Supporting Information

for

Tyrosine-67 in cytochrome *c* is a possible apoptotic trigger controlled by hydrogen bonds *via* conformational transition

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Experimental Section

Mutagenesis, Protein Expression and Purification

The original pBTR1 plasmid, which encodes genes of the yeast iso-1-cytochrome *c* (*CYC*1) and yeast cytochrome *c* heme lyase (*CYC*3), has the native Cys102 residue substituted by a threonine residue to prevent formation of disulphide bonds between cyt *c* molecules, and also contains a mutation of K72A to prevent this residue to serve as a ligand in the alkaline form of the protein.[S1, S2] For the purpose of this work, this variant is used as the reference cytochrome and is referred to as wild-type protein (WT). Mutations of cyt *c* Y67H and Y67R variants were prepared using a QuikChange Site-Directed Mutagenesis Kit (Stratagene). In brief, beginning with two anti-parallel primers carrying codons for the required substitutions. The oligonucleotides used were as follows with mutant codon(s) underlined:

Y67H primer-1: 5'-C GAA AAT AAC ATG TCA GAG CAC TTG ACT AAC CCA GC-3',

Y67H primer-2: 5'-GC TGG GTT AGT CAA GTG CTC TGA CAT GTT ATT TTC G-3';

Y67R primer-1: 5'-AC GAA AAT AAC ATG TCA GAG CGC TTG ACT AAC CCA GCG AA-3',

Y67R primer-2: 5'-TT CGC TGG GTT AGT CAA GCG CTC TGA CAT GTT ATT TTC GT-3'.

The plasmid of pBTR1 was PCR amplified in the elongation process using *PfuTurbo* DNA polymerase according to the manufacture's protocol. The incorporation of the two oligonucleotides primers generates a mutated plasmid containing stagger nicks. The product was then treated with *Dpn* I restriction enzyme, specific for methylated and hemimethylated DNA, then the parental DNA template was digested (almost all DNA isolated from commonly *E. coli* strains is usually dam methylated). The nicked vector DNA containing the desired mutations was then transformed into XL1–Blue super-competent cells. The plasmid DNA was isolated using a QIAprep Spin Miniprep Kit (Qiagen) and the identity of the mutants were confirmed by DNA sequencing.

Expression and purification of iso-1-cyt *c* were performed according to the methods described

previously[S1-S3] with minor modifications.

Cultures were initiated with a single, freshly transformed *E. coli* BL21(DE3)pLysS (purchased from Novagen) that served as the inoculum for 50 mL SB media supplemented with 100 mg/mL ampicillin to 0.1% (V/V). After overnight incubation at 37 °C with vigorous shaking (300 rpm), 5 mL was used to inoculate 800 mL modified SB (tryptone: 10 g/L, yeast extract: 8 g/ L, sodium chloride: 5 g/L, glycerin: 1.5 mL/L, sodium nitrate: 2 g/ L) with 100 mg/L ampicillin in a 1 liter flask and were incubated for 24–36 hrs. The cells were harvested by centrifugation (Sorvall SLA-3000 rotor, 4 °C, 5000 rpm, 15 min) and resuspended in lysis buffer (50 mM Tris-HCl, 1 mM EDTA, pH 8.0) with 3 g/L lysozyme and a few crystals of DNase I and RNase A. The mixture was stirred continuously for 1 hour at 4 °C, and then lysed by sonication on ice. This lysate was cleared by centrifugation (Sorvall SS-34 rotor, 4 °C, 15000 rpm, 20 min.), and the resulting solution was collected. The pellet was resuspended in lysis buffer and centrifugated repeatedly until the pellet and the supernatant were no longer pink. The colored fractions were pooled, and $(NH_4)_2SO_4$ (25% w/v) was added over a period of 30 min while stirring gently at 4 °C. The resulting suspension was centrifuged before dialyzing the supernatant fluid overnight in distilled water (4 °C). The dialysate was centrifuged again, and the protein in the cleared solution was loaded on a column of CM-23 cation exchange resin equilibrated with Buffer A (50 mM sodium phosphate, pH 6.8). After loading, the column was washed with two volumes of Buffer A followed by two volumes of Buffer B (Buffer A plus 75 mM NaCl). Then the recombinant cytochrome was eluted with Buffer C (Buffer A plus 250 mM NaCl) and exchanged into Buffer A by repeated ultrafiltration with YM-5 membrane (Amicon). Samples were oxidized with $K_3[Fe(CN)_6]$ immediately prior to the final purification by cation exchange chromatography with a Pharmacia Mono-S (HR 10/10) column equilibrated with sodium phosphate buffer (20 mM, pH 7.0). The protein was eluted with a linear NaCl gradient of 1-300 mM/mL, Fractions with the R-value (A_{410}/A_{280}) > 4.5 were pooled, concentrated, and exchanged into Buffer A prior to flash-freezing in liquid nitrogen, lyophilized and storage at –20 °C until needed. The Y67H and Y67R mutant proteins were prepared with the same procedure.

Mass spectroscopy

Molecular weight of the wild-type cyt *c*, Y67H variant were measured by electrospray ionization mass spectrometry (ESI-MS) using a Bruker Esquire 3000 Electrospray Mass Spectrometer (Bruker Daltonicsk Germany). The protein solutions were first desalted by ultrafiltration or dialysis, and then dissolved in 10 % formic acid (v/v). (Figure S1-S2).

UV–Visible spectroscopy

The UV-Visible spectra of wild-type cyt *c*, Y67H and Y67R mutant proteins were recorded on a Hewlett-Packard 8453 diode array spectrometer equipped with a Neslab RTE-111 water circulator,

controlled by laboratory software. Proteins were dissolved in 100 mM sodium phosphate buffer, pH 7.0. Protein concentrations were determined by using the pyridine hemochrome method.[S4]

Circular dichroism (CD) spectroscopy

The CD spectra of the wild-type cyt *c*, Y67H and Y67R proteins were collected from 190 to 250 nm (0.1 cm path length) and from 250 to 600 nm (1.0 cm path length), respectively, with a Jasco model J-715 spectropolarimeter equipped with a Neslab RTE-111 water circulator. The instrument was programmed to acquire spectra at 5° internals over the range 15-90°. The protein samples were dissolved in 100 mM sodium phosphate buffer, pH 7.0.

Fluorescence spectroscopy

The fluorescence was measured using a Varian Cary Eclipse fluorescence spectrophotometer with excitation and emission wavelengths of 279 nm and 350 nm. The temperature was controlled by a Neslab RTE-111 water circulator. Proteins were dissolved in 100 mM sodium phosphate buffer, pH 7.0.

Stead-state kinetic studies

The steady-state kinetics of oxidation of guaiacol by hydrogen peroxide were studied with a SF-61 DX2 stopped-flow apparatus (Hi-Tech, UK) thermostated at 16.0 ± 0.1 ˚C and 42.0 ± 0.1 ˚C, respectively. The H_2O_2 solution was prepared with 30% stock solution and its concentration was determined with an absorption coefficient of 39.4 $M^{-1}cm^{-1}$ at 240 nm.[S5] A solution of cyt *c* (2.0 μM) and various concentration of guaiacol (2-500 μM) in 100 mM sodium phosphate buffer (pH 7.0) and a solution of 400 mM H_2O_2 in the same buffer were preincubated at 16.0 ± 0.1˚C or 42.0 ± 0.1˚C for 5 min. Then, both solutions were mixed together in the mixing cell of the stopped-flow instrument to start the oxidation reaction. The steady-state reaction rates were obtained by monitoring the absorbance increase at 470 nm using a molar absorption coefficient of 26.6 $mM^{-1}cm^{-1}$.[S6, S7]

The product formation curve of guaiacol oxidation catalyzed by WT yeast iso-1-cyt *c*, or its variants, is similar to that catalyzed by *Paracoccus versutus* cytochrome *c*-550[S8, S9] as shown in Figure S3, which exhibits an lag phase (I) before a linear phase (II), then followed by a decrease in activity (III) and finally a decrease in absorption. The rate of the steady-state reaction was determined by taking the maximum of the first derivative of the product formation curve (i.e. the linear phase).

[S1] W.B. Pollock, F.I. Rosell, M.B. Twitchett, M.E. Dumont and A.G. Mauk, Biochemistry 37 (1998) 6124-31.

[S2] F.I. Rosell and A.G. Mauk, Biochemistry 41 (2002) 7811-8.

[S3] G. Silkstone, G. Stanway, P. Brzezinski and M.T. Wilson, Biophys Chem 98 (2002) 65-77.

[S4] E.A. Berry and B.L. Trumpower, Anal Biochem 161 (1987) 1-15.

[S5] D.P. Nelson and L.A. Kiesow, Anal Biochem 49 (1972) 474-8.

[S6] D.A. Baldwin, H.M. Marques and J.M. Pratt, J Inorg Biochem 30 (1987) 203-17.

[S7] G.D. DePillis, B.P. Sishta, A.G. Mauk and P.R. Ortiz de Montellano, J Biol Chem 266 (1991) 19334-41.

[S8] R.E. Diederix, M. Ubbink and G.W. Canters, Eur J Biochem 268 (2001) 4207-16.

[S9] R.E. Diederix, M. Fittipaldi, J.A. Worrall, M. Huber, M. Ubbink and G.W. Canters, Inorg Chem 42 (2003) 7249-57.

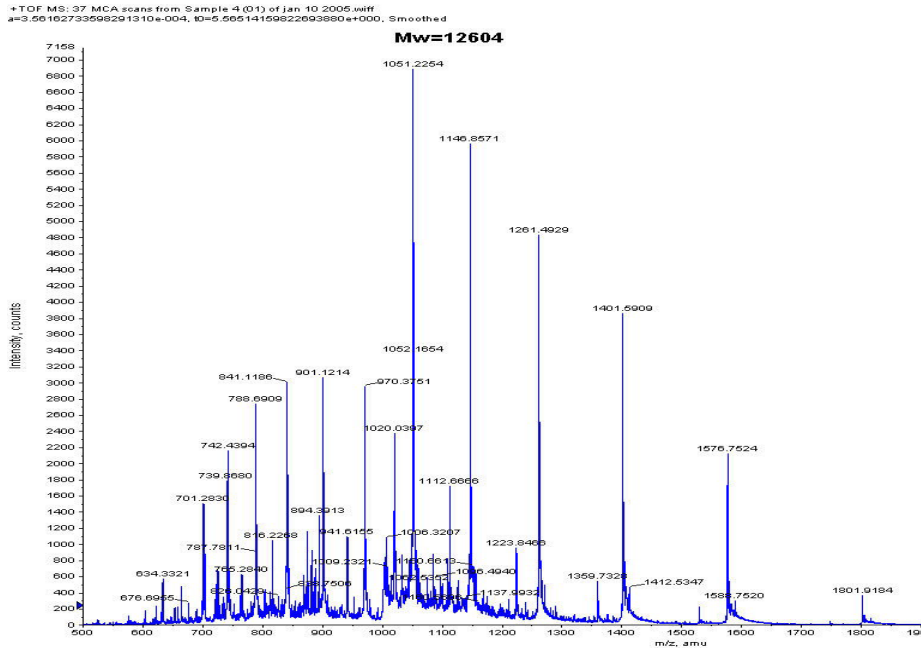

Figure S1. ESI-MS of wild-type yeast iso-1-cytochrome *c*, the measured molecular weight is 12604±2 dalton. (calculated molecular weight: 12606)

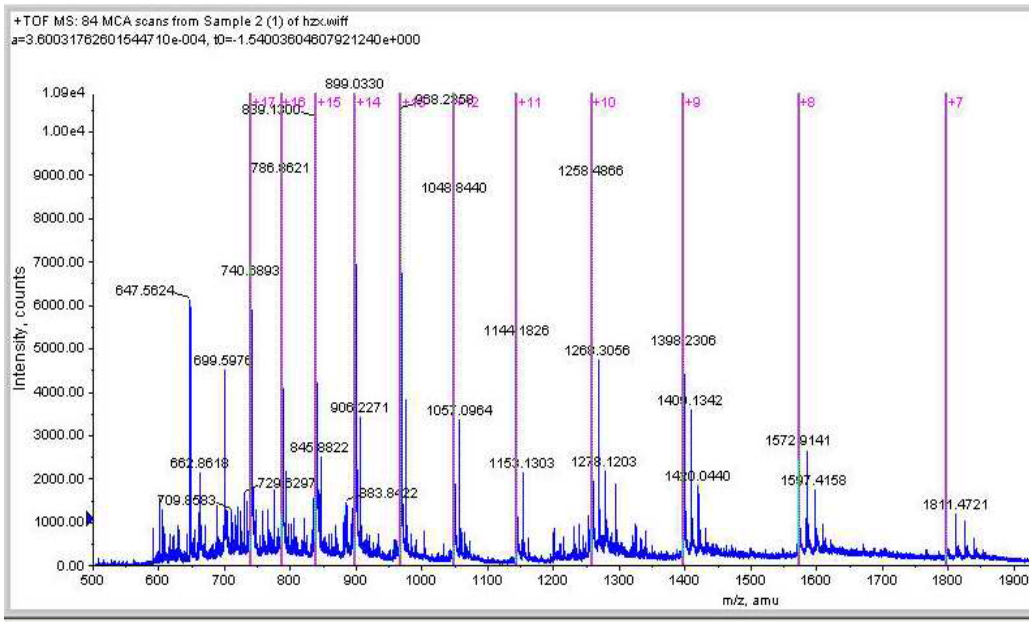

Figure S2. ESI-MS of cyt *c* Y67H variant, the measured molecular weight is 12574±6 dalton. (calculated molecular weight: 12580)

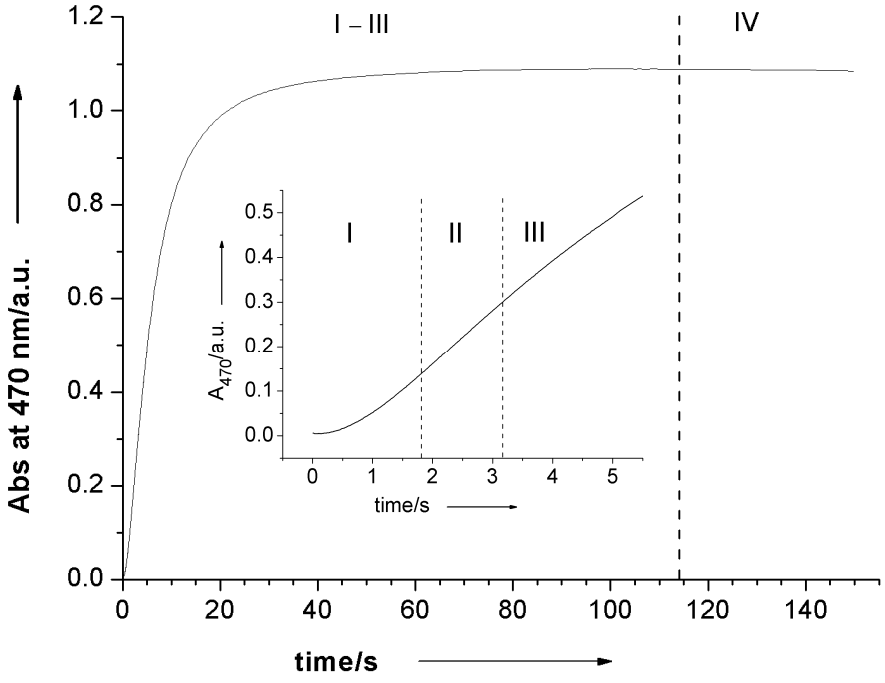

Figure S3. A formation curve of typical guaiacol oxidation product, tetraguaiacol, catalyzed by the cytochrome *c* variant monitored at 470 nm. Conditions: 1 μM protein, 1 mM guaiacol, 75 mM H_2O_2 in 100 mM sodium phosphate buffer (pH 6.0) at 25.0 ± 0.1 ˚C. (Inset) The first 5-second curve of the product formation. The four phases labeled I–IV are explained in text.

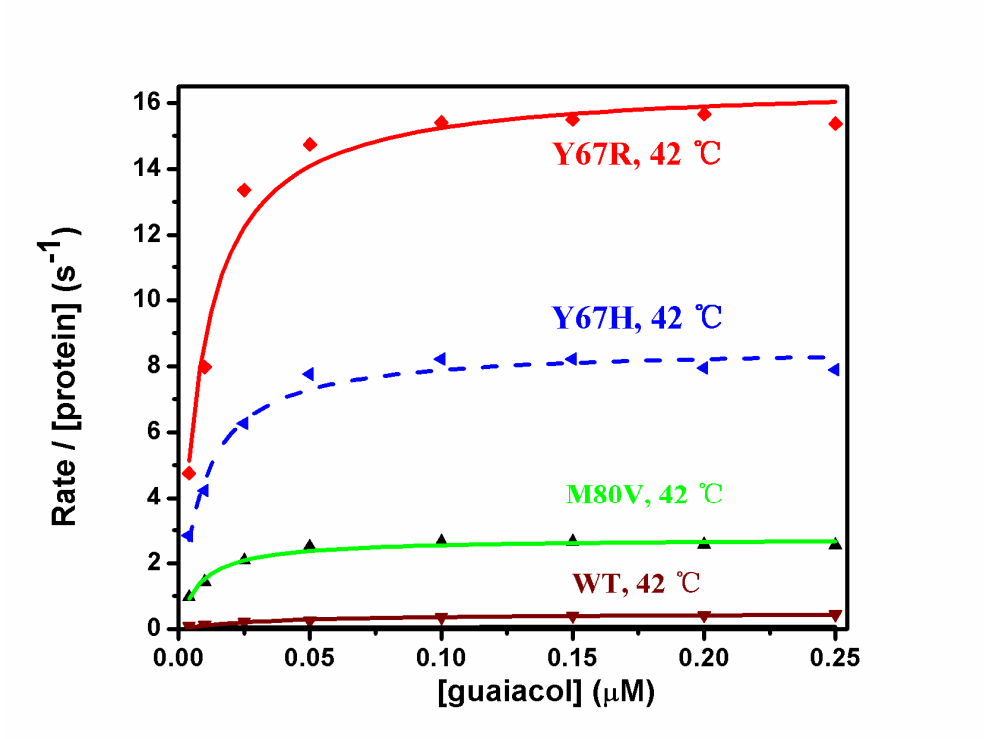

Figure S4. Steady-state rate of the peroxidation as a function of guaiacol concentration at 42ºC.

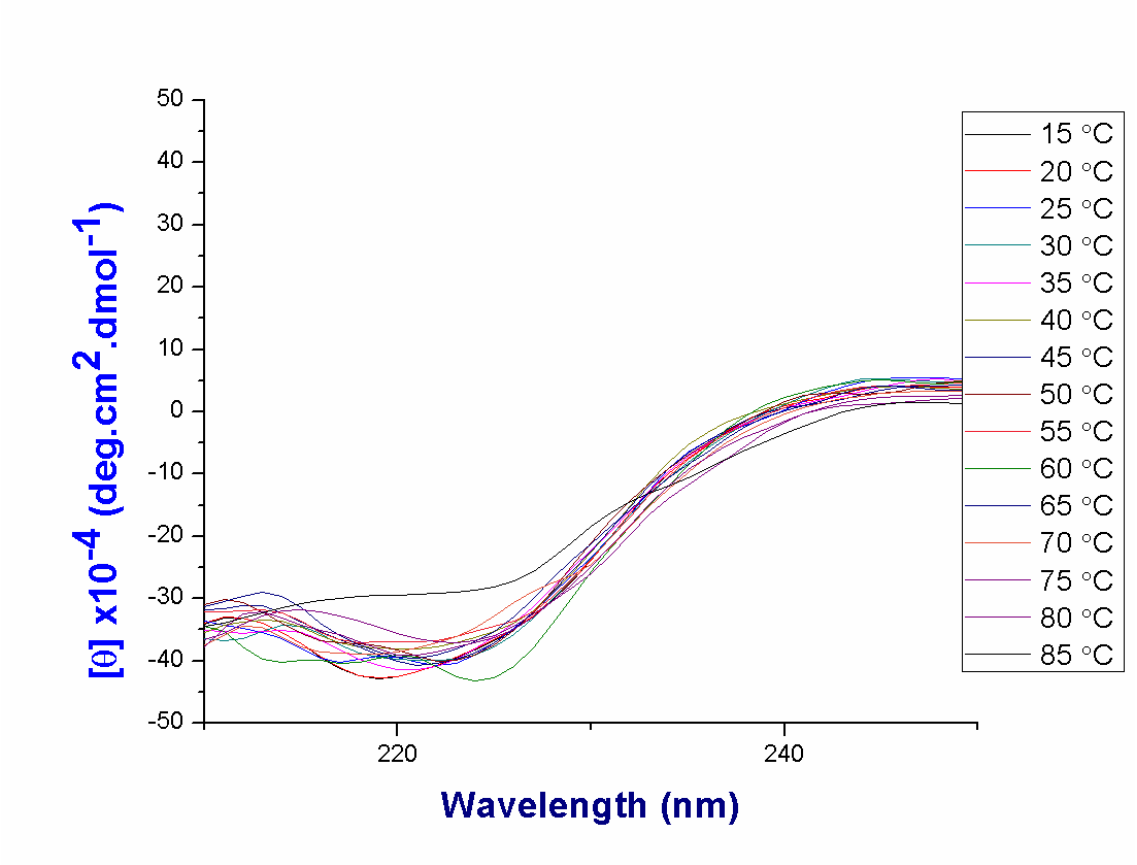

Figure S5. The far-UV CD spectra of WT cyt *c*.

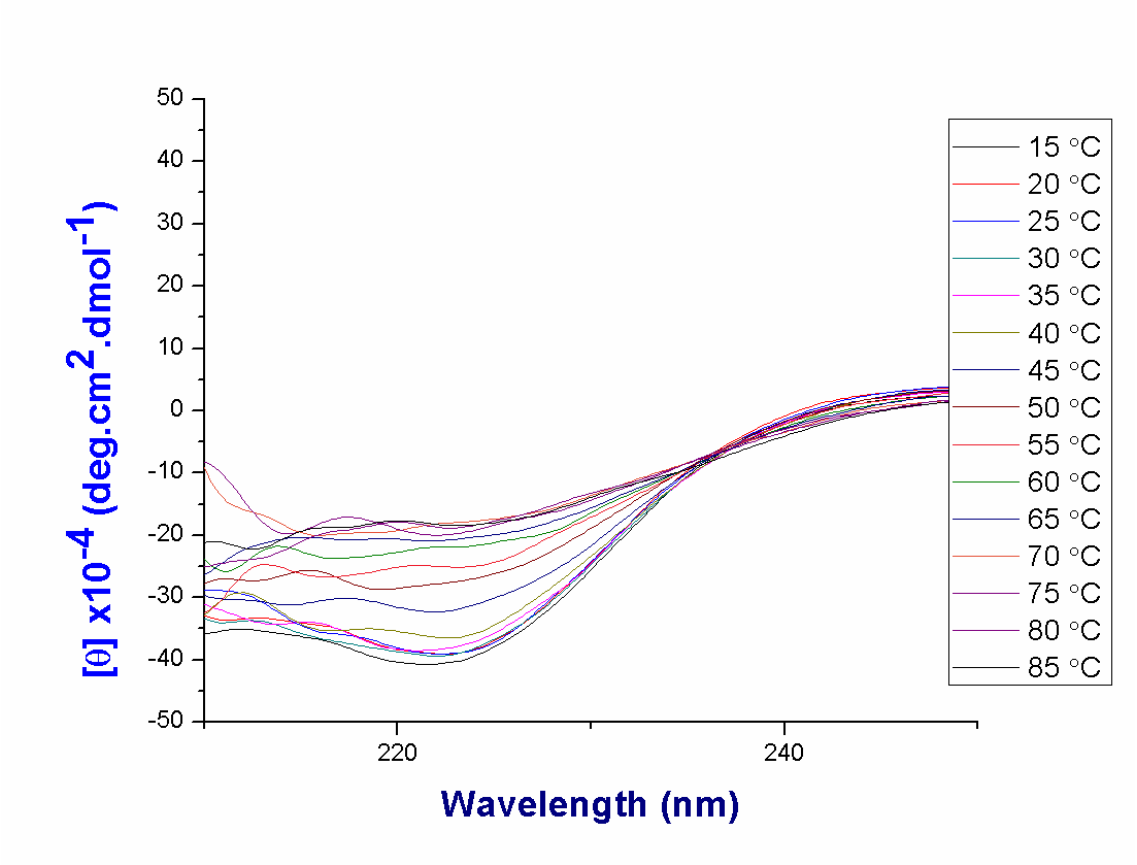

Figure S6. The far-UV CD spectra of the Y67H variant.

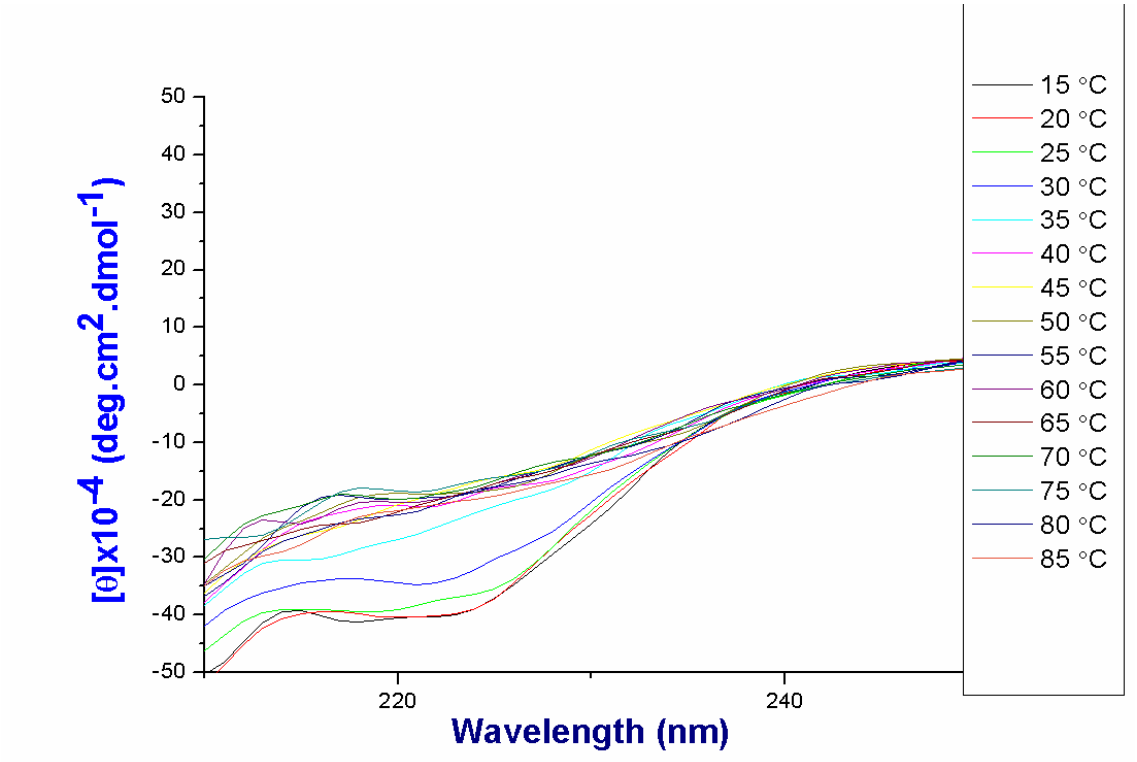

Figure S7. The far-UV CD spectra of the Y67R variant.

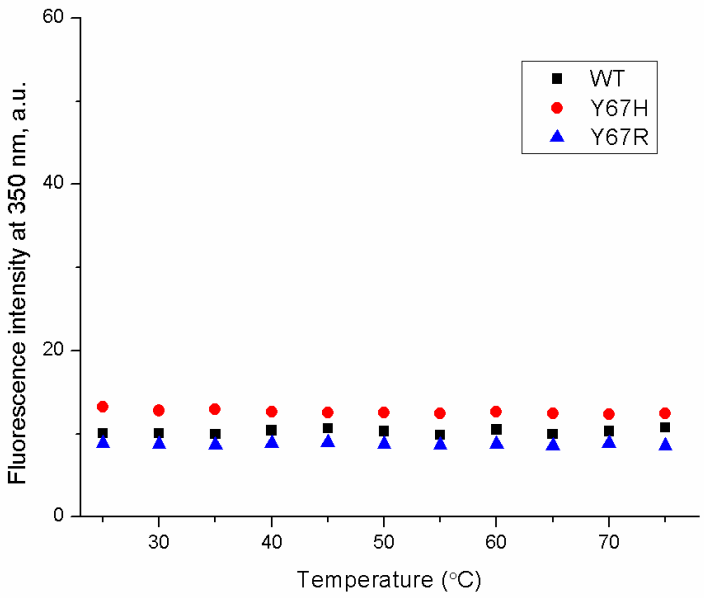

Figure S8. Plots of the change in fluorescence intensity at 350 nm with increasing temperature for the proteins.