# Identifying the Elusive Sites of Tyrosyl Radicals in Cytochrome *c* Peroxidase: Implications for Oxidation of Substrates Bound at a Site Remote from the Heme.

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**Figure S1**. The 285-GHz EPR spectra of Tyr radicals (right panel) formed in the multiple-Trp/Tyr variants of cytochrome *c* peroxidase upon reaction with 2-fold molar excess of hydrogen peroxide. The variants, were designed to include catalytically-relevant Trp sites plus the putative Tyr sites (proposed in various previous reports) in an additive manner, and were named in Pfister *et al*. (28) as follows,

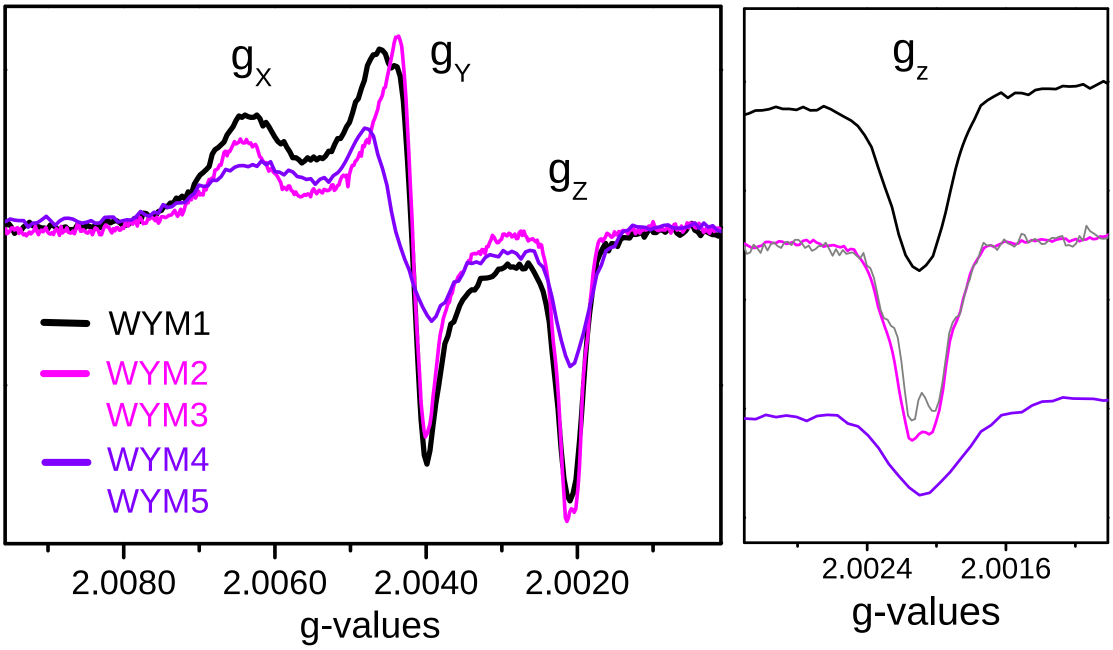
**WYM1** = W191F,

**WYM2** = W191F/W51F,

**WYM3** = W191F/W51F/Y187F,

**WYM4** = W191F/W51F/Y187F/Y229F/Y236F,

**WYM5** = W191F/W51F/Y187F/Y229F/Y236F/Y36F/Y39F/Y42F.



It is of note, that WYM2 and WYM3 variants (left panel, magenta trace) showed identical spectra, hence indicating that Tyr187 does not contribute to the Tyr$^\bullet$ signal. Similarly, the identical spectra of WYM4 and WYM5 variants (left panel, blue trace) indicates that Tyr36, Tyr39 and Tyr42 do not contribute to the Tyr$^\bullet$ signal (see Results section). The zoom-in view (left panel) of the 285 GHz-EPR spectra corresponding to the $g_z$ spectral region of the Tyr$^\bullet$ signal, shows the resolved proton hyperfine couplings of the WYM2 and WYM3 radicals (magenta trace). The Tyr radical spectrum (gray trace) of the W191G variant of CcP-MKT (16) is shown for comparisons. The experimental conditions are described in Figure 1.

**Figure S2.** Zoomed-in view (left panel) of the environment of the Tyr$_{71}^\bullet$ site identified in this work by multi-site Trp/Tyr mutations and multifrequency EPR spectroscopy on cytochrome *c* peroxidase. The view is taken from the protein surface; hence the guaiacol binding site (GUA1) is closer to the reader. The structural water molecule within H-bonding distance (shown in dotted blue line) to the phenolic oxygen of Tyr71, and the short stretch of amino acid residues (Asn82 to Phe89) surrounding the phenol oxygen of Tyr71 and the structural water are also shown. The substrate binding site on the surface (GUA1), located more than 20 Å away from the heme and from Trp191 (see Figure 2), but within 10 Å distance to Tyr71 is also shown (right hand panel). The figure was made using the published structure of the CcP-guaicol complex by Raven and coworkers (25) (PDB accession code 4A6Z).

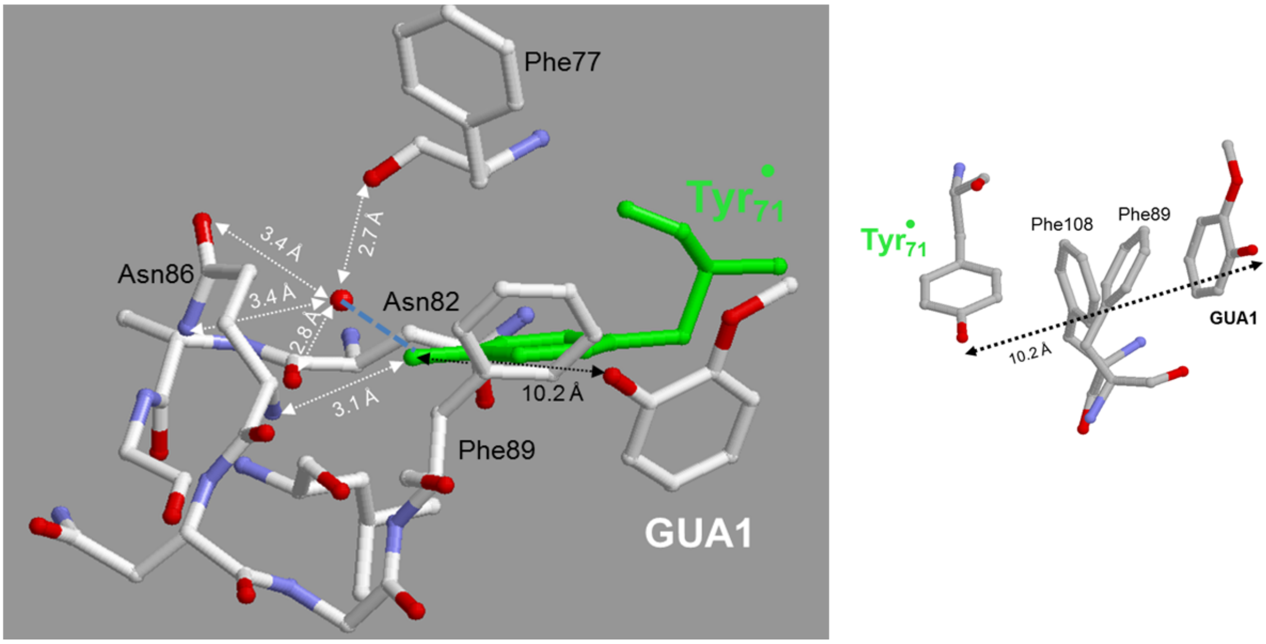


**Figure S3**. (Panel A) The 9-GHz EPR spectra, recorded at 4 K, of the protein-based radicals formed upon reaction of wild-type CcP with hydrogen peroxide, in the absence (black trace) and presence (gray trace) of guaiacol substrate. The (ca. 400 G) broadening of the Trp radical spectrum arises from the weak exchange interaction with the ferryl moiety of the [Fe(IV)=O] intermediate (13-16). The arrow indicated the contribution of the narrow Tyr radical signal, largely masked, at this frequency, by the broad Trp191 radical spectrum (for a detailed description, see (16)). The disappearance on the Tyr radical when the enzyme is incubated with the substrate prior to the reaction with hydrogen peroxide (gray trace) is better quantified when recording the spectra at 40 K and lower microwave power conditions (Panel B), since most of the contribution of the exchange-coupled $Trp_{191}^{\bullet+}$ signal is prevented in these experimental conditions (see (16)). It is of note that the modulation amplitude used to record the spectrum in gray trace (panel B) was three times higher than for the spectrum in black, hence the signal in gray would be three times smaller, if recorded in the same experimental condition than the spectrum in black.