ±30° interval. In these cases, the closest state is indicated.