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Substrate promiscuity of a *de novo* designed peroxidase

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Abstract.

The design and construction of *de novo* enzymes offer potentially facile routes to exploiting powerful chemistries in robust, expressible and customisable protein frameworks, while providing insight into natural enzyme function. To this end, we have recently demonstrated extensive catalytic promiscuity in a heme-containing *de novo* protein, C45. The diverse transformations that C45 catalyses include substrate oxidation, dehalogenation and carbon-carbon bond formation. Here we explore the substrate promiscuity of C45's peroxidase activity, screening the *de novo* enzyme against a panel of peroxidase and dehaloperoxidase substrates. Consistent with the function of natural peroxidases, C45 exhibits a broad spectrum of substrate activities with selectivity dictated primarily by the redox potential of the substrate, and by extension, the active oxidising species in peroxidase chemistry, compounds I and II. Though the comparison of these redox potentials provides a threshold for determining activity for a given substrate, substrate:protein interactions are also likely to play a significant role in determining electron transfer rates from substrate to heme, affecting the kinetic parameters of the enzyme. We also used biomolecular simulation to screen substrates against a computational model of C45 to identify potential interactions and binding sites. Several sites of interest in close proximity to the heme cofactor were discovered, providing insight into the catalytic workings of C45.

Keywords.

Peroxidases, *de novo* proteins, heme, redox enzymology, biomolecular simulation

1. Introduction.

De novo enzymes constructed from standard biotic components that both reproduce and improve upon the chemistry of their natural counterparts are of significant interest as they offer insight into the inner workings of natural enzymes while providing robust and highly versatile catalysts for a multitude of applications [1–6]. To date, the catalytic repertoire of such *de novo* enzymes remains fairly restricted, and while Kemp eliminase [7], retro-aldolase [8], carbonic anhydrase [9], esterase [10], peroxidase [11,12] and carbene transferase [13,14] activities have been successfully introduced into *de novo* designed protein scaffolds, directed evolution is often required to access catalytic parameters approaching the activities of their natural counterparts [8,15]. Most of these examples are cofactor-free, exhibit only a single type of catalytic activity and possess relatively limited substrate scope; these are typically consequences both of the design procedure employed and the type of activity sought for incorporation. While not a problem for achieving the intended

activity *per se*, the singular nature of catalytic function within these enzymes may limit the adaptability, mutability and overall potential of the *de novo* enzymes as broadly active starting points for evolution and downstream application.

In contrast, where the *de novo* enzyme activity is dependent on a protein-bound cofactor such as heme (e.g. peroxidase and carbene transferase activities) [11–13], the *de novo* proteins are imprinted with the cofactor's intrinsic catalytic activities. The interplay between cofactor and protein defines these activities and modulation of the protein environment allows for fine-tuning of their characteristics. For natural heme-containing proteins and enzymes, there are many examples of both catalytic and substrate promiscuity, resulting in versatile multi-functionality that has often been exploited to serve biological function [16–19]. For example, the core function of myoglobin is as an oxygen storage medium and buffer in the muscle tissue of vertebrates [20]; it also has secondary activities as a nitrite reductase [21], nitric oxide dioxygenase [22], peroxidase [23], oxidative dehalogenase [24] and carbene transferase [25]. Though some of these activities are likely not physiologically relevant, this catalytic promiscuity is not unusual for heme proteins and enzymes. Similarly, there are examples of natural heme enzymes that display significant substrate promiscuity for a given function. Heme-containing peroxidases catalyse the oxidation of a diverse variety of small molecule and protein substrates [16,26–28], and given the often highly efficient and rapid catalysis that they can exhibit, they are particularly attractive for industrial application and exploitation [29,30]. Both types of promiscuity are evident in *de novo* designed heme proteins, which can exhibit impressive catalytic activities and reaction scope in minimal scaffolds without the explicit requirement for random or semi-random directed evolution approaches to hone activity [11,13,31]. It is also notable that this promiscuity is exhibited in the burgeoning field of artificial metalloenzymes, where catalytically active metal complexes and organic molecules have been successfully introduced into natural and engineered protein scaffolds [32,33].

We have previously demonstrated that a heme-containing *de novo* protein, C45, functions as a catalytically proficient peroxidase [11], oxidative dehalogenase and carbene transferase [13,14], exhibiting activity *in vitro* and, for homologous ring expansion reactions, in whole cell biocatalysis. C45 is a thermostable four-helix bundle containing a covalently attached, 5-coordinate monohistidine-ligated heme C, which is expressed and fully assembled *in vivo* in *E. coli*. Its design is simple, and does not rely on atomistic recapitulation of natural heme enzyme active sites or catalytic motifs [5,34]. Despite the lack of canonical peroxidase active site residues, C45 exhibits peroxidase kinetics for ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid) oxidation ($k_{\text{cat}} = 1200 \text{ s}^{-1}$, $K_{\text{M}} = 379 \text{ }\mu\text{M}$ $k_{\text{cat}}/K_{\text{M}} = 3.2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$) [11] that match those of horseradish peroxidase ($k_{\text{cat}} = 4100 \text{ s}^{-1}$, $K_{\text{M}} = 800 \text{ }\mu\text{M}$ $k_{\text{cat}}/K_{\text{M}} = 5.13 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$) [35], though with a lower catalytic efficiency for the coupled hydrogen peroxide reduction (C45 $k_{\text{cat}}/K_{\text{M}(\text{H}_2\text{O}_2)} = 1.3 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$; horseradish peroxidase (HRP) $k_{\text{cat}}/K_{\text{M}(\text{H}_2\text{O}_2)} = 4.6 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$) [11,35]. Using presumably similar chemistry, C45 can also catalyse the oxidation of ferrous cytochrome c and the oxidative dehalogenation of trichlorophenol [11]. Most recently, we have demonstrated that the same unmodified enzyme can catalyse the stereoselective insertion of carbenes into olefins, amines and pyrrole, while facilitating the olefination of aldehydes [13,14]. Perhaps surprisingly, NMR spectroscopy reveals that C45 is conformationally dynamic [11]. The role of such flexibility in facilitating or hindering substrate access, peroxide reduction, product release, and ultimately, catalysis within such *de novo* scaffolds remains unresolved. However, such behaviour is not

unusual in this type of simple *de novo* protein, and there are now several examples where dynamic behaviour has been observed in conjunction with desirable functions [36–38]. Irrespective of this issue, C45 represents a robust, catalytically promiscuous and potentially valuable catalyst.

While we have previously reported the peroxidase and carbene transferase activities of C45 [11,13,14], we have not fully explored the substrate promiscuity and scope of its peroxidase-type activity. Here we use a combination of computational and experimental methods to screen the activity of C45 against a structurally diverse panel of common peroxidase and oxidative dehalogenase substrates. While most screened peroxidase substrates are efficiently oxidised by C45 in the presence of hydrogen peroxide despite their structural differences, we find negligible activity against substrates with redox potentials in excess of +1 V (vs NHE). This indicates a likely upper limit for the redox potential of the active oxidising species of C45 during reaction, compounds I and II, and is consistent with the substrate promiscuity and activities observed for natural peroxidases. We also employ *in silico* molecular docking techniques to identify potential substrate binding and interaction sites at the protein surface, finding several clusters of interaction sites within sufficient distance of the heme for rapid electron transfer.

2. Materials and Methods.

2.1 General

All substrates, chemicals and reagents were purchased from Sigma, ThermoFisher or VWR unless otherwise indicated. T7 Express competent cells were purchased from New England Biolabs. UV/visible spectra and initial kinetic scanning experiments were recorded on an Agilent Cary 60 spectrometer (Agilent Technologies).

2.2 Protein expression and purification

C45 was expressed and purified as described previously [11,39]. Glycerol stocks of *E. coli* co-transformed with pEC86 [40] and pSHT-C45 were initially used to inoculate a 50 mL starter culture of lysogeny broth (LB) containing carbenicillin (50 µg/mL) and chloramphenicol (34 µg/mL). After overnight shaking incubation at 37°C and 180 rpm, starter cultures were added to 1 L of aerobic LB, which was then incubated at 37°C and 180 rpm. When the optical density of the cultures at 600 nm reached 0.6-0.7, C45 expression was induced by adding isopropyl-D-1-thiogalactopyranoside (IPTG, Apollo Scientific) to a final concentration of 0.5 mM. After 4 hours of incubation (37°C and 180 rpm), the cells were harvested by centrifugation (at 4,000 xg) and resuspended in lysis buffer (300 mM NaCl, 50 mM sodium phosphate, 20 mM imidazole, pH 8.0). Phenylmethanesulfonylfluoride (PMSF), a serine protease inhibitor, was added to 1 mM and the lysate was kept below 4°C when handling to avoid proteolysis. Three cycles of Sonication (using a Soniprep 150, MSE UK) at 100% amplitude with 30 second pulses separated by 30 seconds of mixing lysed the cells pellets. The aqueous and lipid phases of the lysed sample were subsequently separated by centrifugation at 40,000 xg for 30 minutes. The lysate supernatant was then filtered through a 0.2 µm pore filter and loaded onto a 5 mL nickel affinity chromatography column (HisTrap HP, GE Healthcare, UK) equilibrated with lysis buffer. A red band indicating the his-tagged, heme-containing C45 binds to the column while the majority of cellular proteins flow through. The his-tagged C45 was eluted with a gradient of elution buffer (300 mM NaCl, 50 mM sodium phosphate, 250 mM imidazole, pH 8.0) and the fractions containing C45 were collected and dialysed overnight into TEV cleavage buffer (20 mM Tris, 0.5 mM EDTA,

pH 8.0) in 8,000 MW tubing. The N-terminal His-tag was cleaved during a 4 hour incubation (room temperature in anoxic conditions) with tobacco etch virus n1a (TEV) protease (100 µg/mL) and tris(2-carboxyethyl)phosphine (TCEP, 1 mM). C45 was subsequently loaded onto a 5 mL nickel affinity column equilibrated with lysis buffer, and the flowthrough containing cleaved C45 was harvested. Following concentration of the C45 sample to approximately 4 mL using centrifugal concentrators (Vivaspin 20, 5,000 MWCO), the sample was loaded onto a HiLoad Superdex 75 pg size exclusion column equilibrated with redox buffer (100 mM KCl, 20 mM CHES at pH 8.6) at a flow rate of 1 mL/min. Fractions were then pooled and concentrated to the required concentration for kinetic assays. The pyridine hemochrome assay was then used to accurately determine the heme protein concentration. The assay was performed in a low volume cuvette as follows. 90 µL of C45 (at a concentration of approximately 1 - 10 µM) was added to the cuvette and the UV/visible spectrum was recorded. Then 90 µL of 40 % pyridine in 0.4 M NaOH was added. The solutions were mixed thoroughly and the oxidised spectrum was recorded. Immediately following this, the sample was reduced by the addition of several grains of sodium dithionite and mixed thoroughly. The reduced hemochrome spectrum was recorded and the concentration of heme was calculated by the difference in oxidised and reduced spectrum ($\epsilon A_{550(\text{red})} - \epsilon A_{550(\text{ox})} = 21,840 \text{ M}^{-1} \cdot \text{cm}^{-1}$) [41].

2.3 Steady State Kinetic Assays

All kinetic assays were performed a pH 8.6 in redox buffer at 25°C (100 mM KCl, 20 mM CHES at pH 8.6). In general, stock solutions of the selected substrates were diluted to stock concentrations of 400 µM and 10 µM in Redox buffer prior to use. Michaelis-Menten kinetic data were obtained using a KinetAsyst SF-61DX2 double-mixing stopped-flow spectrophotometer (Hi-Tech Limited, UK) with a 1 cm pathlength. Reactions were initiated by mixing equal volumes of a solution of C45 (0.1 µM) and electron donating substrate in redox buffer, with a solution of hydrogen peroxide (100 µM) in redox buffer. The initial activities were converted to rates using the relevant substrate extinction coefficients, plotted against substrate concentration and fit with either a Michelis-Menten function or a variant of this equation incorporating substrate inhibition. Individual substrate preparations and assays are described below:

40 mg of **2,4,6-tribromophenol (TBP)** was dissolved in 100 µL ethanol and then serially diluted to 400 µM in redox buffer. The extinction coefficients of the three UV-visible peaks of TBP were determined in redox buffer (316, 249 and 214 nm). This was achieved by recording the absorbance peaks at know concentrations of TBP in the range 1 – 150 µM, then using linear regression to determine the gradient. In these conditions TBP is stable over the course of 6 hours. Oxidation of TBP was observed at 249 nm (and 316 nm at substrate concentration above 70 µM) monitoring the decrease in substrate ($\epsilon_{(249\text{nm})} 1,118 \text{ M}^{-1} \cdot \text{cm}^{-1}$, $\epsilon_{(249\text{nm})} 4,994 \text{ M}^{-1} \cdot \text{cm}^{-1}$). The concentrations used were 2.5, 5, 10, 20, 40, 80, 100, 150 µM.

Amplex red was purchased from ThermoFisher (UK). 10 mM stock solutions were prepared in the dark in DMSO and stored at –20°C, as recommended by the supplier's protocol (Amplex Red glutamic acid/glutamate oxidase assay protocol, Invitrogen). Amplex Red was protected from light and kept on ice throughout the assays. 400, 20 and 0.12 µM stocks of Amplex Red were made up in redox buffer. In these conditions only marginal degradation of Amplex Red was observed after 6 hours. Oxidation of Amplex Red was observed at 575 nm, monitoring the increase of the product resorufin ($\epsilon_{(572\text{nm})} 73,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$) [42]. The concentrations used were 0.05, 0.5, 1.2, 2.5, 5, 10, 40, 75, 150 µM.

40 mg of **luminol** was dissolved in 200 μL 1 M KOH, then 800 μL deionized water was added to 1 mL. It was then diluted to 400 μM in redox buffer. The extinction coefficients of the three UV-visible peaks of luminol were determined in redox buffer (348, 302, 221 nm). This was achieved by recording the absorbance peaks at known concentrations of luminol in the range of 1 – 150 μM and using linear regression to determine the gradient. In these conditions luminol is stable over the course of 6 hours. Oxidation of luminol was observed at 302 nm (and 221 nm at substrate concentration above 70 μM) monitoring the decrease in substrate absorbance ($\epsilon_{(302\text{nm})}$ 6,830 $\text{M}^{-1}.\text{cm}^{-1}$, $\epsilon_{(221\text{nm})}$ 27,760 $\text{M}^{-1}.\text{cm}^{-1}$). The concentrations used were 2.5, 5, 10, 20, 40, 80, 100, 150 μM . 1 μL **guaiacol** was diluted to 1 mL in deionized water and then diluted to 400 μM in redox buffer. The concentration was determined spectroscopically ($\epsilon_{(274\text{nm})}$ = 2550 $\text{M}^{-1}.\text{cm}^{-1}$) [43]. In these conditions guaiacol is stable over the course of the experiment. Oxidation of guaiacol was monitored at 470 nm monitoring the increase in tetraguaiacol formation ($\epsilon_{(470\text{nm})}$ = 3,800 $\text{M}^{-1}.\text{cm}^{-1}$). The guaiacol concentrations used were 1, 5, 10, 20, 40, 80, 100, 150 μM . 1 mg **o-dianisidine** was dissolved in 500 μL of deionized water. Following the addition of 100 μL 1 mM KCl, the solution was made up to 1 mL. The concentration was determined spectroscopically in acid ($\epsilon_{(251\text{nm})}$ = 2,550 $\text{M}^{-1}.\text{cm}^{-1}$) and then serially diluted to 400 μM in redox buffer. In these conditions o-dianisidine is stable over the course of the experiment. Oxidation of guaiacol was monitored at 440 nm monitoring the increase in oxidized o-dianisidine formation ($\epsilon_{(440\text{nm})}$ = 11,300 $\text{M}^{-1}.\text{cm}^{-1}$) [44]. The o-dianisidine concentrations used were 1, 5, 10, 20, 40, 80, 100, 150 μM . 40 mg **o-phenylenediamine** was dissolved in ethanol and then diluted to 400 μM in redox buffer. In these conditions, o-phenylenediamine is stable over the course of the experiment. Oxidation of o-phenylenediamine was monitored at 450 nm monitoring the increase in phenazine-2,3-diamine formation ($\epsilon_{(450\text{nm})}$ = 16,300 $\text{M}^{-1}.\text{cm}^{-1}$) [45]. The o-phenylenediamine concentrations used were 1, 5, 10, 20, 40, 80, 100, 150 μM .

2.4 Molecular Dynamics Simulations

Molecular dynamics (MD) simulations were run with AMBER [46] either on the University of Bristol's supercomputer, BlueCrystal phase 3, or a local Linux system. Simulations were carried out in a cuboidal periodic box ($> 18 \text{ \AA}$ from protein) with transferrable intermolecular potential 3 point (TIP3P) explicit solvent and with 150 mM sodium and chloride ions added. The histidine residue in the CXXCH motif of C45 was singly protonated on the N δ (HID). All other ionizable residues were in their physiologically relevant protonation states. Empirically-derived topologies and parameters for c-type heme in the AMBER format were helpfully provided by the Luthey-Schulten group [47]. The MD was setup on a local machine using Visual Molecular Dynamics (VMD) to build a TIP3P water box with ions. VMD was used to patch together the protein and c-type heme parameter, topology and structure files into a coordinate file (pdb) and protein structure file (psf). Chamber within parmed.py (part of AMBERtools16) was used to produce AMBER compatible parameter-topology and coordinate files (.prmtop and .inpcrd). Atom names of the TIP3P water were edited to make them compatible with AMBER naming conventions. A series of preliminary energy minimizations was performed; a minimisation of solvent and hydrogens with protein heavy atoms restrained, a heating of solvent with restraints for the protein, a minimisation of the entire system, a quick heating of the entire system and a final 200 ps NPT ensemble MD equilibration of the system. The production MD was run at 2 fs time steps for a total length of 100 ns using the GPU accelerated AMBER16 code, pmemd.cuda [ref from above]. A weak-coupling algorithm to maintain a constant temperature at 300 K and isotropic Berendsen pressure coupling

at 1 atm were used [48]. Structures were saved every 100 ps. The system was initially run with a non-bonded cutoff of 12 Å. Cpptraj in AMBERTools and VMD were used to analyse and visualise the results.

2.5 Computational substrate binding screen using BUDE

BUDE (The Bristol University Docking Engine) [49] in surface scanning mode was used for 19 ligands on 10 different snapshots from a 100 ns MD simulation of C45. The snapshots were taken every 10 ns of the simulation. The structures of the substrates were taken from the ZINC database online (<https://zinc.docking.org>) and parameterised with the programme acpype.py using Antechamber and the General AMBER Force Field (GAFF) [50–52]. Protonation states of the substrates were defined either by the ZINC database files or available literature pKa values. A single conformer of each ligand was used except for ABTS where 10 different conformers were used, generated by a previous MD simulation. Charges of ligand functional groups were set to their value at pH 8.6. The BUDE setup, BUDE running, processing for MD, MD setup and MD post-processing were scripted where appropriate. Each combination of ligand and snapshot of C45 simulation (receptor) had 1,200 randomly assigned surface positions tested. At each of these positions 3,000 conformational iterations of the starting ligand pose were tested with the top 30 energies selected to be parents. These parent poses were then randomly iterated a further 3,000 times and a new generation of top 30 energy parents selected. This process was repeated for ten generations at each starting pose. In total, 1.08×10^{10} conformations are sampled per ligand with C45, with each generation following a path to stronger binding. The top 50 binding modes per ligand were then individually processed and run as a 5 ns MD simulation using the AMBER setup. VMD was used to process the trajectories aligning all frames from across all ligands by the protein backbone and then calculating the centre of mass for each ligand with respect to the heme iron. For each frame, if the heavy atoms of the ligand were found to be over 5 Å from any part of the protein it was classified as dissociated. This information was then analysed using Python: a three-dimensional cluster analysis was performed on both the BUDE input structures and the MD results after 5 ns, separately. This was to give an approximate quantification of possible binding hotspots. Each of the start and end data sets analyzed the centre of mass of all ligands together. The cluster analysis used sklearn DBSCAN [53]. All ligands marked as dissociated were binned in one cluster for the 5 ns analysis. VMD was also used to generate lists of close interactions (<3 Å) between the heavy atoms of the ligands and protein. The frequency of these interactions on the final structure of the 5 ns MD simulation were binned per protein residue and per protein atom and used to produce ligand binding heat map figures.

3. Results and Discussion.

3.1 Experimental screening of peroxidase activity

Our initial characterisation of peroxidase and oxidative dehalogenase activity in C45 principally focussed on the substrates ABTS, ferrocycytochrome c and 2,4,6-trichlorophenol, establishing typical ping-pong peroxidase kinetics, interprotein electron transfer coupled to peroxide reduction and the potential for using C45 as an active bioremediation catalyst for degrading halogenated phenols [11]. In addition, we highlighted potential substrates that exhibited spectroscopic changes in the presence of C45 and hydrogen peroxide. For this work, we used this possible substrate set as the basis for assembling our panel for activity screening and characterisation, including additional, well-established natural peroxidase substrates. The structural diversity of the selected

substrate panel is highlighted in **Fig. 1**, indicating substrate redox potentials and the conditions in which these data were obtained. Since C45 exhibits a relatively high K_M for hydrogen peroxide ($K_M = 94$ mM), we assessed the steady state kinetics of C45 for the substrates at limiting peroxide concentration (100 μ M), thus minimising peroxide-induced degradation of substrate, and enzyme inactivation at low substrate concentrations. This was consistent with the kinetic analysis we previously applied to the substrates ferrocycytochrome *c* and 2,4,6-trichlorophenol [11]. We also selected buffer conditions (pH 8.6) that previously resulted in optimal C45 activity against ABTS, reasoning that these conditions likely maximise the rate of formation and yield of compound **1**.

Screening this panel of substrates, we found that C45 exhibits substantial promiscuity which is consistent with the behaviour of many natural peroxidases. In particular, C45 exhibited excellent activity against *ortho*-phenylenediamine, guaiacol, *ortho*-dianisidine, Amplex Red, luminol, and 2,4,6-tribromophenol, and we were able to obtain kinetic parameters using simple Michaelis-Menten analysis at limiting peroxide concentrations and the pH optimum for the C45-catalysed oxidation of ABTS (pH 8.6) (**Fig. 2, Table 1**). *ortho*-Phenylenediamine is a common peroxidase substrate, and formation of the 2,3-diaminophenazine product can be monitored spectroscopically at 450 nm (**Fig. 2A**) [54]. Our steady-state kinetic analysis of C45 with OPD revealed kinetic parameters consistent with those obtained for C45-catalysed ferrocycytochrome *c* oxidation ($V/[E]_0 = 1.1$ s⁻¹, $K_M = 6.2$ μ M, $(V/[E]_0)/K_M = 1.8 \times 10^5$ M⁻¹s⁻¹), and we would predict that $V/[E]_0$ and $(V/[E]_0)/K_M$ would significantly increase under non-peroxide-limiting conditions for OPD and indeed, all the substrates for which we measured kinetic parameters. Guaiacol is a small phenolic flavour molecule that is a classical peroxidase substrate [55]. Like natural peroxidases, C45 catalyses the formation of tetraguaiacol which can be monitored at 470 nm, revealing similar kinetic parameters to OPD ($V/[E]_0 = 0.76$ s⁻¹, $K_M = 2.6$ μ M, $(V/[E]_0)/K_M = 2.9 \times 10^5$ M⁻¹s⁻¹) (**Fig. 2B**). *ortho*-Dianisidine is an azo dye precursor and a useful colorimetric peroxidase substrate [56]. While C45 catalyzes the formation of the typical peroxidase induced oxidation product (monitored at 440 nm), we observed significant substrate inhibition in the Michaelis-Menten plot ($V/[E]_0 = 1.04$ s⁻¹, $K_M = 1.9$ μ M, $(V/[E]_0)/K_M = 5.4 \times 10^5$ M⁻¹s⁻¹; $K_i = 30.2$ μ M), possibly indicating that substrate either directly blocks the access of peroxide to the heme or indirectly through conformational changes to the protein (**Fig. 2C**). Amplex Red is a dihydroresorufin derivative that is commonly used in highly sensitive fluorescent assays for peroxide activity *in vitro* and *in vivo*, as peroxidases deacetylate the molecule, liberating the fluorescent resorufin (**Fig. 2D**) [57]. C45 effectively catalyses this deacetylation, exhibiting kinetic parameters consistent with those of the other substrates tested ($V/[E]_0 = 0.41$ s⁻¹, $K_M = 0.53$ μ M, $(V/[E]_0)/K_M = 0.65 \times 10^5$ M⁻¹s⁻¹). Luminol, typically used in crime scene forensics, is a peroxidase substrate that emits light *via* chemiluminescence following a ring-opening oxidation mechanism [58]. C45 also catalyses this chemiluminescent process, functioning essentially as an artificial luciferase, and monitoring the absorbance changes at 302 nm, kinetic parameters were found to be comparable with the other substrates tested ($V/[E]_0 = 0.65$ s⁻¹, $K_M = 2.9$ μ M, $(V/[E]_0)/K_M = 2.3 \times 10^5$ M⁻¹s⁻¹) (**Fig. 2E**). To further probe the oxidative dehalogenation activity of C45, we tested the activity of C45 against 2,4,6-tribromophenol. C45 achieves this reaction with similar kinetics to those exhibited for the chloro- analogue ($V/[E]_0 = 0.5$ s⁻¹, $K_M = 12$ μ M, $(V/[E]_0)/K_M = 4.2 \times 10^4$ M⁻¹s⁻¹) (**Fig. 2F**), again improving on the catalytic efficiency of the natural dehaloperoxidase isoforms from *A. ornata* [59].

The kinetic data are summarised in **Fig. 3**, which includes previously recorded kinetic parameters for the oxidation of ferrocycytochrome *c* and ABTS, and the oxidative dehalogenation of TCP under

the same conditions and at limiting H_2O_2 concentration [11]. While there is some variation in $V/[E]_0$ across the substrates, all fall within the range of 0.4 to 2 s^{-1} . K_M is relatively invariant for all but the halogenated substrates and *ortho*-phenylenediamine, resulting in catalytic efficiencies ($(V/[E]_0)/K_M$) of between 10^5 and $10^6\text{ M}^{-1}\text{s}^{-1}$ for substrates that undergo oxidation or deacetylation, and $10^4\text{ M}^{-1}\text{s}^{-1}$ for those that are oxidatively dehalogenated. As previously noted, these kinetic data should be interpreted with care, as the limiting peroxide concentration prohibits a complete kinetic analysis and leads to a considerable underestimate of the maximum rates of turnover. However, they do provide an indication of C45's relative activity against the substrates and it is notable that C45 exhibits fairly high catalytic efficiencies for most of them.

In addition to these substrates, C45 exhibits detectable activity against the documented peroxidase substrates *p*-anisidine [60], homovanillic acid [61] and Reactive Orange II [62] (**Fig. 4**). However, we were not able to undertake a full kinetic characterisation of these substrates due to practical complications: homovanillic acid is sensitive to light and degrades readily at pH 8.6; and we were unable to fully characterise the oxidation products of *p*-anisidine and Reactive Orange II, or obtain their extinction coefficients. Despite these issues, it was evident that homovanillic acid protects the C45 heme from peroxide-induced degradation, matching the behaviour of substrates such as ABTS [11], and indicating that electron transfer does indeed occur from the substrate to the high valent oxo-iron species, compounds I and II. In contrast, although Reactive Orange II was oxidised by C45, the heme Soret peak bleaches, indicating significant heme degradation over the course of the reaction.

Not all the tested molecules proved to be substrates for C45; negligible peroxidase activity was detected for C45/ H_2O_2 in the presence of *para*-aminobenzoic acid [63], 1,4-dimethoxybenzene [64] or veratrole alcohol [65] (**Fig. 5**). In all cases, we observed bleaching of the heme Soret with no concomitant spectroscopic changes consistent with substrate oxidation. Structurally, these molecules are not dissimilar from those which C45 can oxidise using its peroxidase activity; in fact, prediction of peroxidase and C45 substrates by inspection and structural comparison alone does not unambiguously reveal selection rules for activity, though most successfully characterised substrates are either phenolic or amine-containing. However, comparing the redox potential of the substrate with that of the peroxidase compounds I and II provides a more robust measure of whether the selected peroxidase will have activity against it [66], highlighting the major determinant of their substrate promiscuity. For the substrates that C45 is unable to oxidise in the presence of H_2O_2 , all have high reduction potentials (+1.08, +1.30 and +1.36 V for *para*-aminobenzoic acid [67], 1,4-dimethoxybenzene [68] or veratrole alcohol [69] respectively), representing the highest of the selected substrate panel; only peroxidases with high compound I and II redox potentials (e.g. lignin peroxidase [70]) are capable of catalysing their oxidation. Notably, several of the other peroxidase substrates which we show here to be C45 substrates have reported redox potentials in the range of +800 mV to +1 V, including *o*-dianisidine (+809 mV [71]), 2,4,6-trichlorophenol (+930 mV [72]) and 2,4,6-tribromophenol (+984 mV [73]) (**SI Table 1**). It should be noted that the redox potentials of most of the substrates screened in this study are pH-dependent, and are likely to be lower than the values quoted here under the C45 assay conditions; however, they indicate a threshold substrate potential of between +800 mV and +1 V to facilitate C45-catalysed oxidation. This most likely represents the redox potential of either the C45 CpdI/CpdII or CpdII/ Fe^{3+} couple, which appear to be the most important determinant of peroxidase, and the related oxidative dehalogenase, activities. In comparison, the corresponding

HRP CpdI/CpdII and CpdII/Fe³⁺ couples at pH 7.0 are +949 and +991 mV respectively [74]. Since C45 broadly exhibits similar promiscuity and activity to HRP, it holds that the redox couples of their active oxidising species are comparable. To further confirm this assertion, it may be possible to gain a more precise estimate of C45 CpdI/CpdII and CpdII/Fe³⁺ couples using previously reported methods where peroxidase kinetics are assessed using substituted phenols [66,75]. Future work will therefore focus on assessing the potential of these redox couples through such indirect methods or by direct electrochemistry. For activity against high potential substrates such as veratrole alcohol without mediation or coupled reactions, potentials for these redox couples in excess of +1.3 V would be required, which is only achieved in lignolytic enzymes, such as manganese peroxidase [76] and lignin peroxidase [70]. In future it may be possible to evolve higher potential redox couples for the active oxidising species of C45, though this would likely require extensive computational redesign and directed evolution, and is beyond the scope of this current study.

3.2 Computational substrate binding screen

While the redox potential of the high valent oxo-iron heme species plays a major role in the determination of activity of a peroxidase against a substrate, we were also interested in identifying potential substrate:C45 interactions that might facilitate rapid electron transfer and therefore promote efficient catalysis. For C45, we would anticipate that the rate determining step in catalysis would be the formation of compound I, and while electron transfer from substrate to this species could be very rapid, it will be highly dependent on the location of the principal substrate:protein binding interaction. Mapping potential interaction sites onto our computational model of C45 would allow us to understand better if such interaction sites exist, what their specificity is, if any, and to enable interrogation through mutagenesis.

Using an extended substrate panel of 19 molecules, we performed a computational binding screen to explore potential interaction sites within or on the surface of C45 and gain insight into the peroxidase function of C45 (**Fig. 6, SI Figs. 1 & 2**). We used BUDE [49] to search for low energy binding conformations for each substrate candidate with C45, obtaining a variety of poses principally on the surface of the protein. We then selected the top 50 poses for each substrate and ran 5 ns MD simulations to test whether protein-substrate interactions were maintained, therefore indicating likely binding site:substrate pose combinations. Since all 50 of the poses for TCP were in the same locus, we selected only the top 40, resulting in a total of 940 starting structures for simulation. 18 of these structures produced badly minimised simulations that crashed during production MD and were not investigated further. The remaining 922 structures successfully completed 5 ns MD trajectories; for 609 of these simulations the substrate remained within 5 Å of any part of the protein or heme, and were considered successful poses, and included in further analysis.

It should be noted that only a single protonation state for each potential substrate was considered in these simulations, which may not be representative of substrate protonation states when encountering the protein. However, since this methodology is intended as an initial and relatively rapid screen to determine possible interactions using modest computational resources, we believe it has value in aiding the identification of binding sites. With regards to our computational model of C45, this was constructed as previously described [11], and likely represents one of an ensemble of low energy structures, since C45 is known to exhibit conformational heterogeneity.

However, this structure is consistently reproduced and maintained during our biomolecular simulations, and represents a reasonable framework for investigating substrate:protein interactions.

The results of this computational screen revealed several possible binding hot spots in close proximity to the heme. To probe the interaction of these substrates further and to discern which ligands share similar binding loci, we performed a 3D clustering analysis. **Fig. 7** reveals this clustering analysis following the MD simulations, with substrates represented as centroids, and binding hotspots for the substrates are displayed in the associated pivot table (**Table 2**). Although somewhat extended, cluster 3 above the heme and between helices 1 and 2 is the most frequently and widely sampled location with 52% of successful substrate poses situated here after MD. Small phenolic substrates (TCP, TBP, guaiacol), *o*-phenylenediamine and 1,4-dimethoxybenzene exert a strong preference for binding in a compact region of cluster 3 closest to heme, leading us to define this location as the small phenolic binding pocket (SP pocket). The centre of mass of ligands in this position is around 10 Å from the heme iron, or around 7 Å from the substrates to the heme edge, and substrates typically sit deep within this pocket centred over leucine 22. At this distance, electron transfer between substrate and heme would be very rapid [77], and would likely result in the high catalytic efficiencies observed experimentally for certain substrates. It could also account for the similarities in kinetic parameters that we observed for several of the tested substrates. In total, 287 of the 353 poses starting in the SP pocket remain in this cluster following MD.

The second most frequently sampled binding pocket is cluster 6. It constitutes 6.1% of successful poses and is situated on the opposite side of the protein to the heme, between helix 1 and 3 of our C45 computational model. The centres of mass of the ligands in cluster 6 are, on average, 27.1 Å from the heme iron or about 22 Å from the heme edge. At this distance, direct electron tunnelling from cluster 6-bound substrates to the heme would be slow, and it is unlikely that this would represent a productive location for substrate oxidation to occur. Clusters 5 and 8 comprise 5.4% of successful substrate poses and are situated in a cavity between helices 3 and 4. The centre of mass of ligands in these clusters are on average 11.9 Å from the heme iron or approximately 6 Å from the heme edge. Clusters 1 and 2 are found on the δ and γ heme edges respectively, similar to the location of the proposed phenolic substrate binding site of HRP, with some poses in cluster 1 resulting in direct interactions between substrate and the proximal, heme-coordinating histidine. Clusters 1 and 2 collectively represent 5.2% of successful binding poses. Since they are almost in van der Waals contact with the heme, electron transfer would be exceptionally rapid [77].

Though other clusters were identified, those mentioned above represent almost 70% of the binding poses successfully maintained following MD. Like cluster 6, the locations of other clusters such as 4 and 18 are likely too distant from the heme to facilitate substrate oxidation by the heme's high valent oxo-iron compounds. For some larger substrates, many binding poses resulted in unclustered positions. For instance, 33 of the top binding poses for ABTS were still associated with the protein after 5 ns but the majority were defined by the analysis as unclustered (labelled as cluster-1 in **Table 2**), making it challenging to identify a specific interaction location.

It is possible that the tryptophan side chains in C45 facilitate long range electron transfer from substrate to the C45 compounds I and II. By donating an electron to the high valent heme species, they may form tryptophan-centred radicals that could in turn oxidise surface-bound substrates. Such behaviour has been previously observed in lignin peroxidase, where a surface located tryptophan residue is thought to oxidise substrates binding or transiently interacting at the enzyme surface [5,78]. For C45, tryptophan 43 is located 7 Å from the heme, meaning that it could engage in this behaviour. Since this residue is close to clusters 10, 11 and 13 it may play a role in facilitating substrate oxidation. However, tryptophan 8 and 81 are relatively far from the heme (25 & 26.5 Å respectively). Given the slow rate of electron transfer between them and the heme that would be expected at this distance, it appears unlikely that they would play such a role unless there is further mediation of electron transfer through additional amino acid side chains.

To further visualise these data, we created heat maps demonstrating the frequency of close contacts from substrate atoms to both the heme atoms (**SI Fig. 3**) and all atoms of the protein (**Fig. 8**) following BUDE docking and MD simulation. These frequency maps highlight that the substrates interacting with heme do so mainly on the δ and γ heme edges, as mentioned above, and that the SP pocket is exceptionally well represented as a likely substrate binding site. Mutation of L22, E19 and E47 are predicted to perturb substrate:protein interactions in the SP pocket and should be targeted by mutation to validate the computational screen described here. Future studies will therefore focus on interrogating this binding site and establishing whether the C45 tryptophan residues play an active role in catalysis.

4. Conclusions.

We have demonstrated here that C45 acts as a promiscuous peroxidase, oxidising many typical peroxidase and dehaloperoxidase substrates. Our work indicates that this peroxidase activity is dictated primarily by the comparative redox potentials of substrate and compounds I and II, allowing for substantial variety in the molecular structure of substrates. For C45, and perhaps many of the natural peroxidases, the lack of a well-defined substrate binding site does not hinder catalysis, and it undoubtedly promotes the enzyme's promiscuity, relying on transient surface association with the active oxidising species rather than high affinity binding, reaction and release. In this sense, the peroxidases might be considered an outlier to other classes of enzymes, with substrate promiscuity as an inherent characteristic. However, there are still variations in natural peroxidase activities that may be at least partially explained by preferential substrate binding locations, such as the aromatic binding site of HRP [79] at the heme edge and the surface site of lignin peroxidase at the terminus of an intraprotein electron transfer chain [78]. Our own computational simulations indicate the presence of a small phenolic binding site in close proximity to the heme that may anchor a variety of substrates for long enough to favour rapid electron transfer to the C45 compound I and II. However, cluster analysis reveals a variety of potential locations for substrate association at the C45 surface, and additional studies are required to confirm the presence of these binding sites and establish their respective roles in catalysis.

With regards to *de novo* enzyme design, we have demonstrated that minimal engineering is sufficient to access a very broad palette of chemistries and substrates. As we, and others, have previously stated, such broadly active, small enzymes may provide insight into the catalytic function, activity and properties of primordial enzymes [11,32,80,81]. Catalytic and substrate promiscuity in early enzymes may have provided an economy of scale in a nascent metabolism

or organism, where a single component performing many functions was key to fitness and ultimately survival. Such promiscuity undoubtedly was, and still is, beneficial within a changing environment, enabling organisms to rapidly adapt their metabolisms or detoxify harmful substances to which they are exposed. For both *de novo* and natural peroxidases, achieving a substantial degree of substrate specificity might not be possible without major structural reorganisation to shield heme from bulk solvent, and the creation of well-defined and selective substrate binding sites. However, substrate promiscuity can be expanded by increasing the redox potential of compounds I and II; conversely, lowering these redox potentials could increase substrate specificity at the expense of oxidising power. By doing so, it may be possible to access more challenging chemistries, enabling industrially relevant reactions such as lignin degradation [64] and dye decolorization in industrial effluent [30], while providing a robust platform for exploring monooxygenation reactions. It remains to be seen how far this effective reactivity threshold can be manipulated for *de novo* proteins such as C45, and efforts will focus on both rational and directed evolution to access the full catalytic potential offered by this highly versatile *de novo* heme enzyme.

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Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. This includes the MD trajectories, BUDE docking poses for each substrate, kinetic and spectroscopic data.

Supporting Information

The supporting information contains figures highlighting the computational binding screens for the individual substrates and the frequency of close contacts between substrates and heme, and a table of published redox potentials corresponding to substrates and oxidising species of natural peroxidases. References are provided for the redox potentials in **Fig. 1** and **SI Table 1**.

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Figures.

Figure 1. Structures of peroxidase substrates used in this study. These substrates were selected with a spread of redox potentials and chemistries to probe C45's peroxidase and oxidative dehalogenase activities. Redox potentials are quoted versus the Nernst Hydrogen Electrode (NHE) and the pH at which these measurements were recorded is indicated in parentheses. A referenced list of these potentials and the conditions in which they were obtained is provided in **SI Table 1**. Protonation states displayed in the figure represent those used in the docking and MD simulations.

Figure 2. Steady-state kinetic analysis of substrate turnover by C45. Representative UV/visible spectroscopic changes and Michaelis-Menten steady-state kinetic plots for the turnover of *ortho*-phenylenediamine (**A**), guaiacol (**B**), *ortho*-dianisidine (**C**), Amplex Red (**D**), luminol (**E**) and 2,4,6-tribromophenol (**F**) by C45. Kinetics were measured at 25 °C in 100 mM KCl and 20 mM CHES at pH 8.6, with 100 nM C45 and 100 μ M H_2O_2 . Michaelis-Menten parameters for the data contained in this figure are presented in **Table 1**. 't' denotes the time in seconds after mixing C45 and H_2O_2 with the substrate. All data were collected in triplicate, and error bars represent standard deviations.

Figure 3. Kinetic parameters of substrate turnover by C45. Histograms of (A) $V/[E]_0$, (B) K_M and (C) $(V/[E]_0)/K_M$ values for substrates both tested in this study and previously reported. All data were collected in triplicate, and error bars represent standard deviations.

Figure 4. C45 activity against peroxidase substrates where kinetic parameters could not be obtained. (A) UV-visible spectral changes occurring on mixing 250 nM C45, 100 μ M *para*-anisidine and 100 μ M H_2O_2 . (B) UV-visible spectral changes on mixing 700 nM C45, 100 μ M homovanillic acid and 100 μ M H_2O_2 . (C) UV-visible spectral changes occurring on mixing 100 nM C45, 40 μ M Reactive Orange II and 100 μ M H_2O_2 . Spectra were measured at 25 $^\circ\text{C}$ in 100 mM KCl and 20 mM CHES at pH 8.6. 't' denotes the time in seconds after mixing C45 and H_2O_2 with the substrate.

Figure 5. C45 is inactive against high potential substrates. (A) UV-visible spectral changes on mixing 100 nM C45, 100 μ M *para*-aminobenzoic acid and 100 μ M H_2O_2 . (B) UV-visible spectral changes occurring on mixing 250 nM C45, 100 μ M 1,4-dimethoxybenzene and 100 μ M H_2O_2 . (C) UV-visible spectral changes occurring on mixing 100 nM C45, 40 μ M veratrole alcohol and 100 μ M H_2O_2 . 't' denotes the time in seconds after mixing C45 and H_2O_2 with the substrate.

Figure 6. Illustration of the computational substrate binding screen using BUDE and Molecular Dynamics. Ten MD structures were used as the input into the BUDE calculation. The green dashed box represents the BUDE calculation which iterates through millions of binding poses using a genetic search algorithm. Each generation is composed of a cycle where binding poses are generated or refined, then scored and finally ranked for inclusion in the next cycle. The poses with the top 50 binding energies from the BUDE calculation were then tested in a 5 ns MD simulation.

Figure 7. Cluster analysis of successful substrate binding poses from BUDE/MD analysis. Results from a 3-dimensional cluster analysis of the last frame of 5 ns MD for each successful pose from all tested substrates. Each dot represents the centre of mass of a ligand. The number assigned to each cluster is arbitrary. Ligands that were associated with the protein but not assigned a cluster are not displayed and given the cluster number -1.

Figure 8. Frequency of close contacts between screened substrates and C45. Heat map of C45 displaying the frequency of close contacts between substrates and the amino acid residues of C45. The SP pocket representing a subset of cluster 3 is clearly visible in the surface representation of C45, indicating a likely interaction site between the protein and principally phenolic substrates.

Tables.

Substrate	$V/[E]_0$ (s ⁻¹)	K_M (μM)	$(V/[E]_0)/K_M$ (M ⁻¹ s ⁻¹)
Cyt c	0.51 ± 0.01	1.2 ± 0.6	4.3 ± 2.13 × 10 ⁵
o-PDA	1.1 ± 0.03	6.2 ± 0.7	1.8 ± 0.19 × 10 ⁵
Amplex Red	0.41 ± 0.01	0.53 ± 0.04	7.8 ± 0.65 × 10 ⁵
Guaiacol	0.76 ± 0.01	2.6 ± 0.3	2.9 ± 0.33 × 10 ⁵
Luminol	0.65 ± 0.03	2.9 ± 0.7	2.3 ± 0.52 × 10 ⁵
ABTS	1.95 ± 0.02	3.0 ± 0.19	6.5 ± 0.07 × 10 ⁵
o-Dianisidine	1.0 ± 0.08	1.9 ± 0.42	5.4 ± 1.26 × 10 ⁵
TCP	0.50 ± 0.01	12 ± 1.1	4.2 ± 0.40 × 10 ⁴
TBP	0.43 ± 0.01	11 ± 0.70	4.0 ± 0.25 × 10 ⁴

Table 1. Kinetic parameters of C45 activity against tested and previously reported substrates. Parameters are derived from Michaelis-Menten analysis of substrate turnover. Kinetic data were obtained at limiting H₂O₂ with 100 nM C45 and 100 μM H₂O₂. The ± errors quoted represent the calculated standard deviations. ABTS, cytochrome *c* and TCP data were previously reported in [11]. All data were collected in triplicate, and quoted errors represent standard deviations.

Ligand	3	6	5	1	2	9	16	12	4	15	7	18	8	13	14	17	11	10	-1	Grand Total
<i>p</i> -Anisidine	44							1	1								1		1	48
Amplex Red	5	14	8	4		1			2		2		3			1			5	45
TBP	33	1	6											1					4	45
1,4-Dimethoxybenzene	35		2							1							1		2	41
Dihydroxyphenylalanine	2	10	1	4	2		8									2			12	41
Veratrole alcohol	19		5	1				5			1			1					8	40
TCP	40																			40
Reactive Blue 4					8	7						3							21	39
<i>o</i> -Dianisidine	19	4				1		1	1	1							2		9	38
Isoniazid	30									1								1	5	37
Guaiacol	21		8	1														1	5	36
ABTS	2			5	3														23	33
<i>o</i> -Phenylenediamine	28														1				2	31
Tetramethylbenzidine	7	8		1		2			2					1					9	30
Ascorbate	16									1	1				1				5	24
Luminol	17																		5	22
5-Aminosalicylic Acid				1															8	9
Homovanillic Acid				2											1				4	7
4-Aminobenzoic acid																			3	3
Grand Total	318	37	30	19	13	11	8	7	6	4	4	3	3	3	3	3	3	3	131	609

Table 2. Pivot table derived from the cluster analysis of C45 substrate binding poses. This table correlates the substrate identity with the computationally identified binding clusters. Substrates associated with protein but not clustered are given cluster number -1. Taking *p*-anisidine as an example, 44 top binding poses were associated with the protein at the end of 5 ns in the cluster 3 locus, 3 in other groups, 1 associated but not grouped and 2 dissociated from the protein.