Peroxidase-like activity of cytochrome $b_5$ is triggered upon hemichrome formation in alkaline pH.

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ABSTRACT
In alkaline media (pH 12) a catalytic peroxidase activity of cytochrome $b_5$ was found associated to a different conformational state. Upon incubation at this pH, cytochrome $b_5$ electronic absorption spectrum was altered, with disappearance of characteristic bands of cytochrome $b_5$ at pH 7.0. The appearance of new electronic absorption bands and EPR measurements support the formation of a cytochrome $b_5$ class B hemichrome with an acquired ability to bind polar ligands. This hemichrome is characterized by a negative formal redox potential and the same folding properties than cytochrome $b_5$ at pH 7. The acquired peroxidase-like activity of cytochrome $b_5$ found at pH 12, driven by a hemichrome formation, suggests a role of this protein in peroxidation products propagation.

KEYWORDS
Hemichromes, peroxidase, low spin state, hydrogen peroxide, alkaline pH, cytochrome $b_5$

ABBREVIATIONS
CO - Carbon monoxide; Cytochrome $b_5$ - $Cb_5$; Cyt c - Cytochrome c; EDTA - Ethylene diamine tetra acetic acid; EPR - Electron paramagnetic resonance; HS - High spin; H$_2$O$_2$ - hydrogen peroxide; LS - Low spin; LSpH7 - pH 7 signal; LSpH12 – pH 12 signal; LSpH12' – pH 12 signal'; MPO - Myeloperoxidase; NHE - Normal hydrogen electrode; NO - nitric oxide; NOS - Nitric oxide synthase; OH$^-$ - hydroxide anion; P450 - cytochrome P450.
1. INTRODUCTION

Heme protein autooxidation processes are associated in vivo to many factors (such as, globin matrix, pH, oxygen pressure and denaturant agents) [1,2]. Hemichromes are originated in oxidized heme proteins and present a distorted iron coordination site [3,4]. Although these complexes have been associated to protein denaturation, hemichromes are distinguishable from those generated by denaturation leading to precipitation [5] and loss of the heme [6]. Indeed, a fraction of these complexes is present in the normal heme protein blood population being related to blood diseases [2,7,8,9]. Moreover, in some cases hemichromes have been linked to formation of peroxidation products [10,11]. The most studied protein forming hemichromes upon oxidation is hemoglobin [2,12]. In some of the hemichrome complexes, accessibility of exogenous ligands like water may occur by rearrangement of distal histidines with iron in the heme plane facilitating the access to the heme pocket [2,4,12]. Therefore a role of distal histidines in suitting the barrier for exogenous ligands coordination to the heme has been suggested [2].

Substrate accessibility is crucial for an efficient enzymatic catalysis. In metalloproteins, ligand accessibility is associated to alteration of the coordination properties, sometimes involving conformational changes [8]. In this way, peroxidase activity gained by some heme proteins depends upon a change on the heme coordination state that allows the interaction with peroxides. An interaction of small anions, promoting or inhibiting peroxidase activity in these enzymes has also been described [9,10].

Cytochrome \( b_5 \) (\( Cb_5 \)) enzymatic properties have been overlooked since the labile not covalently heme bond present in the protein has an inherent inefficiency to interact with substrates, due to a full coordination sphere. However, \( Cb_5 \) mutants in the coordinating histidines are capable to form complexes with oxygen [13,14] and CO [15] and a peroxidase-like activity associated to adoption of a non-native state conformation has been reported for an artificial designed \( c \)-type \( Cb_5 \) mutant, with the heme covalently bound to the protein matrix but keeping the coordinating histidines intact [16].

Under alkaline conditions, catalytic activities of metallocompounds have been detected [17-19]. This support that alkaline media is an alternative condition to generate novel reactive species in order to study catalytic reactions mediated in metalloproteins [20,21]. In this communication, we report the acquisition of a peroxidase-like activity by human soluble \( Cb_5 \) driven by a hemichrome formation at alkaline pH. Under these conditions, the oxidized form of the protein undergoes a conformational rearrangement allowing hydrogen peroxide to interact with its redox center and triggers this activity.

2. MATERIAL AND METHODS

2.1 Purification recombinant human erythrocyte \( Cb_5 \)

Purification of recombinant human erythrocyte \( Cb_5 \) was performed by overexpression of the protein using transformed BL21 (DE3) derived strains of \( E. \ coli \) containing the recombinant plasmid [22]. Briefly, after expression, cells were harvested and disrupted by the use of lysozyme, detergents and protease inhibitors. After precipitation in ammonium sulfate 50%, the lysate was
extensively dialyzed against Tris 10 mM, EDTA 1 mM pH 8.1, and loaded in
diethylaminoethyl sepharose column (2.5x30cm) previous equilibrated in 10 mM
Tris, EDTA 1 mM pH 8.1. A step gradient with increasing concentrations of Tris
up to 200 mM was performed in the presence of 0.2% deoxycholate and 0.5%
Triton X-100. Cb₂₅ was eluted from the column using a buffer composed by 10
mM Tris, 1 mM EDTA and 250 mM sodium thiocyanate. A final solution of
ammonium sulfate 1.1 M was added to the sample and after mixing, the solution
was loaded in a CL sepharose 4B column (2.5x10cm) previously equilibrated in
Tris 200 mM, EDTA 1 mM pH 8.1. Cb₂₅ was eluted without retention. To further
purify the protein, the concentrated eluent was loaded in a Sephadex G75
column (2.5x50cm) equilibrated with Tris 150 mM pH 7.5. The sample was
concentrated by filtration and glycerol was added before freezing.

2.2 Unfolding measurement
Tryptophan fluorescence of Cb₂₅ (5 μM) was measured using a fluorescence
spectrophotometer (Perkin Elmer 650–40; Perkin Elmer, Norwalk, CT, USA)
using quartz thermostatted cuvettes (2 ml) at 25 °C. The spectra were recorded
using an excitation wavelength at 290 nm. The excitation and emission slits
were 2 and 5 nm, respectively. The buffer used in the measurements was
potassium phosphate 100 mM, Borate 50 mM, KCl 150 mM, EDTA 1 mM
prepared at different pHs, or urea 10 M, under stirring.

2.3 EPR measurements
X-band EPR spectra were recorded using a Bruker EMX 6/1 spectrometer and
a dual mode ER4116DM rectangular cavity (Bruker); the samples were cooled
with liquid helium in an Oxford Instruments ESR900 continuous-flow cryostat,
fitted with a temperature controller. The spectra were acquired at 10 K, with a
modulation frequency of 100 kHz, modulation amplitude of 0.5 mT and
microwave power of 635 μW. Assays conditions are described in figures
captions.

2.4 Electronic absorption measurement
Cb₂₅ spectra (5 μM) were recorded at 25 °C in buffer: Potassium phosphate 100
mM, Borate 50 mM, KCl 150 mM, EDTA 1 mM at pH 12 using a UV-mini 1240
Shimadzu spectrophotometer.

2.5 Electrochemistry
Cb₂₅ formal potential was calculated by direct electrochemistry, using cyclic
voltammetry (CV); setup: one compartment electrochemical cell, three
electrodes configuration, anaerobic conditions (anaerobic chamber). The gold
electrode was modified by immersion in a 5% mercaptobenzoic acid solution
for 30 min in order to promote the formation of a self-assembled layer on the
surface. The protein was immobilized on the modified gold working electrode
using a cellulose membrane (3.5kDa cut-off), in a thin-layer configuration.
Samples of protein (2.5 mM) were exchanged into potassium phosphate buffer
100 mM, Borate 50 mM, KCl 150 mM, EDTA 1 mM, Neomycin 2.5 mM, used as
supporting electrolyte solution being all reagents analytical grade. Blank assays
were performed using the modified electrode prepared by the same procedure
but without the protein. The experiments were attained using a PGSTAT12
Autolab potentiostat/galvanostat and analysis of the data was performed using
GPES (Eco Chimie) software. To obtain the formal potential, blanks were subtracted to the signals obtained in the presence of the protein before calculation. CV assays were performed at different scan rates to define the best conditions (5mV/s) to measure Cb5 redox features at the selected pH values. Second scans were used for the analysis. All the potentials were converted and are presented in reference to the normal hydrogen electrode scale (NHE).

2.6 Peroxidase activity measurement

Peroxidase activity of Cb5 was measured using a fluorimetric method based on the increase of fluorescence induced by Amplex Red (Invitrogen (Carlsbad, CA) oxidation. The increase of fluorescence was monitored in time using a 2 ml quartz cuvette, after incubation of Cb5 (20 nM), during 10 minutes, at 25 °C in potassium phosphate 100 mM, Borate 50 mM, KCl 150 mM, EDTA 1 mM, prepared at pH 12. The fluorescence increment present upon Amplex red oxidation was measured using a spectrofluorimeter Perkin-Elmer 650-40 with the following set up: excitation and emission wavelengths of 530 nm and 590 nm, with excitation and emission slits of 5 and 10 nm and normal gain. The assay was started by addition of Amplex red and the specific H2O2 concentrations indicated in the figure. A calibration curve was prepared with resorufin (the oxidation product of Amplex Red) to calculate the peroxidase activity. The curves for the peroxidase activity dependence upon H2O2 and Amplex Red concentration have been fitted to a two substrate Michaelis-Menten kinetic model in accordance with a typical peroxidase cycle expected for Cb5 at this pH. The data were analyzed with the following equation:

\[
\nu = \frac{[E]_0}{\left( \frac{1}{k_{cat}} \right) + \left( \frac{K_{mA}}{k_{cat}[A]} \right) + \left( \frac{K_{mB}}{k_{cat}[B]} \right)}
\]

Where \( \nu \) is the initial rate concentration, \( E \) the concentration of enzyme, \( A \) is the concentration of peroxide, \( B \) the reducing agent concentration (in this case Amplex Red) concentration.

3. RESULTS AND DISCUSSION

We characterized the effect of pH on the electronic absorption spectrum of Cb5 (fig. 1). Cb5 electronic spectra at pH 7.0 (panel a) are characterized by the typical \( \alpha \), \( \beta \) and Soret band of this protein at 558, 528 and 413 nm respectively. Identical spectra were obtained in samples prepared from pH 7 to 11 (Supp. Fig. S1). The electronic absorption spectrum of freshly prepared Cb5 at pH 12 (panel b) shows a displacement of the Soret band from 413 to 415 nm, with shifts in \( \alpha \) and \( \beta \) bands to 538 and 575 nm. Cb5 incubation, during 1 hour at pH 12, induced a time dependent disappearance of all bands being the major changes produced after 10-20 minutes incubation. After this time, bands at 395 and 620 nm also appeared. After changing the pH back from 12 to 7 the characteristic spectra of Cb5 at pH 7 was recovered suggesting this experiment that the protein changes acquired at pH 12 are reversible (Supp. Fig. S1).

To characterize the coordination properties of the Cb5 heme at different pH values, we used EPR spectroscopy (fig. 2A). At pH 7, the Cb5 EPR spectrum is dominated by a single low spin (\( S = 1/2 \)) Fe$^{3+}$ species, with \( g_{1,2} = 3.05, 2.20 \)
(hereafter designated "pH 7 signal", abbreviated as LS_{pH7}; Fig. 2A, black line). The experimental determination of the g_{3} value is difficult (because it occurs at high field and is very broad and weak) and its value was calculated from the Taylor's theorem to be 1.36. These g values (g_{1,2,3} = 3.05, 2.20, 1.36) are in agreement with the ones previously described for the pig liver and human erythrocyte Cb_{5} (g_{1,2,3} = 3.03, 2.23, 1.43, [23] and g_{1,2,3} = 3.03, 2.23, 1.39 [24], respectively).

The "Truth Diagram", derived originally by Blumberg and Peisach [25] is useful to predict the heme axial ligands. The "Truth Diagram" correlates the electronic effect of the axial ligands (\Delta/\lambda) with the heme rhombicity (V/\Delta) for various heme complexes and provides empirical guidance for the assignment of axial ligands of unknown samples. The "pH 7 signal" giving species has an axial field strength, |\Delta\lambda|, of 2.73 and a heme rhombicity, |V/\Delta|, of 0.86 and, thus, falls in the "B" region on the "Truth Diagram", what is in agreement with the known bis-histidinyl coordination of the b-type hemes. The EPR spectra of samples prepared from pH 7 to 11 showed an equivalent spectrum.

At pH 12, under aerobic conditions, the EPR spectrum of Cb_{5} is dependent on time (fig. 2A). After 3 min, at pH 12 at room temperature (approximate time needed to freeze the samples), the Cb_{5} spectrum shows, besides the "pH 7 signal" (45%, determined based on the intensity of the g_{1} feature), two other low spin (S=1/2) Fe^{3+} species, one with g_{1,2} = 2.77, 2.25 and g_{3} calculated to be 1.68 and the other with g_{1,2} = 2.83, 2.25 and g_{3} calculated to be 1.62 (hereafter designated "pH 12 signal" and "pH 12' signal", respectively; abbreviated as LS_{pH12} and LS_{pH12'}, respectively; Fig. 2A, red line). A similar signal was described for the pig liver Cb_{5} at pH 12 (g_{1,2,3} = 2.82, 2.28, 1.68) [23]. The species giving rise to both LS_{pH12} and LS_{pH12'} (with |\Delta\lambda| = 3.80 and 3.52 and |V/\Delta| = 0.74 and 0.75, respectively) falls in "H" region of the "Truth Diagram", as also previously described (for pH 12) [12,25,26], supporting the presence of a bis-histidinyl coordinated b-type heme. Moreover, the LS_{pH12} signals are similar to the one described for the hemichrome formed during the autooxidation of oxyhemoglobin [12].

After 60 min at pH 12, at room temperature, the proportion of LS_{pH12} + LS_{pH12'} species relative to the LS_{pH7} increased, but the LS_{pH7} signal did not disappear (Fig. 2A, pink line). In addition to the low spin signals, the Cb_{5} EPR spectrum after 60 min at pH 12 displays a very weak axial high spin (S = 5/2) signal, with the main component at g_{\perp} = 5.8, for which a g_{//} = 2 is expected (not resolved here; abbreviated as HS). A similar spectrum was obtained when Cb_{5} was incubated at pH 12 and room temperature for 10 min (Fig. 2A, blue line). The presence of high spin species at high pH had also been described for the pig liver Cb_{5} (g_{\perp} = 6.2, pH 12), together with a signal equivalent to our LS_{pH12} / LS_{pH12'} (g_{1,2,3} = 2.82, 2.28, 1.68) [23].

The effect of H_{2}O_{2} added to Cb_{5} prepared at pH 12 and 7 is shown in Fig. 2B. Left panel shows the EPR spectra of the protein prepared and immediately frozen, at pH 12 in absence (spectra a) and presence (spectra b) of H_{2}O_{2} (10 mM) in comparison to pH 7 (spectra c) and pH 7 in the presence of H_{2}O_{2} (spectra d). H_{2}O_{2} addition to samples at pH 12 induced a prominent sharp peak (\Delta p=1.1 mT) centered at g = 2.005 (expanded spectra region shown in the right panel), that correlates with the presence of organic radical species.
Moreover, a decrease of the HS signal (g⊥ = 5.8 related to the axial high spin (S = 5/2) signal) was also observed (left panel, indicated by an arrow). Cb5 EPR spectra prepared at pH 7.0, in absence and presence of H2O2 shows no significant changes, correlating H2O2 interaction dependence of the protein with the presence of hemichrome species found at pH 12.

Cb5 maintain a folded state in alkaline buffer (potassium phosphate 100 mM, borate 50 mM, KCl 100 mM, EDTA 1 mM, pH 12) as confirmed by tryptophan fluorescence measurement (fig. 3). As shown by the effect of 10 M urea, which is known to elicit Cb5 denaturation [27], Cb5 unfolding leads to a large increase of the Trp fluorescence intensity like in many other proteins [27,28]. However, the Trp fluorescence intensity of Cb5 prepared at pH 12 and 7 after 60 min (Supp. Fig. S2) incubation in the buffer was the same and much lower than that measured in denaturing 10 M urea conditions.

To further characterize Cb5 properties at pH 12, we observed its electrochemical behavior by cyclic voltammetry and calculated the center formal potential (fig. 4). A shift of the formal potential from +13±1 mV at pH 7 (black line) towards -66±7 mV at pH 12 (red line) was perceived which may be associated to conformational changes, namely a higher solvent exposure [29]. The decrease of reversibility found by the increase of the separation between anodic and cathodic peaks is also in line with such alterations. Lower potentials are usually associated to proteins upon charge, electrostatic and conformational alterations [30,31]. The negative shift of the formal potential of Cb5 observed at pH 12, is, as so, also consistent with conformational changes in the protein and probably a more exposed heme group.

Reaction of H2O2 with Cb5 prepared at pH 12, induced a prominent increase of Amplex Red oxidation associated to the presence of protein, being dependent upon H2O2 concentration. The interaction of Cb5 with H2O2 after hemichrome formation is based on the assumption that this peroxidase follows the classical peroxidase cycle where peroxidases (E) consume peroxide and oxidize organic molecules (like Amplex red) to radicals [32]. Therefore the curve obtained for the dependence upon H2O2 was fitted to a two substrates Michaelis-Menten equation (fig. 5). For the H2O2 dependence with the peroxidase activity, keeping constant protein and Amplex red concentration (AH2) (0.02 μM and 5 μM, respectively) we obtained the following values: kcat = 0.7±0.1 s⁻¹, a KmH2O2 = 951±106 μM and a KmA²H = 4.2±0.6 μM. This equation was also applied for the dependence upon Amplex Red of the peroxidase activity of Cb5, keeping the enzyme and H2O2 concentration constant (0.02 μM and 2 mM, respectively) the following kinetic parameters were obtained: a kcat = 0.8±0.1 s⁻¹, a KmH2O2 = 624±50 μM and a KmA²H = 7.2±0.1 μM. This activity was independent on incubation time of the protein at pH 12 and a practical lack of peroxidase activity was found at pHs below 12 (Supp. Fig. S3), showing the strong correlation with the appearance of the hemichrome signal. The measured peroxidase values obtained were higher than those reported for the same activity found in heme proteins of the b-type family [33].

In summary, the loss of the electronic spectral properties characteristics of Cb5 at pH 7.0 supports the hypothesis that the protein itself is a target for oxidation. In addition, appearance of bands at 538 nm, 575 nm and 620 nm has also been found in hemichromes [4,12,34] being reminiscent to that found for compounds III in peroxidases [36]. The lower redox potential of Cb5 when
incubated at pH 12 also supports the tendency to a more oxidized state of the protein at this pH. Moreover, the more negative formal potential value correlates with the values reported for some heme proteins upon interaction with anions, triggering their peroxidase activity, named, i.e.: Cyt c [36-38] and MPO [39].

The EPR spectra at pH 12 support the formation of a Cb5 hemichrome. Hemichromes can be classified in different classes depending on the ligand associated to the iron. Bis-histidinyl coordinated hemichromes can be categorized in class B, with \( g_1 \) values around 2.83-2.75 and \( g_3 \) 1.69-1.63 with ligand field parameters of reversible hemichrome \( H \) and water and/or its conjugated ions (OH\(^-\) and H\(^+\)) retained in the ligand pocket [12]; and class C with \( g_1 \) values around 3.08-2.89 and \( g_3 \approx 1.51 \) with ligand fields falling in the group of irreversible B and C-type hemichromes, with water ejected from the ligand pocket (Fig. 6) [2]. Therefore our \( \text{LS}_{pH12} \) and \( \text{LS}_{pH12} \) species and the electronic absorption bands found are correlative to the presence of a reversible type B hemichrome suggesting the opening of an interacting site for polar compounds and/or water [4,12,34] (that at pH 12 would be hydroxyl ion). Moreover, similar high spin species to those found in our spectra had also been described in aqua high spin complexes in hemichromes with \( g_{\perp} = 5.86 \) and \( g_{||} \approx 1.99 \) [12].

In addition, measurement of the folding properties of Cb5 through tryptophan fluorescence indicates that the protein keeps its folding properties at pH 12, being this non-native state conformation different from those complexes associated to precipitation or loss of the heme [2,5,6].

4. CONCLUSIONS

Our experimental results support a peroxidase like activity of human soluble Cb5 in alkaline conditions upon formation of a hemichrome. The redox potential and the electronic absorbance spectra found for Cb5, incubated in these conditions are in concordance with the expected for a more oxidized state of the protein. Therefore, the protein has an acquired ability to bind oxygenated species and to be able to catalyze a peroxidase reaction suggesting the opening of ligand site for H\(_2\)O\(_2\) as a substrate. The initial steps that promote peroxidase activity are related to low spin signals assigned to a class B hemichrome, with a reversible acquired accessibility to water like ligands and other polar molecules [4,12,34]. Since hemichromes have been related to formation of peroxidation products [10,11], our results suggest a role of Cb5 hemichromes in amplification of peroxidase reactions upon its formation.

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FIGURE CAPTIONS

Fig. 1. Cb5 electronic spectra were recorded at 25 °C every 5 minutes during 1 hour, using a UV-mini 1240 Shimadzu spectrophotometer from 360 to 650 nm in aerobic conditions.

Fig. 2A. X-band EPR spectra of 2.5 mM Cb5 at pH 7 and 12. The samples at pH 7 and 12 (black and red lines, respectively) were frozen after 3 min (approximate time needed to freeze the samples). In the case of pH 12, the spectrum was also acquired after the sample being incubated at room temperature for 10 and 60 min (blue and pink lines, respectively). The g values are indicated, as well as, their positions. The simulated spectrum at pH 7 (with \( g_{1,2,3} = 3.05, 2.20, \) and 1.36) is present in grey. Spectra were recorded at 10K, with a microwave power of 635 \( \mu W \) and modulation amplitude of 0.5 mT.

Fig. 2B. Organic free radical detection by EPR, after \( \text{H}_2\text{O}_2 \) addition to Cb5 at pH 12. Left panel shows Cb5 EPR spectra recorded as indicated previously and prepared at pH 12.0 (a) and pH 12 + \( \text{H}_2\text{O}_2 \) (10 mM), (b), 7.0 (c), pH 7.0 + \( \text{H}_2\text{O}_2 \) (10 mM) (d). Right panel shows an expanded spectral region obtained at pH 12 + \( \text{H}_2\text{O}_2 \) (10 mM) from 335 to 355 mT (magnetic field region) and obtained at 40 K, other conditions as in caption Fig. 2A.

Fig. 3. Maintenance of the native Cb5 folding state was monitored by measurement of the tryptophan fluorescence intensity. Cb5 (5 \( \mu M \)) was incubated at pH 7 (continuous line) and 12 (dashed line) during 10 minutes and its fluorescence was compared to samples prepared in urea 10 M (dotted line) corresponding to the unfolded protein. Fluorescence measurements were performed at 25 °C with excitation wavelength of 290 nm and excitation and emission slits of 2 and 5 nm, respectively.

Fig. 4. Cyclic voltammograms of Cb5 (continuous lines) vs its respective controls in the absence of protein (dotted lines), recorded at pH 7 (black lines) and pH 12 (red lines) at 5 mV s\(^{-1}\) scan rate, 25 °C. Voltammograms were normalized to the current intensity, for better visualization of the shift in the formal potential.

Fig. 5. Initial rates for Cb5 peroxidase activity dependence upon \( \text{H}_2\text{O}_2 \) were measured with a constant Amplex Red (5 \( \mu M \)) and Cb5 (20 nM) concentration at pH 12 at 25 °C (panel a). In parallel, initial rates for Cb5 peroxidase activity dependence upon Amplex Red were measured with a constant \( \text{H}_2\text{O}_2 \) (2 mM) and Cb5 (20 nM) concentration in buffer at pH 12 at 25 °C (panel b). The kinetic was started after addition of Amplex red or \( \text{H}_2\text{O}_2 \): (black squares). Averages ±SD of triplicate measurements are shown in the figure and the discontinuous line is the non-linear regression fit to the two substrates Michaelis-Menten model as indicated in Material and Methods.

Fig. 6. Bis-histidine coordinated hemichromes complexes [2]. Two classes of bis-histidine coordinated hemichromes have been documented. Reversible
class B (scheme a) with $g_1$ values around 2.83-2.75, $g_3$ 1.69-1.63 and water present in the ligand pocket (scheme a). Class C (scheme b) with $g_1$ values around 3.08-2.89, $g_3 \leq 1.51$ with water ejected from the ligand pocket. In some heme proteins with ability to bind oxygen, superoxide anion has been found to displace oxygen by nucleophilic attack of the distal histidine, being superoxide retained in the ligand pocket (scheme c). In this case, the g values found are similar to the ones or reversible class B hemechrome [4].
SUPPLEMENTARY FIGURE CAPTIONS

Supp. Fig. S1. $Cb_5$ electronic spectra at different pHs and recorded at 25 °C. Panel a shows the spectra from 350 nm to 450 nm. Panel b shows a zoomed (x12) spectra of $Cb_5$ (5 μM) recorded from 500 nm to 650 nm. Panel c and d shows the reversibility of the $Cb_5$ spectra (recorded from 350 nm to 450 nm and from 500 nm to 650 nm, respectively) after incubation of the sample at pH 12 (grey line) in time (1= 0 min, 2= 10 min, 3= 20 min, 4= 60 min) or pH change to 7.0 at those times (black line). $Cb_5$ 50 μM was incubated in buffer at pH 12 at 25 °C and an UV-vis spectrum of the sample was recorded at different times by diluting the sample to 5 μM in a quartz cuvette with buffer at pH 7 or 12. Dashed black line indicates the $Cb_5$ (5 μM) spectrum prepared after dilution at pH 7.0 (time 0 min).

Supp. Fig. S2. $Cb_5$ tryptophan fluorescence intensity dependence upon incubation time at pH 7 (solid square) and 12 (open square), in comparison to the signal of $Cb_5$ prepared in urea 10 M (dotted line). Fluorescence measurements were performed at 25 °C with excitation and emission wavelength of 290 nm and 350 nm respectively and excitation and emission slits of 2 and 5 nm, respectively.

Supp. Fig. S3. Initial rates for the peroxidase activity of $Cb_5$ (20 nM) measured with Amplex red (5 μM) at pH 12 and after protein incubation in the same buffer at different times (panel a). The fluorescence increase was monitored using a spectrofluorimeter Perkin-Elmer 650-40 with the following set up: excitation and emission wavelengths of 530 nm and 590 nm, with excitation and emission slits of 5 and 10 nm and normal gain. After sample incubation in the buffer at different times, $Cb_5$ (20 nM), was added to a 2 ml thermostated quartz cuvette in buffer; potassium phosphate 100 mM, borate 50 mM, KCl 150 mM, EDTA 1 mM, prepared at pH 12 at 25 °C. The measurement was started after $H_2O_2$ addition. Initial rates for the peroxidase activity of $Cb_5$ dependence upon pH was measured showing a practical lack of activity at pHs below 12 (panel b), where no hemichrome formation is formed.
REFERENCES


Figure 1

(a) 10x

528
538
413

(b) 10x

558
613
575
395

Wavelength (nm)

350 400 450 500 550 600 650
Figure 2A

LS_{pH 7}:
- $g = 3.05$
- $g = 2.20$
- $g = 1.36$

HS:
- $g = 5.77$
- $g = 2.83$
- $g = 2.25$
- $g = 1.68$
- $g = 1.36$

Magnetic field (mT)
Figure 3
Figure 5

**Graph a**
- X-axis: Peroxidase activity (μM/s)
- Y-axis: [H₂O₂] (μM)
- Data points and error bars are shown.

**Graph b**
- X-axis: Peroxidase activity (μM/s)
- Y-axis: [Amplex Red] (μM)
- Data points and error bars are shown.
Supp. Fig. S1
Supp. Fig. S3
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