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FERRYL HAEM PROTONATION GATES PEROXIDATIC REACTIVITY IN GLOBINS

Radu Silaghi-Dumitrescu,^{1,2*} Brandon J. Reeder,^{1*} Peter Nicholls¹, Chris E. Cooper¹,
Michael T. Wilson¹

¹*Department of Biological Sciences, University of Essex, Colchester CO4 3SQ, UK and*

²*Department of Chemistry, “Babeş-Bolyai” University, Cluj-Napoca, Romania*

**These authors contributed equally to this work*

Correspondence and requests for materials should be addressed to M.T.W.
(wilsmt@essex.ac.uk).

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Synopsis:

Ferryl (Fe(IV)=O) species are involved in key enzymatic processes with direct biomedical relevance; among others, the uncontrolled reactivities of ferryl myoglobin and haemoglobin have been reported to be central to the pathology of rhabdomyolysis and subarachnoid haemorrhage. Rapid scan stopped-flow methods have been used to monitor the spectra of the ferryl species in myoglobin and haemoglobin as a function of pH. The ferryl forms of both proteins display an optical transition with $\text{pK} \sim 4.7$ and this is assigned to protonation of the ferryl species itself. We also demonstrate for the first time a direct correlation between haemo(myo)globin ferryl reactivity and ferryl protonation status, simultaneously informing on chemical mechanism and toxicity and with broader biochemical implications.

INTRODUCTION

Ferryl species, formally $[\text{Fe}^{4+}=\text{O}^{2-}]^{2+}$, are formed in haem and non-haem iron proteins upon treatment with peroxides or related dioxygen species, as part of their respective catalytic cycles/functions and/or as significant side-reactions under physiological and pathophysiological conditions.[1-9] Such species have long been known to contain an oxo atom bound to Fe(IV), as supported by Mössbauer, resonance Raman, NMR, ENDOR and X-ray absorption (EXAFS) spectroscopies, and by X-ray crystallography (as recently reviewed in Refs [1, 2, 10-13]). In haem proteins, the ferryl species is also referred to as ‘Compound II’; the related ‘Compound I’ species contain an additional oxidizing equivalent located on the porphyrin ring or an adjacent amino acid residue.

Recent experimental and theoretical results have suggested that at least some ferryl species may in fact be protonated, i.e. Fe(IV)-OH rather than Fe(IV)=O. A hydroxo ligand has been implied for the histidine-ligated ferryl group in myoglobin (Mb) Compound II[14] at acidic pH and in cytochrome c peroxidase (CCP) Compound I, based on $\sim 1.9\text{-}\text{\AA}$ iron-oxygen distances determined by atomic-resolution X-ray diffraction crystallography[15] (CCP Compound I is an atypical Compound I, in that the additional oxidizing equivalent is localized on a protein side-chain rather than on the porphyrin; in this respect, CCP Compound I is more akin to typical Compound II species). Yamazaki and co-workers have also shown that Compound II formation in peroxidases is accompanied by uptake of a proton (although they could not demonstrate the exact location of this proton).[16] Green and co-workers have more recently challenged the crystallographic findings on Mb and CCP, using EXAFS and resonance Raman spectroscopies.[13, 17-21] Indeed, Green and co-workers have reported that the Fe-O bond in the quintessential peroxidase HRP (horseradish peroxidase) Compound II is $\sim 1.65\text{ }\text{\AA}$, consistent with a non-protonated ferryl group.[17] They have further shown that the Fe-O stretching frequency, as measured by resonance Raman, does not

significantly change position in the range of pH from 4.5 to neutral and basic values for myoglobin Compound II.[19, 21] On the other hand, the same spectroscopic tools, together with Mössbauer spectroscopy and density functional calculations, have been used by Green and co-workers to establish that chloroperoxidase Compound II and cytochrome P450 Compound II are indeed protonated.[13, 19, 22] Chloroperoxidase and cytochrome P450 feature an endogenous anionic thiolate ligand to the haem, instead of the neutral imidazole ligand found in HRP (though some researchers have interpreted NMR and resonance Raman data for the latter as evidence for imidazolate character[2, 23]) or in myoglobin. Precisely this difference in axial ligation has been proposed to be the reason why HRP Compound II would, unlike CPO Compound II, be unprotonated.[17] A Mössbauer spectroscopy report, complemented by DFT calculations, was also interpreted as evidence that catalase Compound II is at least partially protonated; catalase, like CPO and P450, features an anionic ligand to the haem, namely, a tyrosinate.[24] The accumulated body of evidence from spectroscopic techniques thus currently favours the view that ferryl protonation does not occur within physiologically-relevant pH ranges with histidine-ligated haemoproteins, whereas the ferryl group is at least partially protonated in haemoproteins ligated by thiolate or tyrosinate. Nevertheless, as will be shown below, we believe that the entire body of spectroscopic evidence on histidine-ligated haemoprotein Compound II species may also bear an alternative interpretation.

Mb and haemoglobin (Hb) have long been known to form ferryl (Compound II) species upon treatment of the Fe(III) or Fe(II) state with peroxide.[25-27] Ferryl Mb and Hb are relatively stable at neutral and basic pH, but less so at acidic pH values.[28] This instability at acidic pH parallels an increased reactivity towards reducing substrates and it has been proposed on several occasions that protonation of the ferryl group could be responsible for these trends in reactivity.[7, 8, 28, 29] A recent crystal structure of Mb ferryl at acidic pH has indeed shown a long Fe-O bond (1.9 Å), consistent with an OH ligand rather than the commonly-accepted Fe=O description.[14] Although subsequent spectroscopic work has led to the conclusion that the crystal structure of Mb ferryl must be in error and that Mb ferryl is not protonated,[17], the reason for the pH dependence of ferryl Mb and Hb reactivity is still important, as this species has previously been

detected *in vivo* and is known to be implicated in pathophysiological states involving acute oxidative stress, with this involvement being most manifest in compartments with lowered pH such as kidney and cerebrospinal fluid[7, 8] (although, notably, even normal blood contains detectable amounts of by-products derived from ferryl Hb[9]). Below we present data that demonstrate that the optical absorption spectrum of ferryl haem is distinctly pH-dependent. This optical transition is rapid (sub-millisecond) and, in our view, best interpreted as direct protonation of the ferryl haem species.

EXPERIMENTAL

Horse heart myoglobin (Sigma) and human haemoglobin were used as previously described.[7, 8, 28-30] Ferryl Mb and Hb were prepared as previously described;[28] briefly, a 50 μ M solution of met Mb in 20 mM phosphate buffer at pH=8 was mixed with a 1.5-fold excess of hydrogen peroxide at room temperature; at this pH value, the only observable product is Compound II (as opposed to Compound I).[28] Ferryl formation was monitored in the Soret region as well as in the visible as previously described;[28] within ~20 minutes, the UV-vis spectrum converted to ferryl (Compound II) and remained unchanged throughout the time required for subsequent stopped-flow measurements. Stopped-flow measurements were performed using an Applied Photophysics model SX-18MV instrument (Applied Photophysics, Leatherhead, UK). The buffers used for pH jump experiments were 200 mM phosphate (pH 2, 3, 7 and 8) and acetate (pH 4, 5 and 6); alternatively, for acidic pH's citrate buffers were also used. Diode array-collected data were analyzed within the Pro-K II software (Applied Photophysics). Time courses recorded for up to 20 seconds following mixing of ferryl Mb/Hb with acidic pH solutions could be fitted as an A→B→C sequence. The spectra of A and B were very similar to each other and to the spectrum of Mb/Hb ferryl at pH 8, and also resemble the first spectrum recorded at 1.28 ms after mixing. Spectra of Species A are therefore shown in Figure 1. On the other hand, species C, which accumulates on a slower timescale, featured broadened bands throughout the UV-vis spectrum (cf Supporting Information), suggestive of protein unfolding, most likely accompanied by protonation and dissociation of the proximal histidine ligand. pH dependence data were fitted to the Henderson-Hasselbach equation assuming one-proton processes; for the kinetics data, initial guesses for maximal rates were chosen

assuming a pKa of 4.5, after which both the maximal rates and the pKa values were optimized with a least-squares algorithm within Microsoft Excel 2003; calculated pKa values were ~4.7 for all data sets shown in Figure 2. The rates of decay of ferryl haem via reduction by ascorbate were measured by procedures identical to those used for the data in Figure 1, with the exception that 1 mM ascorbic acid was added at the time of mixing pH 8 ferryl with the respective buffers. Turnover experiments with ascorbate were performed under conditions similar to those used in a standard assay for ascorbate peroxidase,[31] using 600 μ M ascorbate, 1 mM hydrogen peroxide, and 5 μ M myoglobin in 20 mM phosphate pH 7 at room temperature. Urate turnover experiments were similar to ascorbate turnover, except for the use of 500 μ M urate instead of ascorbate, and 8 μ M Hb instead of Mb. Turnover rates varied linearly with Mb/Hb concentrations in these ranges of concentrations (data not shown). Density functional calculations (UBP86/6-31G**) on unsubstituted iron porphyrin adducts listed in Table 1 were performed following protocols previously described; proton affinities are obtained in vacuo, but the trends are readily maintained in solvents;[2, 32] direct computational prediction of pKa values as opposed to simple proton affinities is possible but has inherent pitfalls as recently reviewed in Ref. [33].

RESULTS AND DISCUSSION

Figure 1 shows optical spectra collected on rapidly mixing ferryl myoglobin (produced by standard procedures at basic pH, where it is relatively stable) with solutions of varying pH in a stopped-flow spectrophotometer. Following mixing, the time courses of spectral changes were recorded, from which the initial spectra ($t=0$) could be constructed (see Methods). The latter therefore report the spectra of the ferryl form at any given pH value before decay of the ferryl species or protein structural changes occurred. These spectra show a pH dependence with a pKa of ≤ 5 . Likewise, a pKa of ≤ 5 is obtained for Hb ferryl in similar pH-jump experiments.

The optically-detected pKa values for the globin haem ferryl species are entirely consistent, as shown in Figure 2, with previously-measured[7, 8, 28-30, 34, 35] pH

profiles for Mb ferryl *reactivity* in autoreduction, or in oxidative reactions such as HPODE consumption, isoprostane formation, or for ascorbate or urate reduction. These latter reactivity data hold direct physiological relevance; 13(S)-hydroperoxy-9,11(cis,trans)-octadecadienoic acid (HPODE), is generated in vivo from linoleic acid by lipoxygenases and has been used as a model lipid peroxide to explore the mechanism of met-globin-induced damage to lipid membranes;[30] isoprostane F₂ formation from low density lipoprotein (LDL) has been implicated in Mb- and acidosis-driven renal failure[36]; finally, ascorbate and urate are known to detoxify met and ferryl states of myoglobins and haemoglobins.[9, 37-39] These pH profiles are also identical to those previously measured by Skibsted and co-workers for the reactivity of ferryl Mb towards ABTS (a typical peroxidase substrate) as well as towards chlorogenate.[40, 41] Free haemin in solution exhibits an identical pH dependence to myoglobin in the HPODE assay,[28] suggesting that this reactivity pattern is an intrinsic property of the haem rather than being due to protonation of a protein residue. Taken together, these data establish a direct link between biologically-relevant acid-induced reactivity of ferryl globins and a distinct spectral change in the optical spectrum of their haems.

The pH-dependent spectral changes shown in Figure 1 for ferryl Mb are consistent with spectra previously reported for the same species at basic pH vs. pH 3.5.[42] These differences were shown to be paralleled by changes in the MCD spectra that were irreconcilable with simple changes in hydrogen bonding patterns, and could only be explained as arising from protonation of an iron ligand at acidic pH.[42] This ligand was proposed by Thomson and co-workers[42] to be the proximal histidine, i.e. $[\text{imidazole-haem-Fe}^{4+}\text{-O}^{2-}]^0 \leftrightarrow [\text{imidazolate-haem-Fe}^{4+}\text{-O}^{2-}]^{-1}$. The pK_a of this proximal histidine is ~11 in ferric Mb, i.e. $[\text{imidazole-haem-Fe}^{3+}\text{-OH}]^0 \leftrightarrow [\text{imidazolate-haem-Fe}^{3+}\text{-OH}]^{-1}$. [42] Thomson and co-workers have pointed out that a pK_a shift of several pH units for the imidazole ligand may be expected upon going from the ferric-hydroxide state to the ferryl.[42] Nevertheless, especially in view of the recent crystallographic data on myoglobin Compound II,[14] an attractive alternative interpretation is that the myoglobin ferryl pK_a may be due to protonation of the oxygen atom, rather than to protonation of an imidazolate axial ligand. Notably, in the

myoglobin Compound II crystal structure, besides the iron-oxygen bond length being consistent with a protonated ferryl, the iron-imidazole bond length is consistent with a *protonated* imidazole (i.e., it is ~ 2.1 Å as opposed to the ~ 1.9 Å predicted for an imidazolate).[2, 14]

In order better to estimate the relative likelihood of the two sites (imidazolate vs. oxygen atom) being protonated in Mb ferryl, Table 1 shows a list of similar $[X\text{-haem-Fe-Y}]^0 \leftrightarrow [X\text{-haem-Fe-Y}]^{-1}$ processes (i.e. similar charge balances), where either one of the haem axial ligands X or Y undergoes deprotonation in Mb as well as in selected related proteins. Table 1 lists known pKa values or known protonation states where experimental data is available, and it also lists *calculated* proton affinities for models of the myoglobin active site as well as of the chloroperoxidase active site (see Experimental section for an account of how these models are treated computationally). For the cases where both experimental data and calculated proton affinities are available, theory and experiment appear to correlate well, without exception. Thus, calculated proton affinities in excess of 330 kcal/mol always correlate with a protonated state being observed at physiological pH, while calculated proton affinities of 250-260 kcal/mol appear consistent with the deprotonated state becoming populated in the physiological pH range. Table 1 thus notably shows that the proton affinity of $[\text{Imidazolate-Fe(IV)=O}]^{-1}$ is very similar to those of other species, with the same overall charge and present in the same protein pocket, known to be protonated at neutral pH. Computations thus offer no underlying electronic structural reason to assign the ferryl Mb/Hb pKa as due to protonation of the histidine ligand, rather than the ferryl itself. Table 1 also shows that the proton affinity of the oxygen atom in an imidazole-ligated ferryl group, i.e. $[\text{Im-Fe(IV)-OH}]^{1+} \rightarrow [\text{Im-Fe(IV)=O}]^0$, would be slightly lower than the proton affinity of the ferric-hydroxo adduct, i.e. $[\text{Im-Fe(III)-OH}_2]^{1+} \rightarrow [\text{Im-Fe(III)-OH}]^0$ (comparing two protonation processes with the same overall charge balance). Ferric Mb and Hb feature an aqua ligand with a pKa of $\sim 8\text{-}9$;^[43] the pKa of the ferryl Mb/Hb should therefore be expected to be lower than that of ferric Mb/Hb. This prediction is in good agreement with the experimental data in Figures 1 and 2 (note, that our interpretation of the computational results focuses on trends and relative values, while the *exact value* of the difference in pKa values is not quantitatively addressed by

our gas-phase calculations). It thus follows that the iron ligand protonating at pH~5 in ferryl Mb is most likely the ‘oxo’ oxygen. This now provides an atomic-level insight into the origin of the known[7, 8, 28, 29] physiologically-relevant pH-dependence of the myoglobin ferryl reactivity. We have argued elsewhere that the alternative electronic description of Fe(IV)-OH as Fe(III)+hydroxyl radical would recommend protonated ferryl as a particularly reactive species.[28, 29, 32]

As stated in the introduction, a large body of spectroscopic evidence has recently been interpreted as evidence for a non-protonated ferryl in myoglobin, and for a protonated ferryl in anion-ligated haemoproteins (chloroperoxidase, cytochrome P450). We feel that this spectroscopic evidence bears an alternative interpretation, which is also in line with our own data reported herein, as follows:

- (1) Green and co-workers find that using resonance Raman they can detect the Fe=O (non-protonated) stretching frequency for ferryl Mb even at pH values as low as 4.5. This is entirely consistent with our measured pKa value of 4.7: there should indeed be significant amounts of unprotonated ferryl present even at pH 5[13, 21, 22]
- (2) A history of decades of unsuccessful attempts with chloroperoxidase has shown that detection of the Fe-OH stretching frequency for a protonated ferryl can be extremely problematic.[2, 21] Green and co-workers have recently remarkably succeeded to detect this Fe-OH frequency in chloroperoxidase Compound II.[21] However, in parallel Mössbauer and DFT studies, they have also shown that ~30% of chloroperoxidase Compound II is in fact non-protonated;[19] neither they nor others have so far been able to detect this non-protonated CPO ferryl using resonance Raman spectroscopy. One lesson that we can therefore learn from CPO Raman spectroscopy is that detection of the ferryl iron-oxygen stretching frequency by resonance Raman can be problematic even after sustained effort. The failure so far to detect a protonated ferryl group in myoglobin by resonance Raman is therefore not a strong argument against such protonation.

- (3) Green and co-workers advocate that an anionic ligand (thiolate) in ferryl Compound II results in protonation of the ferryl form at physiological pH values.[17] If one were to interpret our measured pK_a value in terms of the basic form of Mb Compound II containing an anionic imidazolate ligand, then we would also have to conclude, by analogy with thiolate-ligated CPO and P450 (and perhaps also by analogy with tyrosinate-ligated catalases[24]), that the basic, imidazolate-ligated, form of Mb ferryl contains a hydroxide ligand. This, however, would be at odds with the resonance Raman data, showing that even at pH 4.5 at least part of the sites contain a non-protonated ferryl.
- (4) EXAFS data on horseradish peroxidase Compound II has recently been interpreted to show an iron-oxygen bond of 1.65 Å.[17] This can be safely interpreted as proof for a non-protonated ferryl group. However, as previously discussed by us elsewhere,[2] an older, independent EXAFS study cites a 1.9-Å distance for the same bond length, consistent now with a *protonated* ferryl.[44] Furthermore, a ~0.1-Å difference in the other iron-axial ligand bond length (iron-histidine) also exists between the two EXAFS measurements.[17, 44] In fact the earlier EXAFS measurement citing an Fe-O bond length at 1.9 Å and an iron-histidine bond length at 2.1 Å, is also entirely consistent with the atomic-resolution crystal structure of Mb ferryl,[14] while the more recent EXAFS study[17] is at odds with crystallography both in terms of the Fe-O distance and in terms of the Fe-histidine distance; the latter is important to note, as the larger number of atoms in the histidine ligand makes it less likely to be affected by crystallographic disorder or partial occupation compared to the Fe-O distance. There are now three reports of high-resolution crystal structures for three Compound II species (Mb - 1.35-Å resolution,[14] CCP - 1.3 Å,[15] HRP - 1.6 Å[45]), all of which concur in indicating a protonated ferryl group and an ~2.1-Å Fe-histidine bond length.

Based on arguments 1-4 presented above, our proposed interpretation of the Mb pKa as being due to ferryl protonation appears to be in reasonable agreement with other spectroscopic evidence as well as with X-ray crystallography.

Thomson and co-workers have compared the MCD spectra of acidic and basic ferryl myoglobin, and found that the *acid* form closely resembled typical peroxidase Compound II species, whereas the basic form did not.[42] If we accept that acid ferryl Mb contains an Fe-OH bond, the implication is that most histidine-ligated Compound II species are also protonated. It has long been known that Compound II formation in peroxidases is accompanied by uptake of a proton;[16] our findings now seem to indicate that this proton is located on the ferryl moiety. In Mb/Hb, protonation brings about increased reactivity (cf Figure 2); a non-protonated ferryl Mb/Hb at neutral pH presents an evolutionary advantage, limiting potentially deleterious reactivity in this dioxygen-carrying protein. It is also an evolutionary advantage that in peroxidases, enzymes the function of which is precisely to form ferryl and efficiently use its oxidizing power, the ferryl is protonated at neutral pH. In peroxidases, the ferryl pKa is likely modulated by a carboxylate side-chain hydrogen-bonded to the proximal imidazole[5]; this added negative charge should increase proton affinity and shift the pKa towards basic values. By contrast, the proximal imidazole/His ligand in Mb is hydrogen-bonded to a neutral backbone carbonyl group, implying a smaller proton affinity. The general conclusion is that Nature gates the reactivity of ferryl species by controlling their protonation state.

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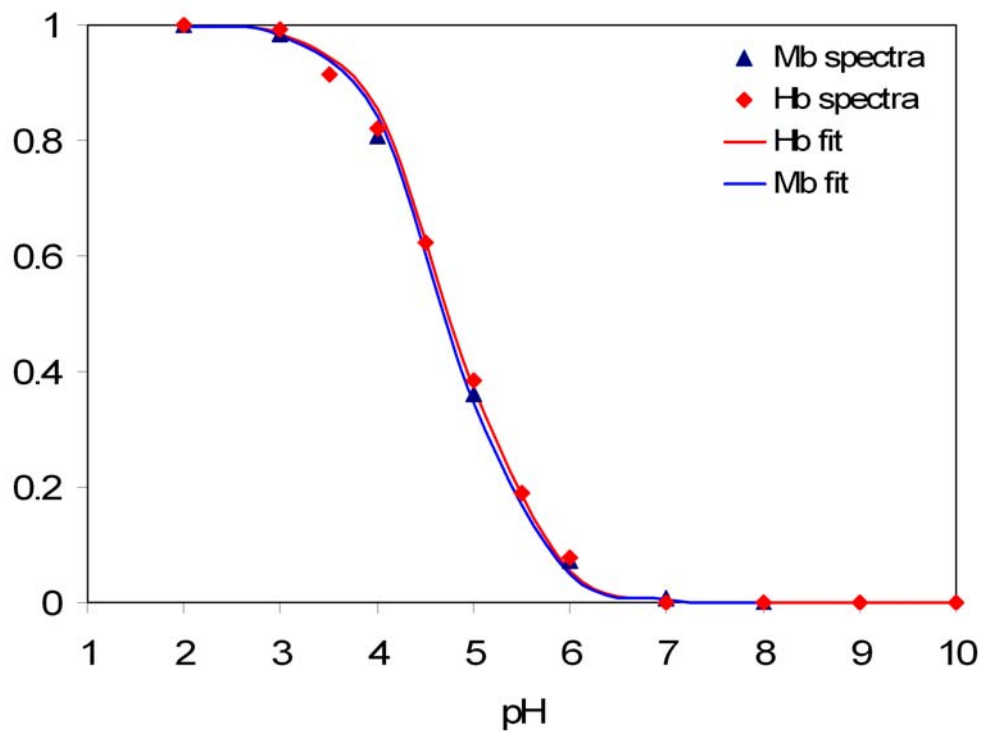
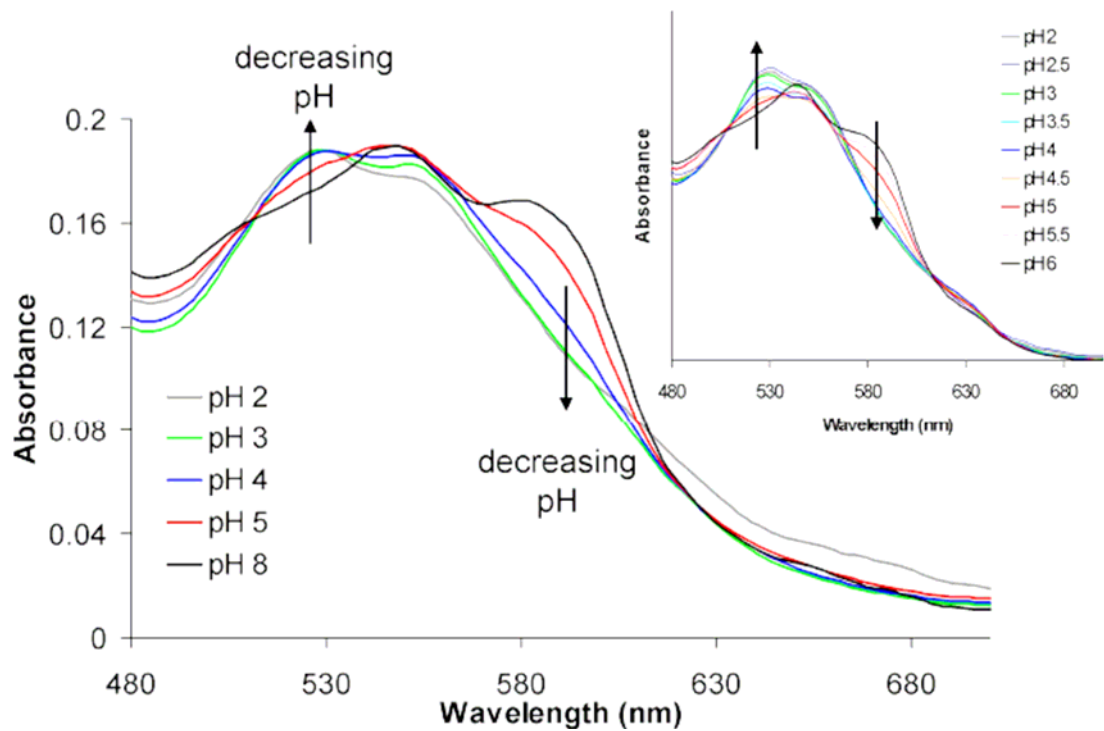


Figure 1. Upper panel: spectra of ferryl Mb collected at known pH values; the corresponding haemoglobin ferryl spectra are shown as inset. Lower panel: normalized pH dependence of A(529)-A(588) for Mb and Hb; fits to the Henderson equation yield pKa values of ~4.7 for both proteins.

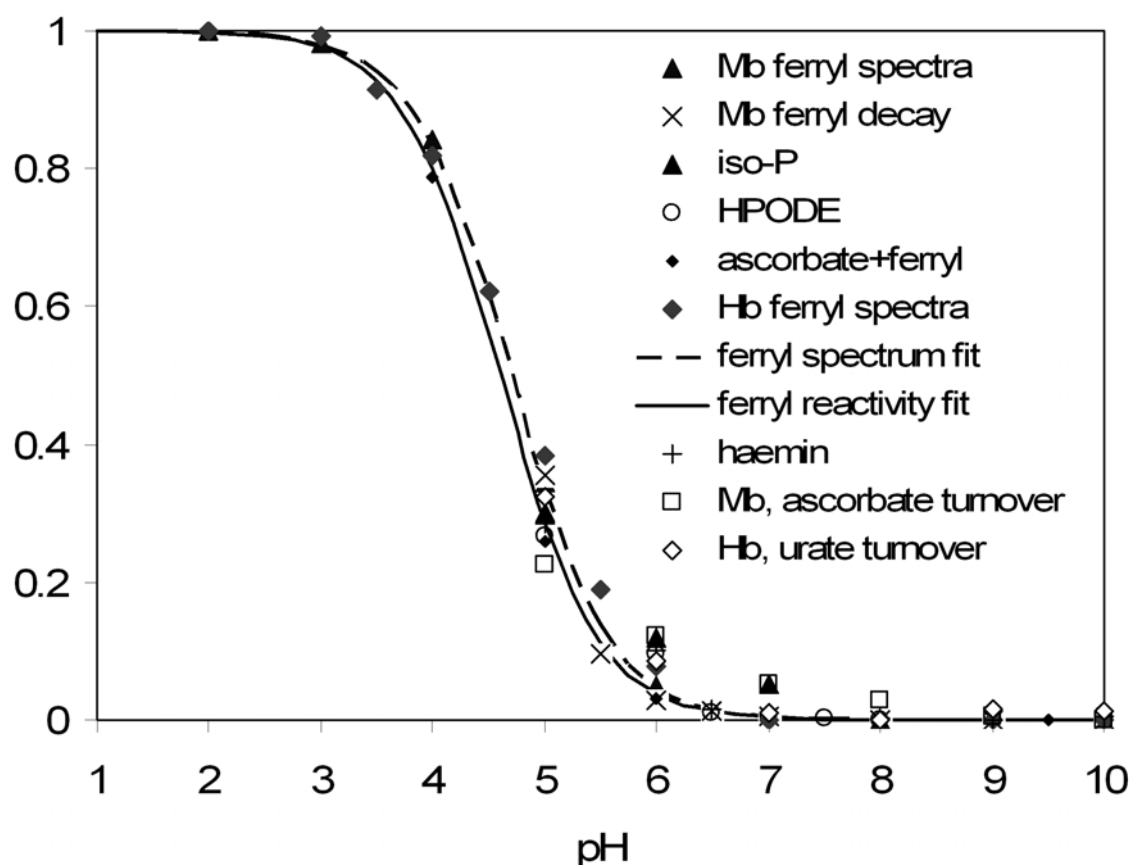


Figure 2. Comparison of the optically-detected pKa of Mb and Hb ferryl with pH dependant kinetics of decay with or without exogenous substrates added. The reactivity data points refer to reactions listed in text: 'iso-P' refers to isoprostane F₂ formation by Mb,[36] 'HPODE' refers to HPODE (13(S)-hydroperoxy-9,11(cis,trans)-octadecadienoic acid) consumption by Mb,[30] 'ascorbate+ferryl' refers to the rate of ferryl reduction by ascorbate, 'haemin' refers to HPODE consumption by free haemin,[28] and 'Mb, ascorbate turnover' and 'Hb, ascorbate turnover' refer to catalytic ascorbate and urate oxidation by Mb and Hb, respectively, in the presence of hydrogen peroxide.

Table 1. Room temperature pKa values (at neutral pH, room temperature, for Mb where otherwise not specified, and for chloroperoxidase) and calculated proton affinities (for models of the corresponding active sites, cf. Experimental section, this work) for relevant deprotonation processes.

Charge balance	Experimental protonation state/pKa,	Calculated proton affinity (kcal/mol)
+1 -> 0		
$[\text{Im-Fe(III)-OH}_2]^+ \rightarrow [\text{Im-Fe(III)-OH}]^0$	Partially protonated (pKa~8 in Hb and 9 in Mb)[43]	258 kcal/mol
$[\text{Im-Fe(IV)-OH}]^+ \rightarrow [\text{Im-Fe(IV)=O}]^0$	Partially protonated;proposed interpretation of Figure 2 data	256 kcal/mol
+0 -> -1		
$[\text{Im-Fe(III)-OH}]^0 \rightarrow [\text{Imidazolate-Fe(III)-OH}]^{-1}$	protonated, pKa~11[42]	
$[\text{Im-Fe(III)-OH}]^0 \rightarrow [\text{Im-Fe(III)=O}]^{-1}$	protonated	
$[\text{Im-Fe(II)-OO}]^0 \rightarrow [\text{Imidazolate-Fe(II)-OO}]^{-1}$	protonated;	407 kcal/mol
$[\text{Im-Fe(III)-OOH}]^0 \rightarrow [\text{Im-Fe(III)-OO}]^{-1}$	protonated;[46]	374 kcal/mol
$[\text{Im-Fe(III)-OOH}]^0 \rightarrow [\text{Imidazolate-Fe(III)-OOH}]^{-1}$	protonated;[46]	338 kcal/mol
$[\text{Im-Fe(III)-OO}]^0 \rightarrow [\text{Imidazolate-Fe(III)-OO}]^{-1}$	protonated;[46]	373 kcal/mol
$[\text{Im-Fe(IV)=O}]^0 \rightarrow [\text{Imidazolate-Fe(IV)=O}]^{-1}$	alternative interpretation of Figure 2 data, based on Ref. [42]	337 kcal/mol
$[\text{thiolate-Fe(IV)-OH}]^0 \rightarrow [\text{thiolate-Fe(IV)=O}]^{-1}$ (chloroperoxidase)	protonated[17];	345 kcal/mol
$[\text{thiolate-Fe(III)-OH}_2]^0 \rightarrow [\text{thiolate-Fe(III)-OH}]^{-1}$ (P450)	protonated[3]	

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