Supplementary Information

XFEL Crystallography Reveals Catalytic Cycle Dynamics during Non-Native Substrate Oxidation by Cytochrome P450BM3

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Supplementary Note S1. Structural Integrity of P450cam in References 23 and 24. The difference in the hydrogen bonding network between the reported and current structures is not due to the effect of radiation damage: The structural analysis in reference 24 was based on the diffraction data collected in-house, where the radiation effect was suppressed to a minimum. In reference 23, the radiation effect was carefully controlled by tuning the X-ray wavelength and observing dose-dependent structural alterations while using synchrotron radiation. Consequently, the structures and findings in references 23 and 24 were consistent, ensuring the existence of the hydrogen bonding network in P450cam, although the use of XFEL is now a straightforward approach for damage-free analysis, particularly for determining the coordination structure of haem.

Supplementary Table S1. Bond length and angle between the haem and O_2 in the oxygen-bound form of the P450BM3 mutants determined by XFEL crystallography

	Chain	Fe-O_A[1]	O_A-O_B	∠Fe-O_A-O_B	∠O_A-Fe-N_D
F393H	A	1.97 Å	1.27 Å	143°	97°
	B	1.97 Å	1.26 Å	141°	96°
F87A/F393H	A	1.92 Å	1.24 Å	133°	93°
	B	1.93 Å	1.25 Å	130°	91°

[1]O_A and O_B are the proximal and distal oxygen atoms of the haem-bound O_2, respectively. N_D is the nitrogen atom at haem pyrrole D.

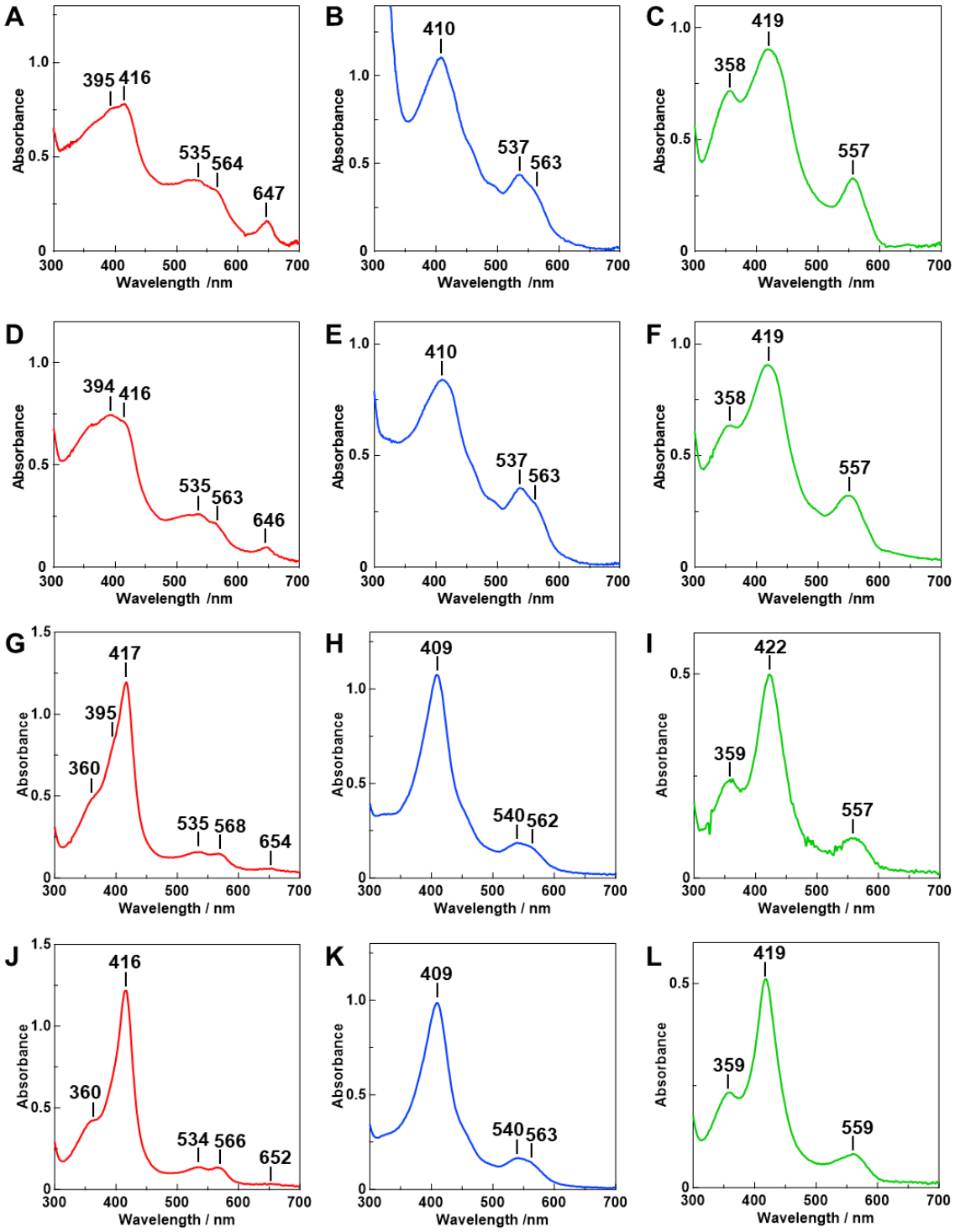
Supplementary Table S2. Bond length and angle between the haem and O_2 in the oxygen-bound form of F87A/F393H of P450BM3 determined by synchrotron X-ray crystallography

	Chain	Fe-O_A[1]	O_A-O_B	∠Fe-O_A-O_B	∠O_A-Fe-N_D
F87A/F393H	A	2.05 Å	1.33 Å	112°	89°
	B	2.08 Å	1.39 Å	116°	88°

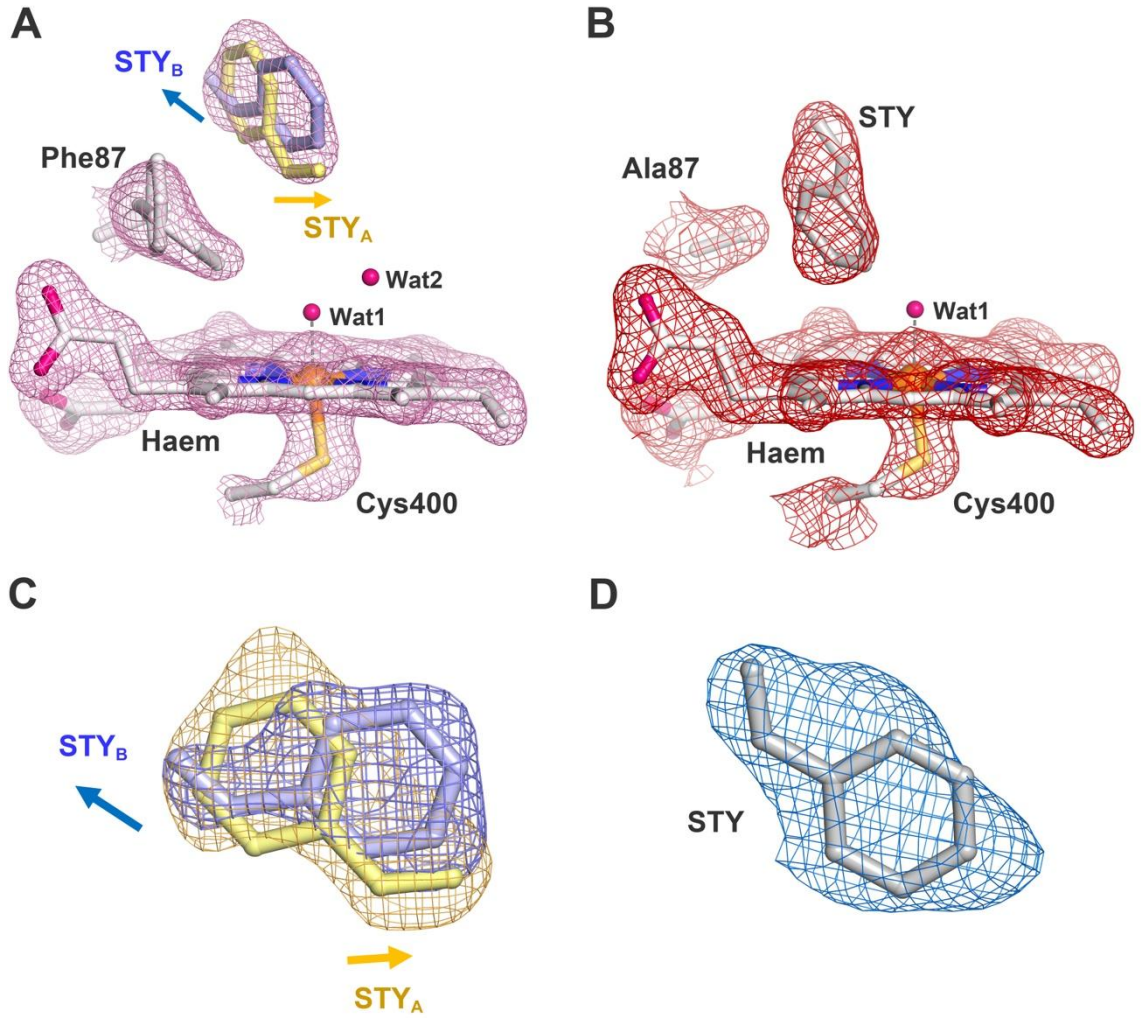
[1]O_A and O_B are the proximal and distal oxygen atoms of the haem-bound O_2, respectively. N_D is the nitrogen atom at haem pyrrole D.

Supplementary Table S3. The real space correlation coefficients of styrene in the structures of P450BM3 mutants determined by XFEL crystallography

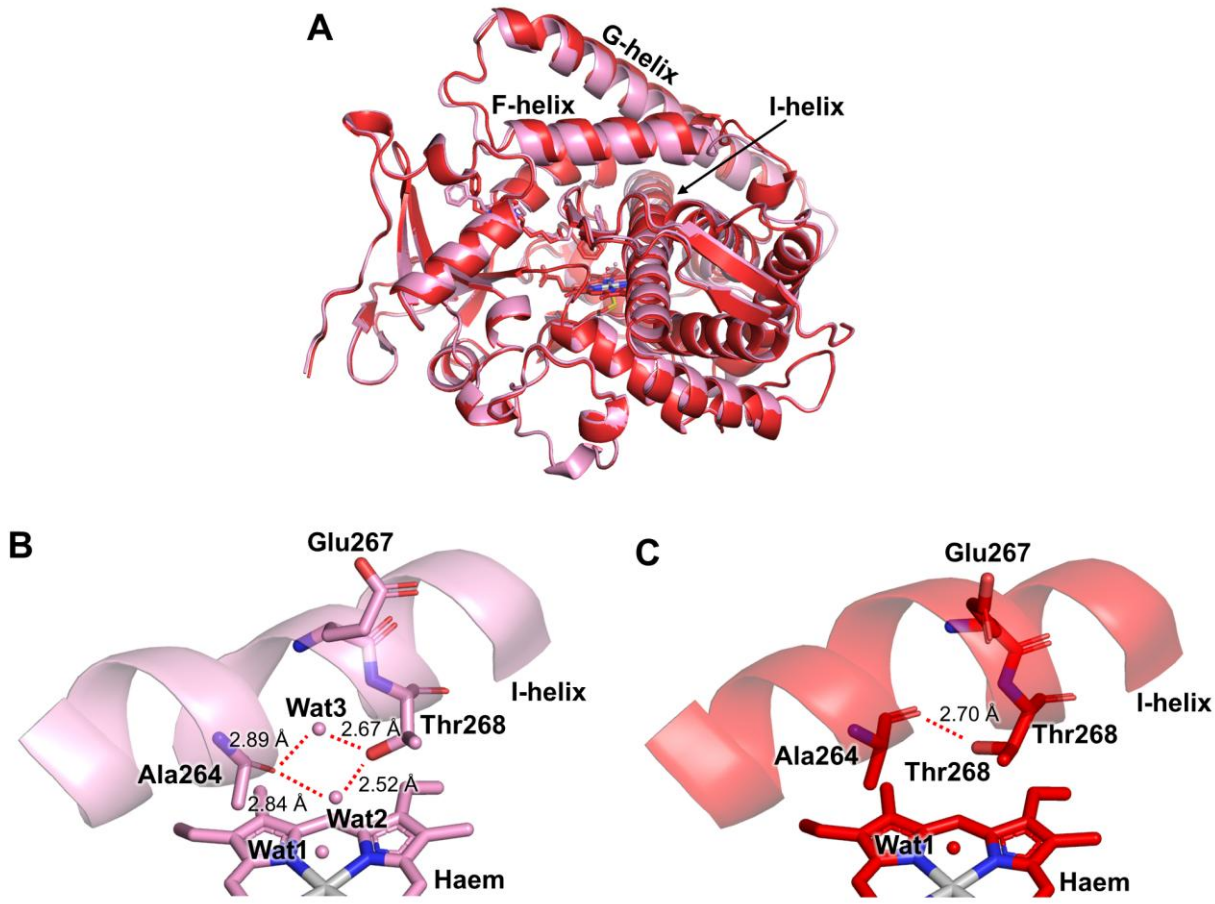
	Oxidised	Reduced	Oxygen-bound
F393H	0.86 (STY_A)	0.91 (STY_A)	0.93
	0.85 (STY_B)	0.92 (STY_B)	
F87A/F393H	0.78	0.91 (STY'_A)	0.86
		0.87 (STY'_B)	



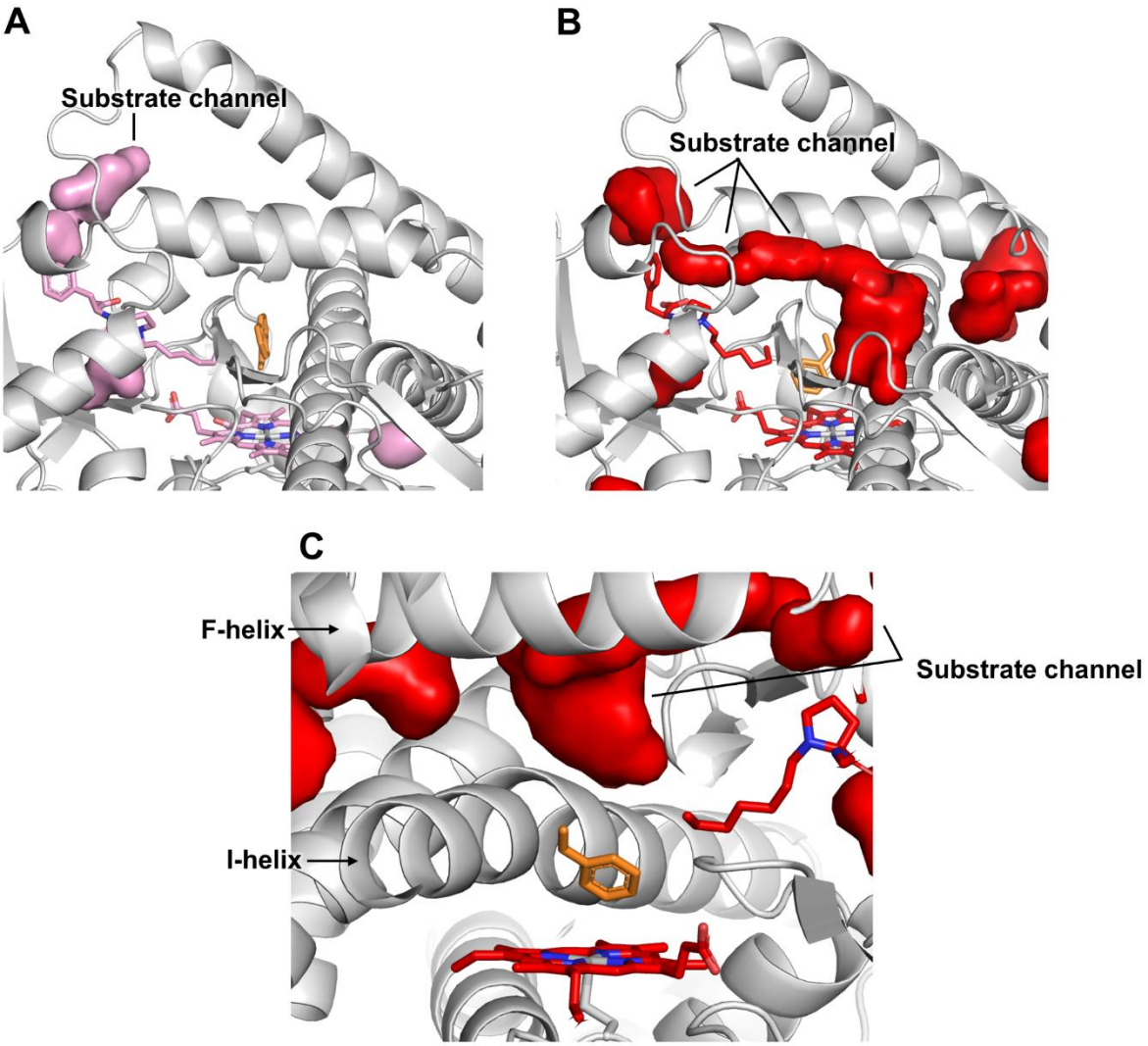
Supplementary Fig. S1. UV-visible absorption spectra of F393H (A–C and G–I) and F87A/F393H (D–F and J–L) of P450BM3 in microcrystals (A–F) and solutions (G–L). The oxidised (A, D, G, and J), reduced (B, E, H, and K), and oxygen-bound (C, F, I, and L) forms are shown in red, blue, and green, respectively. In the oxidised form, the haem existed as a mixture of low-spin and high-spin states in microcrystals, whereas the low-spin haem was dominant under solution conditions due to the limited concentration of styrene.



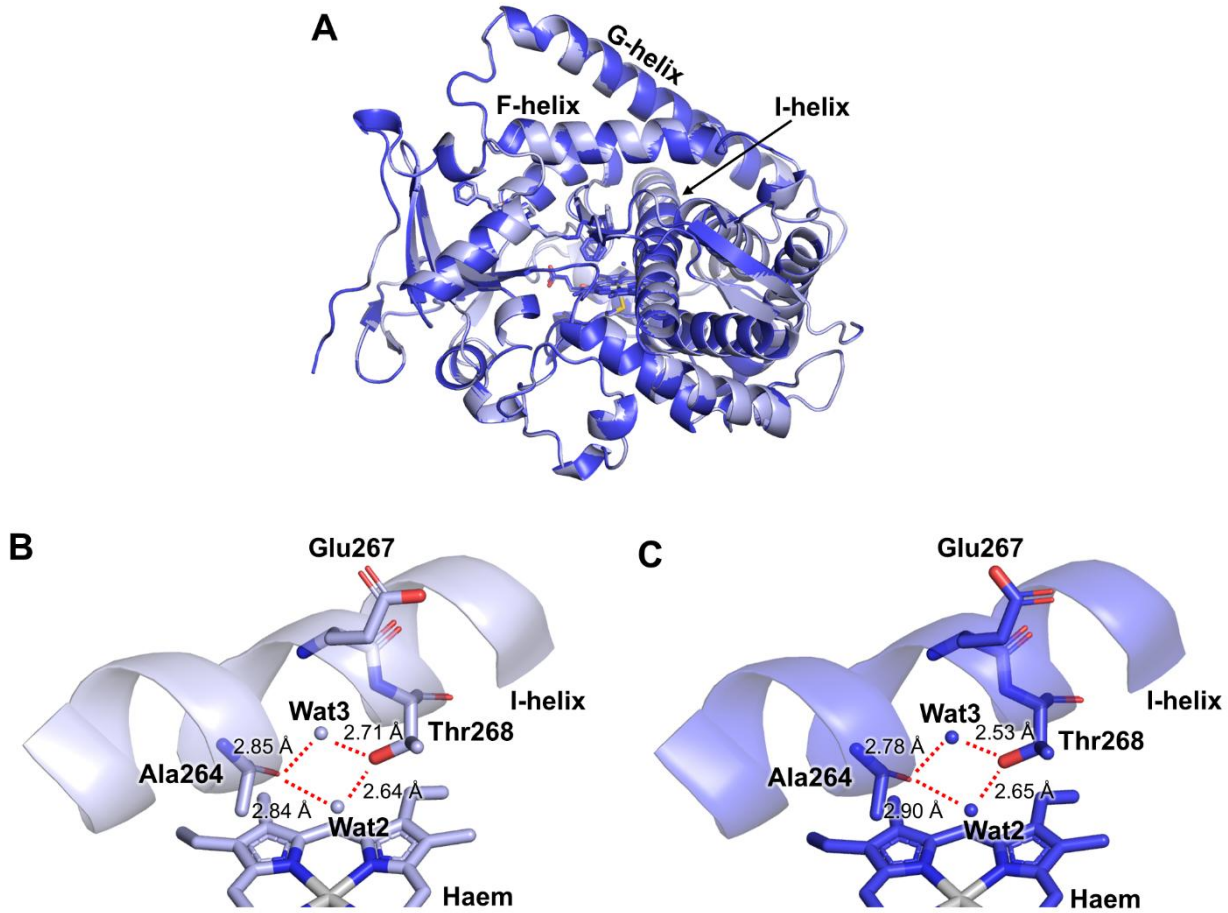
Supplementary Fig. S2. $2F_o - F_c$ electron density maps of the XFEL data for the oxidised form of P450BM3 F393H at 1.80 Å resolution (A) and of F87A/F393H at 1.85 Å resolution (B). The maps are shown as a mesh and contoured at 1.5σ. The haem, styrene, and side chains of Phe87, Ala87, and Cys400 are shown by the stick model. Water molecules are shown by small spheres. The directions of the vinyl group of styrene (STY) with the two orientations are shown by arrows (A and C). The polder omit maps for each alternative orientation (STY_A and STY_B) of styrene in F393H (C) and for the single model of STY in F87A/F393H (D) are shown as a mesh contoured at 3.5σ.



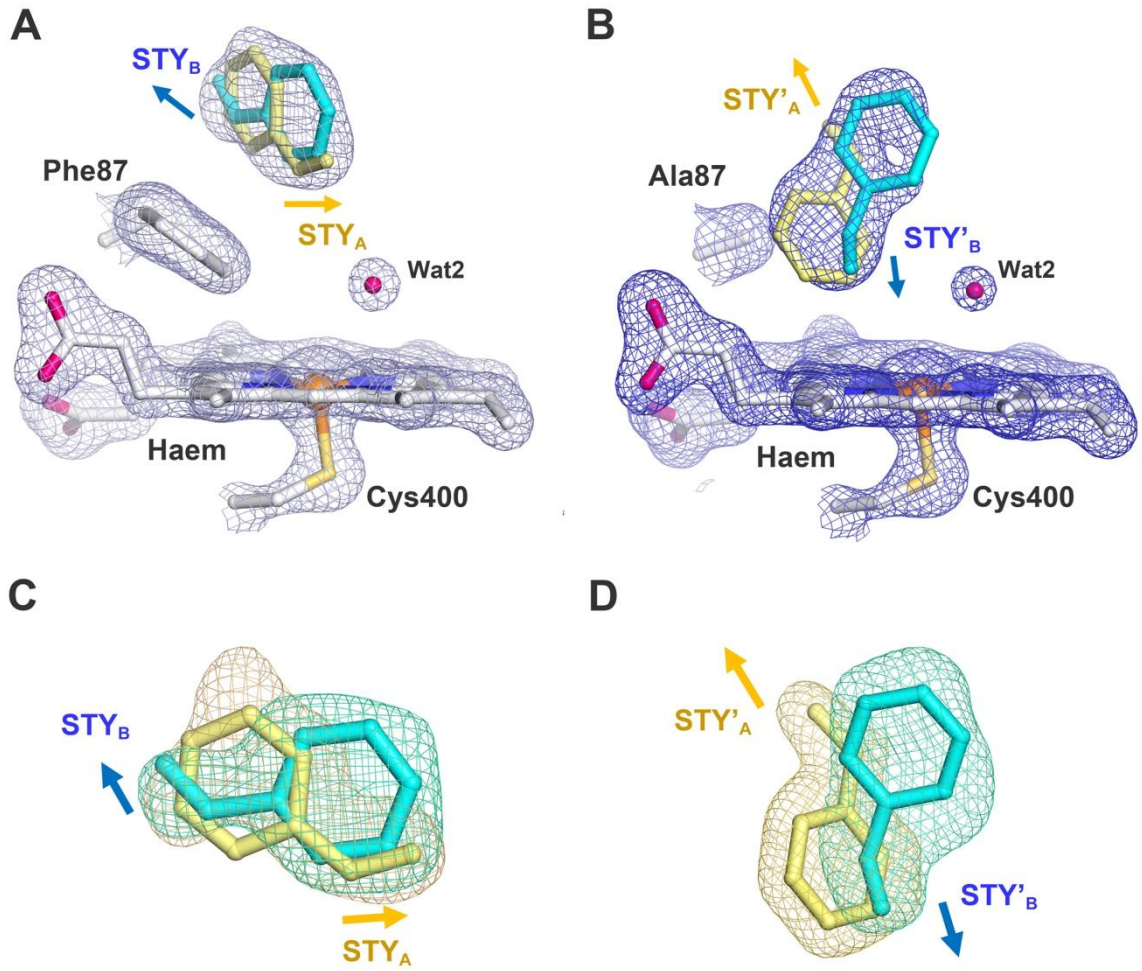
Supplementary Fig. S3. Crystal structures of the oxidised form of F393H (pink) and F87A/F393H (red) of P450BM3. Overall structures (A) and structures around Thr268 in the I-helix (B, C). In (A), the two structures are superimposed. The haem, Ala264, Glu267, and Thr268 are shown by the stick model. Water molecules near the haem are shown by small spheres. Hydrogen bonds are shown by red dotted lines.



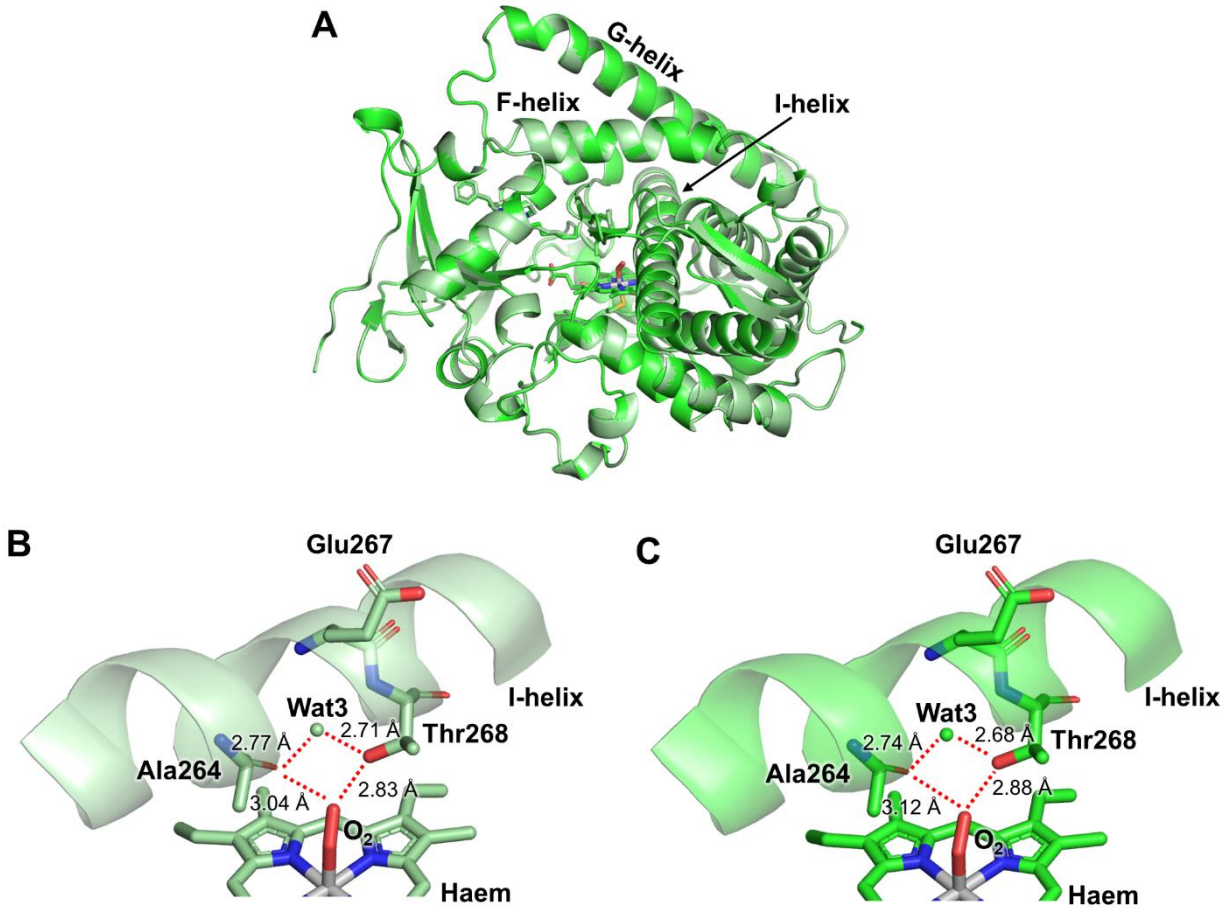
Supplementary Fig. S4. Substrate channel in F393H (A) and F87A/F393H (B) of P450BM3. The cavities are shown by the surface model. The haem, C7ProPhe, and styrene are shown by the stick model. Styrene is highlighted in orange. In F393H, the cavities are almost completely abolished. A magnified view of the channel end in F87A/F393H is shown in (C).



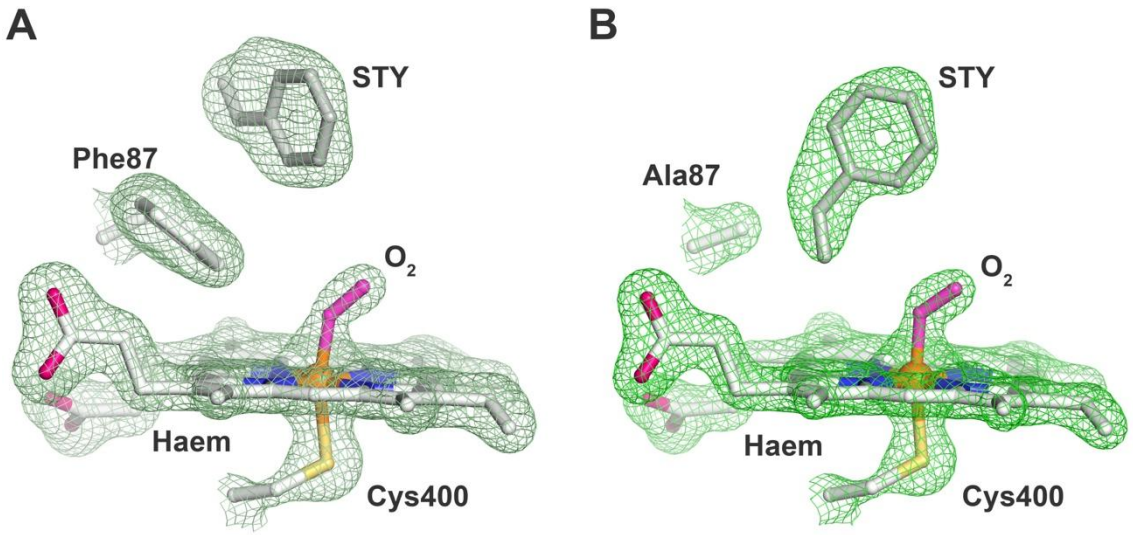
Supplementary Fig. S5. Crystal structures of the reduced form of F393H (pale blue) and F87A/F393H (blue) of P450BM3. The overall structures (A) and the structures around Thr268 in the I-helix (B, C). In (A), the two structures are superimposed. The haem, Ala264, Glu267, and Thr268 are shown by the stick model. Water molecules near the haem are shown by small spheres. Hydrogen bonds are shown by red dotted lines.



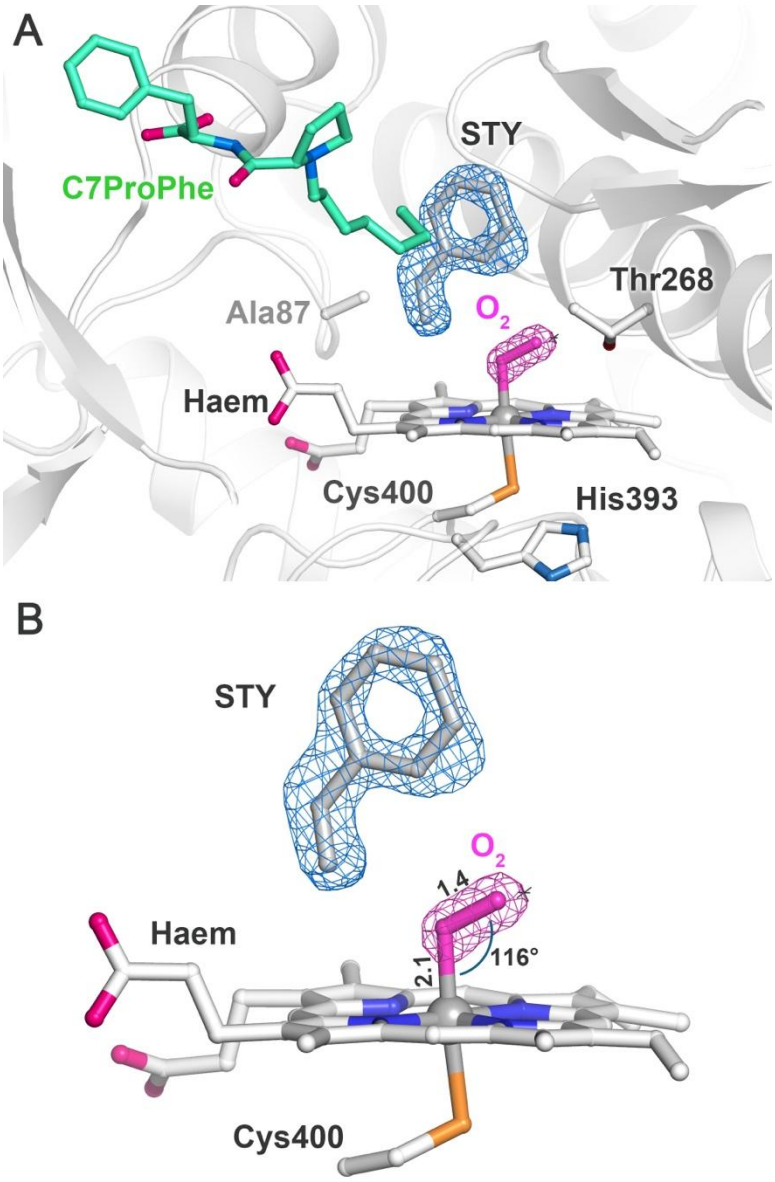
Supplementary Fig. S6. $2F_o - F_c$ electron density maps of the XFEL data at 1.60 Å resolution for the reduced form of P450BM3 F393H (A) and F87A/F393H (B) contoured at 1.5σ. The haem, styrene, and side chains of Phe87, Ala87, and Cys400 are shown by the stick model. The directions of the vinyl group of styrene with the two orientations (STY'$_A$ and STY'$_B$) are shown by arrows. The polder omit maps for each alternative orientation of styrene in F393H (C) and F87A/F393H (D) are shown as a mesh contoured at 3.5σ.



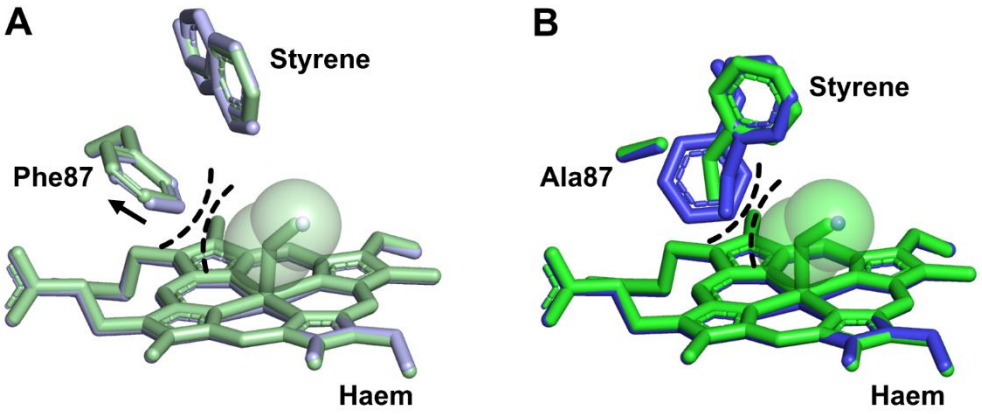
Supplementary Fig. S7. Crystal structures of the oxygen-bound form of F393H (pale green) and F87A/F393H (green) of P450BM3. Overall structures (A) and the structures around Thr268 in the I-helix (B, C). In (A), the two structures are superimposed. The haem, Ala264, Glu267, and Thr268 are shown by the stick model. Water molecules near the haem are shown by small spheres. Hydrogen bonds are shown by red dotted lines.



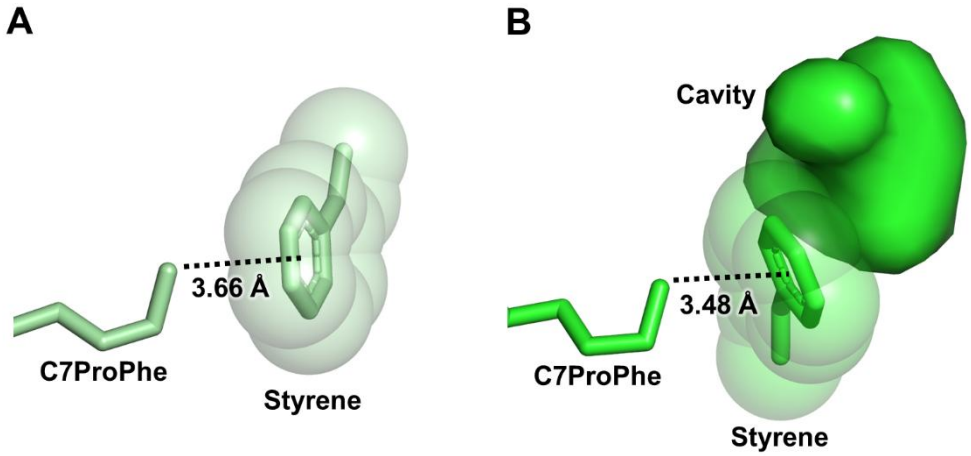
Supplementary Fig. S8. $2F_o - F_c$ electron density maps of the XFEL data at 1.50 Å resolution for the oxygen-bound form of P450BM3 F393H (A) and F87A/F393H (B) contoured at 1.5σ and 2.0σ, respectively. The haem, O_2, styrene, and side chains of Phe87, Ala87, and Cys400 are shown by the stick model.



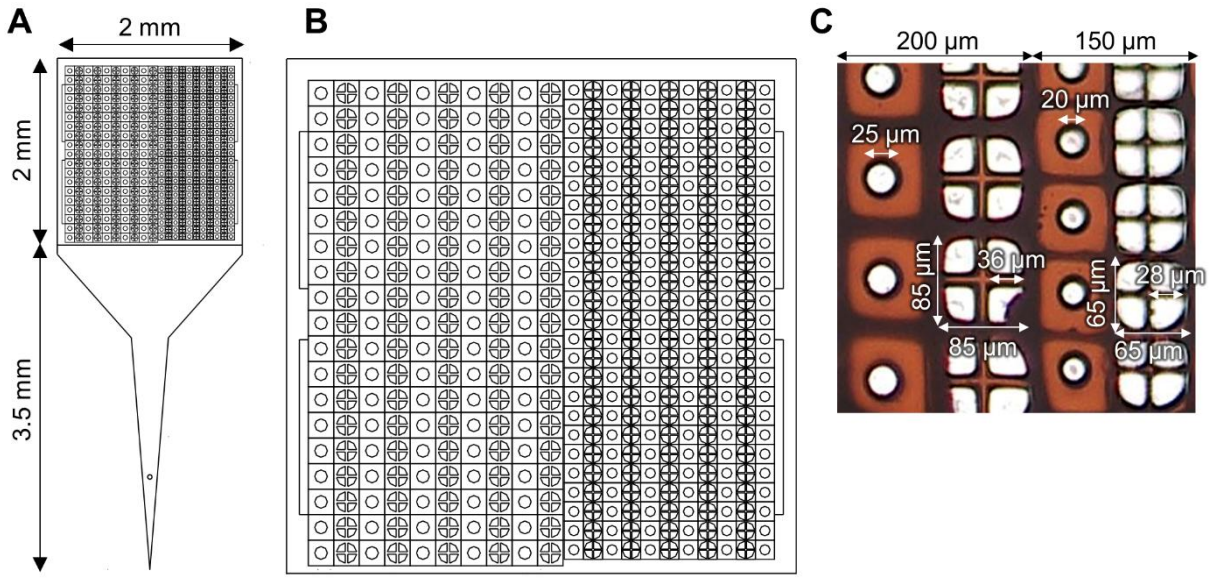
Supplementary Fig. S9. Crystal structure of the oxygen-bound form of P450BM3 F87A/F393H analysed by synchrotron X-ray crystallography at 1.60 Å resolution. The X-ray dose during data collection was 2 MGy. The polder omit maps for styrene (STY) and haem-bound O_2 in chain B are shown as a mesh contoured at 4σ. An enlarged view of the haem-O_2 moiety is shown in (B). Details of the haem-O_2 geometry are provided in **Supplementary Table S2**.



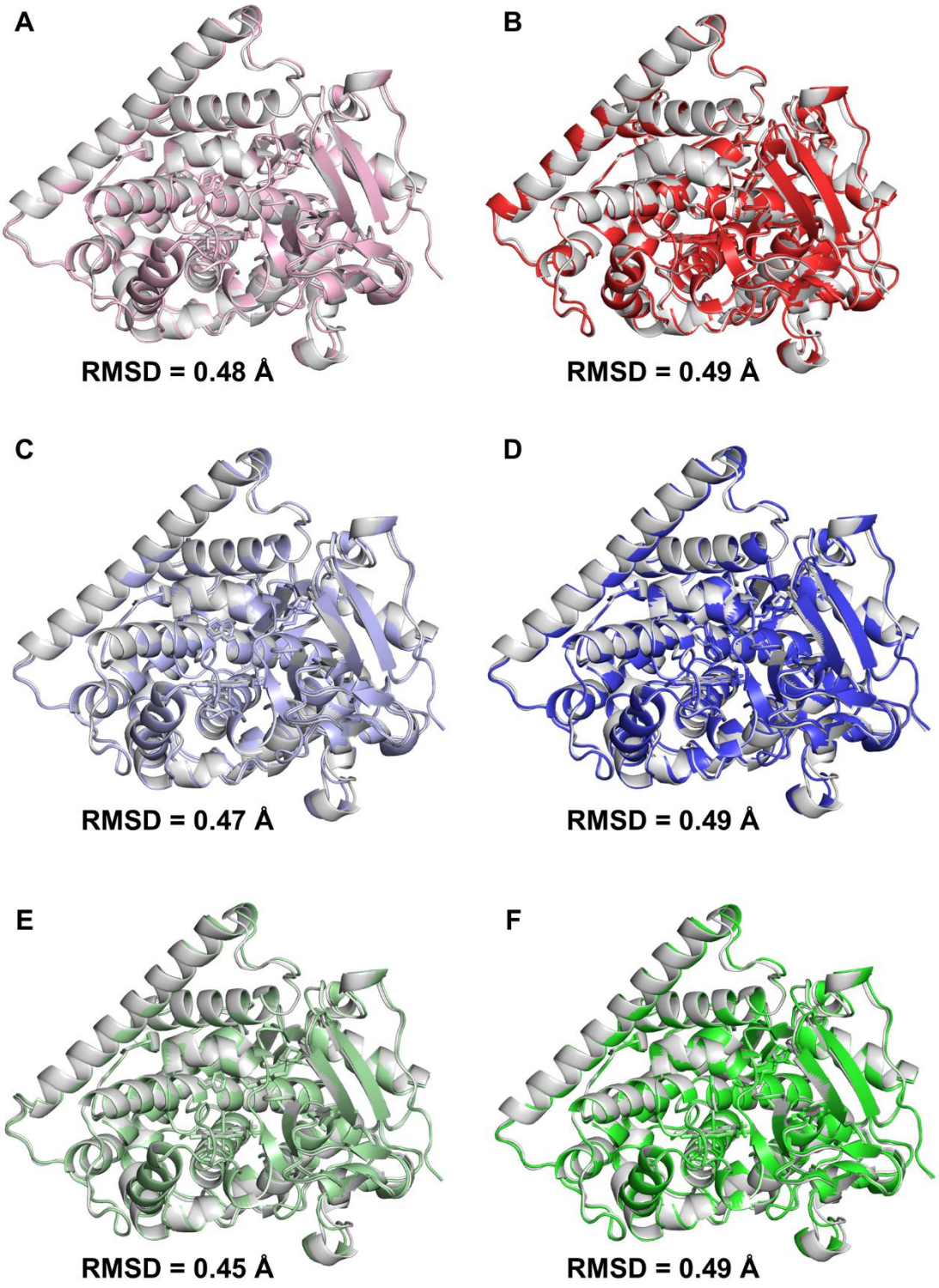
Supplementary Fig. S10. Superimposed haem active site structures of the oxygen-bound (green) and reduced (blue) forms of F393H (A) and F87A/F393H (B) of P450BM3. The haem, styrene, O_2, and side chains of Phe87 and Ala87 are shown by the stick model. The van der Waals radius of O_2 is shown by the sphere model. The interactions between O_2 and Phe87 (A) and between O_2 and styrene (B) are shown by broken curves. The movement of Phe87 in (A) is shown by an arrow.



Supplementary Fig. S11. Interaction between C7ProPhe and styrene in the oxygen-bound form of F393H (A) and F87A/F393H (B) of P450BM3. C7ProPhe and styrene are shown by the stick model. The van der Waals radius of styrene is shown by the sphere model. The cavity next to styrene is shown by the surface model in (B). The distance between the terminal carbon atom of C7ProPhe and the center of the phenyl group of styrene is shown in the figure.



Supplementary Fig. S12. Custom-ordered 2 × 2 mm^2 mesh loop designed for efficient data collection in diffraction measurements. Overall (A) and expanded (B) drawing of the loop and image of the mesh (C).



Supplementary Fig. S13. Comparison of the overall structures of chain A and chain B of F393H (A, C, E) and F87A/F393H (B, D, F) P450BM3 in their oxidised (A and B), reduced (C and D), and oxygen-bound (E and F) forms. The structure of chain A (shown in grey) is superimposed on that of chain B (shown in colour).