Switching metalloporphyrin binding specificity of a *b*-type cytochrome to fluorogenic zinc by design.

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Supporting information is at the end of the main manuscript.

Abstract

Metalloporphyrins play important roles in areas ranging from biology to nanoscience. Biology uses a narrow set of metal centres comprising mainly of iron and magnesium. Here, we convert metalloporphyrin specificity of cytochrome *b*₅₆₂ from iron (haem) to fluorogenic zinc protoporphyrin IX (ZnPP). Through a computationally guided iterative design process, a variant with a near total preference for ZnPP was generated representing a switch in specificity. The new variant greatly enhanced (≥60 fold) the negligible aqueous fluorescence of free ZnPP *in vitro* and *in vivo*.

Introduction

Here, we describe how an iterative design process can change the metalloporphyrin specificity of cytochrome *b*₅₆₂ (cyt *b*₅₆₂), a *b*-type haem binding protein unit, from iron to fluorogenic and photochemically active zinc. Metalloporphyrins¹ represent an important class of organometallic compounds and play a major role in biology as essential supplements to enable protein function in processes ranging from light harvesting to electron transport to enzyme catalysis to O₂ transport². Binding to a protein tunes and modulates the metalloporphyrin physiochemical properties. The metal centres used in biology are Fe (e.g. haem), Mg (e.g. chlorophyll) and, to a much lesser extent Co (Vitamin B₁₂) and Ni (F₄₃₀). This does not however represent the full metal repertoire available to porphyrins (e.g. Cu, Pt, Ag, Cd, Ir, Zn)¹. There is currently great interest in incorporating new metal cofactors into proteins. Such abiotic cofactors have recently been shown to confer new enzymatic catalytic routes³⁻⁴. Zinc porphyrins such as zinc protoporphyrin IX (ZnPP, Figure 1a) are one such class, playing important roles in clinical (including cancer therapy) and nanoscience settings. While ZnPP is not naturally utilised in biology as a protein cofactor, its aberrant presence in blood is commonly used to diagnose haem metabolism disorders⁵. The photo-induced electron and energy transfer properties of zinc porphyrins make them particularly attractive for nanoscale applications, ranging from photosensitizers in solar cells to molecular optoelectronic components⁶⁻⁹. ZnPP is inherently fluorescent but substantially quenched in aqueous solution¹⁰⁻¹¹. Designer protein components have the potential to further enhance ZnPP use through: (i) activation of fluorogenic properties¹² in aqueous conditions as an aid to clinical sensing and cell imaging; (ii) providing an organised, defined and tunable self-assembling scaffold for nanodevice construction^{6, 13-17}. ZnPP incorporation within a protein scaffold has classically been achieved by either: (i) metal centre exchange (in the case of covalently attached porphyrins) that require harsh conditions unsuitable for most biological settings^{13, 18-19}; (ii) facile exchange in the case of non-covalent porphyrin-protein complexes^{11, 20}. With regards to the latter, affinity for ZnPP is inherently low (Table S1), with preference for the original haem cofactor; little attention is paid to optimising the protein interaction despite the structural and physicochemical variations on exchanging the porphyrin metal centre (Fig 1a). Here we show how computational design can be used to switch cyt *b*₅₆₂

specificity from its normal iron porphyrin (haem) to zinc. The new protein can activate ZnPP fluorescence both *in vitro* and *in vivo*.

Figure 1. Porphyrin structure and cyt *b*₅₆₂ variants. (a) Structure of haem (cyan) and ZnPP (green). The metalloporphyrin structure geometries were optimised using GAMESS-US²¹. (b) Sequences of the cyt *b*₅₆₂ variants and their corresponding nomenclature. The methionine that coordinate Fe in haem is highlighted with *. (c) Model of ZnPP-bound cyt *b*₅₆₂^{ZnPP} with the residues mutated that shift metalloporphyrin specificity highlighted. (d) Cell pellets of recombinantly expressed cyt *b*₅₆₂^{ZnPP} and the wild-type protein.

Results and discussion

Cyt *b*₅₆₂ (Figure S1) is a small, helical bundle protein that binds haem non-covalently²²⁻²⁴. It is an important model for electron transfer²⁵⁻²⁸, engineering novel components²⁹⁻³⁴ and assembling supramolecular structures^{6, 14, 35}. ZnPP can replace haem through passive exchange^{20, 33, 36} but the environment is non-ideal resulting in relatively low affinity binding (404 nM ± 11 *K*_D; Table S1) and a significant background haem binding *in vivo* (Figure 1). Factors include non-optimal metal coordination ligands and change in tetrapyrrole structure and planarity due to the larger ionic radius of Zn²⁺ (Fig 1a)³⁷⁻³⁸.

The first stage in the design process was to optimise Zn coordination. Zn coordination is normally through soft ligands such as the cysteine thiol group and the imidazole group of histidine, or base ligands from the carboxylate groups of aspartate and glutamate; the native M7 S-methyl thioether group is not an ideal axial ligand. This was confirmed through computational analysis with cysteine considered

the best, albeit still relatively poor, alternative (the M7C mutation). The second native axial ligand, the imidazole group of H102, was considered optimal so was not changed. The M7C variant was generated and the measured ZnPP and haem affinity (dissociation constant, K_D) were comparable (181 ± 11 nM for ZnPP and 206 ± 2 nM for haem; Table S1) so generating a starting mutant with slightly improved ZnPP binding and lower haem affinity. Replacement of Met7 with either His (a second potential coordinating ligand) or Gly lowered ZnPP binding affinity to that of wild-type cyt b_{562} with the *bis*-His mutant retaining specificity for haem (Table S2). The absorbance spectrum of cyt b_{562}^{M7C} (Fig S4) confirms ZnPP binding to the protein as indicated by the red shift in the Soret peak at 418nm for free ZnPP to a sharper peak at 431nm. The Soret and α/β band peaks of cyt b_{562}^{M7C} are slightly blue shifted (8-10 nm) compared to the ZnPP bound to wild-type cyt b_{562} . The Soret peak is also broader for wt cyt b_{562} suggesting a mixed binding population compared to cyt b_{562}^{M7C} . The ZnPP α/β peaks ratio also switches on cyt b_{562} binding.

To further optimise ZnPP binding, a more systematic *in silico* mutagenesis approach was adopted. Using the wt holo cyt b_{562} crystal structure ³⁹ as a starting point, every residue from 1 to 20 was replaced to every possible residue and energy minimised to avoid clashes. The structures derived from the energy minimisation were used as the starting point for docking simulations using AutoDock ⁴⁰ and Rosetta LigandDock ⁴¹. As a test for the accuracy, the *in silico* derived binding energy of the wt cyt was compared with the measured K_D . The measured binding energy for wt cyt b_{562} (where $\Delta G = RT \ln K_D$) is -8.7 kcalmol⁻¹ for ZnPP, and -11 kcalmol⁻¹ and for haem. These are close to the binding energies predicted from our modelling approach (Table S1). The *in silico* design process identified a variant with improved ZnPP binding energies termed cyt b_{562}^{ZnPP} . In addition to the M7C mutation, four other mutations were identified (L3K, E4D, T9C, L10R). The affinity of variant for ZnPP was much lower (<10 nM) compared to wt cyt b_{562} (Table S1 and Figure S3) with minimal haem binding observed both *in vivo* (Figure 1d) and *in vitro* (Figure S3a). This represents a near total switch in specificity from haem to ZnPP. The lowest scoring models were chosen for verification with Rosetta LigandDock, followed by molecular dynamics for 2 ns to ensure that the shape of the binding pocket was accurate. There was no significant change in the shape of the binding pocket when comparing before and after the molecular dynamics run. Residue 10 lies arose in the

later stages of the design and buried in the holo form of the protein (Fig 1c) so it was surprising that not only was the L10R mutation tolerated but improved binding. Mutating residue 10 to either alanine or serine reduced affinity to >100 nM. Analysis of the model suggests that the guanidinium group of R10 lies within 2-3 Å of the C7 thiol group (Fig 1c and S4); proton abstraction by R10 may generate a thiolate group, which is a better ligand for Zn binding than the C7 thiol. Cyt b_{562}^{ZnPP} was analysed in more detail.

Cyt b_{562}^{ZnPP} had a characteristic absorbance spectrum indicative of formation of ZnPP holo protein (Fig 2a), with an extinction coefficient of $158 \text{ mM}^{-1} \text{ cm}^{-1}$ at 424 nm, 5 nm blue shifted compared to wt cyt b_{562} . On binding cyt b_{562}^{ZnPP} , ZnPP fluorescence intensity increased >60 fold (Fig 2b). Excitation at 431 nm gave the expected dual peak emission characteristic of ZnPP ¹⁹ (λ_{EM} 590 and 640 nm) with a Stoke's shift of 160 nm and 210 nm (Fig 2b). The peak ratio of the two emission peaks was *circa* 2:1 (590:640 nm). Protein-bound ZnPP could be excited at 431 nm, 550nm and 590 nm (Fig S5). While the brightest emission originated from excitation at 431 nm the ability to excite at multiple and longer wavelengths has obvious benefits for biological imaging. These fluorescence characteristics were observed for wt cyt b_{562} and cyt b_{562}^{M7C} (Fig S6) suggesting that the mutations affected only binding affinity. The quantum yield for the combined emission peaks was measured to be $\sim 92\%$ generating a protein with a brightness of $146 \text{ mM}^{-1} \text{ cm}^{-1}$, higher than many autofluorescent proteins ⁴²⁻⁴⁴. ZnPP fluorescence is quenched in aqueous solutions to the extent that the quantum yield could not be accurately measured

Figure 2. Absorbance and fluorescence properties of cyt b_{562} ZnPP binding variants. (a) absorbance spectra of free ZnPP (dashed red line), wt cyt b_{562} (blue line), and cyt b_{562}^{ZnPP} (black line). Inset is the Soret peak (top) and α/β bands (bottom). (b) fluorescence emission spectra (on excitation at 431 nm) of ZnPP in the presence (black line) and absence (red dashed line) of cyt b_{562}^{ZnPP} . (c) Rate of fluorescence emission increase (excitation 431 nm) of *E. coli* cell culture

incubated with ZnPP with cells containing (black line) or without (red line) plasmid-based *cyt b₅₆₂^{ZnPP}*. Inset is the fluorescence emission spectra after 1 hr.

Coupled with the fluorogenic properties, *cyt b₅₆₂^{ZnPP}* could make a useful genetically encoded imaging agent. A prerequisite for cell imaging is that ZnPP should pass through membranes and have no significant fluorescence in the cell until it binds specifically to *cyt b₅₆₂^{ZnPP}*. *E. coli* cells expressing *cyt b₅₆₂^{ZnPP}* were colourless in comparison to those expressing wt *cyt b₅₆₂* (Figure 1d), which are a pink-red hue due to binding available haem with high affinity³¹. This confirms *cyt b₅₆₂^{ZnPP}* reduced affinity for haem and thus a decreased capacity to bind endogenous haem compared to the wt *cyt b₅₆₂*. On addition of ZnPP to *E. coli* cells expressing *cyt b₅₆₂^{ZnPP50}*, fluorescence increased over time until plateauing after ~40-50 min (Fig 2c). Cells with no plasmid-encoded *cyt b₅₆₂* exhibited low baseline fluorescence on addition of ZnPP, which remained steady over the course of the incubation.

Figure 3. ZnPP binding to *cyt b₅₆₂^{ZnPP}*. (a) REES profile (emission wavelength, λ_{EX} , versus excitation wavelength, λ_{EM}) of free ZnPP (red line) and ZnPP-bound *cyt b₅₆₂^{ZnPP50}* (black line). EX-1 and EX-2 refer to the two separate emission peaks. (b) CD spectra of apo-*cyt b₅₆₂^{ZnPP50}* (black dotted line) and *cyt b₅₆₂^{ZnPP50}* bound to ZnPP (black line).

Red edge excitation shift (REES) analysis was performed to ascertain the effect of ZnPP binding to *cyt b₅₆₂^{ZnPP}* (Fig 3a). REES is an optical phenomenon where decreasing the excitation energy (increasing the excitation wavelength, λ_{EX}) gives rise to a red shift in the maximum of the fluorescence emission (λ_{EM}). Such shifts can arise where the fluorophore exists in a range of discrete solvation environments that are sampled as part of an equilibrium of solvent-solute interaction energies⁴⁵. In some cases, decreasing the energy of excitation allows for photo-selection of the discrete states that are red shifted (lower energy). We have recently demonstrated that for a single fluorophore (tryptophan) containing protein, the REES effect can

inform on the equilibrium of conformational states accessible to the fluorophore ⁴⁶. The REES data for free and protein-bound ZnPP are shown in Figure 3a and have been extracted for the α/β emission maximum shown in Figure 2b, λ_{EM-1} and λ_{EM-2} , respectively. Free ZnPP exhibits a significant REES effect that is reduced on binding to cyt b_{562}^{ZnPP} ; the change in λ_{EM-1} and λ_{EM-2} respectively being $0.56 \pm 0.02 \text{ nm}^{-1}$ and $0.21 \pm 0.02 \text{ nm}^{-1}$ for free ZnPP, which reduces to $0.12 \pm 0.01 \text{ nm}^{-1}$ and $-0.04 \pm 0.03 \text{ nm}^{-1}$ on binding cyt b_{562}^{ZnPP50} . These data therefore suggest that the free ZnPP exists in an equilibrium of discrete solvation states and that this equilibrium collapses to essentially a single state on binding to the protein. These data therefore suggest tight ZnPP binding within the core of the protein, with limited solvent access, akin to haem binding. To further confirm ZnPP binding to cyt b_{562}^{ZnPP} , CD spectroscopy was used to monitor the expected transition from a partially folded helical structure to a folded 4-helix bundle on co-factor binding. On addition of ZnPP to cyt b_{562}^{ZnPP} , the deepening of troughs at $\sim 208 \text{ nm}$ and 222 nm was observed confirming the increase in helical character, with the spectra of holo-cyt b_{562}^{ZnPP50} similar to that observed for wt holo-cyt b_{562} (Fig 3b).

Using systematic computationally guided engineering, the metalloporphyrin specificity of cyt b_{562} can be switched to ZnPP; the affinity is on a par with the natural haem cofactor and represented a near total switch in specificity. A fluorogenic effect was observed generating a protein complex with high brightness that exceeds that of many autofluorescent proteins. Higher affinity and thus more stable binding make holo-cyt b_{562}^{ZnPP50} a more useful photo-electronic nanodevice ^{15, 33}; a protein scaffold can also tune facets such as electronic excitation and emission. More generally, it provides a route for improving affinity of various different haemoproteins for ZnPP and other metalloporphyrins, in which the haem can be replaced by the photosensitizer effect of ZnPP ^{13, 47} or the catalytic properties of Ir and Cu porphyrins ³⁻⁴. The design process has shown changing metal coordinating ligands is not the defining step but that optimising residues adjacent to the metal coordination site is critical to success.

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References

1. Kadish, K.; Smith, K. M.; Guillard, R., *The porphyrin handbook*. Academic Press: New York, 1999.
2. Messerschmidt, A., *Handbook of metalloproteins*. Wiley: Chichester, 2001.
3. Dydio, P.; Key, H. M.; Nazarenko, A.; Rha, J. Y. E.; Seyedkazemi, V.; Clark, D. S.; Hartwig, J. F., An artificial metalloenzyme with the kinetics of native enzymes. *Science* **2016**, 354 (6308), 102.
4. Key, H. M.; Dydio, P.; Clark, D. S.; Hartwig, J. F., Abiological catalysis by artificial haem proteins containing noble metals in place of iron. *Nature* **2016**, 534 (7608), 534-7.
5. Labbe, R. F.; Vreman, H. J.; Stevenson, D. K., Zinc protoporphyrin: A metabolite with a mission. *Clin Chem* **1999**, 45 (12), 2060-72.
6. Brodin, J. D.; Carr, J. R.; Sontz, P. A.; Tezcan, F. A., Exceptionally stable, redox-active supramolecular protein assemblies with emergent properties. *Proceedings of the National Academy of Sciences of the United States of America* **2014**, 111 (8), 2897-902.
7. Li, L. L.; Diau, E. W., Porphyrin-sensitized solar cells. *Chem Soc Rev* **2013**, 42 (1), 291-304.
8. Mathew, S.; Yella, A.; Gao, P.; Humphry-Baker, R.; Curchod, B. F.; Ashari-Astani, N.; Tavernelli, I.; Rothlisberger, U.; Nazeeruddin, M. K.; Gratzel, M., Dye-sensitized solar cells with 13% efficiency achieved through the molecular engineering of porphyrin sensitizers. *Nature chemistry* **2014**, 6 (3), 242-7.
9. Sedghi, G.; Garcia-Suarez, V. M.; Esdaile, L. J.; Anderson, H. L.; Lambert, C. J.; Martin, S.; Bethell, D.; Higgins, S. J.; Elliott, M.; Bennett, N.; Macdonald, J. E.; Nichols, R. J., Long-range electron tunnelling in oligo-porphyrin molecular wires. *Nat Nanotechnol* **2011**, 6 (8), 517-23.
10. Hu, Y.; Geissinger, P.; Woehl, J. C., Potential of protoporphyrin IX and metal derivatives for single molecule fluorescence studies. *Journal of Luminescence* **2011**, 131 (3), 477-481.
11. Leonard, J. J.; Yonetani, T.; Callis, J. B., A fluorescence study of hybrid hemoglobins containing free base and zinc protoporphyrin IX. *Biochemistry* **1974**, 13 (7), 1460-4.
12. Bruchez, M. P., Dark dyes-bright complexes: fluorogenic protein labeling. *Current opinion in chemical biology* **2015**, 27, 18-23.
13. Anderson, J. L.; Armstrong, C. T.; Kodali, G.; Lichtenstein, B. R.; Watkins, D. W.; Mancini, J. A.; Boyle, A. L.; Farid, T. A.; Crump, M. P.; Moser, C. C.; Dutton, P.

- L., Constructing a man-made c-type cytochrome maquette : electron transfer, oxygen transport and conversion to a photoactive light harvesting maquette. *Chem Sci* **2014**, 5 (2), 507-514.
14. Brodin, J. D.; Ambroggio, X. I.; Tang, C.; Parent, K. N.; Baker, T. S.; Tezcan, F. A., Metal-directed, chemically tunable assembly of one-, two- and three-dimensional crystalline protein arrays. *Nature chemistry* **2012**, 4 (5), 375-82.
 15. Oohora, K.; Hayashi, T., Hemoprotein-based supramolecular assembling systems. *Current opinion in chemical biology* **2014**, 19, 154-61.
 16. Oohora, K.; Mashima, T.; Ohkubo, K.; Fukuzumi, S.; Hayashi, T., Energy migration within hexameric hemoprotein reconstituted with Zn porphyrinoid molecules. *Chemical communications* **2015**, 51 (55), 11138-40.
 17. Sontz, P. A.; Song, W. J.; Tezcan, F. A., Interfacial metal coordination in engineered protein and peptide assemblies. *Current opinion in chemical biology* **2014**, 19, 42-9.
 18. Anni, H.; Vanderkooi, J. M.; Mayne, L., Structure of zinc-substituted cytochrome c: nuclear magnetic resonance and optical spectroscopic studies. *Biochemistry* **1995**, 34 (17), 5744-53.
 19. Vanderkooi, J. M.; Adar, F.; Erecinska, M., Metallocytochromes c : Characterization of electronic absorption and emission spectra of Sn⁴⁺ and Zn²⁺ cytochromes c. *European Journal of Biochemistry* **1976**, 64, 381-387.
 20. Della Pia, E. A.; Chi, Q.; Elliott, M.; Macdonald, J. E.; Ulstrup, J.; Jones, D. D., Redox tuning of cytochrome b562 through facile metal porphyrin substitution. *Chemical communications* **2012**, 48 (86), 10624-6.
 21. Schmidt, M. W.; Baldrige, K. K.; Boatz, J. A.; Elbert, S. T.; Gordon, M. S.; Jensen, J. H.; Koseki, S.; Matsunaga, N.; Nguyen, K. A.; Su, S.; Windus, T. L.; Dupuis, M.; Montgomery, J. A., General atomic and molecular electronic structure system. *Journal of Computational Chemistry* **1993**, 14 (11), 1347-1363.
 22. Arnesano, F.; Banci, L.; Bertini, I.; Faraone-Mennella, J.; Rosato, A.; Barker, P. D.; Fersht, A. R., The solution structure of oxidized Escherichia coli cytochrome b562. *Biochemistry* **1999**, 38 (27), 8657-70.
 23. Feng, Y.; Sligar, S. G.; Wand, A. J., Solution structure of apocytochrome b562. *Nat Struct Biol* **1994**, 1 (1), 30-5.
 24. Lederer, F.; Glatigny, A.; Bethge, P. H.; Bellamy, H. D.; Matthew, F. S., Improvement of the 2.5 Å resolution model of cytochrome b562 by redetermining the primary structure and using molecular graphics. *Journal of molecular biology* **1981**, 148 (4), 427-48.
 25. Della Pia, E. A.; Chi, Q.; Jones, D. D.; Macdonald, J. E.; Ulstrup, J.; Elliott, M., Single-molecule mapping of long-range electron transport for a cytochrome b(562) variant. *Nano letters* **2011**, 11 (1), 176-82.
 26. Della Pia, E. A.; Chi, Q.; Macdonald, J. E.; Ulstrup, J.; Jones, D. D.; Elliott, M., Fast electron transfer through a single molecule natively structured redox protein. *Nanoscale* **2012**, 4 (22), 7106-13.
 27. Wittung-Stafshede, P.; Lee, J. C.; Winkler, J. R.; Gray, H. B., Cytochrome b562 folding triggered by electron transfer: approaching the speed limit for formation of a four-helix-bundle protein. *Proceedings of the National Academy of Sciences of the United States of America* **1999**, 96 (12), 6587-90.
 28. Zuo, P.; Albrecht, T.; Barker, P. D.; Murgida, D. H.; Hildebrandt, P., Interfacial redox processes of cytochrome b562. *Physical chemistry chemical physics : PCCP* **2009**, 11 (34), 7430-6.

29. Brodin, J. D.; Medina-Morales, A.; Ni, T.; Salgado, E. N.; Ambroggio, X. I.; Tezcan, F. A., Evolution of metal selectivity in templated protein interfaces. *Journal of the American Chemical Society* **2010**, *132* (25), 8610-7.
30. Della Pia, E. A.; Macdonald, J. E.; Elliott, M.; Jones, D. D., Direct binding of a redox protein for single-molecule electron transfer measurements. *Small* **2012**, *8* (15), 2341-4.
31. Jones, D. D.; Barker, P. D., Design and characterisation of an artificial DNA-binding cytochrome. *ChemBiochem* **2004**, *5* (7), 964-71.
32. Takeda, S.; Kamiya, N.; Nagamune, T., Rational design of a protein-based molecular device consisting of blue fluorescent protein and zinc protoporphyrin IX incorporated into a cytochrome b562 scaffold. *Biotechnol Lett* **2004**, *26* (2), 121-5.
33. Tokita, Y.; Yamada, S.; Luo, W.; Goto, Y.; Bouley-Ford, N.; Nakajima, H.; Watanabe, Y., Protein photoconductors and photodiodes. *Angewandte Chemie* **2011**, *50* (49), 11663-6.
34. Arpino, J. A.; Czapinska, H.; Piasecka, A.; Edwards, W. R.; Barker, P.; Gajda, M. J.; Bochtler, M.; Jones, D. D., Structural basis for efficient chromophore communication and energy transfer in a constructed didomain protein scaffold. *Journal of the American Chemical Society* **2012**, *134* (33), 13632-40.
35. Radford, R. J.; Tezcan, F. A., A superprotein triangle driven by nickel(II) coordination: exploiting non-natural metal ligands in protein self-assembly. *Journal of the American Chemical Society* **2009**, *131* (26), 9136-7.
36. Onoda, A.; Kakikura, Y.; Uematsu, T.; Kuwabata, S.; Hayashi, T., Photocurrent generation from hierarchical zinc-substituted hemoprotein assemblies immobilized on a gold electrode. *Angewandte Chemie* **2012**, *51* (11), 2628-31.
37. Scheidt, W. R.; Mondal, J. U.; Eigenbrot, C. W.; Adler, A.; Radonovich, L. J.; Hoard, J. L., Crystal and Molecular-Structure of the Silver(I) and Zinc(II) Derivatives of Meso-Tetraphenylporphyrin - an Exploration of Crystal-Packing Effects on Bond Distance. *Inorganic Chemistry* **1986**, *25* (6), 795-799.
38. Tezcan, F. A.; Crane, B. R.; Winkler, J. R.; Gray, H. B., Electron tunneling in protein crystals. *Proceedings of the National Academy of Sciences of the United States of America* **2001**, *98* (9), 5002-6.
39. D'Amelio, N.; Bonvin, A. M.; Czisch, M.; Barker, P.; Kaptein, R., The C terminus of apocytochrome b562 undergoes fast motions and slow exchange among ordered conformations resembling the folded state. *Biochemistry* **2002**, *41* (17), 5505-14.
40. Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J., AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *J Comput Chem* **2009**, *30* (16), 2785-91.
41. Meiler, J.; Baker, D., ROSETTALIGAND: protein-small molecule docking with full side-chain flexibility. *Proteins* **2006**, *65* (3), 538-48.
42. Shaner, N. C.; Steinbach, P. A.; Tsien, R. Y., A guide to choosing fluorescent proteins. *Nat Methods* **2005**, *2* (12), 905-9.
43. Tsien, R. Y., The green fluorescent protein. *Annual review of biochemistry* **1998**, *67*, 509-44.
44. Zhang, J.; Campbell, R. E.; Ting, A. Y.; Tsien, R. Y., Creating new fluorescent probes for cell biology. *Nat Rev Mol Cell Biol* **2002**, *3* (12), 906-18.
45. Demchenko, A. P., The red-edge effects: 30 years of exploration. *Luminescence* **2002**, *17* (1), 19-42.
46. Catici, D. A. M.; Amos, H. E.; Yang, Y.; van den Elsen, J. M. H.; Pudney, C. D., The red edge excitation shift phenomenon can be used to unmask protein

structural ensembles: implications for NEMO-ubiquitin interactions. *FEBS J* **2016**, *In Press*.

47. Komatsu, T.; Wang, R. M.; Zunszain, P. A.; Curry, S.; Tsuchida, E., Photosensitized reduction of water to hydrogen using human serum albumin complexed with zinc-protoporphyrin IX. *Journal of the American Chemical Society* **2006**, *128* (50), 16297-301.
48. Chen, Z.; Wang, Y.; Zhai, Y. F.; Song, J.; Zhang, Z., ZincExplorer: an accurate hybrid method to improve the prediction of zinc-binding sites from protein sequences. *Mol Biosyst* **2013**, *9* (9), 2213-22.
49. Schrodinger, LLC, The PyMOL Molecular Graphics System, Version 1.3r1. 2010.
50. Berweger, C. D.; van Gunsteren, W. F.; Müller-Plathe, F., Force field parametrization by weak coupling. Re-engineering SPC water. *Chemical Physics Letters* **1995**, *232* (5), 429-436.
51. Hanwell, M. D.; Curtis, D. E.; Lonie, D. C.; Vandermeersch, T.; Zurek, E.; Hutchison, G. R., Avogadro: an advanced semantic chemical editor, visualization, and analysis platform. *Journal of cheminformatics* **2012**, *4* (1), 17.
52. Hornak, V.; Abel, R.; Okur, A.; Strockbine, B.; Roitberg, A.; Simmerling, C., Comparison of multiple Amber force fields and development of improved protein backbone parameters. *Proteins* **2006**, *65* (3), 712-25.
53. Arpino, J. A.; Rizkallah, P. J.; Jones, D. D., Crystal structure of enhanced green fluorescent protein to 1.35 Å resolution reveals alternative conformations for glu222. *PloS one* **2012**, *7* (10), e47132.
54. Reddington, S. C.; Driezis, S.; Hartley, A. M.; Watson, P. D.; Rizkallah, P. J.; Jones, D. D., Genetically encoded phenyl azide photochemistry drives positive and negative functional modulation of a red fluorescent protein. *RSC Advances* **2015**, *5*, 77734-77738.

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Supporting Information

Supporting Methods.

In silico design.

ZincExplorer

In silico protein design was performed by initially using the native wild-type cyt *b*₅₆₂ sequence with the ZincExplorer web server⁴⁸ (<http://protein.cau.edu.cn/ZincExplorer>). Successive mutations were assessed by selecting existing residues, replacing them and resubmitting them to the server. The relative scores returned from the server were used to determine if a mutation was likely to improve Zinc protoporphyrin IX (ZnPP) binding (see Figure S1). The first design phase focused on replacing the M7 co-ordinating residue with alternatives to identify a better potential axial ligand residue. The second design round built on the best scoring variant from the first round introduced secondary mutations around residue 7.

Mutant Modelling

Using the published crystal structure of wild type cyt *b*₅₆₂ (PDB:256B²⁴) as a starting point, *in silico* mutagenesis was performed using the mutagenesis application within MacPyMol⁴⁹ to change a single residue to another. This process was automated using shell scripts to allow for the creation of a library of structure files that replaced residue 1-20 with every other possible residue. Each initial model was used as the starting point and energy minimised to avoid any clashes caused by the mutation introduction. The starting structure was then placed within a triclinic box with dimension of 6.4x6.1x7.8 nm. This was populated using the Simple Point Charge (SPC) water model⁵⁰ to solvate the system to a total number of 16986 solvent molecules. The system was first energy minimised by performing 500 steps of steepest descent method followed by 500 steps of conjugant gradient method. The lowest energy state of the system was used as the starting conformation for the docking simulations.

Ligand Docking with AutoDock

Structure files for ZnPP were made using Avogadro⁵¹. Geometry optimization calculations were performed using GAMESS-US²¹ at the HF/6-31G* level in order to be consistent with the AMBER99sb force field⁵². The optimised structure was used as the ligand file during docking simulations. The standard AutoDock4⁴⁰ protocol was used as described in the software documentation. The scripts prepare_receptor4, prepare_ligand4, prepare_gpf4, prepare_dpf42 and prepare_flexreceptor4 (distributed with the software) were used to prepare the energy minimised mutated structure files for docking. For each variant 50 models

were made and the lowest 5% of the predicted free binding energies were taken and averaged to give an approximate score for each mutation, these were then ranked and the lowest scoring mutant was chosen as the starting point for another round of mutations.

Ligand Docking with Rosetta LigandDock

The five lowest scoring variants from the AutoDock docking simulations for each round were chosen to be confirmed using the Rosetta LigandDock program ⁴¹. Docking was carried out using the Automatic RosettaLigand Setup script supplied with the program. The predicted binding energies and Rosetta score were compared with the results from the AutoDock docking analysis to inform the choice of which mutation would be used as the starting point of the next round of mutations.

Cyt *b*₅₆₂ mutagenesis and protein production.

Construction of cyt *b*₅₆₂^{ZnPP} variants described in this paper was performed using site directed mutagenesis, essentially as outlined previously ³⁰. Cyt *b*₅₆₂ and its variants were expressed and purified as outlined previously ³⁰.

When required, purified cyt *b*₅₆₂ was converted to apo-form before ZnPP replacement by using organic solvent extraction approach ³¹. The proteins were buffer exchanged into water and the pH lowered to 1.5 before mixing with ice-cold 2-butanone. The mixture was then centrifuged for 2 minutes at 13,000x rpm in a bench top microfuge. The organic phase was removed and the preceding steps were repeated 5 times. The pH was returned to pH 6 before buffer exchanging back into 10mM Tris buffer.

ZnPP and hemin was purchased from Sigma Aldrich. The molecules were dissolved in 1 M NaOH prior to use to a final ZnPP concentration of 1 mM. ZnPP was stored in the dark at 4 C and centrifuged prior to use in experiments to prevent molecular aggregation and photodegradation. The ZnPP solution was then added to 10 mM Tris buffer to a final concentration of 200 μ M. This was then added to a solution of 100 μ M apo- cyt *b*₅₆₂ in 10 mM Tris buffer and incubated for 12 hours at 25°C to form holo cyt *b*₅₆₂ ZnPP.

Biophysical analysis of cyt *b*₅₆₂ variants

UV-Vis Spectrophotometry and porphyrin titration

The absorbance spectra were monitored using Cary UV-Vis Spectrophotometer. The porphyrin titration experiments were performed using 20 μ M apo-cyt *b*₅₆₂ in 1ml of 10 mM phosphate buffer pH 6.2 with either 10 μ M ferricyanide (for oxidised spectra) or 10 μ M ascorbic acid (for reduced spectra). Either hemin (20 μ M stock) or ZnPP (20 μ M stock) were titrated against the protein solution. Apo-cyt *b*₅₆₂ concentration was determined by measuring the absorbance at 280 nm (molar absorbance coefficient 2.98 mM⁻¹ cm⁻¹) and the holo-cyt *b*₅₆₂ concentration was determined by monitoring 430 nm for ZnPP titration and 418 nm for oxidised hemin or 428 nm for reduced hemin. The resultant titration curves were then fitted to determine dissociation constant (see Figure S3 for examples), as described previously ²⁰.

Fluorescence Spectroscopy

Excitation and emission spectra were recorded using a Varian Cary Eclipse spectrophotometer and the corresponding Cary Eclipse software. Samples of 750 μ L comprising of 2.5-10 μ M protein/porphyrin were analysed in a 5 × 5 mm QS quartz cuvette (Hellma, Müllheim, Germany). Spectra were recorded at a scan rate of either

120 or 600 nm/min with a slit width of 5 nm. Emission spectra were recorded up to 700 nm from a fixed excitation wavelength (corresponding to λ_{MAX} from the absorbance spectrum [~430 nm and ~590 nm] with an associated emission peak) for each variant. Quantum Yield studies were carried out using Alexa Fluor 431, essentially as described previously⁵³⁻⁵⁴. For the *in vivo* analysis, liquid cell cultures were grown at 37°C to an A_{600} of 0.6. The cells were pelleted by centrifugation in a microfuge for 20 min at 4°C. ZnPP (20 μ M) in 10 mM Tris-HCl (pH 8.0) was used to resuspended the cell pellet. A cell culture without the plasmid encoding the cyt b_{562} variants was used as a control. Fluorescence readings were taken periodically to determine rate of ZnPP uptake and incorporation.

Red edge excitation spectra (REES) analysis

All fluorescence measurements were performed using a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies) with a 5×5mm QS quartz cuvette (Hellma UK). The fluorimeter was baselined with 10mM sodium phosphate buffer (pH6.2) and all measurements were taken at 20°C. Excitation and emission slit widths were 5 nm and a scan rate of 600 or 120nm/min was used. Samples were incubated for 30 minutes at the given conditions prior to recording measurements. The red edge excitation scans were monitored from 413 to 437 nm with increments of 2 nm in the excitation wavelength. The wavelength of maximum emission, was extracted from the emission spectra by fitting to a sum (two) of skewed Gaussians of the form:

$$FI = FI_0 + Ae^{-e^{-\left(\frac{\lambda_{EM}-\lambda_{EM}^{max}}{w}\right)}} \frac{(\lambda_{EM}-\lambda_{EM}^{max})}{w} + 1 \quad \text{Eq 1}$$

Where FI is the measured fluorescence intensity at an emission wavelength, λ_{EM} . The spectra are then characterised by the amplitude, A of the spectrum at the maximum of fluorescence intensity, λ_{EM}^{max} , spectral width at half maximal, w and the minimum of the fluorescence emission, FI_0 , which was ~ zero for the buffer subtracted spectra.

CD spectroscopy

Circular dichroism spectroscopy was performed on a Jasco J-710 as described previously²⁰.

Table S1. Metalloporphyrin affinity of cyt *b*₅₆₂ variants

Variant	Predicted Affinity (kcal/mol) ^a		Measured Affinity (nM)	
	ZnPP	Haem	ZnPP	Haem
Wild Type	-8.7	-12.3	404	10 ^b
cyt <i>b</i> ₅₆₂ M7C	-8.9	-8.9	181	206
cyt <i>b</i> ₅₆₂ ^{ZnPP}	-9.5	-9	<10 ^c	ND ^d

^a calculated using Autodock. ⁴⁰; ^b determined previously ²⁰; ^c affinity too tight to measure accurately by current approach so a upper estimate is given; ^d affinity too weak to measure.

Table S2. Binding affinities for additional cyt *b*₅₆₂ variants for ZnPP and haem.

Variant	Measured binding affinity (nM)	
	ZnPP	Haem
cyt <i>b</i> ₅₆₂ M7H	458	100
cyt <i>b</i> ₅₆₂ M7G	409	472
cyt <i>b</i> ₅₆₂ ^{ZnPP} L(R)10A	152	200
cyt <i>b</i> ₅₆₂ ^{ZnPP} L(R)10S	108	248
Cyt <i>b</i> ₅₆₂ ^{ZnPP} L(R)10C	51	250

Figure S1. Cytochrome b_{562} structure. The protein is shown as green cartoon with haem shown in the stick representation and coloured cyan; the Fe centre is coloured brown. On the right hand side, the residues targeted for mutagenesis in helix H1 are shown.

Fig S2. Absorbance spectra of free ZnPP (black dashed line) and bound to wt cyt b_{562} (blue line) and cyt b_{562}^{M7C} . Inset are enlarged images of the Soret peak (top) and α/β band (bottom).

Fig S3. Binding affinity for haem and ZnPP. (a) cyt *b*₅₆₂^{ZnPP} (red) and wt cyt *b*₅₆₂ haem binding capacity. Protein (5 μ M) was incubated with 6 μ M haem and binding monitored. The free haem spectrum (dashed black lines) is shown for reference. Less than 10% haem binding to cyt *b*₅₆₂^{ZnPP} was observed. (b) Haem and (c) ZnPP titration curves. The wild-type cyt *b*₅₆₂ (black line, circles), cyt *b*₅₆₂^{M7C} (blue line, diamonds) cyt *b*₅₆₂^{ZnPP} (red) are shown. The data is based on triplicate reading (errors bars are removed for clarity). The data was fit and the K_D was calculated using a previously described approach ^{20, 30}.

Figure S4. Model of the cyt *b*₅₆₂^{ZnPP50} binding pocket. The potential interaction between the introduced guanidinium group of R10 and the new thiol group that can coordinate Zn²⁺ is shown as a red dashed line.

Figure S5. Relative fluorescence emission profiles of ZnPP bound to cyt b_{562}^{ZnPP} on excitation at the wavelengths stated in the figure.

Figure S6. Fluorescence emission profile for cyt b_{562} variants bound to ZnPP. Red line, cyt b_{562}^{ZnPP} ; dashed green line, cyt b_{562}^{M7C} ; blue dotted line, wt cyt b_{562} ; dotted black line free ZnPP.

Supporting References.

1. Chen, Z.; Wang, Y.; Zhai, Y. F.; Song, J.; Zhang, Z., ZincExplorer: an accurate hybrid method to improve the prediction of zinc-binding sites from protein sequences. *Mol Biosyst* **2013**, 9 (9), 2213-22.
2. Lederer, F.; Glatigny, A.; Bethge, P. H.; Bellamy, H. D.; Matthew, F. S., Improvement of the 2.5 Å resolution model of cytochrome b562 by redetermining the primary structure and using molecular graphics. *Journal of molecular biology* **1981**, 148 (4), 427-48.
3. Schrodinger, LLC, The PyMOL Molecular Graphics System, Version 1.3r1. 2010.

4. Berweger, C. D.; van Gunsteren, W. F.; Müller-Plathe, F., Force field parametrization by weak coupling. Re-engineering SPC water. *Chemical Physics Letters* **1995**, 232 (5), 429-436.
5. Hanwell, M. D.; Curtis, D. E.; Lonie, D. C.; Vandermeersch, T.; Zurek, E.; Hutchison, G. R., Avogadro: an advanced semantic chemical editor, visualization, and analysis platform. *Journal of cheminformatics* **2012**, 4 (1), 17.
6. Schmidt, M. W.; Baldrige, K. K.; Boatz, J. A.; Elbert, S. T.; Gordon, M. S.; Jensen, J. H.; Koseki, S.; Matsunaga, N.; Nguyen, K. A.; Su, S.; Windus, T. L.; Dupuis, M.; Montgomery, J. A., General atomic and molecular electronic structure system. *Journal of Computational Chemistry* **1993**, 14 (11), 1347-1363.
7. Hornak, V.; Abel, R.; Okur, A.; Strockbine, B.; Roitberg, A.; Simmerling, C., Comparison of multiple Amber force fields and development of improved protein backbone parameters. *Proteins* **2006**, 65 (3), 712-25.
8. Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J., AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *J Comput Chem* **2009**, 30 (16), 2785-91.
9. Meiler, J.; Baker, D., ROSETTALIGAND: protein-small molecule docking with full side-chain flexibility. *Proteins* **2006**, 65 (3), 538-48.
10. Della Pia, E. A.; Macdonald, J. E.; Elliott, M.; Jones, D. D., Direct binding of a redox protein for single-molecule electron transfer measurements. *Small* **2012**, 8 (15), 2341-4.
11. Jones, D. D.; Barker, P. D., Design and characterisation of an artificial DNA-binding cytochrome. *Chembiochem* **2004**, 5 (7), 964-71.
12. Della Pia, E. A.; Chi, Q.; Elliott, M.; Macdonald, J. E.; Ulstrup, J.; Jones, D. D., Redox tuning of cytochrome b562 through facile metal porphyrin substitution. *Chemical communications* **2012**, 48 (86), 10624-6.
13. Arpino, J. A.; Rizkallah, P. J.; Jones, D. D., Crystal structure of enhanced green fluorescent protein to 1.35 Å resolution reveals alternative conformations for glu222. *PloS one* **2012**, 7 (10), e47132.
14. Reddington, S. C.; Driezis, S.; Hartley, A. M.; Watson, P. D.; Rizkallah, P. J.; Jones, D. D., Genetically encoded phenyl azide photochemistry drives positive and negative functional modulation of a red fluorescent protein. *RSC Advances* **2015**, 5, 77734-77738.