Supporting Information

Supplemental Table 1. Crystallographic Data[a]

STRUCTURE	CYTC (WT) MOUSE[b]	CYTC (T28A) MOUSE	CYTC (T28E) MOUSE	CYTC (Y47F) HUMAN[c]
PDB CODE	**5C0Z**	**5C9M**	**5DF5**	**3ZOO**
CRYSTALLIZATION				
Iron	Oxidized	Oxidized	Oxidized	Oxidized
Protein	15 mg/mL Cytc (WT) + 5 mM $K_3Fe(CN)_6$ in water	22 mg/mL Cytc (T28A) + 5 mM $K_3Fe(CN)_6$ in water	15 mg/mL Cytc (T28E) + 5 mM $K_3Fe(CN)_6$ in water	12.5 mg/mL oxidized protein in 22.5% (w/v) PEG 1000, 50 mM KH_2PO_4, pH 7.0
Well	25% PEG 4K, 8% isopropanol, 0.1 M Na acetate, pH 6.5	25% PEG 4K, 8% isopropanol, 0.1 M Na acetate, pH 6.5	30% PEG 1500, pH 4.5	26-31% (W/V) PEG 1000, 40 mM, KH_2PO_4 pH 7.0
Drop	1:1 protein:well	1:1 protein:well	1:1 protein:well	n/a
Cryoprotectant	30% PEG 4K, 8% iso propanol, 0.1M Na acetate, 20% ethylene glycol, 10 min soak with 5 mM $K_3Fe(CN)_6$	30% PEG 4K, 8% iso propanol, 0.1 M Na acetate, 20% ethylene glycol, 10 min soak with 5 mM $K_3Fe(CN)_6$	35% PEG 1500, 20% ethylene glycol	26-31% (w/v) PEG 1000, 40 mM, KH_2PO_4 pH 7.0, 15% glycerol
CRYSTAL DATA				
Space group:	P1	P1	P1	P1
Unit cell: a	34.401	34.517	34.609	36.367
b	52.471	52.567	51.739	53.952
c	61.647	61.771	61.748	58.95
Alpha	110.04	109.86	110.01	76.55
Beta	92.77	92.93	93.09	88.73
Gamma	92.02	92.20	91.88	71.86
Chains per A.U.	4	4	4	4
Matthews Coeff.	2.24	2.27	2.25	2.3
Solvent %	45.12	45.7	45.26	46.7
X-RAY DATA				
Resolution-high (Å)	1.124	1.36	1.30	1.35
Resolution-low (Å)	49.22	57.96	57.88	30.59
Beamline	APS 21-ID-F	APS 21-ID-F	APS 21-ID-D	DIAMOND 103 (UK)
Wavelength	0.97872	0.97872	1.07812	0.9762
Reflections	127840	76544	90455	81707
Completeness	87.4 (44.66)	92.4 (66.2)	96.6 (89.38)	94.73 (80.71)
Average I/sigma	14.4 (2.0)	14.1 (2.2)	14.9 (2.4)	8.70 (n/a)
Redundancy	3.9 (3.7)	3.9 (3.8)	5.3 (4.2)	n/a
Rmerge	0.05 (0.548)	0.052 (0.580)	0.081 (0.504)	0.07
REFINEMENT				
Rfactor	0.132 (0.239)	0.129 (0.229)	0.148 (0.256)	0.13821

Rfree	0.159 (0.240)	0.170 (0.303)	0.178 (0.252)	0.17935
Avg. B-factor (Å^2)	15.97	21.41	19.32	17.703
Protein atoms per A.U.	3228	3220	3236	3981
Water molecules	537	472	334	417
Bond RMSD	0.017	0.016	0.018	0.017
Angle RMSD	1.903	1.803	1.884	1.99
Chiral RMSD	0.124	0.122	0.131	0.125
FC6 OCCUPANCY				
202a/A	0.28	0.35	–	
202b/A	0.37	0.34	–	
202/B	0.93	0.98	0.92	
203/B	–	–	0.48	
203a/B	0.30	0.39	–	
203b/B	0.32	0.28	–	
202/C	0.90	1.00	0.89	
HEME LINKS:				
C14 SG - HEM CAB (Å)				
Chain-A	1.86	1.92	1.91	1.93
Chain-B	1.86	1.93	1.90	1.96
Chain-C	1.91	1.94	1.95	1.96
Chain-D	1.95	2.02	1.94	1.93
Average	1.90	1.95	1.93	1.95
C17 SG - HEM CAC (Å)				
Chain-A	1.98	2.09	1.96	2.06
Chain-B	1.94	2.07	1.99	2.21
Chain-C	1.97	2.12	2.07	2.16
Chain-D	2.04	2.21	2.06	2.08
Average	**1.98**	**2.12**	**2.02**	**2.13**
H18 NE2 - FE2 (Å)				
Chain-A	2.02	2.03	2.00	2.00
Chain-B	2.03	2.00	2.02	2.06
Chain-C	2.02	2.04	2.03	2.01
Chain-D	2.01	2.07	2.02	2.02
Average	**2.02**	**2.04**	**2.02**	**2.02**
M80 SD - FE2 (Å)				
Chain-A	2.29	2.27	2.28	2.29
Chain-B	2.28	2.27	2.27	2.29
Chain-C	2.30	2.28	2.30	2.24
Chain-D	2.31	2.27	2.29	2.29
Average	**2.30**	**2.27**	**2.29**	**2.28**

[a]All three structures have 4 molecules in a P1 unit cell with very similar dimensions. When the

twelve independently refined Cyt*c* molecules are overlapped with each other using their main chain atoms, the average RMSD value among the 66 unique pairs is 0.189 Å, and the highest value is 0.280 Å between chain-A in WT and chain-D in Thr28Glu. Comparing equivalent chains, the three A-chains have the smallest RMSD (0.080 Å) followed by the B-chains (0.107 Å), the D-chains (0.0.113 Å) and the C-chains (0.140 Å). The three Cyt*c* "tetramers" reported here have a similar orientation in the P1 unit cell. The Thr28Ala and Thr28Glu mutants have RMSD values, based on backbone atoms, of 0.151 Å and 0.330 Å, respectively, when superposed on the WT structure as tetramers. The Thr28Ala structure is more similar to the WT structure because it was crystallized at pH 6.5 from the exact same conditions as the WT protein, whereas the Thr28Glu mutant was crystallized from a different solution at pH 4.5. The Thr28Glu mutant only crystallized at pH 4.5 because the four Glu28 residues are arranged in closely adjacent pairs in the "native" tetramer and destabilize the crystal packing if they are deprotonated (see Supplemental Figures 3 and 4). The Thr28Glu crystal structure has the same two major FC6 ligands (FC6 202/B and FC6/C) as the WT structure and a third, unique site (FC6 203/B), which is possibly related to the lower pH. It has no significant density for the two minor sites (FC6 202/A and FC6 203/B) in the WT and Thr28Ala crystal structures. The lower occupancies of the minor FC6 sites suggest they are weakly bound and were probably lost from the Thr28Glu crystals during the crystal freezing procedure, which used a cryo-solution soak that did not include the FC6 reagent.

[b]Note that the mouse and rat Cyt*c* amino acid sequences are identical.

[c]Mouse and human Cyt*c* monomers are similar but their tetramers differ: The mouse WT structure was solved using chain-A from the crystal structure of human Cyt*c* (see Methods), and the two refined chains have a RMSD of 0.266. Also, the three mouse structures, like the human structure, were solved and refined using the free heme moiety (PDB ligand code: HEM) and finished with very similar, unconstrained bond distances for the dative bonds to heme iron and the covalent links to Cys14 and Cys17. In contrast, the tetramers observed in both crystal structures, have a RMSD overlap of 17 Å because the Cyt*c* molecules have very different orientations within the tetramer (not shown). The tetramers are crystal entities and do not exist in solution. All three Cyt*c* monomers – WT, Thr28Ala, Thr28Glu – have a region with strong positive electrostatic potential (ESP) (Supplemental Figure 4E and F). In the three structures reported here, the monomers are arranged with their positive ESP regions adjacent to each other in pairs. This arrangement is possible because the FC6 ligands with their -3 charge balance the strong positive ESP. The human Cyt*c* lacks these ligands. Consequently, the monomers are rotated within the human tetramer so the ESP potentials are distant from each other (not shown).

Supplemental Figures

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                10        20        30        40        50        60        70        80        90        100
                *         *         *         *         *         *         *         *         *         *
Human     GDVEKGKKIFIMKCSQCHTVEKGGKHKTGPNLHGLFGRKTGQAPGYSYTAANKNKGIIWGEDTLMEYLENPKKYIPGTKMIFVGIKKKEERADLIAYLKKATNE
Horse     GDVEKGKKIFVQKCAQCHTVEKGGKHKTGPNLHGLFGRKTGQAPGFSYTDANKNKGITWKEETLMEYLENPKKYIPGTKMIFAGIKKKTERADLIAYLKKATNE
Bull      GDVEKGKKIFVQKCAQCHTVEKGGKHKTGPNLHGLFGRKTGQAPGFSYTDANKNKGITWGEETLMEYLENPKKYIPGTKMIFAGIKKKGERADLIAYLKKATNE
Pig       GDVEKGKKIFVQKCAQCHTVEKGGKHKTGPNLHGLFGRKTGQAPGFSYTDANKNKGITWGEETLMEYLENPKKYIPGTKMIFAGIKKKGERADLIAYLKKATNE
Goat      GDVEKGKKIFVQKCAQCHTVEKGGKHKTGPNLHGLFGRKTGQAPGFSYTDANKNKGITWGEETLMEYLENPKKYIPGTKMIFAGIKKKGERADLIAYLKKATNE
Dog       GDVEKGKKIFVQKCAQCHTVEKGGKHKTGPNLHGLFGRKTGQAPGFSYTDANKNKGITWGEETLMEYLENPKKYIPGTKMIFAGIKKTGERADLIAYLKKATKE
Mouse     GDVEKGKKIFVQKCAQCHTVEKGGKHKTGPNLHGLFGRKTGQAAGFSYTDANKNKGITWGEDTLMEYLENPKKYIPGTKMIFAGIKKKGERADLIAYLKKATNE
Rat       GDVEKGKKIFVQKCAQCHTVEKGGKHKTGPNLHGLFGRKTGQAAGFSYTDANKNKGITWGEDTLMEYLENPKKYIPGTKMIFAGIKKKGERADLIAYLKKATNE
Bat       GDVEKGKKIFVQKCAQCHTVEKGGKHKTGPNLHGLFGRKTGQAPGFSYTDANKNKGITWGEATLMEYLENSKKYIPGTKMIFAGIKKSAERADLIAYLKKATNE
Dolphin   GDIEKGKKIFVQKCAQCHTVEKGGKHKTGPNLHGLFGRKTGQAVGFSYTDANKNKGITWGEETLMEYLENPKKYIPGTKMIFAGIKKKXERADLIAYLKKATNE

Bull-T    ADAEAGKKIFIQKCAQCHTVEKGGKHKTGPNLWGLFGRKTGQAPGFSYTEANKNKGIIWGEQTLMEYLENPKKYIPGTKMIFAGLKKKSEREDLIEYLKQATSS
Mouse-T   GDAEAGKKIFVQKCAQCHTVEKGGKHKTGPNLWGLFGRKTGQAPGFSYTDANKNKGVIWSEETLMEYLENPKKYIPGTKTIFAGIKKKSEREDLIKYLKQATSS
Rat-T     GDAEAGKKIFIQKCAQCHTVEKGGKHKTGPNLWGLFGRKTGQAPGFSYTDANKNKGVIWTEETLMEYLENPKKYIPGTKMIFAGIKKKSEREDLIQYLKEATSS
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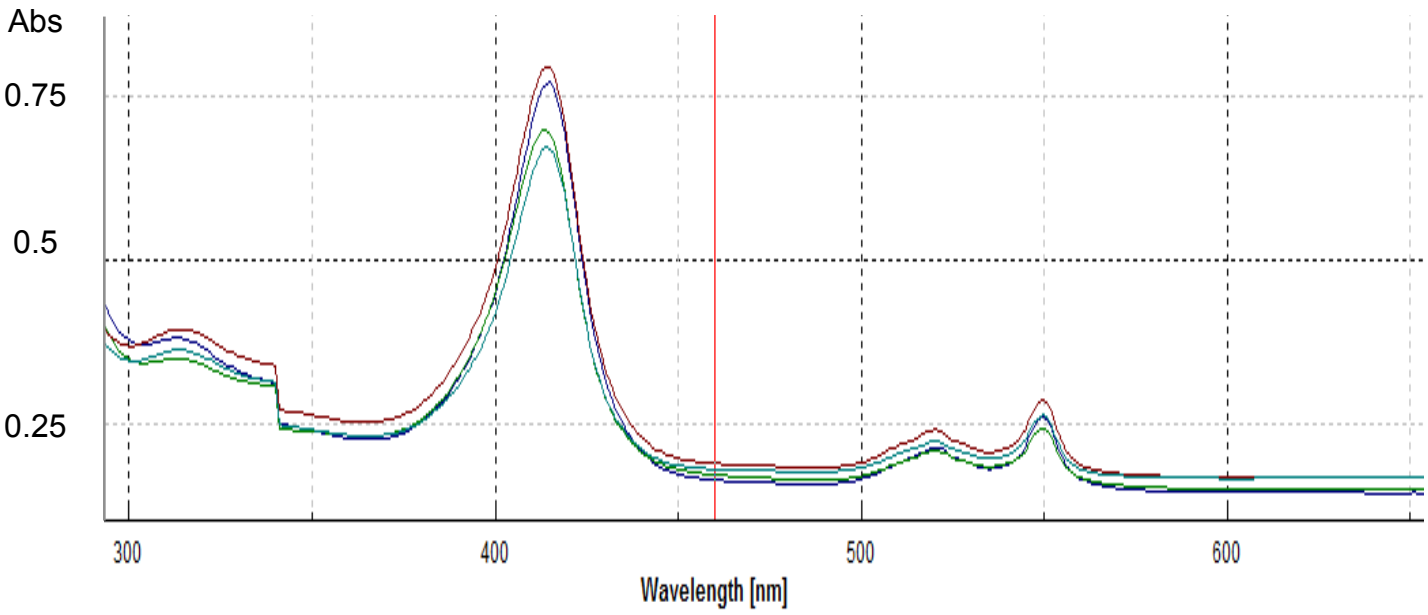
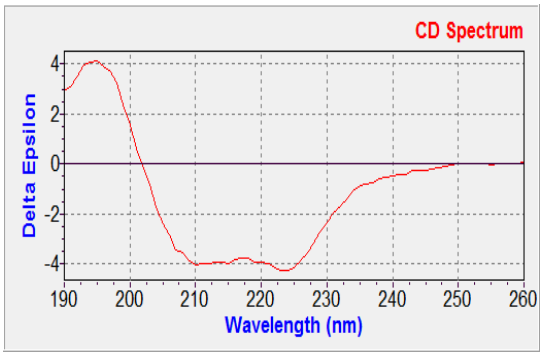
Supplemental Figure 1A. Alignment of mammalian somatic (top) and testes (T, bottom) Cyt*c* sequences. Conserved amino acids are highlighted in yellow. Thr28 is highlighted in bold print. Accession numbers: NP_061820 (human); NP_001157486 (horse); NP_001039526 (cow); NP_001123442 (pig); NP_001183974 (dog); CAA25899 (mouse); NP_036971 (rat); EPQ18492 (bat); AJF48831 (goat); AFN27378 (dolphin); AAI02715 (bull testes); AAH59728 (mouse testes); NP_036972 (rat testes).

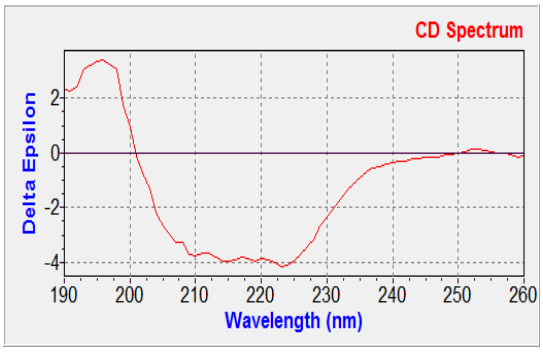


Supplemental Figure 1B. Spectral analysis of overexpressed and purified reduced WT (blue), Thr28Ala (green), Thr28Glu (red), and cow heart Sigma Cyt*c* as control (cyan) indicates correct folding and functionality of the proteins.

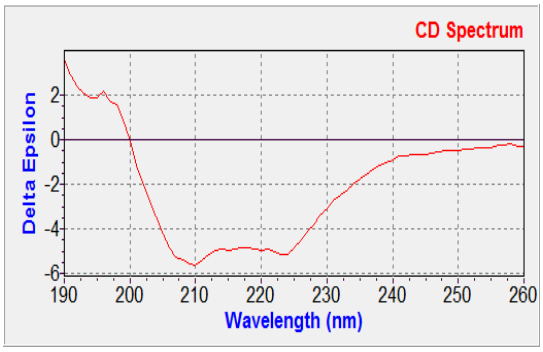
WT



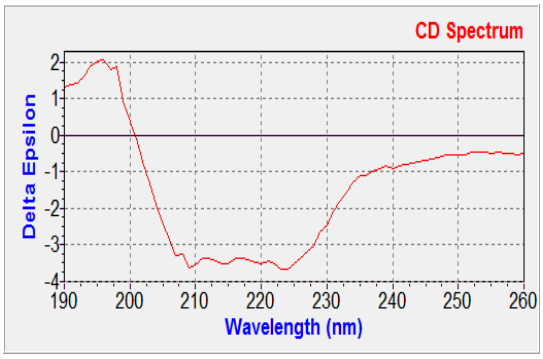
Thr28Glu



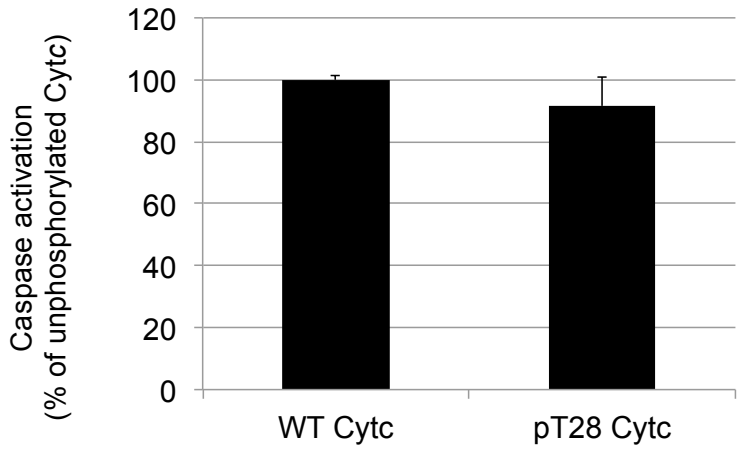
Thr28Ala



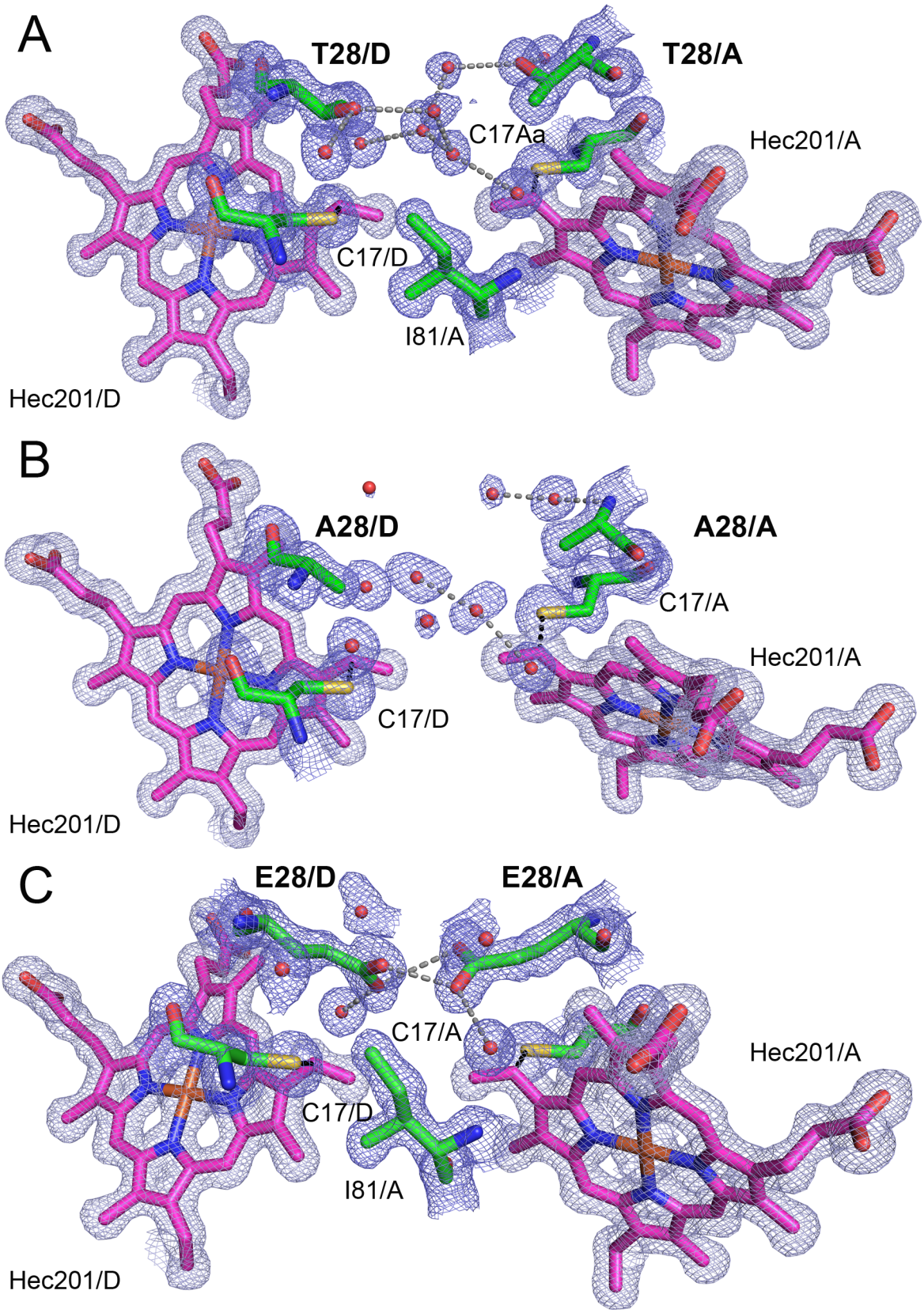
SCC



Supplemental Figure 1C. Circular dichroism spectra are similar for WT, Thr28Glu, and Sigma Cyt*c* (SCC), whereas the Thr28Ala mutant shows spectral differences as lower wavelengths.

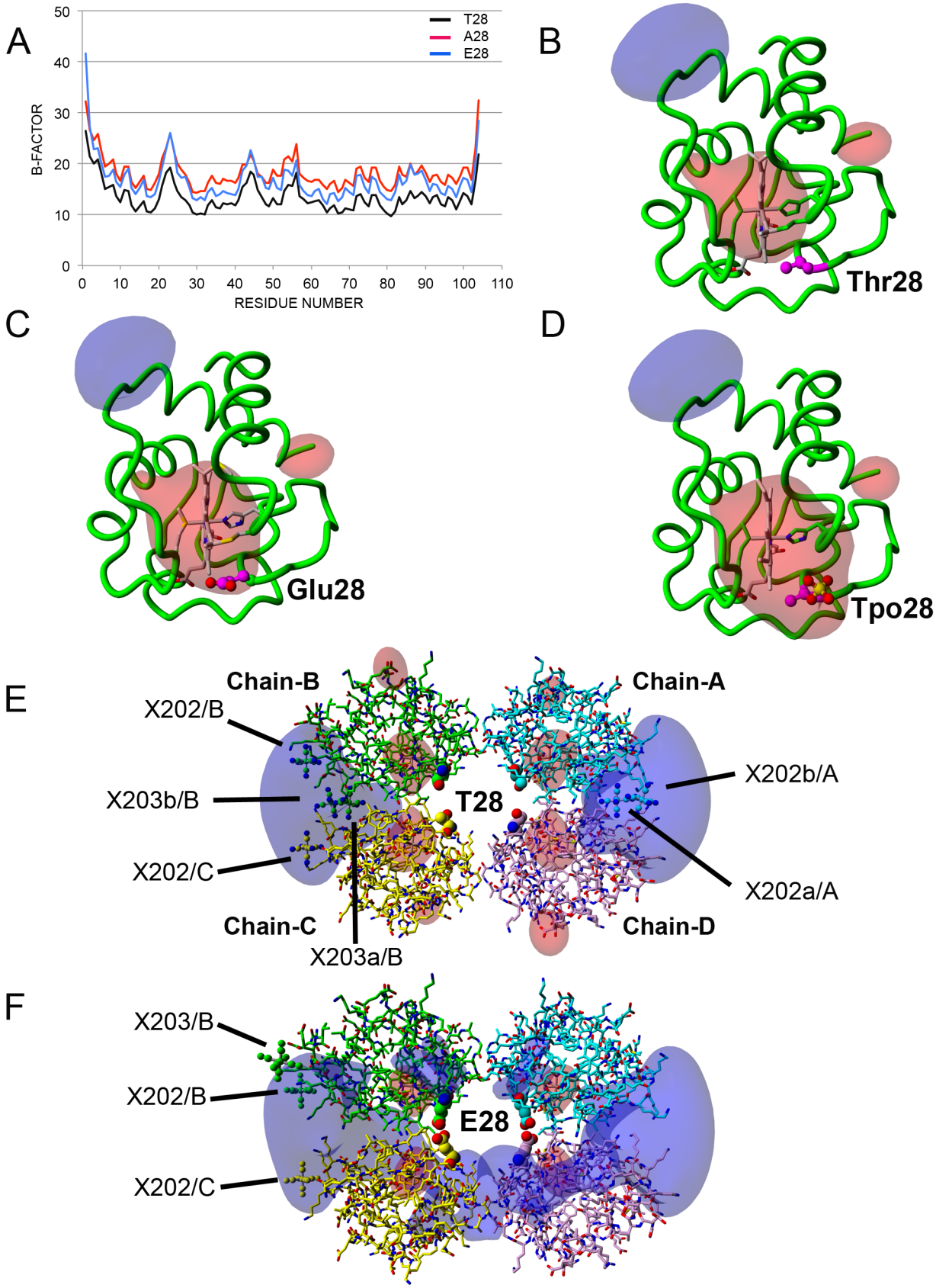


Supplemental Figure 2. Unphosphorylated (WT) and in vivo phosphorylated (pT28) Cyt*c* have a similar ability to induce caspase 3 activation in a cell free caspase assay.


A
T28/D
T28/A
C17Aa
Hec201/A
C17/D
I81/A
Hec201/D
B
A28/D
A28/A
C17/A
Hec201/A
C17/D
Hec201/D
C
E28/D
E28/A
C17/A
Hec201/A
C17/D
I81/A
Hec201/D

Supplemental Figure 3.

A. Residues and water molecules near Thr28 in the WT protein (green carbon atoms) are shown with their electron density contoured at 1.0 sigma. To minimize the effects of differing side chain lengths and to make this figure for WT comparable to those for T28A and T28E, it includes all groups within 4 Å of Glu28 superposed onto Thr28. The heme (PDB code Hec) groups have magenta carbon atoms for contrast. The covalent links from Cys17 SG to the Hec vinyl groups (see Supplemental Table 1) are shown as black dashes. Hydrogen bonds are indicated with gray dashed lines. **B.** Equivalent to (A) above but for the T28A structure (5C9M.pdb), residues and water molecules near Ala28 (green carbon atoms) are shown with their electron density contoured at 1.0 sigma. To make this figure for T28A comparable to those for WT and T28E, it includes all groups within 4 Å of Glu28 superposed onto Ala28. Ile81/A, which is shown in (A) and (C) is omitted here because it is at a distance of 4.1 Å from Glu28 in the superposed structure of T28E. **C.** Equivalent to A above but for the T28E structure (5DF5.pdb), residues and water molecules within 4Å of Glu28 (green carbon atoms) are shown with their electron density contoured at 1.0 sigma. The terminal oxygen atoms of the two Glu28 residues share two hydrogen bonds (2.72 & 3.25 Å) and are presumed to be protonated.


A
B-FACTOR
RESIDUE NUMBER
T28
A28
E28
B
Thr28
C
Glu28
D
Tpo28
E
Chain-B
Chain-A
X202/B
X203b/B
X202/C
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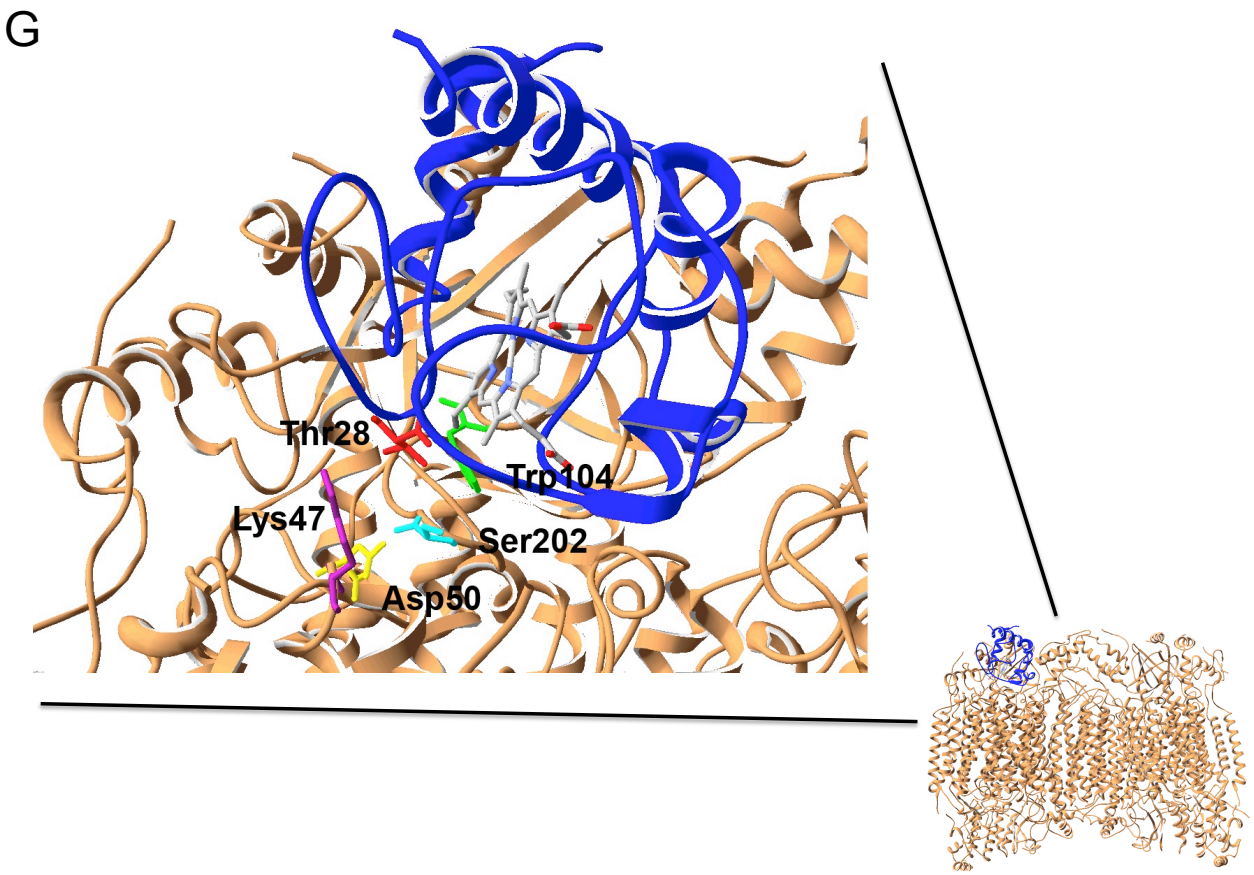
X202/B
X202/C
E28



Supplemental Figure 4.

A. Average B-Factors. The average temperature factor (B-factor) for the main chain atoms in WT (black), Thr28Ala (red), and Thr28EGlu (blue) are shown plotted by residue position. **B.** The negative (red) and positive (blue) electrostatic potential (ESP) are shown contoured at 20 kcal/mole for the WT protein shown as a "tube" tracing. The side chain atoms (magenta) of Thr28 are shown as balls. The ESP was calculated by YASARA using the recommended Nova force field *in vacuo* at pH 7.4. The ESP plot for the T28A mutant (not shown) is indistinguishable from this figure. **C.** The negative (red) and positive (blue) electrostatic potential (ESP) are shown contoured at 20 kcal/mole for the T28E mutant shown as a "tube" tracing. The side chain atoms (magenta) of Glu28 are shown as balls. The ESP was calculated by YASARA using the recommended Nova force field *in vacuo* at pH 7.4. **D.** The negative (red) and positive (blue) electrostatic potential (ESP) are shown contoured at 20 kcal/mole for the phosphor-Thr model (Tpo28) shown as a "tube" tracing. The side chain atoms (magenta) of Tpo28 are shown as balls. The ESP was calculated by YASARA using the recommended Nova force field *in vacuo* at pH 7.4. **E.** The entire WT "tetramer" in the unit cell of space group P1 is shown with its six hexacyanoferrate(3-) ligands (PDB code FC6) identified with "X" as a one letter code in this figure. X202a&b/A and X203a&b/B are pairs of FC6 ligands with partial occupancies and alternate positions (Supplemental Table 1) that

physically overlap with each other and cannot be occupied simultaneously. The ESP was calculated by YASARA using the recommended Nova force field *in vacuo* at pH 7.4. The T28A tetramer, which is not shown, was crystallized under the same conditions as the WT protein, and has very similar FC6 ligands (Supplemental Table 1) and the same ESP contours. **F.** The entire T28E "tetramer" in the unit cell of space group P1 is shown with its three hexacyanoferrate(3-) ligands (PDB code FC6) identified with "X" as a one letter code. The ESP was calculated by YASARA using the recommended Nova force field *in vacuo* at pH 4.5. Given the close proximity and presumed hydrogen bonding between pairs of Glu28 residues (Supplemental Figure 3C) their pKa values were manually set to 7.0 so they would be protonated during the calculation of the ESP. **G.** Docking model of Cyt*c* and C*c*O (1). Residues on C*c*O within a distance of 7 Å from Thr28 (red) are highlighted (Lys47 of C*c*O subunit 7c, magenta; Asp50 of C*c*O subunit I, yellow; Trp 104, green, and Ser 202, cyan, of C*c*O subunit II).

REFERENCES

1. Roberts, V. A., and Pique, M. E. (1999) *J Biol Chem* 274, 38051-38060