Unveiling the bases of alkaline stability of an evolved versatile peroxidase

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Short title: Investigating the bases of stability in an evolved VP variant

Summary statement: We provide a structural basis for the improved alkaline stability of a versatile peroxidase obtained by directed evolution. New interactions promoted by three basic residues contribute to stabilize the heme and its environment helping to maintain the enzyme's structural integrity.

Key words: pH stability, versatile peroxidase, directed evolution, structure-function relationships

A variant of high biotechnological interest (called 2-1B) was obtained by directed evolution of the *Pleurotus eryngii* VP expressed in *Saccharomyces cerevisiae* (García-Ruiz et al. Biochem. J. 441, 487, 2012). 2-1B shows seven mutations in the mature protein that resulted in improved functional expression, activity and thermostability, along with a remarkable stronger alkaline stability. The latter is highly demanded for biorefinery applications. Here we investigate the structural bases behind the enhanced alkaline stabilization of this evolved enzyme. In order to do this, several VP variants containing one or several of the mutations present in 2-1B were designed, and their alkaline stability and biochemical properties determined. In addition, the crystal structures of 2-1B and one of the intermediate variants (both expressed in *Escherichia coli*) were solved and carefully analyzed, and molecular dynamics simulations for both native VP and 2-1B were carried out. We concluded that the introduction of three basic residues in VP (Lys-37, Arg-39 and Arg-330) led to new interactions at the heme-helix B (where the distal histidine is located) interface, and formation of two-three new salt bridges that avoided the hexacoordination of the heme iron. These new structural determinants stabilized the heme and its environment, helping to maintain the structural enzyme integrity (with pentacoordinated heme iron) under alkaline conditions. Moreover, the reinforcement of the solvent-exposed area around Gln-305 in the proximal side, prompted by the Q202L mutation, further enhanced the stability.

Abbreviations: ABTS, 2, 2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); CDS, coding DNA sequence; CT, charge transfer (bands in electronic absorption spectra); DMP, 2,6-dimethoxyphenol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GP, generic peroxidase; IPTG, isopropyl- β -D-thiogalactopyranoside; LiP, lignin peroxidase; MnP, manganese peroxidase; RB5, Reactive Black 5; VA, veratryl alcohol; VP, versatile peroxidase.

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The coordinates and structure factors for the 2-1B and EHG variants of the *Pleurotus eryngii* VP have been deposited in the PDB under codes 5fnb and 5fne, respectively.

INTRODUCTION

Lignin is the main reservoir of aromatic compounds in living organisms and a valuable feedstock for the sustainable production of chemicals and materials [1;2]. Due to its aromatic and heterogeneous nature, lignin is highly resistant to chemical and biological degradation [3]. White-rot fungi produce ligninolytic peroxidases involved in lignin degradation, and they are the only organisms in nature being able to extensively mineralize lignin [4;5]. Ligninolytic peroxidases include three families: lignin peroxidases (LiP, E.C. 1.11.1.14), which oxidize high redox potential substrates through an exposed tryptophan radical formed by electron transfer to the heme [6]; manganese peroxidases (MnP, E.C. 1.11.1.13), which oxidize Mn^{2+} to Mn^{3+} at a specific Mn-binding site near one of the heme propionates [7;8]; and versatile peroxidases (VP, E.C. 1.11.1.16), which combine the catalytic properties of LiPs and MnPs (due to the simultaneous presence of the exposed tryptophan and the Mn-binding site, mentioned above) at the same time that they oxidize the typical substrates of generic peroxidases (GPs, E.C. 1.11.1.7) at the main heme access channel [9;10].

The above enzymes are naturally designed to overcome the recalcitrant nature of lignin taking advantage from their high redox potential, unspecific oxidation mechanism and other catalytic properties. Their use in industrial processes that require the oxidation of lignin and other phenolic and non-phenolic aromatic compounds and dyes is, therefore, very promising [11]. In this way, ligninolytic peroxidases could be used in the deconstruction of the lignocellulosic biomass and production of biofuels, materials and chemicals in lignocellulose biorefineries; in bioremediation processes for the treatment of recalcitrant dye wastes; or in bleaching applications in paper pulp manufacture [2;12;13]. However, there are some drawbacks that prevent the industrial application of these enzymes as they are produced in nature. Among them, it is possible to mention their relative low stability towards some conditions of pH, temperature or hydrogen peroxide concentration, in which industrial processes are often carried out (including the alkaline conditions often used in lignocellulose processing) [2].

2-1B is an enzyme variant obtained by directed evolution of the VP from *Pleurotus eryngii* in *Saccharomyces cerevisiae* [14]. During the evolutionary process, seven mutations were introduced in the sequence of mature VP. As a consequence, 2-1B showed enhanced expression levels, increased thermo and alkaline stability, and improved oxidation of some substrates. In the present work, five VP variants containing one or several of the mutations present in 2-1B were designed, and their biochemical properties carefully evaluated, at the same time that the crystal structures of two of the most relevant variants were solved and used in molecular simulations. The final objective was to understand how the substitutions introduced by directed molecular evolution modulate the alkaline stability and catalytic properties of the enzyme, with the aim of using this information in future peroxidase engineering work.

MATERIALS AND METHODS

Chemicals

Isopropyl- β -D-thiogalactopyranoside (IPTG), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), hemin, oxidized glutathione, veratryl alcohol (VA), manganese (II) sulphate, Reactive Black 5 (RB5), 2,6-dimethoxyphenol (DMP), sodium tartrate and other chemicals were purchased from Sigma-Aldrich; urea and hydrogen peroxide were from Merck; and 2, 2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) from Roche.

Directed mutagenesis

All VP variants but 2-1B (see below) were produced using the QuikChangeTM Site-Directed Mutagenesis kit (Stratagene). Each mutation was introduced by PCR using the expression plasmid pFLAG1 (International Biotechnologies Inc.) containing the coding DNA sequence (CDS) of *P. eryngii* VPL (allelic variant VPL2; GenBankTM AF007222), named pFLAG1-VPL2, or the same plasmid containing already mutated CDSs as templates and two primers, a direct and a reverse

primer designed complementary to opposite strands of the same DNA region containing the desired mutation.

The mutagenic primers E37K_p, H39R_p and G330R_p (Supplementary Table S1) were used to obtain the corresponding single variants. The double variant E37K/H39R was obtained using the plasmid pFLAG1-VPL2 as template and the primers E37K/H39R_p containing both E37K and H39R mutations. The double variant E37K/G330R (hereinafter EG) was obtained using the plasmid pFLAG1-VPL2 harbouring the G330R mutation as template and the primers E37K_p. Similarly, the triple variant E37K/H39R/G330R (hereinafter EHG) was achieved using the plasmid pFLAG1-VPL2 containing the E37K and H39R mutations as template and the primers G330R_p. In the same way, the multiple variants E37K/H39R/Q202L/G330R (hereinafter EHGQ) and E37K/H39R/T184M/G330R (hereinafter EHGT) were obtained using the template pFLAG1-VPL2-E37K/H39R/G330R with Q202L_p or T184M_p as primers, respectively.

PCR reactions were carried out in an Eppendorf Mastercycler Pro S using 10 ng of template DNA, 250 µM each dNTP, 125 ng of both direct and reverse primers, 2.5 units of *Pfu* Turbo AD polymerase (Stratagene) and the manufacture's reaction buffer. Reaction conditions were as follows: (i) a "hot start" of 95°C for 1 min; (ii) 18 cycles at 95°C for 50 s, 55°C for 50 s and 68°C for 10 min; and (iii) a final cycle at 68°C for 10 min. Clones harbouring mutations were transformed into *Escherichia coli* DH5α. One positive clone of each variant was selected, sequenced (Perkin-Elmer ABI PRISM 377) and checked to confirm that the desired mutations had been properly introduced.

Heterologous expression

Wild-type recombinant (hereinafter native) VP and its directed variants were expressed in *E. coli* W3110 after transformation with the corresponding plasmids. The gene encoding the 2-1B variant was obtained by digesting the plasmid used for *S. cerevisiae* expression [14], cloned into pFLAG1 (resulting in the pFLAG-VPL2-2-1B construction) and transformed into *E. coli* W3110 for expression.

Cells were grown in Terrific Broth [15] at 37°C until OD₅₀₀~1 (~3 h). Then protein expression was induced with 1 mM IPTG and cells were grown for a further 4 h. The apoenzyme was produced as inclusion bodies and was recovered in a 50 mM Tris-HCl (pH 8.0) solution containing 8 M urea, 1 mM EDTA and 1 mM DTT. The subsequent *in vitro* folding of the solubilised apoenzyme was carried out in a solution of 0.16 M urea, 20 µM hemin, 5 mM CaCl₂, 0.1 mM DTT, 0.5 mM oxidized glutathione and 0.1 mg/ml protein concentration in 20 mM Tris-HCl buffer, at pH 9.5, at room temperature overnight. The refolded enzyme was purified by Resource-Q chromatography using a 0-0.3 M NaCl gradient (2 ml/min, 20 min) in 10 mM sodium tartrate (pH 5.5) containing 1 mM CaCl₂. Finally, the purified enzyme was dialyzed against 10 mM sodium tartrate (pH 5). The proteins showed Reinheitszahl values (R_z , A_{407}/A_{280})~4 confirming their high purity. Their UV-visible spectra in the 300-700 nm range confirmed that they were correctly folded [16]. Enzyme concentrations were determined from the Soret absorbance (ϵ_{407} = 150 mM⁻¹ cm⁻¹) [17].

pH stability studies

Native VP and its directed variants (1 µM) were incubated at different pH values (from 2 to 9) in 0.1 M Britton-Robinson (B&R) buffer [18] at 4°C for 120 h. At different times (1 min, 1 h, 4 h, 24 h and 120 h), aliquots were taken and their residual activity was evaluated by measuring the oxidation of ABTS (2 mM) using 0.1 mM H₂O₂ and 0.01 µM of enzyme in 0.1 M sodium tartrate (pH 3.5). The activity obtained from samples incubated for 1 min at pH 5 was taken as reference (maximum activity) to calculate the percentage of residual activity. Native VP and those variants showing improved alkaline stability (2-1B, EHG and EHGQ) (4 µM and 8 µM) were incubated at pH 5 or pH 8 in 0.1 M B&R buffer for 120 h at 4°C, and their electronic absorption spectra (in the 300-800 nm range) were recorded using an Agilent 8453 diode array UV/visible spectrophotometer.

Kinetic constants

Oxidation of Mn^{2+} (Mn^{3+} -tartrate complex ϵ_{238} 6500 $\text{M}^{-1} \text{cm}^{-1}$) was estimated at pH 5; that of VA (veratraldehyde ϵ_{310} 9300 $\text{M}^{-1} \text{cm}^{-1}$) at pH 3; and those of RB5 (ϵ_{598} 30000 $\text{M}^{-1} \text{cm}^{-1}$), ABTS (cation radical ϵ_{436} 29300 $\text{M}^{-1} \text{cm}^{-1}$) and DMP (coerulignone dimeric product ϵ_{469} 55000 $\text{M}^{-1} \text{cm}^{-1}$) at pH 3.5. All enzymatic activities were measured as initial velocities taking linear increments of absorbance due to the appearance of the reaction product (decreases in the case of RB5). Reactions were performed in 0.1 M tartrate buffer, at 25 °C, in the presence of 0.1 mM H_2O_2 using a variable wavelength Shimadzu UV-1800 spectrophotometer.

Steady-state kinetic constants were calculated from the estimated oxidation of increasing substrate concentrations until enzyme saturation was observed. Values and standard errors for affinity constant (Michaelis constant, K_m) and maximal enzyme turnover (catalytic constant, k_{cat}) were obtained fitting the experimental measurements to the Michaelis-Menten model using SigmaPlot 12.0 software. Fitting of these constants to the normalized equation $v = (k_{\text{cat}}/K_m) [\text{S}] / (1 + [\text{S}]/K_m)$, where $[\text{S}]$ is the substrate concentration, yielded the catalytic efficiency values (k_{cat}/K_m) with their corresponding standard errors.

Crystallization, data collection and crystal structure determination

Crystallization trials were carried out by the sitting drop vapor diffusion method, using 96-well MRC2 plates with 50 μl reservoir solution and the commercially available screenings from Emerald (Wizard classic crystallization screens I, II and III) and Jena Biosciences (JBScreen Classic Kits 1-10). Drops consisted of 0.2 μl of protein solution (10 mg/ml in 10 mM sodium tartrate buffer at pH 5.0) and 0.2 μl of reservoir solution. Crystallization was carried out at 22°C. Crystals of 2-1B were obtained in 0.1 M Tris-HCl (pH 7.8), 5 % PEG3350, 1.25 M $(\text{NH}_4)_2\text{SO}_4$ and 7.5 % glycerol; and cryoprotected raising the amount of glycerol up to 30 %. Crystals of EHG were obtained in 0.1 M Na-acetate (pH 4.5), 1.26 M $(\text{NH}_4)_2\text{SO}_4$ and 0.2 M NaCl; and cryoprotected with 0.8 M Li_2SO_4 .

Crystals were mounted in nylon loops and flash-frozen in liquid nitrogen in the mother liquor containing the cryoprotectant indicated above. All diffraction data were obtained at 100 K. X-ray diffraction intensities were collected at the X06DA beam line at the Swiss Light Source (Villigen, Switzerland) using a wavelength of 1.0000 Å. Diffraction data were indexed, integrated, merged and scaled using the program XDS [19]. Data collection statistics are shown in supplementary **Table S2**.

The structures of the two VP variants were solved by molecular replacement using the crystal structure of *P. eryngii* VPL (PDB entry 3FMU) as the search model and the program PHASER implemented in the PHENIX package [20]. The final models were obtained by consecutive rounds of refinement, performed with the PHENIX package; followed by manual model building, performed with Coot [21] using σ_A weighted 2Fo-Fc and Fo-Fc electron density maps. Solvent molecules were introduced in the structure automatically in the refinement as implemented in the PHENIX package and visually inspected. A total of 5 % of reflections was used to calculate the R_{free} value throughout the refinement process. The structures were validated using MolProbity [22;23]. Refinement and final model statistics are summarized in supplementary **Table S2**. The coordinates and structure factors have been deposited with the Protein Data Bank. All figures were produced with PyMOL (<http://www.pymol.org/>).

Molecular dynamics

Structural and dynamical differences between wild type VP and its 2-1B variant were studied by molecular dynamics (MD) simulations. Hydrogen addition, ligand optimization, completion of the two terminal residues and protonation state adjustment of ionizable residues (Glu, His and Asp) in both crystal structures were performed with the protein preparation wizard [24] at pH 8. Molecular dynamics were then produced with the Desmond software and its Maestro interface from Schrödinger Inc. Enzymes were solvated in an orthorhombic box with at least 10 Å from the protein surface, followed by NaCl addition for an ionic force of 0.15 M. The default relaxation protocol in Desmond was performed followed by a 20 ns trajectory production with the OPLS-2005 [25] force

field at 300K. The NPT ensemble with Nose-Hoover thermostat [26] and a Martyna–Tobias–Klein barostat [27] were employed.

RESULTS

pH stability of the different variants

VP 2-1B (containing E37K/H39R/V160A/T184M/Q202L/D213A/G330R mutations) expressed in *E. coli* showed improved alkaline stability (**Figure 1D**) compared with native VP (**Figure 1A**). After 24 and 120 h of incubation at pH 8, it retained 82% and 78% of residual activity, respectively, whereas native VP was fully inactivated. Moreover, after 1 h of incubation at pH 9, native VP retained only 5% of the initial activity while 2-1B maintained 36% of activity. The differences in stability between both enzymes at neutral and most acidic pH conditions (in the pH 3.5-7 range) were minimal, and 2-1B was less stable at the lowest pH value (pH 3) assayed.

To analyze the effect of each of the individual mutations in 2-1B, and the effect of combining them, several single and multiple VP variants were designed (**Table 1**), expressed in *E. coli* and characterized. The H39R variant showed high instability at acidic and alkaline pH values compared with native VP (**Figure S1A**). Native VP maintained around 90% of the initial activity in the range of pH 4-7 after 120 h of incubation, while H39R only showed 41%, 68% and 21% activity at pH 4, 5 and 6 respectively (and no activity at pH 7). The following VP variant analyzed, EG containing mutations E37K/G330R, exhibited similar pH stability than native VP and no improvement in the alkaline range was observed (**Figure S1B**).

Surprisingly, the EHG variant, which combined the mutations of the two variants previously analyzed (E37K/H39R/G330R), displayed an increase in the alkaline stability compared with native VP (**Figure 1B**). This triple variant maintained 55% of the initial activity after 120 h of incubation at pH 8, whereas native VP not only showed no activity under these conditions but it was inactivated after 24 h of incubation (**Figure 1A**). In a similar way, after 1 h of incubation at pH 9, EHG exhibited a 46% of the initial activity, whereas native VP only retained 5%. Moreover, this variant maintained a similar stability compared with native VP at acidic and neutral pH values. However, the values of pH-stability observed for EHG were not as high as those obtained for 2-1B, which retained 78% of activity after 120 h of incubation at pH 8.

The next VP variant analyzed was EHGQ (E37K/H39R/Q202L/G330R), which harboured the three mutations of EHG plus the Q202L mutation, also found in 2-1B. EHGQ showed 67% of residual activity after 120 h of incubation at pH 8 (**Figure 1C**), a value higher than that of EHG (55% of activity). EHGQ also retained a low percentage of the initial activity (24%) after 1 h incubation at pH 9 compared with native VP, which was completely inactivated. Therefore, it was determined that Q202L substitution also contributed to the enhancement of the pH stability in 2-1B. As previously described for EHG, its stability at neutral and acidic pH was similar to that of the native VP.

The last variant tested, EHGT (E37K/H39R/T184M/G330R), harboured the three mutations introduced in EHG plus the T184M mutation, also found in 2-1B. After 120 h at pH 8, EHGT displayed 30% of the initial activity (**Figure S1C**) compared with 55% of activity retained by EHG. In consequence, we concluded that the T184M substitution did not contribute to the alkaline stability of 2-1B.

pH effect on the UV-visible spectra

The electronic absorption spectra of native VP and its variants were recorded after incubation at alkaline pH 8, and at pH 5 at which all of them are stable, to check the eventual modification of the heme interactions with its protein environment. As expected, all the spectra at pH 5 exhibited a Soret maximum at 407 nm, maxima at 505 nm and 637 nm corresponding to ligand-to-metal charge transfer bands (CT2 and CT1 respectively), and the β band at 541 nm (**Figure 2**, black lines), which correspond to the typical spectrum of the ferric enzyme with high-spin pentacoordinated heme iron.

However, the spectrum of native VP after incubation at pH 8 suffered important changes (**Figure 2A**, gray lines). The Soret maximum was red-shifted to 413 nm and showed less intensity, the CT

bands at 505 nm and 637 nm disappeared and a new maximum rose at 538 nm (β band) together with a shoulder at 559 nm (α band). In contrast, 2-1B preserved the spectrum mostly unaltered when incubated at pH 8 (**Figure 2D**, gray lines), maintaining the Soret maximum at 407 nm, as well as the CT1, CT2 and β bands at 637, 505 and 541 nm, respectively. In the same way, the EHG and EHGQ variants also preserved the ferric high-spin heme spectrum with all maxima unaffected (**Figure 2B and 2C**, gray lines, respectively).

Catalytic properties

Different substrates are oxidized at specific VP sites: i) high redox potential VA and RB5 are oxidized at the catalytic tryptophan; ii) low redox potential ABTS and DMP are oxidized both at the catalytic tryptophan (high efficiency site) and at the heme channel in direct contact with the heme edge (low efficiency site); and iii) Mn^{2+} is oxidized at the Mn^{2+} -binding site, in direct contact with a heme propionate. The steady-state kinetic constants for the oxidation of the above five substrates by native VP and four variants were measured (**Table 2**).

In general, the oxidation of substrates in the catalytic tryptophan was not affected by the substitutions introduced in 2-1B, since the kinetic constants for the oxidation of VA, RB5, and ABTS and DMP (in the high efficiency site) were mostly unaltered in this and the other variants. The only exception was EHGT which exhibited a 3-fold higher catalytic efficiency ($559 \text{ s}^{-1}\text{mM}^{-1}$) for DMP oxidation, compared with that of native VP ($186 \text{ s}^{-1}\text{mM}^{-1}$), due to a decrease in the affinity constant (K_m).

Mn^{2+} oxidation was impaired in 2-1B and the rest of analyzed variants since their efficiency values were decreased around 60-fold, changing from $1640 \text{ s}^{-1}\text{mM}^{-1}$ in native VP to $25\text{-}30 \text{ s}^{-1}\text{mM}^{-1}$ in the variants. The worsening in the oxidation was mainly due to the increase of the K_m values.

Interestingly, the oxidation of ABTS and DMP at the low efficiency site was improved in 2-1B and EHGT. The efficiency for the oxidation of ABTS showed a 9-12-fold increase in both variants, mainly due to a reduction of the K_m values. In a similar way, the efficiency in the oxidation of DMP exhibited a 5-fold increase. In EHGT, this improvement was mainly due to the decrease in the K_m . However, in 2-1B, a decrease of the K_m value combined with an increase of the k_{cat} was observed.

In contrast to the results obtained for EHGT, EHG showed no improvement in ABTS and DMP oxidation. Since the only difference between these two variants is the T184M mutation present in EGHT, it was concluded that this substitution is involved in the improved oxidation of low redox potential substrates by EGHT (and probably by 2-1B).

Analysis of crystal structures

The crystal structures of 2-1B and EHG were obtained, analyzed and compared with that of native VP (PDB entry 2BOQ) to explain, from a structural point of view, both the enhanced alkaline stability and the modified catalytic properties of these variants. The simultaneous presence of H39R, E37K and G330R mutations was determinant for the higher alkaline stability found in 2-1B. Residues 37 and 39 are partially solvent-exposed, close to the heme (at 7.1 and 3.3 Å, respectively, in native VP structure) (**Figure 3A**) and close to the Mn^{2+} -binding site (formed by Glu-36, Glu-40 and Asp-175). They belong to helix B, which is located directly above the heme and harbours the distal histidine (His-47) and the distal arginine (Arg-43) that are involved in catalysis. Moreover, the distal histidine is involved in the hexacoordination of the heme iron observed at alkaline pH. The new Arg-39 in EHG and 2-1B (**Figure 3C and D**) showed a different orientation of its side chain relative to the position of His-39 in native VP (**Figure 3B**). The new orientation enabled the formation of a new H-bond with the distal propionate of the heme, being this interaction absent in native VP. Instead, in VP, His-39 formed two H-bonds with Cys-34 and Pro-190, which were lost in the improved variants.

The E37K substitution changed an acidic residue for a basic one. The new lysine forms a salt bridge with Glu-40 in 2-1B (**Figure 3D**) and a double salt bridge with Glu-36 and Glu-83 in EHG (**Figure 3C**). In contrast, the side chain of Glu-37 in VP does not make this type of interactions (**Figure 3B**). On the other hand, Glu-37 is close ($< 7 \text{ Å}$) to several acidic residues (such as Glu-40

and Glu-36 on helix B, Glu-83 on helix C, Glu-26 on helix A and Asp-30 and Asp-175 on two loops). Therefore, the introduction of the new Lys-37 in 2-1B and EHG variants contributed to reduce the putative destabilizing acid-acid interactions in the region where this mutation is located.

G330R was also involved in the pH-stability improvement of 2-1B. This is the penultimate residue in the VP C-terminal tail, and its position could not be determined in the crystals of native VP and its mutated variants due to the high mobility of this area. For this reason, we could not verify in which way this mutation contributes to enhance the stability. Nonetheless, it is interesting to note that residue 330 is close to residues 37 and 39, all of them around the Mn^{2+} -binding site.

Q202L, at the bottom of the molecule (**Figure 4A**), was the last mutation identified as stabilizing VP under alkaline conditions. The introduction of Leu-202 in 2-1B led to the disappearance of two H-bonds present in native VP, one with Ala-235 and other with Glu-304 (**Figure 4B and C**). As a consequence, the position of the side chain of some surrounding residues changed in the 2-1B variant, especially that of Gln-305 forming three new interactions with Gln-245, Cys-307 and Thr-310 in 2-1B. These interactions reinforced and joined two solvent-exposed regions of the protein: i) the helix I; and ii) the loop between the 3_{10} helix (from Leu-300 to Asp-302) and the C-terminal tail. None of these interactions appeared in native VP.

Regarding the structural calcium ions, described as essential for pH stability, both of them appeared in the 2-1B and EHG structures coordinated in the same way as in the native VP.

Finally, the T184M mutation was analyzed in the 2-1B structure since it was involved in the enhanced oxidation of ABTS and DMP at the low efficiency site. Residue 184 is solvent exposed and located below the Mn^{2+} -binding site. No significant differences were observed between native VP and 2-1B neither in the position of the surrounding residues nor in the interactions among them.

Molecular dynamics

Taking advantage of the solved crystal structures, the dynamics of native VP and 2-1B in solution were studied by MD simulations at pH 8. Since alkaline inactivation has been related to the formation of a hexacoordinated iron complex with the distal histidine, we monitored the evolution of this distance aiming at possible correlations with the stability of the enzyme. **Figure 5A** shows the iron to His-47 distance along a 20 ns molecular dynamics for both enzymes. Although this distance is similar in both crystals, the addition of thermal fluctuations at room temperature reveals a different behavior, with a higher distance ($\sim 1\text{\AA}$ difference on average) for the 2-1B variant. Such distance elongation is the result of a new hydrogen bond between Arg-43 and His-47 (**Figure 5C**), which is not formed in native VP because of the long distance between these two residues (**Figure 5B and 5D**). Overall, inspection of the simulated trajectory indicates a slight shift (along with a higher mobility) in the Arg-43 position, facilitating its interaction with His-47. This deviation towards the histidine is mainly due to the electrostatic repulsion produced by the introduction of three new positive residues in the propionate region (E37K, H39R, G330R). In addition, the H39R mutant, introduces a stronger interaction with Glu-40, weakening the contact of this glutamic acid with Arg-43. In native VP, Arg-43 interacts mostly with Glu-40, keeping it away from His-47 and allowing a closer distance of the distal histidine with the heme iron, facilitating the inactivation of the enzyme.

DISCUSSION

The 2-1B variant of *P. eryngii* VP contains seven mutations, introduced during the evolution process in *S. cerevisiae*, that conferred, among other properties, significantly improved alkaline stability and enhanced activity on some low redox potential substrates [14]. In the present work we identified some of the structural bases of the alkaline stabilization of this VP variant, by analyzing the stability at basic pH and catalytic properties of a series of (single and multiple) variants expressed in *E. coli*.

First, we confirmed that 2-1B expressed in *E. coli* retains the improved properties in terms of alkaline stability and activity at acid pH on low redox potential compounds observed when expressed in *S. cerevisiae*. However, it is possible to mention that, although 2-1B produced in *E.*

coli showed a significantly increased alkaline stability (retaining 80% and 20% activity after 120 h at pH 8 and pH 9, respectively) compared with native VP (which was completely inactivated), this improvement was not as high as found when the variant was expressed in *S. cerevisiae* (100% and 60% activity at pH 8 and pH 9, respectively) [14]. These differences are likely due to the lack of glycosylation of 2-1B expressed in *E. coli*, as reported for temperature stability of *Phanerochaete chrysosporium* ligninolytic peroxidases [28]. In general, glycosylation has been described to give robustness, thermostabilization and protection against enzyme inactivation [29;30] and, therefore, could reinforce the alkaline stabilizing effect of the mutations introduced in 2-1B.

Explaining the alkaline stability

During alkaline inactivation, ligninolytic peroxidases lose their structural calcium ions responsible for maintaining the architecture of the heme environment. This loss leads to a relaxation of the protein structure and hexacoordination of the heme involving the distal histidine [31]. These changes are reflected in the electronic absorption spectrum, which is indicative of the high or low spin states of the heme iron and its coordination state [32]. The *P. eryngii* native VP was inactivated in 24 h upon alkaline incubation (pH 8), showing the spectrum of a low-spin hexacoordinated heme iron. This is characterized by the red-shifted Soret band and the appearance of the α and β bands. Similar changes have been reported for *Bjerkandera adusta* VP [33] and other ligninolytic peroxidases, such as LiP [31] and MnP [32] from *P. chrysosporium*. In contrast, the EHG variant showed improved alkaline stability and conserved the UV-visible spectrum (including the Soret maximum and the CT1 and CT2 bands) unchanged during incubation at pH 8. This spectrum is indicative of a peroxidase with a high-spin ferric state of the heme iron, which is completely functional [34;35]. From these data, it can be inferred that the three new basic residues present in EHG (Lys-37, Arg-39 and Arg-330) reinforced the heme environment enabling the maintenance of pentacoordinated heme iron at pH 8.

The stability improvement achieved with the simultaneous introduction of these basic residues could be rationalized by comparative structural analysis and MD simulations of the EHG, 2-1B and native VP crystal structures. The new Arg-39 appeared hydrogen-bonded to the heme distal propionate in the molecular structure of the two variants. The connections of the heme with the apoprotein have been described as essential for stability [36;37] and engineering new connections with the heme has been proposed as an strategy to increase stability [38]. The heme in ligninolytic peroxidases is non-covalently bound and shows no polar interactions with the apoprotein, with the exception of those that the propionates make. So, the new connection made by Arg-39 seems to be important for the stability achieved, as it anchors the heme to the protein (helix B) and contributes to fix the relative position of His-47 with respect to the iron ion.

On the other hand, the new Lys-37 forms new salt bridges. These interactions have been positively correlated with stability in different families of proteins [39-42]. Those of Lys-37 with Glu-36 and Glu83 in EHG and with Glu-40 in 2-1B reinforced the structure of helix B, located directly above the heme and harbouring distal histidine (His-47) and arginine (Arg-43), which are essential in the reaction of the enzyme with H_2O_2 [43] and in the stabilization of the oxidized forms of the heme iron [44;45]. Moreover, as in the case of H39R mutation, fixing the helix B position reduces the possibilities of His-47 to approach the heme and generate the inactive form with hexacoordinated iron. Helix B also contains Asp-48, one of the residues coordinating the distal calcium, which is required for pH stability. In addition, a high number of acidic residues were observed around residue 37. At alkaline pH 8, these residues would be deprotonated. In consequence, a high negative charge would characterize this region. The introduction of a basic residue (Lys-37) would reduce the negative charge avoiding detrimental interactions and reinforcing the structure of VP at alkaline pH. At acidic pH, however, the acidic residues will be protonated and no accumulation of negative charge would be produced, explaining the lack of acidic stability improvement of EHG and 2-1B.

The G330R mutation also contributed to the improved stability found in 2-1B and EHG. Although the role played by Arg-330 could not be studied in the crystal structures, it is likely that

this residue located in the same area as residues 37 and 39 forms new interactions stabilizing the surrounding region containing a high number of acidic residues.

It was remarkable the fact that the stabilizing effect produced by Lys-37, Arg-39 and Arg-330 was only detected when they appeared together, since the H39R variant was unstable and the E37K/G330R variant showed a similar stability to that of the native VP. The sum of the new interactions formed by these residues results in the stabilization of VP but, individually, they make the protein instable due to the vulnerability of the region where they are (near the heme and the Mn^{2+} -binding site), being a clear effect of positive epistasis unveiled by directed evolution [14]. According to the structural analysis described above, this effect is caused by the three basic residues simultaneously contributing to stabilize the relative position of the distal histidine. The MD simulations performed under alkaline conditions were quite enlightening in this regard. They revealed that the electrostatic repulsion, formed as a consequence of the introduction of Lys-37, Arg-39 and Arg-330, along with other interactions in which these and other surrounding residues are involved, is able to maintain the distal histidine for longer in a position far enough from the porphyrin to keep the heme iron in a pentacoordinate active state.

The EHGQ (E37K/H39R/G330R/Q202L) variant showed higher stability than EHG and almost achieved the alkaline stability found in 2-1B, so it was concluded that: i) the Q202L mutation also contributed to the enzyme stabilization; and ii) the four mutations introduced in EHGQ are responsible for the most part of the enhancement towards alkaline stability found in 2-1B. From the analysis of the crystal structure of 2-1B, it was observed that the Q202L substitution promoted the reorganization of Gln-305 and the formation of new interactions in the area which could explain the improvement observed due to this mutation. Although residue 202 is in the core of the protein, the residues affected by the new interactions are solvent exposed. The solvent exposed regions are very sensitive to pH changes, so the reinforcement of these areas could be determinant to strengthen the structure and avoid the alkaline inactivation at pH 8. Moreover, residue 202 would be an example of a residue located far from the heme and the rest of catalytic sites that is able to increase the stability of the enzyme.

Explaining the modified catalytic properties

Regarding the catalytic properties of 2-1B, it was found that this variant is more efficient than the native enzyme oxidizing low redox potential substrates, mainly due to an increased affinity (a decreased K_m) in the low efficiency site. The same behaviour was previously reported for 2-1B expressed in *S. cerevisiae* [14]. With the data in hand, this fact was attributed to the presence of the T184M mutation. It is possible to speculate that the substitution introduced could help to a better substrate accommodation at the main heme access channel. However, the absence of structural differences in the environment of the residue at position 184 after comparative structural analysis of the native enzyme and 2-1B, has not allowed us to give a rational explanation to the effect produced by this mutation on the catalytic activity.

The simultaneous worsening in manganese oxidation is explained by the introduction of Lys-37 and Arg-39 [14]. These two residues could compromise the Mn^{2+} binding by forming salt bridges with Glu-36 and Glu-40 (two of the three residues involved in Mn-coordination together with Asp-175) in EHG and 2-1B, respectively. Moreover, it is also probable that the new interaction established between the heme internal propionate and the apoprotein in 2-1B and EHG affects the Mn^{2+} binding and oxidation, considering that this propionate is involved in these two events [46]. Interestingly, the kinetic constants for Mn^{2+} oxidation in 2-1B and the rest of variants were similar to that obtained for VP E36D and E40D variants [46], which suffered a shortening of the lateral chain of Glu-36 and Glu-40 (substitution by aspartic acid). These substitutions led to a worsening in the Mn^{2+} oxidation reflected in a high increase (25-fold) of the K_m and a consequent decrease in the efficiency. In the same way, 2-1B and derived variants showed K_m values 43-54-fold higher. It is likely that salt bridges formed by Lys-37 in EHG and 2-1B were limiting the mobility of Glu-36 and Glu-40 leading to an impaired activity towards manganese in a similar way as happened with the E36D and E40D variants. Finally, substrate oxidation at the catalytic tryptophan was not

impaired by the mutations introduced in 2-1B given that the oxidation of high redox compounds such as VA and RB5 by this variant was similar to that found in native VP. This is indicative of the maintenance of the high redox potential of the enzyme, determined by the position of the proximal histidine and by the strength of the interaction between this residue and the heme iron [47;48].

Conclusions

We were able to provide a structural-functional basis for the improved alkaline stability of the 2-1B variant by designing and characterizing several (individual and multiple) intermediate variants with the same mutations found in 2-1B. The *E. coli* expression system used not only facilitated variant generation by site-directed mutagenesis, but also enabled crystallization of the non-glycosylated forms of two of them. In this way, the introduction of three basic residues in VP (Lys-37, Arg-39 and Arg-330) resulted in new interactions at the heme environment that avoided the hexacoordination of the heme iron, which leads to enzyme destabilization at pH 8. Moreover, the reinforcement of the solvent-exposed area around Gln-305, in the proximal side, further enhanced the stability. Interestingly, the biochemical characterization of the intermediate variants revealed discrete stability improvements, and the combination of most of them was required to stabilize VP. This stabilization could be obtained by random combination of mutations using directed molecular evolution, and would be impossible by a rational approach since the basis for the synergistic improvements observed could not be predicted "a priori".

Author contributions

V. Sáez-Jiménez performed most of the experimental biochemical work. S. Acebes and V. Guallar contributed to the molecular dynamics analyses. F.J. Medrano and A. Romero crystallized VP variants. All authors participated in the interpretation and discussion of results. V. Sáez-Jiménez, A.T. Martínez and F.J. Ruiz-Dueñas contributed data integration and writing.

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Table 1

Seven mutations present in 2-1B and their combination in the five intermediate variants analyzed in this study

Variants	Mutations
2-1B	E37K / H39R / V160A / T184M / Q202L / D213A / G330R
H39R	H39R
EG	E37K / G330R
EHG	E37K / H39R / G330R
EHGQ	E37K / H39R / Q202L / G330R
EHGT	E37K / H39R / T184M / G330R

Table 2

Kinetic constants - K_m (μM), k_{cat} (s^{-1}) and k_{cat}/K_m ($\text{s}^{-1} \text{mM}^{-1}$) - for oxidation of VA, RB5, Mn^{2+} , ABTS and DMP by native VP, 2-1B and three intermediate variants (see Table 1 for mutations in each of them)^a

		VP	EHG	EHGQ	EHGT	2-1B
VA	K_m	2600 $\pm$ 190	2110 $\pm$ 150	1780 $\pm$ 60	5910 $\pm$ 190	2710 $\pm$ 270
	k_{cat}	5.8 $\pm$ 0.1	5.2 $\pm$ 0.1	4.4 $\pm$ 0.04	5.9 $\pm$ 0.1	3.8 $\pm$ 0.1
	k_{cat}/K_m	2.2 $\pm$ 0.1	2.5 $\pm$ 0.1	2.5 $\pm$ 0.1	1.0 $\pm$ 0.1	1.4 $\pm$ 0.1
RB5	K_m	3.4 $\pm$ 0.3	4.3 $\pm$ 0.5	3.5 $\pm$ 0.3	2.4 $\pm$ 0.1	7.6 $\pm$ 0.6
	k_{cat}	5.5 $\pm$ 0.3	7.8 $\pm$ 0.4	6.7 $\pm$ 0.3	5.1 $\pm$ 0.1	6.7 $\pm$ 0.3
	k_{cat}/K_m	1.6 $\pm$ 0.1	1.8 $\pm$ 0.1	1.1 $\pm$ 0.1	2.2 $\pm$ 0.1	0.8 $\pm$ 0.1
ABTS (high efficiency)	K_m	3.0 $\pm$ 0.2	2.6 $\pm$ 0.2	2.3 $\pm$ 0.2	13 $\pm$ 3	nd ^b
	k_{cat}	8.1 $\pm$ 0.2	8.3 $\pm$ 0.3	7.2 $\pm$ 0.3	39 $\pm$ 7	nd ^b
	k_{cat}/K_m	2700 $\pm$ 140	3100 $\pm$ 180	3200 $\pm$ 150	3050 $\pm$ 160	nd ^b
ABTS (low efficiency)	K_m	1020 $\pm$ 70	400 $\pm$ 40	280 $\pm$ 20	61 $\pm$ 3	98 $\pm$ 9
	k_{cat}	208 $\pm$ 6	89 $\pm$ 2	90 $\pm$ 2	117 $\pm$ 2	234 $\pm$ 8
	k_{cat}/K_m	204 $\pm$ 10	219 $\pm$ 15	320 $\pm$ 20	1920 $\pm$ 70	2370 $\pm$ 150
DMP (high efficiency)	K_m	38 $\pm$ 4	17 $\pm$ 2	16 $\pm$ 3	11 $\pm$ 2	27 $\pm$ 7
	k_{cat}	7.1 $\pm$ 0.1	2.9 $\pm$ 0.1	4.1 $\pm$ 0.1	6.3 $\pm$ 0.3	2.9 $\pm$ 0.4
	k_{cat}/K_m	186 $\pm$ 16	168 $\pm$ 18	250 $\pm$ 38	559 $\pm$ 80	112 $\pm$ 18
DMP (low efficiency)	K_m	10500 $\pm$ 400	34500 $\pm$ 6050	34100 $\pm$ 5290	3070 $\pm$ 420	7470 $\pm$ 380
	k_{cat}	30 $\pm$ 1	40 $\pm$ 4	56 $\pm$ 4	46 $\pm$ 2	91 $\pm$ 2
	k_{cat}/K_m	2.8 $\pm$ 0.1	1.1 $\pm$ 0.1	1.6 $\pm$ 0.1	15 $\pm$ 2	12 $\pm$ 1
Mn^{2+}	K_m	130 $\pm$ 11	7020 $\pm$ 190	6790 $\pm$ 200	5590 $\pm$ 371	5710 $\pm$ 350
	k_{cat}	211 $\pm$ 4	208 $\pm$ 2	188 $\pm$ 2	160 $\pm$ 4	143 $\pm$ 3
	k_{cat}/K_m	1640 $\pm$ 130	30 $\pm$ 1	28 $\pm$ 1	29 $\pm$ 1	25 $\pm$ 1

^a Reactions were carried out at 25 °C in 0.1 M sodium tartrate (pH 3 for VA, pH 5 for Mn^{2+} , and pH 3.5 for RB5, ABTS and DMP). Means and 95% confidence limits of replicate assays. ABTS and DMP oxidation showed biphasic kinetics enabling calculation of two sets of constants (for high and low efficiency sites). ^b Not determined because the kinetic curve of the high efficiency site overlapped with that of the low efficiency site.

Figure 1 pH stability of native VP and three mutated variants

Residual activities of VP (A) and the EHG (B) EHGQ (C) and 2-1B (D) variants, after 1 h (gray bars), 4 h (white bars), 24 h (black bars), and 120 h (striped bars) of incubation in 0.1 M B&R buffer at different pH values (3-9) and 4°C. Residual activity measured as ABTS (2 mM) oxidation in 0.1 M sodium tartrate (pH 3.5) using 0.01 µM enzyme and 0.1 mM H₂O₂. Means and 95% confidence limits of replicate assays. See **Table 1** for the mutations present in each of the variants.

Figure 2 Electronic absorption spectra of native VP and variants after treatments at pH 5 and pH 8

UV-visible spectra of native VP (A) and the EHG (B), EHGQ (C) and 2-1B (D) variants incubated for 120 h in 0.1M B&R buffer at pH 5 (black) and pH 8 (gray), at 4°C. Amplified (x 5) 450-800 nm regions are shown. Main maxima are indicated.

Figure 3 E37K and H39R mutations in 2-1B and EHG crystal structures

Crystal structure of 2-1B (A) with details of the region in front of the heme propionates in native VP (B) and the EHG (C) and 2-1B (D) variants showing the heme, the mutated residues at positions 37 and 39, and other surrounding residues (in CPK colours). Hydrogen bonds are shown as green dashed lines, calcium ions as green spheres and secondary structure as colour cartoon.

Figure 4 Q202L mutation in 2-1B crystal structure

Crystal structure of 2-1B (A) with a detail of the region around residue 202 in native VP (B) and the 2-1B (C) variant. Analyzed residues are shown as CPK sticks, hydrogen bonds as green dashed lines, calcium ions as green spheres and secondary structure as coloured cartoon.

Figure 5 Molecular dynamics simulations.

Distance profiles between the heme iron and the distal His-47 (A) and between the distal His-47 and Arg-43 (B); and representative snapshots, with the average distances, extracted from the MD simulations of 2-1B (C) and native VP (D). All distances are in Angstroms.

FIGURE 1 (2 columns)

FIGURE 2 (2 columns)

FIGURE 3 (2 columns)

FIGURE 4 (2 columns)

FIGURE 5 (2 columns)

SUPPLEMENTARY ONLINE DATA

Unveiling the bases of alkaline stability of an evolved versatile peroxidase

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SUPPLEMENTARY TABLES AND FIGURE

Table S1 Oligonucleotide primers used in mutagenic PCRs for the production of the VP variants

Only the direct sequences are shown with indication of the changed triplets (underlined) and the mutations introduced (bold).

Primer	Sequence 5'-3'	Mutation
E37K _p	GCCCAGTGTGGAGAA <u>AAG</u> GTGCACGAGTCCC	E37K
H39R _p	GGAGAAGAGGTG <u>C</u> CGAGTCCCTTCGTTTGACTTTCCACG	H39R
G330R _p	CCCTCCCGTCCCT <u>AGAT</u> CGTAAGATCTCTCG	G330R
Q202L _p	CTCAATTCTTCATCGAAACG <u>CTC</u> CTTAAAGGCAGACTCTTCCC	Q202L
T184M _p	CCCATCCGATTCTGGAAT <u>TGCC</u> ATTGATTCAACCCCCGG	T184M
E37K/H39R _p	GCCCAGTGTGGAGAA <u>AAG</u> GTG <u>C</u> CGAGTCCCTTCG	E37K/H39R

Table S2 Crystallographic data collection and refinement statistics of VP EHG and VP 2-1B. Data in parenthesis correspond to the last resolution layer.

Data collection	EHG	2-1B
Space group	P 2 ₁ 2 ₁ 2	P 2 ₁ 2 ₁ 2 ₁
Cell constants	a = 55.2, b = 104.2, c = 76.6 Å	a = 55.1, b = 106.0, c = 107.6 Å
Resolution range (Å)	50.00 - 1.50 (1.59 - 1.50)	50.00 - 1.80 (1.90 - 1.80)
N° of total reflections	920257	777990
N° of unique reflections	71617	59335
R _{merge} (%)	26.6 (324.2)	41.2 (330.8)
Completeness (%)	99.5 (97.2)	98.6 (94.2)
<I/s(I)>	8.5 (0.8)	6.8 (0.7)
Multiplicity	12.8 (12.0)	13.1 (12.0)
CC(1/2)	99.7 (28.8)	99.0 (30.9)
Solvent content (%) / Matthews coef.	55.33 / 2.75	37.48 / 1.97
Subunits per asymmetric unit	1	2
Wilson B factor (Å ²)	16.0	26.8
Refinement		
Resolution range	50.0 - 1.50 Å	50.0 - 1.80 Å
Working reflections	71227	58796
R _{work} / R _{free}	19.2 / 22.9 %	18.5 / 21.9 %
Protein atoms (non H)	2440	4740
Heme group	1	2
Ca ²⁺	2	4
Water molecules	367	618
SO ₄ ions	10	2
Mean B factors (Å ²)		
Protein atoms (non H)	20.07	21.41
Heme group	15.37	17.85
Ca ²⁺	14.63	15.57
Water molecules	30.98	38.23
SO ₄ ions	62.93	30.51
Deviations from ideality		
rmsd bond lengths	0.006 Å	0.007 Å
rmsd angles	1.126°	1.104°
Ramachandran plot statistics		
Preferred %	98.47	98.43
Allowed %	1.53	1.57
Outliers %	0.00	0.00
PDB code	5fne	5fnb

Figure S1 pH stability of H39R, EG (E37K/G330R) and EHGT (E37K/H39R/G330R/T184M) VP variants

Residual activities of H39R (A), EG (B) and EHGT (C) after incubation in 0.1 M Britton & Robinson buffer at different pH values (3-9) and 4°C for 1 h (gray bars), 4 h (white bars), 24 h (black bars), and 120 h (striped bars). Residual activity measured as ABTS (2 mM) oxidation in 0.1 M sodium tartrate (pH 3.5) using 0.01 μ M enzyme and 0.1 mM H_2O_2 . Means and 95% confidence limits of replicate assays.